Recent Advances in **Molecular Biology and Biochemistry**

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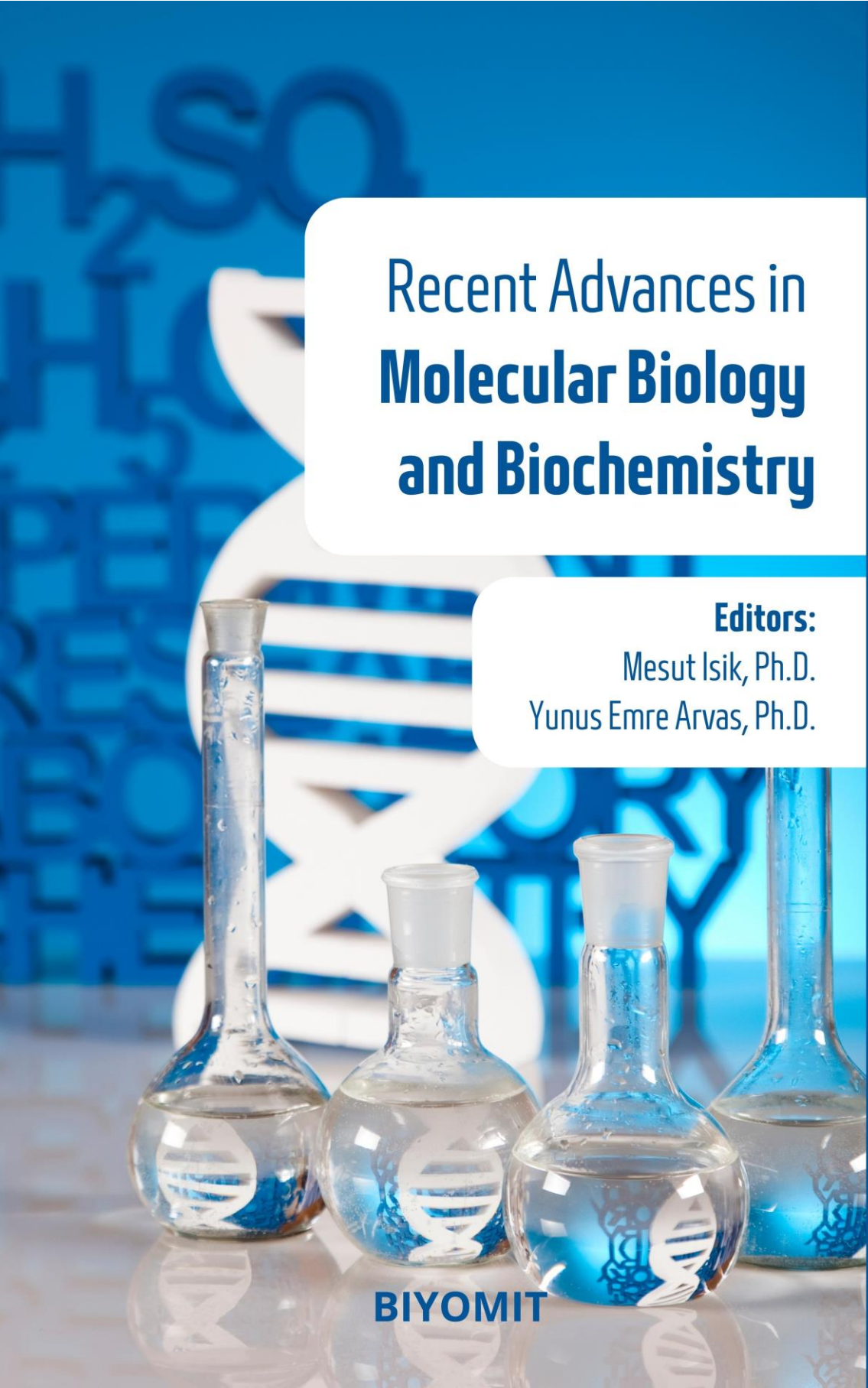

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Recent Advances in Molecular Biology and Biochemistry

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First Edition

©August 2023

ISBN

978-605-71699-4-5

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Turkey Address: Çifte Havuzlar Mah. Eski Londra Asfaltı Cad. Kuluçka Merkezi A1 Blok No:151/1C/B35, Esenler, İSTANBUL

Web: www.biyomit.com

E-mail: biyomit@gmail.com

Certificate No: 53687

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Editors

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Preface

This book covers a wide range of essential topics, from genetic expression profiles to toxicological analysis, and provides an overview of many different fields, from plants to microorganisms, from cancer therapies to biological diagnostics.

In the first chapter, you will explore how the gene expression profile of drought stress resilience relates to morphological traits. Next, you will learn about the latest developments in cancer therapy by examining the apoptotic effects of gamma-tocotrienol on different types of cancer. Next-generation sequencing techniques and applications that have revolutionized the genetic analysis of plants will also be waiting for you in this book.

Detailed information on the diversity and effect of bacterial alkane hydroxylase is presented for those wondering about the role of microorganisms in hydrocarbon degradation. You will also review the latest advances in the molecular diagnosis of infectious diseases and look at exosome research to understand its role in spreading cancer cells.

There is also a section on molecular-based biomonitoring and toxicant analysis for those interested in the characterization and monitoring of toxins in aquatic ecosystems. Exciting topics such as the pharmacological potential of adaptogen plants and MXen nanomaterials used in cancer therapy are also addressed.

You will also examine the antidiabetic effects of selected Cucurbitaceae plant species used in the fight against diabetes and learn to evaluate enzyme activity with nanoparticles. You will also learn about MicroRNAs that modulate the autophagy pathway.

Following the chapter that provides an overview of animal and plant genome research, you will examine the mechanisms of oxidative stress-induced toxicity and explore the therapeutic potential of toxic peptides.

This book provides essential information for anyone interested in the natural sciences. We hope it will benefit both students and teachers as a target audience in terms of careful explanation of key concepts and a source of commentary and references to additional sources of information.

Sincerely,

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CHAPTER 1

Relationships Among Gene Expression Profiles and Stress Tolerance Indices of Morphological Traits of Accessions of Cowpea Screened Under Differential Drought Stress

Alaba Emmanuel Gbadamosi,
Abiola Toyin Ajayi

Relationships Among Gene Expression Profiles and Stress Tolerance Indices of Morphological Traits of Accessions of Cowpea Screened Under Differential Drought Stress

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Abstract

This study investigated drought tolerance in cowpea accessions through morphological traits and gene expression profiles. The experiment utilized a randomized complete block design (CRD) with three replicates in a screen house. Accessions were subjected to drought stress and well-watered conditions. Stress tolerance indices (STIs) were calculated for plant height (PHSTI), number of leaves (NLSTI), dry shoot weight (DSWSTI), dry root weight (DRWSTI), and combined (ComSTI) traits. Gene expression profiling of *vucapx* and *ptgs2* was conducted under drought stress (T) and control (A) conditions. Correlation analysis was performed by biplot analysis. The mean square values for gene expression profiles showed significant variation among accessions and treatments for both *vucapx* and *ptgs2* genes. Accession and treatment interaction also contributed significantly to the variation in gene expression. Also, significant differences existed in stress tolerance indices of the studied morphological traits. Highly positive correlations were observed among the expression of *vucapx* under drought stress and all the STIs. Similarly, higher expression of *vucapx* under control was strongly related to higher *ptgs2* under drought stress with both weakly correlated to *vucapx* under drought stress, plant height, and dry root weight STIs. The significant correlations between gene expression

profiles and stress tolerance indices suggest that these genes may play a crucial role in conferring stress tolerance in cowpea. This study identified the best accessions (AC22, AC23, and AC13) among the highly tolerant which can be included in the breeding program of cowpea for drought tolerance to bring a positive outcome. These findings suggest that *vucapx* and *ptgs2* gene expression may contribute to drought tolerance in cowpea. Further research on the functional roles of these genes could provide valuable insights into the molecular mechanisms underlying drought resistance in cowpea, aiding breeding programs for improved drought tolerance.

Keywords: Climate change, Drought tolerance, productivity, *ptgs2*, *vucapx*, yield

1. Introduction

Cowpea is an important source of protein for food and feeds in the tropical and sub-tropical regions of the world [1]. It is adapted to diverse ranges of environmental conditions compared to other legumes and possesses a tremendous capability to enhance soil fertility through nitrogen fixation thereby making it a key crop in crop rotation [2, 3]. The protein content in cowpea can range between 23 to 28% [4] making it an important grain legume for resource-poor people of the world; its ability to fix up to 80 kg/ha [5] of nitrogen in the soil can cause a dramatic reduction in the use of chemical fertilizers in agriculture. Nigeria is the highest producer of cowpea in the world with over 3.6 million tonnes in 2021 [6], where the yield per hectare stands at 771.7 kg/ha and falls below the expected yield due to many production constraints such as infestation by diseases, insect pest attacks, and drought stress.

Drought is one of the consequences of climate change and a major limiting factor to cowpea productivity in Nigeria [7]. Plants can adopt various mechanisms functioning autonomously or together in coping with drought conditions making it a complex trait [8]. The major drought coping mechanisms include drought escape, drought avoidance, drought tolerance,

and drought recovery [8]. Nonetheless, various demerits are associated with these mechanisms leading to a compromise in yield. Hence, the adaptation of crops to drought must display a balance among all coping mechanisms while sustaining adequate yield [9].

Several approaches have been deployed to screen for the level of drought tolerance in cowpea and many other crops. These include physiological, morphological, biochemical, and molecular, as well as differential expression of candidate genes under drought stress [9]. Unfortunately, information regarding the expression of candidate genes under stress in cowpea is limited in Nigeria. Hence, a better understanding of the genetic differences of cowpea for expression of various drought genes under differential drought stress and their relationships with stress tolerance indices (STIs) will significantly enhance the breeding program of cowpea for drought tolerance. Many drought-responsive genes have been identified in cowpea and many other crops. Many of these genes have been implicated in the biosynthesis pathway of abscisic acid (ABA) and other biochemicals under stress and thereby conferring drought tolerance [10, 11]. A candidate gene such as *vucapx* for the biosynthesis of the enzyme ascorbate peroxidase has been reported to be highly expressed under drought stress amongst susceptible genotypes of cowpea compared to the tolerant ones. However, its relationship with STIs under differential drought stress in cowpea has not been determined [11]. Another crucial gene, *ptgs2* (glutamine synthase gene) for biochemical pathways of nitrogen supply in higher plants has been reported to enhance drought tolerance in the tree poplar [12]. However, limited information exists for this gene regarding its role in drought tolerance in crop species.

The purpose of this study was to determine the genotypic differences for the differential expressions of the candidate genes *vucapx* and *ptgs2* among accessions of cowpea under differential drought stress and their relationships with stress tolerance indices (STIs) of seedling morphological traits.

1.1 Materials and Methods

The plant materials utilized for the study are presented in Table 1.1. The setup was done in the Department of Plant Science and Biotechnology screen house, Akungba-Akoko, Nigeria between January and March 2017.

1.2. Drought Stress Treatment

Seeds were sown in pots filled with 7 kg topsoil and at emergence, three seedlings were maintained in each pot by thinning for unstressed and drought stressed. The study was laid out in a completely randomized design (CRD) with three replicates. The field capacity of the soil was predetermined at approximately 500 ml [13]. Each pot received 500 ml of water for three weeks, afterwards watering was withheld for ten days for the drought-stressed treatment only. On day ten of imposed stress, leaflets were collected for RNA extraction.

1.3. Profiling for Gene Expression

The response of the genes among the seedlings of cowpea under differential drought stress was analyzed through quantitative PCR according to Ajayi [14]. Total RNA was extracted from the middle leaflets of the terminal leaves of stressed and unstressed plants using an RNA Snap kit adhering to the manufacturer's protocol. The quality and yield of RNA were determined by spectrometry and cDNA was synthesized according to the manufacturer's protocol using the MMLV Reverse Transcriptase cDNA Synthesis Kit (NEB). Then, exponential amplification of cDNA with gene-specific primer pairs: *vuapx* and *ptgs2* (self-designed using the SnapGene software) was done with a reference gene (*p-Actin*) as an endogenous control. The quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) products were determined on 0.5% agarose gel, and the bands' intensity was determined with the software Image-J (2.3.0 V, Mac version). The primers used for the study are shown in Table 1.2.

1.4. Stress Tolerance Indices (STIs) Determination

A part of the STIs is published in Ajayi [14]. Plant height, number of leaves per plant, shoot dry weight, and root weight of one vigorous plant per pot across the three replicates were utilized for STI determination in line with [14]: (Mc × Ms)/ $(GMc)^2$; where Mc, Ms, and GMc, respectively are mean values in control, drought-stressed, and grand mean of control. The procedures for determining the morphological traits have been previously described [14].

1.5. Statistical Analysis

All data were subjected to statistical analysis. The bar chart of the relative expression of the candidate genes was done on an Excel platform. Means in triplicates were subjected to analysis of variance (ANOVA) in SPSS version 20. Biplot analysis was done using PAST statistics version 4.03.

Table 1.1. List of selected cowpea accessions screened for differential expression of *vucapx* and *ptgs2* under differential drought stress

S/N	Accession ID	Accession code	Biological status	Origin
1	TVu-7362	AC01	Landrace	Ghana
2	TVu-185	AC02	Landrace	Nigeria
3	TVu-199	AC03	Breeding material	USA
4	TVu-207	AC04	Breeding material	USA
5	TVu-218	AC05	Breeding material	USA
6	TVu-224	AC06	Breeding material	USA
7	TVu-235	AC07	Breeding material	Ghana
8	TVu-236	AC08	Breeding material	Ghana
9	TVu-239	AC09	Breeding material	South Africa
10	TVu-241	AC10	Breeding material	USA
11	IT98K-205-8	AC11	Unknown	Nigeria

12	IT98K-555-1	AC12	Unknown	Nigeria
13	TVu-4886	AC13	Landrace	Niger
14	TVu-4866	AC14	Landrace	Niger
15	TVu-8660	AC15	Landrace	Benin
16	TVu-9225	AC16	Landrace	Tanzania
17	TVu-11986	AC17	Landrace	Sudan
18	TVu-9256	AC18	Landrace	Burkina Faso
19	TVu-9252	AC19	Landrace	Burkina Faso
20	TVu-11979	AC20	Landrace	Sudan
21	IT97K-568-18	AC21	Landrace	Nigeria
22	IT89K-288	AC22	Unknown	Nigeria
23	IT96-610	AC23	Unknown	Nigeria
24	IT81-994	AC24	Unknown	Nigeria
25	IT89K-391	AC25	Unknown	Nigeria

Table 1.2. The primer sequence of the candidate genes for differential expression among selected cowpea accessions under differential drought stress

Target genes	Forward	Reverse
vucapx	GGAAAGCCTGAGACTAAATA	GCCAAACTGCAATAATAATC
ptgs2	TTCGGGAGCACAACAGAGTG	TGAAGTGGTAACCGCTCAGG
p-Actin	TGCCAAGAACAGCTCCTCAG	GAAGCACTTCCTGTGGACGA

2. Results and Discussion

2.1. Variation for the Expression of *vucapx* and *ptgs2* Among Cowpea Accessions Under Differential Drought Stress

ANOVA revealed the existence of highly significant differences among the accessions for the expression of the genes. The effect of drought treatment as well as the interaction between drought treatment and accessions were also highly significant (Table 1.3). Overall, the results indicate that both

vucapx and *ptgs2* genes show significant variation in expression levels among different cowpea accessions and are significantly affected by drought stress. Additionally, there seems to be an interaction effect between the cowpea accessions and the treatment conditions, implying that the gene expression response to drought stress may vary among different accessions. The gel pictures and expression patterns of the genes are presented in Figures 1.1 and 1.2, respectively. All figures showed that *vucapx* and *ptgs2* were expressed in all accessions of cowpea in both stressed and control conditions. Drought stress repressed the expression of both genes significantly in all accessions except in AC10, AC11, AC12, AC13, AC14, AC15, and AC16 for *vucapx* and AC21, AC22, and AC23 for *ptgs2* where the genes expressions were significantly higher in the drought stress conditions. Repression percentage under drought stress ranged between 26.25% in AC17 and -47.36% (increase) in AC12 for *vucapx* while it ranged between 12.03% in AC20 and -29.45% (increase) in AC21 for *ptgs2*. Generally, accessions AC10, AC20, AC21, AC22, and AC23 had an above-average expression of *vucapx* under both stressed and control conditions while AC20, AC21, and AC22 had an above-average expression of *ptgs2* in both stressed and control conditions. While *vucapx* had been previously confirmed to be induced by drought stress in cowpea [10, 11, 17] there is no such record of the expression of *ptgs2* in cowpea except in the tree poplar species [12, 15]. And in both cases, higher expression of the genes indicated higher levels of drought tolerance. The highly significant variation among the selected accessions of cowpea for the expression of the genes indicates that selection for higher drought-tolerant genotypes could be done among the present collection for breeding schemes.

Previous studies involving the genes have indicated that their higher expressions are obtainable under the stress conditions compared to control in contrast to the present observations in most of the accessions screened for this study. This may likely be linked to the extended period of exposure of the plants to drought stress as this has been reported to cause the downregulation of certain genes due to feedback inhibition of

abscisic acid (ABA) under steadily progressing drought conditions [16, 18, 19]. Similar observations were reported for the expression of the *nced1* gene among the same accessions of cowpea [14].

2.2. Variation for Stress Tolerance Indices (STIs) Among Cowpea Accessions Under Differential Drought Stress

There are significant differences in stress tolerance indices of the studied morphological traits (PHSTI, DSWSTI, DRWSTI, NLSTI, and ComSTI) among the cowpea accessions screened under different levels of drought stress. These findings suggest the presence of genetic variation among the accessions, which could be further explored to identify drought-tolerant cowpea varieties. The STIs for plant height, number of leaves, dry shoot weight, dry root weight, and combined are presented in Figures 1.3, 1.4, 1.5, 1.6, and 1.7, respectively. STIs were not determined for AC05 and AC19 due to an inadequate number of plants across replicates as a result of poor germination. The combined STIs ranged from the highest (0.84) in accession AC13 to the lowest (0.21) in AC01. Generally, thirteen accessions had above-average values (> 0.47) for the combined STIs; these include accessions AC03 (0.51), AC07 (0.52), AC08 (0.50), AC10 (0.53), AC11 (0.48), AC12 (0.54), AC13 (0.84), AC15 (0.56), AC16 (0.55), AC20 (0.55), AC21 (0.48), AC22 (0.55), and AC23 (0.54) and are regarded as the highly drought tolerant accessions, while others are susceptible accessions. Among the tolerant accessions, only accession AC20 performed consistently above average for all the STIs while AC16 had an above-average performance for plant height, number of leaves, and dry weight of shoot STIs while others exhibited above-average performance in one or two of the STIs. Among the existing drought tolerance indices, STI is one of the most effective for its ability to pinpoint the best-performing genotypes under both control and drought stress conditions [20]. Therefore, accessions with higher STIs for morphological traits are those with superior performances for those traits in both stressed and unstressed conditions. In the present study, the tolerant accessions possessed the combination of higher root dry weight,

taller height, a higher number of leaves, and higher shoot dry weight under both stressed and unstressed conditions compared to the sensitive accessions. Hence, selection for higher STIs of these traits will automatically enhance the drought tolerance level in cowpea.

2.3. Relationships Between the Expression Profiles of *vuapx* and *ptgs2* and STIs Among Cowpea Accessions Screened Under Differential Drought Stress

Biplot analysis for measured gene expression profiles and STIs of morphological traits of accessions of cowpea under differential drought stress is presented in Figure 1.1. The possession of more than 63% of the total variance in the first two Principal Component axes indicates an effective biplot [21], which suggests a high level of variation among the accessions for drought responses. Further, the length of vectors as well as the angles among them are properties of utmost importance in a biplot analysis. Acute angles among vectors signify positive correlations, obtuse angles denote negative correlations while a right angle denotes a lack of correlation [22]. This biplot displays the link between the differential expressions of the gene in some of the accessions to their STIs. For instance, tolerant accessions such as AC10, AC20, AC21, AC22, and AC23 mostly combined above-average expression of the genes in both conditions with above-average values for the combined STIs. This informed the highly positive correlations among expression of *vucapx* under drought stress and all the STIs. Therefore, selection based on higher *vucapx* under stress will improve drought tolerance among the accessions of cowpea. Similarly, higher expression of *vucapx* under control was strongly related to higher *ptgs2* under drought stress with both weakly correlated to *vucapx* under drought stress, plant height, and dry root weight STIs suggesting that selection based on these parameters will create moderate improvement for drought tolerance in cowpea.

All the highly tolerant accessions were linked to the right hand of the biplot with AC10, AC07, AC15, AC22, AC23, AC20, and AC21 in quadrant I with high correlation to *ptgs2*

and *vucapx* under stress and control respectively. Accessions AC23 and AC22 are vertex accessions in this quadrant being the best-performing accessions for the two genes in all conditions except *ptgs2* under control. Also, many of the tolerant accessions such as AC13, AC12, AC16, AC11, and AC25 were linked to most of the STIs in quadrant II of the biplot where AC13 was a vertex accession (the best for the STIs sectors) while the susceptible accessions fell at the left hand of the biplot. The inclusion of accessions AC22, AC23, and AC13 in the breeding program of cowpea for drought tolerance would bring a positive outcome. Similar trends were observed among the same accessions for STIs and expression of *nced1* under differential drought stress by Ajayi et al [14]. Quadrant III consisted of susceptible accessions such as AC08, AC17, AC04, AC03, AC18, AC02, AC09, and AC06 not correlated with any of the STIs and the gene expression profiles. Accessions in quadrant IV included susceptible accessions such as AC24, and AC01 which were highly correlated with *ptgs2* under control conditions.

3. Conclusion

Overall, the results indicate that both *vucapx* and *ptgs2* genes show significant variation in expression levels among different cowpea accessions and are significantly affected by drought stress. The significant correlations between gene expression profiles and stress tolerance indices suggest that genes, such as *vucapx* and *ptgs2*, may play a crucial role in conferring stress tolerance in cowpea. These genes could serve as potential genetic markers for selecting plants with enhanced stress tolerance.

Table 1.3. Mean square values for the gene expression profiles of the candidate genes among selected cowpea accessions screened under differential drought stress

Source of variation	Degree of freedom	*vucapx*	*ptgs2*
Accession	24	24705.10**	5820.14**
Treatment	1	320795.55**	204989.19**
Accession × Treatment	24	18753.40**	4611.38**
Error	100	0.001	0.001

*****: Significant at P≤0.01***

Also, by selecting plants with positive correlations among different stress tolerance indices, breeders can combine multiple stress tolerance traits, developing cowpea varieties with enhanced overall stress tolerance.

This study identified the best accessions (AC22, AC23, and AC13) among the highly tolerant which can be included in the breeding program of cowpea for drought tolerance to bring a positive outcome. These findings suggest that *vucapx* and *ptgs2* gene expression may contribute to drought tolerance in cowpea. Further research on the functional roles of these genes could provide valuable insights into the molecular mechanisms underlying drought resistance in cowpea, aiding breeding programs for improved drought tolerance.

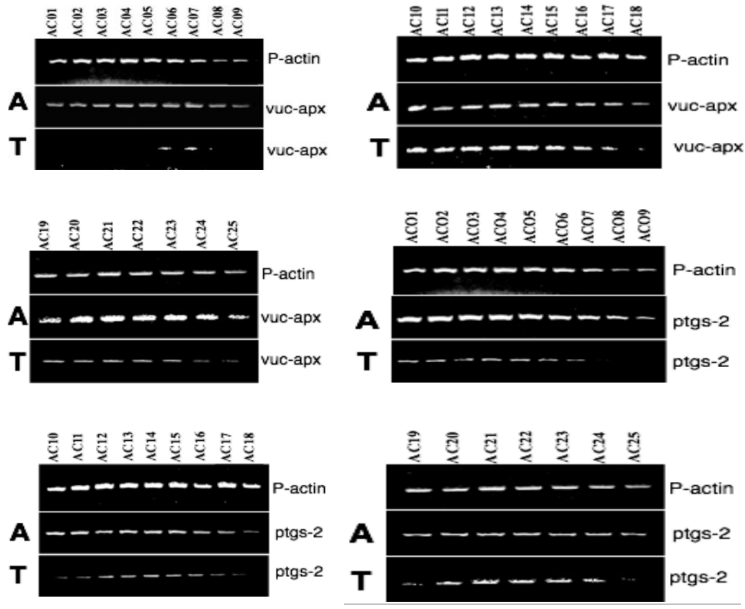
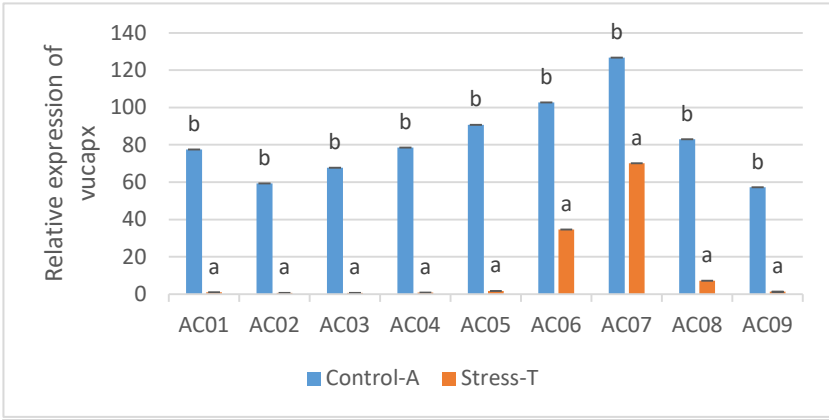
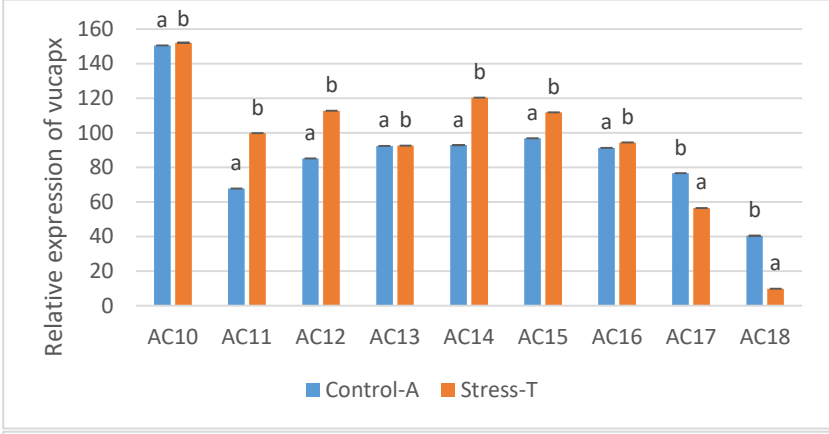
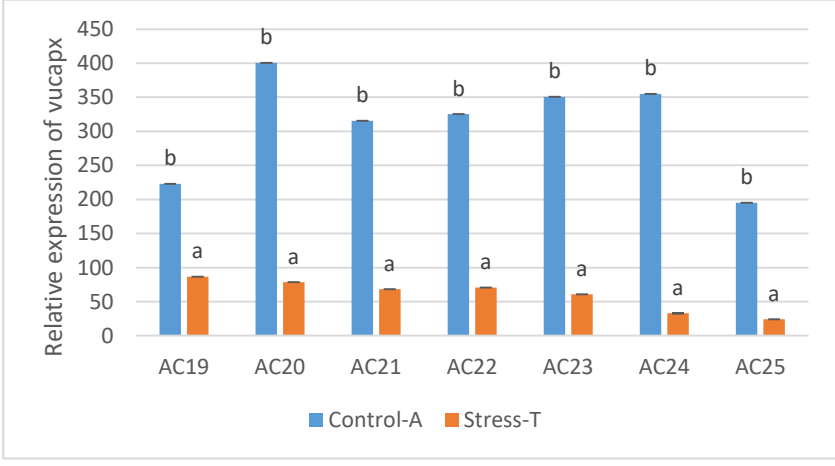
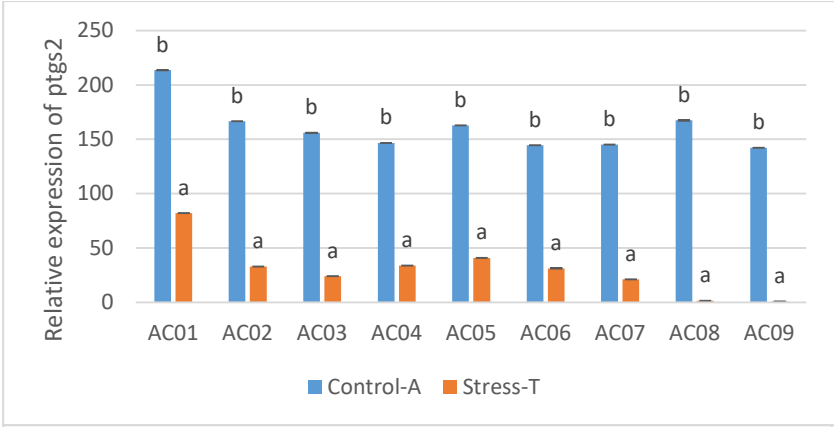


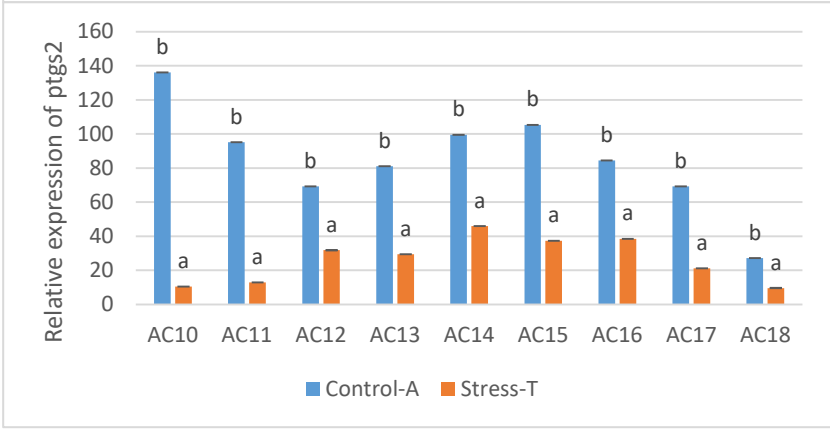
Figure 1.1. The gel image of expression patterns of *vucapx*, *ptgs2*, and *P-Actin* (internal control) genes among selected cowpea accessions screened under differential drought stress. A and T respectively are control and drought-stressed conditions. AC01 – AC25 are accessions of cowpea


Relative expression of vucapx
140
120
100
80
60
40
20
0
AC01 AC02 AC03 AC04 AC05 AC06 AC07 AC08 AC09
Control-A
Stress-T


Relative expression of vucapx
160
140
120
100
80
60
40
20
0
AC10 AC11 AC12 AC13 AC14 AC15 AC16 AC17 AC18
Control-A
Stress-T


Relative expression of vucapx
450
400
350
300
250
200
150
100
50
0
AC19 AC20 AC21 AC22 AC23 AC24 AC25
Control-A
Stress-T


Relative expression of ptgs2
250
200
150
100
50
0
b
a
AC01
AC02
AC03
AC04
AC05
AC06
AC07
AC08
AC09
Control-A
Stress-T


Relative expression of ptgs2
160
140
120
100
80
60
40
20
0
b
a
AC10
AC11
AC12
AC13
AC14
AC15
AC16
AC17
AC18
Control-A
Stress-T

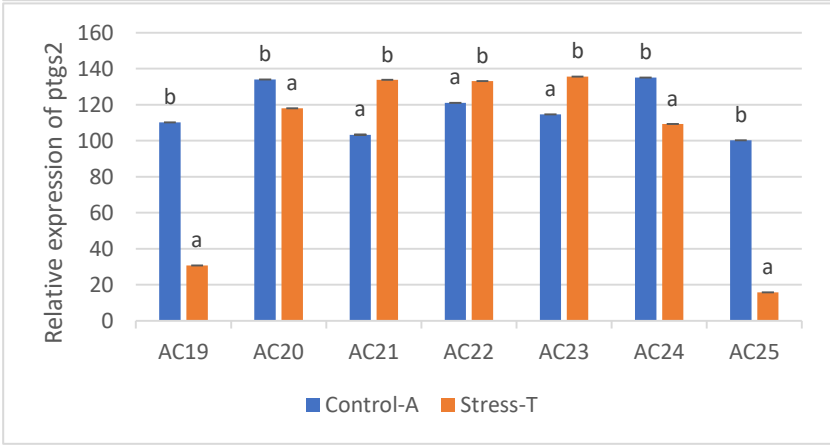

Relative expression of ptgs2
160
140
120
100
80
60
40
20
0
b
a
AC19
AC20
AC21
AC22
AC23
AC24
AC25
Control-A
Stress-T

Figure 1.2. Relative expression patterns of *vucapx* and *ptgs2* genes among selected cowpea accessions screened under differential drought stress. A and T respectively are control and drought-stressed conditions. AC01 – AC25 are accessions of cowpea. Each bar stands for the mean value ± standard error (n = 3, where n indicates the number of replicates). Means with different alphabets indicate significant differences between treatments at $P \leq 0.01$ according to least significant difference (LSD)

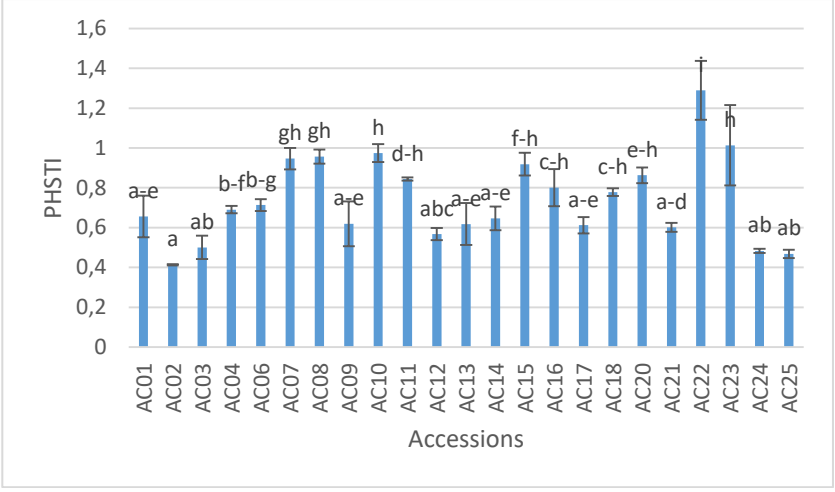


Figure 1.3. Stress tolerance index of plant height among selected cowpea accessions screened under differential drought stress. AC01 – AC25 are accessions of cowpea. Each bar stands for the mean value ± standard error (n = 3, where n indicates the number of replicates). Means with different alphabets indicate significant differences between accessions at $P \leq 0.05$ according to DMRT

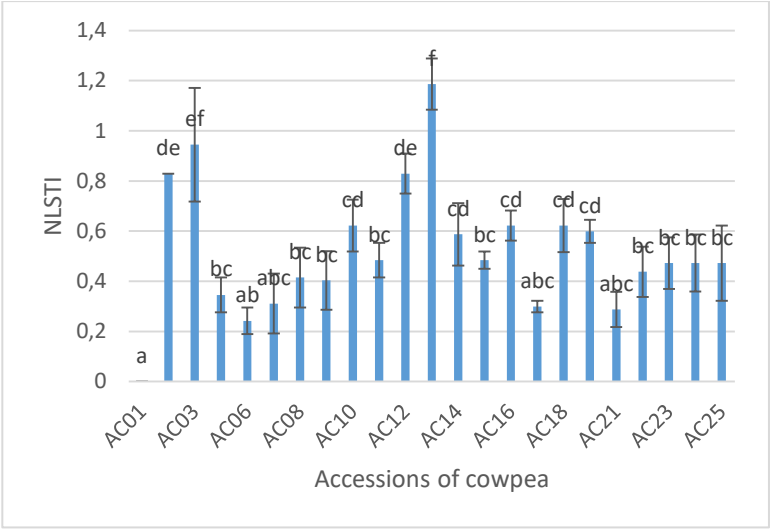


Figure 1.4. Stress tolerance index of number of leaves among selected cowpea accessions screened under differential drought stress. AC01 – AC25 are accessions of cowpea. Each bar stands for the mean value ± standard error (n = 3, where n indicates the number of replicates). Means with different alphabets indicate significant differences between accessions at $P \leq 0.05$ according to DMRT

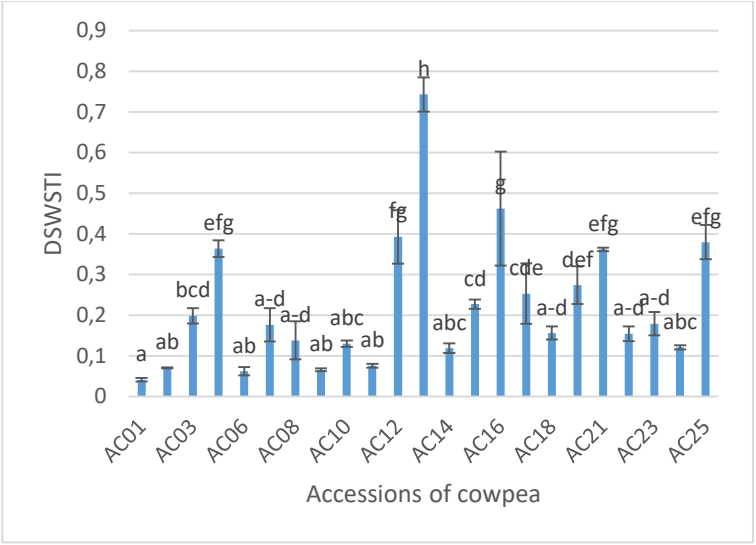


Figure 1.5. Stress tolerance index of dry shoot weight among selected cowpea accessions screened under differential drought stress. AC01 – AC25 are accessions of cowpea. Each bar stands for the mean value ± standard error (n = 3, where n indicates the number of replicates). Means with different alphabets indicate significant differences between accessions at $P \leq 0.05$ according to DMRT

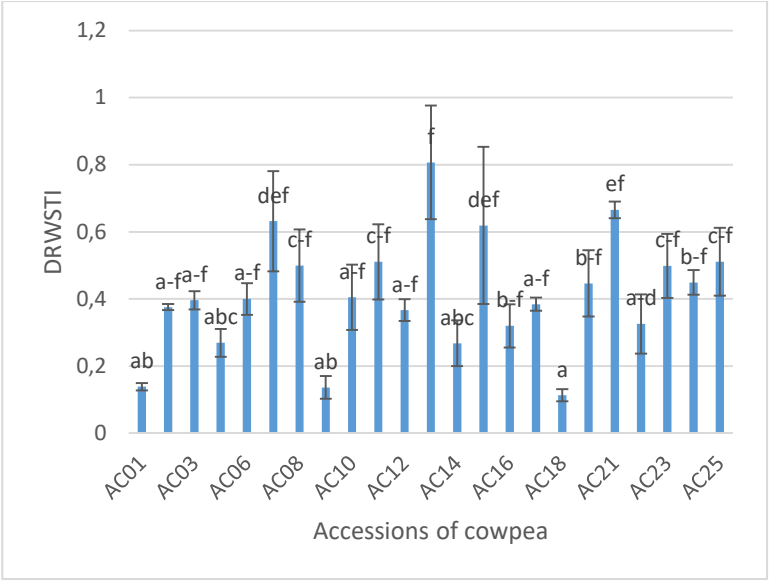


Figure 1.6. Stress tolerance index of dry root weight among selected cowpea accessions screened under differential drought stress. AC01 – AC25 are accessions of cowpea. Each bar stands for the mean value ± standard error (n = 3, where n indicates the number of replicates). Means with different alphabets indicate significant differences between accessions at $P \leq 0.05$ according to DMRT

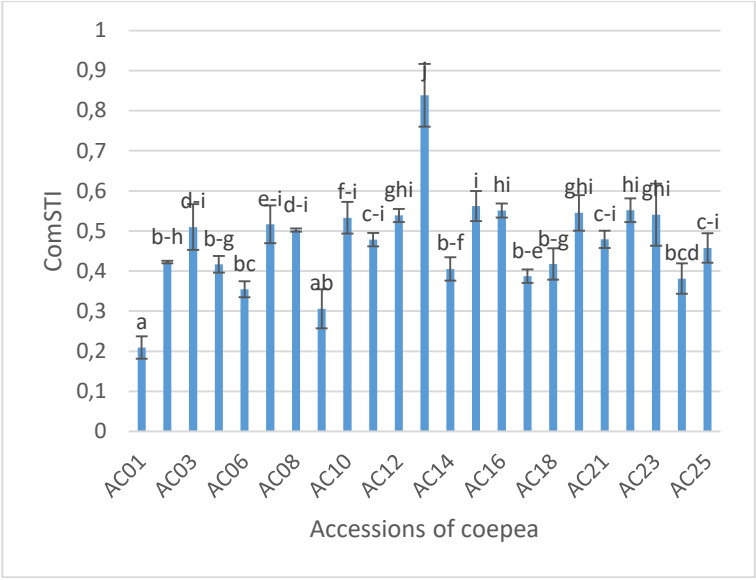


Figure 1.7. Combined stress tolerance indices among selected cowpea accessions screened under differential drought stress. AC01 – AC25 are accessions of cowpea. Each bar stands for the mean value ± standard error (n = 3, where n indicates the number of replicates). Means with different alphabets indicate significant differences between accessions at $P \leq 0.05$ according to DMRT

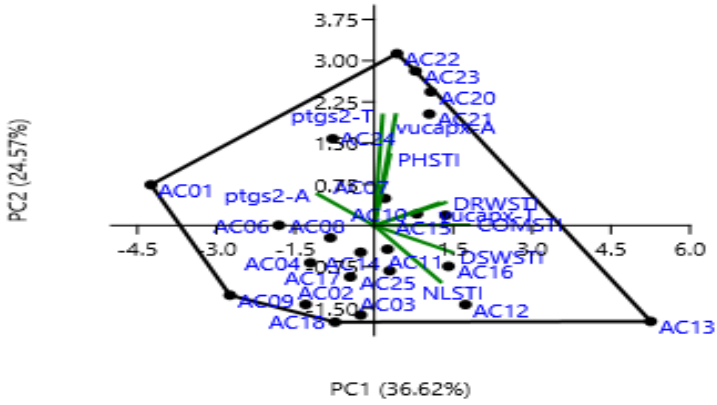


Figure 1.8. Biplot analysis showing relationships between the expression profiles of *vucapx* and *ptgs2* and STIs among cowpea accessions under differential drought stress. AC01 – AC25 are accessions of cowpea

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgment

Prof. Idowu Olaposi Omotuyi of Afe Babalola University, Nigeria provided his expertise for gene expression profiling. The Genetic Resources Center (GRC), International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria is acknowledged for supplying the cowpea accessions.

References

[1] Kebede E, Bekeko Z. Expounding the production and importance of cowpea (Vigna unguiculata (L.) Walp.) in

Ethiopia. Cogent Food and Agriculture. 2020; 6(1): 1769805. DOI: 10.1080/23311932.2020.1769805
[2] Gomes AMF, Nhantumbo N, Ferreira-Pinto M, Ramalho JC, Ana Ribeiro-Barros A. Breeding elite cowpea varieties for improved food security and income in Africa: Opportunities and Challenges. In: El Esawi M. A, editor, Legume Crops–Characterization and Breeding for Improved Food Security. IntechOpen; 2019. p. 1–14. DOI: 10.5772/intechopen.84985
[3] Ajayi AT, Gbadamosi, AE, Osekita OS, Taiwo BH, Fawibe AB, Adedeji I, Omisakin TF. Genotype × environment interaction and adaptation of cowpea genotypes across six planting seasons. Frontiers in Life Sciences and Related Technologies. 2022; 3(1): 7–15. DOI: 10.51753/flsrt.1036051
[4] Gerrano AS, Jansen van Rensburg WS, Venter SL, Shargie NG, Amelework BA, Shimelis HA, Labuschagne MT. (2019). Selection of cowpea genotypes based on grain mineral and total protein content. Acta Agriculturae Scandinavica Section B: Soil and Plant Science. 2019; 69(2): 155–166. DOI: 10.1080/09064710.2018.1520290
[5] Gerrano AS, Jansen van Rensburg WS, Mathew I, Shayanowako AIT, Bairu MW, Venter SL, Swart W, Mofokeng A, Mellem J, Labuschagne M. Genotype and genotype × environment interaction effects on the grain yield performance of cowpea genotypes in dryland farming system in South Africa. Euphytica. 2020; 216(5): 80. DOI: 10.1007/s10681-020-02611-z
[6] FAOSTAT. Food and agricultural organization of the United Nations. 2022. Available from http://faostat.fao.org. FAOSTAT_data_8-29-2020-Excel [accessed 2023-May-21].
[7] Adetumbi AJ, Solomon A, Akinyosoye T, Agbeleye A, Titilope K, Olubusola K, Oduwaye F, Adebayo GG. Genetic variability in the agronomic traits, the inheritance pattern of seed coat colour, and response to brown blotch disease among cowpea hybrids. Euphytica. 2019; 215: 142. DOI: 10.1007/s10681-019-2466-6
[8] Zhou L, Liu Z, Liu Y, Kong D, Li T, Yu S, Mei H, Xu X, Liu H, Chen L, Luo L. (2016). A novel gene OsAHL1 improves both drought avoidance and drought tolerance in rice. Scientific Reports. 2016; 6: 1–15. DOI: 10.1038/srep30264

[9] Agbicodo EM, Fatokun CA, Muranaka S, Visser RGF, Linden van der CG. Breeding drought tolerant cowpea: constraints, accomplishments, and prospects. Euphytica. 2009; 167: 353–370. DOI 10.1007/s10681-009-9893-8
[10] Muchero W, Ehlers JD, Roberts PA. Restriction site polymorphism-based candidate gene mapping for seedling drought tolerance in cowpea [Vigna unguiculata (L.) Walp.]. Theoretical and Applied Genetics. 2010; 120(3): 509–518. DOI: 10.1007/s00122-009-1171-6
[11] Carvalho M, Lino-Neto T, Rosa E, Carnide V. Cowpea: a legume crop for a challenging environment. Journal of the Science of Food and Agriculture. 2017; 97(13): 4273–4284. DOI: 10.1002/jsfa.8250
[12] El-Khatib RT, Hamerlynck EP, Gallardo F, Kirby EG. Transgenic poplar characterized by ectopic expression of a pine cytosolic glutamine synthetase gene exhibits enhanced tolerance to water stress. Tree Physiology. 2004; 24(7): 729–736. DOI: 10.1093/treephys/24.7.729
[13] Ogbaga CC, Stepien P, Johnson GN. Sorghum (Sorghum bicolor) varieties adopt strongly contrasting strategies in response to drought. Physiologia Plantarum. 2014; 152(2): 389–401. DOI: 10.1111/ppl.12196
[14] Ajayi AT, Gbadamosi AE, Olumekun VO, Omotuyi IO. Variable expression of the candidate gene NCED1 among cowpea accessions under different drought stress conditions. J Genet Resour. 2021; 7(1): 133–143. DOI: 10.22080/jgr.2021.20645.1233
[15] Castro-Rodríguez V, García-Gutiérrez A, Canales J, Avila C, Kirby EG, Cánovas FM. The glutamine synthetase gene family in Populus. BMC Plant Biology. 2011; 11: 1–16. DOI: 10.1186/1471-2229-11-119
[16] Changan SS, Ali K, Kumar V, Garg NK, Tyagi A. Abscisic acid biosynthesis under water stress: anomalous behavior of the 9-cis-epoxy carotenoid dioxygenase1 (NCED1) gene in rice. Biologia Plantarum. 2018; 62(4): 663–670. DOI: 10.1007/s10535-018-0807-2
[17] D'Arcy-Lameta A, Ferrari-Iliou R, Contour-Ansel D, Pham-Thi AT, Zuily-Fodil Y. Isolation and characterization of four ascorbate peroxidase cDNAs responsive to water deficit in

cowpea leaves. Annals of Botany. 2006; 97(1): 133–140. DOI: 10.1093/aob/mcj010
[18] Muñoz-Espinoza VA, López-Climent MF, Casaretto JA, Gómez-Cadenas A. Water stress responses of tomato mutants impaired in hormone biosynthesis reveal abscisic acid, jasmonic acid, and salicylic acid interactions. Frontiers in Plant Science. 2015; 6.997: 1–14. DOI: 10.3389/fpls.2015.00997
[19] Tian L, DellaPenna D, Zeevaart JAD. Effect of hydroxylated carotenoid deficiency on ABA accumulation in Arabidopsis. Physiologia Plantarum. 2004; 122(3): 314–320. DOI: 10.1111/j.1399-3054.2004. 00409.x
[20] Al-rawi IMD. Study of drought tolerance indices in some bread and durum wheat cultivars. Jordan Journal of Agricultural Sciences. 2016; 12(4): 1125–1139.
[21] Ajayi AT, Olumekun VO, Nwosu PO. GT biplot analysis of shoot traits indicating drought tolerance in cowpea [Vigna unguiculata (L.) Walp] accessions at the vegetative stage. International Journal of BioSciences and Technology. 2020; 13(2): 18–33. DOI: 10.5281/zenodo.4019163
[22] Atnaf M, Tesfaye K, Dagne K, Wegary D. Genotype by trait biplot analysis to study associations and profiles of Ethiopian white lupin (Lupinus albus L.) landraces. Australian Journal of Crop Science. 2017; 11(1), 55–62. DOI: 10.21475/ajcs.2017.11.01.pne226

CHAPTER 2

Gamma-Tocotrienol Therapy and Its Apoptotic Effects on Different Cancer Types

Tugce Nur Eralp, Akin Sevinc

Gamma-Tocotrienol Therapy and Its Apoptotic Effects on Different Cancer Types

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Abstract

Cancer is among leading causes of death worldwide, and researchers predict that approximately 17 million people will be diagnosed with cancer by 2050. Development of treatment alternatives against cancer are gaining momentum worldwide and these accurate molecular targets are gaining importance with an unprecedented pace. Owing to its significant public health burden, these developments turn out to be crucial for healthcare and economical systems, worldwide. In addition to this, development of conjugation therapy strategies is further important since they further augment the effectiveness and efficacy of these treatment strategies. In this regard, vitamin derivatives gain considerable attention due to their familiar metabolism in human physiology. Accumulating evidence point to the possible involvement of Vitamin E and Vitamin D derivatives as such candidates. Vitamin E family consists of tocotrienols and tocopherols, of which has 4 isoforms, α, β, γ and δ, with different biological properties. Even though the structure and functional studies of these molecular have been ongoing for a considerable amount of time, it is now becoming apparent that these molecules might have other biological functions, where there is a significant potential benefit in cancer therapies. We, therefore, aimed to elucidate the mechanisms of

anti-cancer actions of these molecules, with a specific emphasis on their pro-apoptotic activities.

Keywords: Vitamin E, Gamma-tocotrienol (GT3), cancer, drug resistance, apoptosis.

1. Introduction

1.1 Gamma Tocotrienol Structure

Tocotrienols belong to the vitamin E family; are naturally occurring compounds that can be found in plant seeds such as rice bran, palm oil, and annatto [1]. Tocotrienol and tocopherols which form the Vitamin E family of molecules, are structurally similar that they all have a common chromanol ring and a side chain at the C2 position, with the only difference that the saturated wick tail in tocopherols is replaces with an unsaturated isoprenoid side chain in the tocotrienols [2]. Depending on the number and position of methyl substituents on the chromanol ring, tocotrienol and tocopherol are further classified into α, β, γ, and δ isoforms [1].

1.2 Apoptotic Pathways

Apoptosis is the overarching name given to plethora of molecular pathways leading to programmed death of cells with critical roles in developmental and homeostatic pathways [3]. Elimination of unnecessary or unwanted cells is among principal advantages of apoptotic processes that begins with the normal developmental process and is regulated through highly controlled molecular mechanisms. There are many conditions that can lead to activation of these apoptotic pathways, including excessive DNA damage or uncontrolled proliferation, where apoptotic pathways can be activated by both intracellular and extracellular stimuli [4]. There are two distinct pathways that can activate apoptotic pathways: intrinsic and extrinsic pathways, depending on the source of the signal. Depending on the source of the initiating factor, these pathways are also referred to as mitochondrial and receptor mediated apoptosis

pathways, respectively. Excessive DNA damage, deprivation of growth factor and/or cytokine stimulation are among common intracellular signals leading to activation of the apoptotic pathways, whereas extracellular signals mostly produced by cytotoxic T cells of the immune system in response to damage of infected cells [5].

Caspases, an important family of endo-proteases, also known as the cysteine-aspartyl specific proteases, are central molecules in the apoptotic pathways [4]. The activity of caspase proteases is essential for successful apoptosis as they metabolize numerous different targets. Among these, there are four initiator caspases (caspase-2, -8, -9, 10) and three executioner caspases (caspase-3, -6, -7) in cells. Executor caspases, activated by the initiator caspases cleave target proteins, leading to cell death. These caspase cascades are highly coordinated at the molecular and cellular level, so that apoptosis occurs only when signaled [5]. The intrinsic apoptotic pathway is regulated specifically by the B-cell lymphoma-2 (Bcl-2) family of proteins, which includes proapoptotic effector proteins, proapoptotic Bcl2 homology domain-3 (BH3)-containing proteins, and antiapoptotic Bcl-2 proteins. Whereas antiapoptotic Bcl-2 proteins inhibits apoptosis by inhibiting the proapoptotic BCL-2 family member protein constructs, the BCL-2-associated X protein (Bax) and the BCL-2 homologous antagonist killer (Bak). It is important to note that, BH3-only proteins can inhibit antiapoptotic Bcl-2 proteins [6].

Extrinsic stimulus of the apoptotic pathways involves ligands such as tumor necrosis factor (TNF), Fas ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL), their corresponding receptors, such as TNFR1, CD95/Apo-1, and TRAIL- R, where activation starts with their binding to the extracellular domains of these receptors. These receptors molecular, named death receptors become activated upon ligand binding and in turn bind to adapter proteins such as Fas-associated death domain (FADD) and TNF receptor-associated deatch domain (TRADD) via their intracellular death domains. These adapter proteins also contain another protein interaction

domain called the Death Effector Domain (DED), which is also found in the procapase-8 protein, leading to its interaction with the DED domain of FADD. Upon these interactions, a death-inducing signaling complex (DISC) is formed, which leads to the automatic catalytic activation of procaspase-8, where activated caspase-8 leads to activation of downstream effector/executive caspases that will initiate pathways leading to cell death by damaging or destructing the nucleus and other intracellular structures [7].

On the other hand, a number of internal stimuli such as excessive DNA damage, growth factor and cytokine deprivation can also cause an increase in mitochondrial outer membrane permeability (MOMP). MOMP is most commonly mediated by various protein-membrane and protein-protein interactions of the Bcl-2 family. The Bcl-2 protein family consists of 25 pro- and anti-apoptotic members, and cell survival is a process guided by the balance of these pro-apoptotic and anti-apoptotic Bcl-2 proteins. Bcl-2-family members are characterized by the presence of one or more Bcl-2 homology (BH) domains. Those with all four Bcl-2 homology domains are Bcl-2, Mcl-1, A1/ Bfl-1, Bcl-B/ Bcl2L10 and Bcl -xL. These proteins function in the cell as anti-apoptotic molecules that block apoptosis by inhibiting corresponding pro-apoptotic counterparts. Proapoptotic Bcl-2 family members are classified according to the number of BH domain that they contain. Bax, Bak, and Bok (Bcl-2 ovarian killer) are the effector proteins with more than one BH domain, whereas Bid, Bim, Puma, Noxa, Bik, Bad, Hrk, and Bmf proteins contain a single BH3 domain [7].

Following the apoptotic stimuli, Bcl-2 family proteins (Bax and Bak) with single BH3 domain are activated, which leads to translocation of cytochrome c and apoptosis inducing factor (AIF) from the outer mitochondrial membrane, which leads to release of Smac/DIABLO (low PI caspase /second mitochondria-derived activator of direct IAP binding protein) and serine protease Omi / HtrA2 (high temperature requirement protein A2) by the activity of endonuclease G [4]. Among these, cytochrome c, among principal players of apoptosis, when

released into the cytosol, interacts with apoptosis protease activating factor 1 (Apaf-1) and forms a complex known as the apoptosome [8].

Owing to the central role of apoptosis in cellular metabolism, the apoptosome complex, a sevenfold ring-shaped multiprotein structure, plays a central role in the control and augmentation of the apoptotic response through activation of an initiator caspase such as Caspase 9, which in turn activates the effector caspase-3 and initiates the caspase cascade that eventually leading to the destruction of the cellular structures. Both AIF and endonuclease G can induce condensation of chromatin structures and fragmentation of nuclear DNA, promoting subsequent caspase-independent cell death. Other mitochondrial proteins that are released to the cytosol are Smac/DIABLO and Omi/HtrA2, which stimulate additional mechanisms for caspase activation. Smac/DIABLO and Omi/HtrA2 interact and inhibit IAP proteins, which leads to further caspase activation [9].

Consistent with its central role in human physiology, and consistent with its potential impact, apoptotic pathways must be tightly regulated to maintain a desired balance between cell death and survival. In this regard, it is well-documented that carcinogenesis utilizes apoptosis avoidance as an important feature [10].

Apoptotic pathways can be manipulated at transcriptional, translational, and post-translational levels by cancer cells, where cancer cells can employ a number of different strategies simultaneously to block apoptosis. Various proteins exerting pro- or anti-apoptotic activity in the cell are present as mentioned earlier, the ratio of these pro- and anti-apoptotic proteins plays an important role in the regulation of cell death, and the disruption of the balance of pro- and anti-apoptotic proteins contributes to carcinogenesis by reducing apoptosis in malignant cells. For example, the disruption of this balance in favor of pro-apoptotic proteins can promote cancer cell survival, which leads to the formulation of chemotherapeutic strategies exploiting BH3 mimetics [7].

1.3 Gamma Tocotrienol Conjugation Therapy

Targeting the apoptotic pathways in cancer cells is regarded as an attractive approach to effectively eliminate cancer cells without causing inflammation. The proapoptotic effects of tocotrienols have been studied previously in breast, lung, colon, brain, liver, cervix, blood and skin cancers, where it has been shown that tocotrienols (T3) have roles in inducing mitochondrial apoptosis pathway. For example, they can contribute to the direct displacement of the Bcl-2 molecule by acting as a BH3 mimetic. On the other hand, they may also affect the transcriptional regulation of Bax gene expression. They can provide inhibition of the IAP family or induction of the caspase-independent apoptotic pathways which are normally activated upon mitochondrial damage [11].

It is important to note that solely targeting the death pathways of cancer cells may not be sufficient to eliminate carcinogenesis. Tocotrienols have previously been shown to simultaneously activate cell death programs and prosurvival factors such as nuclear factor kappa B (NFKB), phosphoinositide 3-kinase (PI3K)/Akt, Wingless and INT-1 (Wnt), and mitogen-activated protein kinases (MAPKs), and subsequent signaling pathways [11].

Caspase family of proteins has been well-documented to play a central role in regulation and execution of the apoptotic processes. Caspases are synthesized as proenzymes and cleaved at certain aspartate residues leading to the conversion of the proenzymes into biologically active cysteine proteases. Active caspases either eliminate or activate the function of their protein targets. Among these, caspase-3 and -7 are among the best studied players at the initiation stages of apoptosis [12].

Considering the central role of caspases in promoting apoptosis, it is hypothesized that the regulation of pro- or active enzymes can enable us to predict the susceptibility of cells to cell death and in turn the clinical success of anticancer strategies [12].

PARP1 (poly-ADP ribose polymerase), on the other hand, is cleaved by the active Caspase 3 and 7 into a ~25 kDa N-terminal fragment containing a DNA binding domain (DBD) and a ~89 kDa and C-terminal fragment that retains basal enzymatic activity but cannot be induced by DNA binding [13]. This truncation is necessary to abolish PARP1 activation in response to DNA fragmentation, to protect cells from ATP depletion and subsequent necrotic death, and to prevent futile attempts for DNA repair. Therefore, PARP1 cleavage enables cells to go to the apoptotic pathway [14]. Therefore, PARP1 plays a central role in the determination of the cell fate [15].

In a study conducted by Jiang *et al.* to show the effect of different vitamin E forms on the viability of LnCap and PC3 prostate cancer cell lines, GT3 was found to be the most potent pro-apoptotic agent among the isoforms. In this study, while an increase in apoptotic protein expression was observed with GT3 application, no significant change was observed in phosphorylated Akt protein expression. These results suggest that inhibition of PI3K signaling is not a causative factor in GT3-induced death of PC3 and LnCap cells [16]. In a subsequent study, it was shown that GT3 leads to the suppression of mesenchymal markers in pancreatic tumor cell lines and the restoration of E-cadherin and γ-catenin expression, which is associated with suppression of cell invasion capacity [17].

Recent studies in malignant mammary epithelial cells have shown that the antiproliferative effects of gamma-tocotrienol are through phosphorylation and activation of PDK1 and Akt [18]. In another study conducted in neoplastic +SA mammalian cancer cells, it was shown that GT3 inhibits neoplastic breast epithelial cell proliferation by reducing Akt activity [19].

Furthermore, when MDA-MB-231, SUM159 and SUM149 triple negative breast cancer cell lines were investigated, it has been observed that 20 μM GT3 most effectively activated apoptosis by docosahexaenoic acid. At the same time, administration of GT3 alone and doxohexanoic acid both

resulted in an increase in Cut Caspase 9 and Cut PARP in these cells [20].

In a separate study using breast cancer cell lines with different molecular profiles, GT3 exposure caused the inhibition of caspase 9 and PARP activities [21]. Additionally, GT3 application at a concentration of 2 μM impacted the expression of numerous proteins, among which were the PARP and Caspase 3 [22].

In a study conducted in 2008, it was revealed that GT3 administration in the gastric cancer cell line SGC-7901 decreased the expression of p-ERK1/2 proteins. In this study, GT3-induced apoptosis in SGC-7901 cells was found to be associated with a suppression of mitogen-activated protein kinase signaling. Upon analysis of apoptotic markers, Bcl-2 family members and MAPK family members were identified upon GT3 administration. It further downregulated the activation of Raf-ERK signaling pathway and downregulated c-Myc by reducing the expression of Raf-1 and p-ERK1/2 proteins. This study suggests that the key regulators in GT3-induced apoptosis may be Bcl-2 families and caspase-3 in SGC-7901 cells through downregulation of the Raf-ERK signaling pathway [23].

In a study in which it was revealed that the administration of 20 μM gamma- and delta- tocotrienol in HepG2 cells leads to the inhibition of proliferation via inhibition of the MAPK pathway. In this study, the expression of genes associated with the MAPK pathway was also examined and it was shown that the inhibitory effect of GT3 on cell proliferation was through modification of MAPK upstream signaling. Downregulation of proto-oncogene H-Ras gene expression was also detected by administration of GT3 at 20 μM concentration in HepG2 cells. Simultaneous analysis of the gene expression profiles of other genes associated with the MAPK pathway; revealed that Grb2, Sos-1, c-Src and Shc2, the signaling elements located upstream of Ras-Raf-MEK-ERK, were significantly downregulated upon application of 20 μM GT3. Also in this study, proto-oncogene

Hras and its associated upstream signal protein expressions were measured by immunoblotting. GT3 administration in HepG2 cells significantly caused downregulation of H-Ras, Grb2, Sos-1, phospho-Src (Tyr416) and phospho-Shc (Tyr239 / 240) protein expressions [24].

When MDA-MB-231, SUM159 and SUM149 triple negative breast cancer cell lines were used, it was revealed that 20 μM GT3 enhanced the effect of apoptosis by docosahexaenoic acid trigger. At the same time, administration of GT3 alone and doxohexanoic acid both resulted in an increase in Cut Caspase 9 and Cut PARP in these cells [20].

In CEM-SS cells, the effect of GT3 was most prominent with48-hour period exposure at a dose of 16 μg/ml GT3 [25]. In lung cancer cell lines, A549 and U87MG cells, 2 μM and 3 μM GT3 drug doses were determined as IC50, respectively [26]. When MDA-MB-231, SUM159 and SUM149 triple negative breast cancer cell lines were used, it has been shown that 20 μM GT3 increased the effect of apoptosis by docosahexaenoic acid [20]. In the study of Gopalan et al., MCF7 and SUM158 breast cancer cell lines with different molecular profiles were exposed to GT3 and the effective dose was chosen as 10 μM in these cells [21].

In the +SA cell line, which shows a high degree of malignancy, 2 μM GT3 application was determined as the most effective dose and the expression of many proteins was analysed by immunoblotting method [22].

It is important to note that, GT3-induced apoptotic cell death was accompanied by upregulation of Bax expression and an increase in Bid and caspase 8 fragments. These data revealed that GT3 induces apoptosis in Hep3B cells through caspase-8 and caspase-9 activation. In summary, these results indicate that GT3 regulates apoptosis of Bax and Bid in Hep3B cell line [27].

Bcl-2 leads to cell survival function in response to a wide variety of apoptotic stimuli through inhibition of mitochondrial

cytochrome c release [28]. It also plays a role in modulating mitochondrial Ca2+ homeostasis and proton flux. Several phosphorylation sites have been identified in Bcl-2, including Thr56, Ser70, Thr74, and Ser87 [29]. It has been suggested that these phosphorylation sites may be targets of the ASK1/MKK7/JNK1 pathway and that phosphorylation of Bcl-2 may be a marker for mitotic events [30]. Mutation of Bcl-2 at Thr56 or Ser87 has also been shown to inhibit its anti-apoptotic activity during glucocorticoid-induced apoptosis of T lymphocytes [31]. It has been revealed that Bcl-2 phosphorylation induced by interleukin-3 and JNK may be required for enhanced anti-apoptotic functions of Bcl-2 [32]. In a study, the cytotoxic effects of alpha, gamma and delta-tocotrienols in both A549 and U87MG cancer cells were first determined in terms of cell viability and morphology. It has been found out that it changes [26].

In a subsequent study evaluating the antitumor activity of GT3 on human hepatoma Hep3B cells, the effect of GT3 on the proliferation of human hepatoma Hep3B cells was examined, and it has been found to increase in caspase 3, -8 and -9 activity. Furthermore, GT3-induced apoptotic cell death was accompanied by upregulation of Bax and an increase in Bid and caspase 8 fragments. These data revealed that GT3 induces apoptosis in Hep3B cells, and that caspase-8 and caspase-9 are involved in this apoptotic induction. These results show that GT3 regulates apoptosis through Bax and Bid regulation in Hep3B cell line [27].

GT3-induced apoptosis in human gastric cancer SGC-7901 cells was found to be associated with a suppression of mitogen-activated protein kinase signaling, along with downregulation of Bcl-2, upregulation of Bax, and activation of caspase-3 which was followed by cleavage of PARP. These results demonstrate that up- or down-regulation of Bcl-2 family proteins plays an important role in the initiation of GT3-induced apoptosis as an activator of caspase-3 [23].

Phosphorylated pAkt, the activated form of Akt, also known as protein kinase B, is an important downstream effector of the phosphatidylinositide 3-kinase (PI3K) pathway. It plays a crucial role in the development, progression and metastatic spread of many cancer types. Extensive preclinical evidence has shown that inhibition of the PI3K/Akt pathway can sensitize breast cancer cells to doxorubicin [33]. It has been further shown that increased pAKT expression is associated with radiation resistance in cervical cancer [34].

In a study conducted to show the effect of different vitamin E forms on the viability of LnCap and PC3 prostate cancer cell lines, GT3 was found to be the most potent among the forms. In this study, while an increase in apoptotic protein expression was observed with GT3 application, no significant change was observed in phosphorylated Akt protein expression. These results suggest that inhibition of PI3K signaling is not a causative factor in GT3-induced death of PC3 and LnCap cells [35]. Recent studies in malignant mammary epithelial cells have shown that the antiproliferative effects of gamma-tocotrienol are through phosphorylation and activation of PDK1 and Akt [18]. In another study conducted in neoplastic +SA mammalian cancer cells, it was revealed that GT3 inhibits neoplastic breast epithelial cell proliferation by reducing Akt activity [19].

PPARs have been proven to represent a prominent molecular target of tocotrienols, PPAR gamma (PPARγ) is thought to have a tumor suppressive role in HCC [36]. It has also been shown that PPARγ agonists not only have cell growth inhibitory effects, but can also inhibit migration, invasion and metastasis of hepatoma cells. Activation of PPARγ has been revealed to trigger apoptosis and inhibition of cell growth and metastasis in hepatocellular carcinoma. Cell growth inhibition is hypothesized to be induced by increased levels of cell cycle proteins such as cdc25c, cdc2, p21, p27 and CITED2, and/or decreased levels of cell cycle triggering-related proteins such as cyclin D1. Apoptosis can also be induced by intrinsic (increased Bax and PARP and activated caspase 3, 7, 9) or extrinsic (increased Fas and TNF-α and activated caspase-8) pathways,

and PPAR-mediated inhibition of metastasis is also associated with TIMP3, PAI-1 and induced through upregulation of E-cadherin and/or downregulation of MMP2, 9, 13 and HPSE [36]. 42/p44 MAPK, extracellular signal-regulated kinase 2/1 (ERK2/1) s are also involved in various biological processes including cell proliferation, differentiation and apoptosis. Therefore, both of these enzymes are potential therapeutic targets for cancer therapy [37].

In a study conducted in 2008 it was revealed that GT3 administration in the SGC-7901 cell line led to decreased expression of p-ERK1/2 proteins. In this study, GT3-induced apoptosis in human gastric cancer SGC-7901 cells led to the suppression of mitogen-activated protein kinase signaling and upon analysis of apoptotic markers, Bcl-2 family members and MAPK family members, it has been shown that GT3 led to the downregulation of Raf-ERK signaling pathway and downregulation of c-Myc by reducing the expression of Raf-1 and p-ERK1/2 proteins. Therefore, Bcl-2 family and Caspase-3 proteins were among key regulators for GT3-induced apoptosis in SGC-7901 cells through downregulation of the Raf-ERK signaling pathway [23].

Burdeos et al. showed that the administration of 20 μM GT3 and Delta tocotrienol in HepG2 cells led to the downregulation of MAPK pathway and subsequent inhibition of proliferation. Simultaneously, expression of genes associated with the MAPK pathway was examined by qRT-PCR and it was revealed that the inhibition effect of GT3 on cell proliferation was realized by changing MAPK upstream signaling. Downregulation of H-Ras proto-oncogene expression was obtained by administration of GT3 at 20 μM concentration in HepG2 cells. Analysing the expression of other genes associated with the MAPK pathway; it was shown that Grb2, Sos-1, c-Src and Shc2, the signaling elements located upstream of Ras-Raf-MEK-ERK, were significantly downregulated upon application of 20 μM GT3. Also in this study, proto-oncogene Hras and its associated upstream signal protein expressions were measured by immunoblotting. GT3 administration in HepG2 cells; It

significantly caused downregulation of H-Ras, Grb2, Sos-1, phosphorylated-Src (Tyr416) and phosphorylated-Shc (Tyr239 / 240) protein expressions [24].

NF-KB proteins constitute a family of inducible transcription factors that play a central role in the regulation of cell proliferation, cell survival, tumor development as well as malignant transformation. EGF-stimulated activation of the PI3K / PDK-1 / Akt mitogenic pathway has been shown to increase NFKB transcriptional activity [18].

In a study conducted in neoplastic +SA mammalian cancer cells, it was shown that GT3 inhibits neoplastic breast epithelial cell proliferation by decreasing NF-KB activity [18]. Experimental evidence indicates that the inhibitory effects of tocotrienol on NF-KB activation are due to a reduction in IKKalpha/beta and suppression of IkB binding and NF-KB transcriptional activity [38]. Since Akt and NF-KB play a critical role in the development and progression of breast tumorigenesis, overexpression of activated Akt and NF-KB is associated with the development of resistance to chemotherapy in the majority of breast cancer cases [38].

These findings reveal the potential value of tocotrienols as a chemotherapeutic agent against multidrug-resistant forms of breast cancer [18].

2. Conclusion

Owing to its great burden on public health, development of therapeutic strategies for cancer treatment is of utmost importance. Effectiveness of traditional cancer drugs such as cisplatin, doxorubicin, tamoxifen, 5- FU in clinical use is still not reaching its desired levels due to side effects and resistance to these drugs. For this reason, it is imperative to develop novel combination therapy strategies to increase the effectiveness of these agents. In recent studies, it has been shown that vitamin E family of molecules, such as tocopherol and tocotrienols have potential therapeutic potential as adjuvant molecules. In some of

cancer cells, γ-tocotrienol (GT3) has been shown to trigger apoptosis and restore sensitivity to chemotherapeutic chemicals. Evidence on the interplay of these effects with the biochemistry of cancer cells is shown in many studies. Studies have shown that GT3 combination therapy leads to an increase in the rate of apoptosis of cancer cells. GT3 and, its use with traditional reagents stands as a novel approach due to its cytotoxic, antioxidant and apoptotic effects in different cancer cell lines.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by the Altınbaş University Scientific Research Projects Unit.

References

[1] Kannappan R, Gupta SC, Kim JH, & Aggarwal BB. Tocotrienols fight cancer by targeting multiple cells signaling pathways. Genes & Nutrition. 2011; 7(1), 43–52. DOI: 10.1007/s12263-011-0220-3.
[2] Sailo BL, Banik K, Padmavathi G, Javadi M, Bordoloi D, Kunnumakkara AB. Tocotrienols: The promising analogues of vitamin E for cancer therapeutics. Pharmacological Research. 2018; 130, 259-272. DOI: 10.1016/j.phrs.2018.02.017.
[3] Danial NN, Korsmeyer SJ. "Cell death: critical control points ". Cell. 2004; 116, 205-219. DOI: 10.1016/s0092-8674(04)00046-7.
[4] Schleich K, Lavrik IN. Mathematical modeling of apoptosis. Cell Communication and Signaling. 2013; 11, 44. DOI: 10.1186/1478-811X-11-44.
[5] Zaman S, Wang R, Gandhi V. Targeting the apoptosis pathway in hematologic malignancies. Leukemia&Lymphoma. 2014; 55, 1980-1892. DOI: 10.3109/10428194.2013.855307.

[6] Lopez J, Tait SW. Mitochondrial apoptosis: killing cancer using the enemy within. British Journal of Cancer. 2015;112: 957-962. DOI: 10.1038/bjc.2015.85.
[7] Goldar S, Khaniani MS, Derakhshan SM, Baradaran B. Molecular mechanisms of apoptosis and roles in cancer development and treatment. Asian Pacific Journal of Cancer Prevention. 2015;16, 2129-2144. DOI: http://dx.doi.org/10.7314/APJCP.2015.16.6.2129.
[8] Zou H, Henzel WJ, Liu X, et. al. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 1997; 90, 405-413. DOI: https://doi.org/10.1016/S0092-8674(00)80501-2.
[9] Vande Walle L, Lamkanfi M, Vandenabeele P. The mitochondrial serine protease HtrA2/Omi: an overview. Cell Death and Differentiation. 2018; 15, 453- 460. DOI: https://doi.org/10.1038/sj.cdd.4402291.
[10] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144, 2011, 646-674. DOI: 10.1016/j.cell.2011.02.013.
[11] Tham SY, Loh HS, Mai CW, & Fu JY. Tocotrienols Modulate a Life-or-Death Decision in Cancers. International Journal of Molecular Sciences. 2019, 20, 2, 372. DOI: 10.3390/ijms20020372.
[12] Grigoriev MY, Pozharissky KM, Hanson, KP. et.al. Expression of Caspase-3 and -7 Does Not Correlate with the Extent of Apoptosis in Primary Breast Carcinomas, Cell Cycle, 2002; 1:5, 326-331. https://doi.org/10.4161/cc.1.5.152.
[13] Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE. and Poirier GG. Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Research. 1993, 53, 17, 3976–3985.
[14] Soldani C. and Scovassi AI. Poly (ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis. 2002; 7, 2002, 321–328. DOI: 10.1023/a:1016119328968.
[15] Edinger AL. and Thompson CB. Death by design: apoptosis, necrosis and autophagy, Current Opinion in Cell Biology. 2004; 16: 663–669. DOI: 10.1016/j.ceb.2004.09.011.
[16] Jiang Q, Rao, X, Kim, CY, Freiser, H, Zhang, Q, Jiang, Z, Li, G. Gamma-tocotrienol induces apoptosis and autophagy in

prostate cancer cells by increasing intracellular dihydrosphingosine and dihydroceramide. International Journal of Cancer. 212; 130:685-693. DOI: 10.1002/ijc.26054.
[17] Yap WN, Chang PN, Han HY, Lee DTW, Ling MT, Wong YC and Yap YL. γ-Tocotrienol suppresses prostate cancer cell proliferation and invasion through multiple signalling pathways. British Journal of Cancer. 2008; 99(11): 1832–1841. DOI: 10.1038/sj.bjc.6604763.
[18] Sylvester PW, Shah SJ. Mechanisms mediating the antiproliferative and apoptotic effects of vitamin E in mammary cancer cells. Frontiers in Bioscience. 2005; 10B, 699-709. DOI: 10.1038/sj.bjc.6604763.
[19] Shah SJ, Sylvester PW. Gamma-tocotrienol inhibits neoplastic mammary epithelial cell proliferation by decreasing Akt and nuclear factor kappaB activity. Experimental Biology and Medicine (Maywood). 2005; 230A:235-241. DOI: 10.1177/153537020523000402.
[20] Xiong A, Yu W, Tiwary R, Sanders BG, & Kline K. Distinct roles of different forms of vitamin E in DHA-induced apoptosis in triple-negative breast cancer cells. Molecular Nutrition & Food Research, 2012; 56:923–934. DOI: 10.1002/mnfr.201200027.
[21] Gopalan A, Yu W, Jiang Q, Jang Y, Sanders BG, Kline K. Involvement of de novo ceramide synthesis in gamma-tocopherol and gamma-tocotrienol-induced apoptosis in human breast cancer cells. Molecular Nutrition & Food Research. 2012; 56:1803-1811. DOI: 10.1002/mnfr.201200350.
[22] Ayoub NM, Bachawal SV, Sylvester PW. γ-Tocotrienol inhibits HGF-dependent mitogenesis and Met activation in highly malignant mammary tumor cells. Cell Proliferation. 2011; 44: 516-526. DOI: 10.1111/j.1365-2184.2011.00785. x.
[23] Sun W, Wang Q, Chen B, Liu J, Liu H, Xu W. Gamma-tocotrienol-induced apoptosis in human gastric cancer SGC-7901 cells is associated with a suppression in mitogen-activated protein kinase signalling. British Journal of Nutrition. 2008;99(6):1247-1254. DOI: 10.1017/S0007114507879128.
[24] Burdeos GC, Ito J, Eitsuka T, Nakagawa K, Kimura F, Miyazawa T. δ and γ tocotrienols suppress human hepatocellular carcinoma cell proliferation via regulation of Ras-Raf-MEK-

ERK pathway-associated upstream signaling. Food&Function. 2016, 7: 4170-4174. DOI: 10.1039/c6fo00826g.
[25] Wong, RS, Radhakrishnan, AK, Ibrahim TA, Cheong, SK. δ- and γ-tocotrienols induce classical ultrastructural apoptotic changes in human T lymphoblastic leukemia cells. Microscopy and Microanalysis. 2012, 18 :462-469.
[26] Lim SW, Loh HS, Ting KN, Bradshaw TD, Zeenathul NA. Cytotoxicity and apoptotic activities of alpha-, gamma- and delta-tocotrienol isomers on human cancer cells. BMC Complementary Alternative Medicine. 2014; 6: 469. DOI: 10.1186/1472-6882-14-469.
[27] Sakai M, Okabe M, Tachibana H, Yamada K. Apoptosis induction by gamma-tocotrienol in human hepatoma Hep3B cells. Journal of Nutritional Biochemistry. 2006; 17: 672-676. DOI: 10.1016/j.jnutbio.2005.11.001.
[28] Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene. 2003, 24: 8590-607. DOI: 10.1038/sj.onc.1207102.
[29] Maundrell K, Antonsson B, Magnenat, E, Camps, M, Muda, M, Chabert, C, Gillieron C, Boschert U, Vial-Knecht E, Martinou JC, Arkinstall S. Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. Journal of Biological Chemistry. 1997, 3 :25238-25242. DOI: 10.1074/jbc.272.40.25238.
[30] Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G (2)/M. Molecular and Cellular Biology. 1999, 19 :8469-8478. DOI: 10.1128/MCB.19.12.8469.
[31] Huang ST, Cidlowski JA. Phosphorylation status modulates Bcl-2 function during glucocorticoid-induced apoptosis in T lymphocytes. FASEB Journal. 2002, 16: 825-832. DOI: 10.1096/fj.01-0852com.
[32] Deng X, Xiao L, Lang W, Gao F, Ruvolo P, May WS Jr. Novel role for JNK as a stress activated Bcl2 kinase. Journal of Biological Chemistry. 2001, 276: 23681-23688. DOI: 10.1074/jbc.M100279200.

[33] Pérez-Tenorio G and Stål O; Southeast Sweden Breast Cancer Group. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. British Journal of Cancer. 2002, 86: 540–545. DOI: 10.1038/sj.bjc.6600126.
[34] Kim TJ, Lee JW, Song SY, Choi JJ, Choi CH, Kim BG, Lee JH, & Bae DS. Increased expression of pAKT is associated with radiation resistance in cervical cancer. British Journal of Cancer.N 2006, 94: 1678–1682. DOI: 10.1038/sj.bjc.6603180.
[35] Jiang Q. Natural forms of vitamin E: metabolism, antioxidant, and anti-inflammatory activities and their role in disease prevention and therapy. Free Radical Biology and Medicine. 2014; 72: 76–90. doi: 10.1016/j.freeradbiomed.2014.03.035.
[36] Hsu HT, Chi CW. Emerging role of the peroxisome proliferator-activated receptor-gamma in hepatocellular carcinoma. Journal of Hepatocellular Carcinoma.2014; 1: 127-135.
[37] Bourcier C, Jacquel A, Hess J, Peyrottes I, Angel P, Hofman P, Auberger P, Pouysségur J, Pagès, G. p44 mitogen-activated protein kinase (extracellular signal-regulated kinase 1)-dependent signaling contributes to epithelial skin carcinogenesis. Cancer Research. 2006; 66: 2700-277. DOI: 10.1158/0008-5472.CAN-05-3129.
[38] Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annual Reviews of Immunology. 1996, 14: 649-683. DOI: 10.1146/annurev.immunol.14.1.649.

CHAPTER 3

Next Generation Sequences and Its Application in Plant Science

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Next Generation Sequences and Its Application in Plant Science

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Abstract

The process of determining the sequence of the nucleotides A, C, G, and T in a strand of DNA is known as DNA sequencing. Although sequencing studies are important in molecular study, their importance is undoubtedly higher in elucidating the molecular mechanisms of environmental product losses experienced in recent years. The Sanger DNA sequencing method utilizes electrophoresis to randomly integrate chain-terminating dideoxynucleotides through DNA polymerase activity during in vitro DNA replication. Next-Generation Sequencing (NGS), a massively parallel sequencing technology, offers exceptional throughput, scalability, and rapidity. This innovative approach enables the determination of nucleotide sequences for complete genomes and specific DNA or RNA segments. Since 2010, extensive research has been conducted on employing NGS techniques to obtain the entire genome of various plant species. Prior to this period, the sequencing of several plant genomes was only accomplished partially. This chapter serves as a valuable resource for investigating the utilization of both Sanger sequencing and NGS methodologies in plant research, highlighting the significance of NGS in the field of plant genomics.

Keywords: DNA, next-generation sequencing, plant, RNA, Sanger sequencing

1. Introduction

DNA sequencing is the procedure employed to decipher the arrangement of nucleotides A, C, G, and T within a DNA strand. In its early stages, DNA sequencing was an arduous and time-consuming endeavor. Determining the precise order of letters or bases in DNA and the sequential arrangement of amino acids in protein molecules posed significant technical challenges. The scarcity of resources and the necessity for intricate reaction conditions contributed to the sluggish pace of sequencing advancements. Consequently, the process of sequencing a single gene or even a couple of genes spanned several years [1]. Studies on extracting the entire genome of plants with next-generation sequencing methods have been carried out since 2010. Before this date, only a part of the genomes of some plants could be sequenced [2]. Research has been conducted to determine which next-generation sequencing methods are better for plants. Due to the complexity of the plant genome, Sanger sequencing and Micro-bead methods were compared in some studies, and these comparisons proved that the determination of Single Nucleotide Polymorphisms (SNPs) with the micro-bead method was more accurate and detected in a short time [3].

1.1. Sanger Sequencing and Micro-Bead Methods

The Sanger sequencing technique involves using electrophoresis to randomly integrate chain-terminating dideoxynucleotides into the DNA polymerase during in vitro DNA replication. Developed by Frederick Sanger and colleagues in 1977, this method became the most widely employed sequencing technique for over four decades. Applied Biosystems made it commercially available starting in 1986. However, Next-Generation Sequencing (NGS) methods have recently gained prominence and replaced Sanger sequencing, particularly for large-scale and automated genome analyses. Nonetheless, the Sanger method continues to be extensively used for smaller-scale projects and validation of profound sequencing outcomes. In recognition of their contributions,

Walter Gilbert and Frederick Sanger were jointly awarded the Nobel Prize in Chemistry in 1980 [4].

The genome of Heterodera glycines, a Soybean cyst nematode responsible for significant soybean damage, comprising 400 million bases, was sequenced using both the Sanger and microbead methods, with a comparative analysis of the two techniques [5]. The microbead method, which relies on the size of the microbeads, is widely recognized as one of the most prevalent approaches for microbead separation. Conventional membrane-based microfilters are commonly employed for microfiltration. Multicomponent particle filtration can be achieved by employing membranes with varying pore sizes. In theory, this technique has the capability to separate microbeads of any size by employing the appropriate pore size. Sun et al. (2018) [6] developed a method using modified microbeads that exhibit preferential binding to target cells, thereby enhancing the capture efficiency of small cells or particles by enlarging their size. Their approach focused on the size amplification of cells through the attachment of 3 μm microbeads. Additionally, Wong et al. (2013) [7] designed a 3D micro-trap array to filter out smaller diameter beads while retaining larger ones selectively, offering potential applications in bead microarray assays. Single-nucleotide polymorphism (SNPs) in the nematode genome is known to affect nematode virulence and host selection. Therefore, the identification of SNPs is vital in agricultural control. When the sequence analysis results of both methods were compared using the genomic DNA of the two subtypes of the nematode, it was shown that the SNPs in the genome could be detected much more quickly and accurately with the microbeads method [6].

1.2. Next-Generation Sequencing (NGS)

Next-Generation Sequencing (NGS) represents a groundbreaking massively parallel sequencing technology that delivers unparalleled throughput, scalability, and speed levels. This innovative methodology empowers researchers to unravel the nucleotide sequence of complete genomes or targeted DNA

and RNA fragments. The advent of NGS has sparked a transformative revolution within the biological sciences, enabling laboratories to undertake diverse tasks and explore biological systems with unprecedented depth. In the face of today's intricate genomics inquiries, the information depth required surpasses the capacities of traditional DNA sequencing techniques. NGS has emerged as the indispensable tool that bridges this gap, evolving into the standard apparatus for addressing these complex genomic questions [8].

1.3. Applications of NGS

Rapid whole-genome sequencing has emerged as an alternative to standard genome sequencing, offering the potential for swift and accurate diagnosis of rare genetic diseases in critically ill infants within a timeframe of fewer than 50 hours. Clinical studies have demonstrated that rapid whole-genome sequencing (rWGS), also known as Rapid Whole Genome Sequencing, achieved a positive diagnosis rate ranging up to 57% in cases involving pediatric patients admitted to the Intensive Care Unit (ICU), subsequently informing medical management in 30% to 72% of these cases. However, the clinical utility of rWGS has faced limitations regarding scalability and the ability to resolve complex structural variations, contributing to approximately 20% of genetic diagnoses in pediatric ICU settings [9].

Deep sequencing of target regions provides unique insights into specific areas of interest within the genome. This powerful application finds useful applications in various disease areas, including oncology, inherited diseases, immunology, and infectious diseases. Researchers can effectively investigate these disease areas by precisely and efficiently targeting specific genes, coding regions, and even chromosomal segments. RNA sequencing (RNA-Seq) is employed to discover novel RNA variants, splice sites, and quantify mRNA levels for gene expression analysis. Gene expression refers to the process by which genetic information encoded in genes is transformed into proteins through messenger RNA (mRNA). The genotype

encompasses the complete set of genes within an organism, containing alleles that, when expressed, determine the traits or phenotypes observed in the organism [10].

Analyzing epigenetic factors such as genome-wide DNA methylation and DNA-protein interactions provides valuable insights. DNA methylation, histone modifications, noncoding RNA, nucleosome positioning, and DNA sequence collectively contribute to the epigenetic landscape. DNA methylation, catalyzed by DNA methyltransferases (DNMTs), is a stable repressive mark and plays a crucial role in the epigenetic regulation of transcriptional silencing. DNA methylation can coordinate chromatin status through interactions with other modifications and components of the associated molecular machinery [11].

Cancer sequencing utilizing Next-Generation Sequencing (NGS) techniques yields extensive information in less time compared to conventional single-gene and array-based methodologies. Using NGS, researchers can perform tasks such as whole-genome analyses, targeted gene profiling, and tumor-normal comparisons. NGS is highly sensitive to detecting circulating DNA fragments, tumor subclones, and rare somatic variations. The study of the human microbiome has gained significant attention to comprehend the roles played by symbiotic microorganisms and their impact on human health [12].

In clinical microbiology laboratories, metagenomic analysis based on NGS is increasingly adopted to identify novel pathogens in clinical samples. This agnostic approach enables the detection of unexpected or previously unknown pathogenic microorganisms. NGS technologies facilitate high-throughput parallel sequencing of nucleic acid fragments in blood and tissues without predefined targets. Bioinformatics software can reassemble the sequenced fragments into a microbial genome and identify mutations, resistance markers, and virulence factors by comparing the sequences with existing databases [9].

1.4. Advances in NGS Technology

Significant advancements have been made in Illumina's Next-Generation Sequencing technology, as highlighted by recent breakthroughs [8, 13]:

1) Semiconductor sequencing: The iSeq 100 System integrates a complementary metal-oxide semiconductor (CMOS) chip with one-channel Sequencing by Synthesis (SBS) technology, resulting in a compact system that delivers highly accurate data.

2) Patterned flow cell technology: This innovation provides exceptional throughput for various sequencing applications, enhancing the efficiency and productivity of sequencing processes.

3) Up to 6 terabases (Tb): The NovaSeq 6000 System offers a tunable output of up to 6 Tb, enabling researchers to generate vast amounts of sequencing data in approximately two days.

4) 75 breakthrough innovations: The NextSeq 1000 and 2000 Systems offer remarkable flexibility for emerging applications, featuring a streamlined workflow and rapid data analysis capabilities, with results obtainable in as little as two hours. Third-generation sequencing technologies offer several advantages over existing sequencing technologies, including:

- Higher yield: These technologies provide increased sequencing output, allowing for the generation of more data.

- Faster turnaround time: The turnaround time for obtaining sequencing results is significantly reduced compared to previous methods.

- Long read rates: Third-generation sequencing technologies can produce longer sequencing reads, enabling the study of complex genomic regions and structural variations.

- High compromise accuracy: Despite the longer read lengths, the accuracy of third-generation sequencing is still relatively high, ensuring reliable and precise results.

- Ability to work with small quantities of starting material: These technologies require smaller amounts of initial sample material, making them suitable for applications where sample availability is limited.

- Cost-effectiveness: Third-generation sequencing technologies have the potential to be more cost-effective, offering efficient sequencing solutions at a reduced cost.

Overall, these advancements and advantages in Next-Generation Sequencing technologies have significantly expanded the capabilities and applications of genomic research and analysis.

1.5. Applications of NGS Technique in Plants

NGS methods have been applied in many plants. Studies have been conducted to develop insertion-deletion (inDel) markers using next-generation sequencing methods made with sesame (*Sesamum indicum* L.) from industrial plants. The results showed that there is a positive correlation between chromosome size and inDel number in sesame (*S. indicum*) [14]. Studies have been carried out to understand better the Black Leg disease, which is a threat in Canola (*Brassica napus* L.), an industrial plant, and to eliminate the disease by resistance gene transfer [15]. There are many stress studies on legumes, which is the most essential food product after grains in the world. Establishing transcriptome profiles of legumes has made it possible to understand and obtain information on the analysis of plant responses to various stresses at the molecular level. In recent years, transcriptome analyzes showing changes in various stress conditions have been reported in many legume species [16]. Another essential food crop in the world is rice (*Oryza sativa* L.). NGS methods have contributed to the development of new varieties, rice evolution and improvement in rice (*O. sativa*)

breeding due to the difficulties caused by changing climatic conditions and increasing population [17].

Next-generation sequencing methods have made significant contributions to the study of tropical plants. In the case of the tropical fruit banana (*Musa acuminata* L.), a next-generation sequencing approach was employed to investigate pests and diseases, leading to advancements in crop improvement and production increase studies [18]. Similarly, the complete genome sequencing of 'Hicaznar' and '33N262' pomegranate cultivars (*Punica granatum* L.), which are tropical plants, was accomplished using next-generation sequencing techniques [19].

In Brazil, a study utilized next-generation sequencing technologies to enhance tropical plants' diversity and disease resistance. The objective was to leverage genomic tools in tropical forage breeding programs to accelerate genetic progress and reduce the time required to develop new varieties suitable for tropical environments. The critical challenges in tropical forage production include enhancing productivity, resistance to pests and diseases, nutritional value, adaptability to climate change, and addressing internal obstacles like apomixis [20].

Overall, the application of next-generation sequencing in tropical plant research has opened up avenues for understanding and improving the genetic characteristics of these plants, leading to advancements in crop productivity, disease resistance, and the development of new varieties tailored to tropical conditions. Next-generation sequencing studies have made DNA extraction processes from stubborn plants easy and reliable. It has been observed that this method provides convenience in studies where DNA isolation is difficult due to the presence of phenolics and polysaccharides in the leaves of stubborn plants [21]. Recognition of the genetic structure of viruses and viroids that cause disease in plants, identification of known and unknown species, and control of the diseases they cause have made it possible to examine at the genomic level with next-generation sequencing [22]. Developing biotechnology-based breeding methods can create specially designed plants by providing

diversity with genomic design studies in medicinal plants [23]. In recent years, studies have been started to determine the phytochemicals of medicinal plants with new-generation sequencing technologies. The aim here is to recognize the biosynthetic pathways of medicinal plants and the enzymes involved in these pathways. With extensive genomic and transcriptomic studies, important genes that play a role in the biosynthesis of the compounds they contain will be able to be identified [24].

There are also studies to identify the causative mutation in plants. Causal mutations are divided into two; They are (1) spontaneous mutations and (2) mutations that occur without external factors. Induced mutations are mutations produced by mutagens [25]. Genetic approaches based on next-generation sequencing techniques were used. Mutations were identified and mapped with advanced bioinformatics tools [26].

2. Conclusion

Due to the complexity and size of the plant genome, when Sanger sequencing technology is used, plant projects are limited to large-scale sequencing centers, as the method is technologically limited, and the cost is high. Next-Generation Sequencing offers high data quality with long and highly accurate reads and is very useful in plant studies due to its affordable speed and cost.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Emara K, Aiyer A, Rogero R. What Is the Role of Molecular Techniques for Detection of Pathogen Deoxyribonucleic Acid (DNA) (Polymerase Chain Reaction [PCR] or Next-Generation Sequencing) in Patients with Infected Total Ankle Arthroplasty

(TAA)? Foot & Ankle International. 2019; 40(1_suppl): 29S-31S.
[2] Dönmez D, Şimşek Ö, Kaçar, YA. Yeni Nesil DNA Dizileme Teknolojileri ve Bitkilerde Kullanımı. Türk Bilimsel Derlemeler Dergisi. 2015; 1: 30-37.
[3] Üstek D, Abacı N, Sırma S, Çakiris A. Yeni Nesil DNA Dizileme. Deneysel Tıp Araştırma Enstitüsü Dergisi. 2011; 1(1): 11-18.
[4] Shendure J, Ji H. Next-generation DNA sequencing. Nature Biotechnology. 2008: 26(10): 1135-1145.
[5] Nagaratnam N, Nagaratnam K, Cheuk G. Geriatric diseases: evaluation and management. New York, NY: Springer; 2018. No. 144426.
[6] Sun N, Li X, Wang Z, Li Y, Pei R. High-purity capture of CTCs based on micro-beads enhanced isolation by size of epithelial tumor cells (ISET) method. Biosensors and Bioelectronics. 2018; 102: 157-163.
[7] Wong CC, Liu Y, Wang KY, Rahman ARA. Size based sorting and patterning of microbeads by evaporation driven flow in a 3D micro-traps array. Lab on a Chip. 2013; 13(18): 3663-3667.
[8] Takagi J. Correlation Technology for the Next-generation Structural Life Science. Trends in The Sciences. 2012; 17(12): 12-37. https://doi.org/10.5363/tits.17.12_34.
[9] Fishman JA. Next-Generation Sequencing for Identifying Unknown Pathogens in Sentinel Immunocompromised Hosts. Emerging Infectious Diseases. 2023; 29(2): 431.
[10] Byron SA, Van Keuren-Jensen KR, Engelthaler DM, Carpten JD, Craig DW. Translating RNA sequencing into clinical diagnostics: opportunities and challenges. Nature Reviews Genetics. 2016; 17(5): 257-271.
[11] Jin B, Li Y, Robertson KD. DNA methylation: superior or subordinate in the epigenetic hierarchy? Genes and Cancer. 2011; 2(6): 607-617.
[12] Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, NIH HMP Working Group. (2009). The NIH human microbiome project. Genome research. 2009; 19(12): 2317-2323.

[13] Anonymous, 2022. What are the 4 steps of next generation sequencing (NGS)? [Internet]. 2022. Available from: https://www.aatbio.com/resources/faq-frequently-asked-questions/What-are-the-4-steps-of-next-generation-sequencing-NGS [Accessed: 2023-04-18].
[14] Uzunoğlu S. Susamda Yeni Nesil Dizileme Verileri Kullanılarak Indel Markerlerin Geliştirilmesi [thesis]. Antalya: Akdeniz University; 2020.
[15] Thomas WJ, Amas JC, Wu T, Cantila AY, Ton LB, Batley J. The Impact of Genomics in Identifying Blackleg Disease Resistance Genes in Canola. Next-generation Sequencing and Agriculture. 2022; 12: 24.
[16] Morgil H. Kuraklık Stresi Uygulanmış Mercimek (*Lens Culinaris* Medik.) Bitkisinin RNA Dizileme Yöntemi ile Transkripsiyonel Analizi [thesis]. İstanbul: İstanbul University; 2020.
[17] Junliang ZV, Dilin L. Rice Genetics, Genomics and Breeding Revolutionized by Next-Generation Sequencing. Next-generation Sequencing and Agriculture. 2022; 12: 39.
[18] Stephan G, Dugdale B, Deo P, Harding R, Dale J, Visendi P. The Use of Next-Generation Sequencing to Study Banana Traits, Pests and Diseases in Tropical Agriculture. In Next-Generation Sequencing and Agriculture. GB: CABI. 2022; 1-23.
[19] Şimşek Ö, Dönmez D, İmrak B, Özgüven AI, Kaçar, YA. Narda (*Punica granatum* L.) Yeni Nesil Dizileme Teknolojisi Kullanılarak SSR Markırlarının Geliştirilmesi. Uluslararası Tarım ve Yaban Hayatı Bilimleri Dergisi. 2018; 4(2): 161-167.
[20] Pereira JF, Azevedo ALS, Pessoa-Filho M, Romanel EADC, Pereira AV, Vigna BBZ, Machado JC. Research priorities for next generation breeding of tropical forages in Brazil. Crop Breeding and Applied Biotechnology. 2018; 18: 314-319.
[21] Healey A, Furtado A, Cooper T, Henry RJ. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. Plant methods. 2014; 10(1): 1-8.
[22] Hadidi A, Flores R, Candresse T, Barba M. Next-generation sequencing and genome editing in plant virology. Frontiers in microbiology. 2016; 7: 1325.

[23] Niazian M. Application of genetics and biotechnology for improving medicinal plants. Planta. 2019; 249: 953-973.
[24] Younessi-Hamzekhanlu M, Ozturk M, Jafarpour P, Mahna N. Exploitation of next generation sequencing technologies for unraveling metabolic pathways in medicinal plants: A concise review. Industrial Crops and Products. 2022; 178: 114669.
[25] Löwdin PO. Proton tunneling in DNA and its biological implications. Reviews of Modern Physics. 1963; 35(3): 724.
[26] Sahu PK, Sao R, Mondal S, Vishwakarma G, Gupta SK, Kumar V, Das B K. Next generation sequencing based forward genetic approaches for identification and mapping of causal mutations in crop plants: A comprehensive review. Plants. 2020; 9(10):1355.

CHAPTER 4

Bacterial Alkane Hydroxylase; Diversity, Distribution and Influence in Degradation of Hydrocarbons

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Bacterial Alkane Hydroxylase; Diversity, Distribution and Influence in Degradation of Hydrocarbons

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Abstract

The main components of hydrocarbons, which are found in fossil fuels like crude oil, are hydrogen and carbon. Although being natural chemicals, the process of refining crude oil creates commercial goods with unique physical and chemical properties, which can make them more complex and poisonous and hinder economic growth except they were degraded. A necessary condition for an efficient homeostatic response to the different hydrocarbons that contaminate ecosystems is the presence of bio-diverse natural microbial communities. Its elimination, however, is contingent on the polluted compartment (water, sediment, or soil), their molecular weight, and the extent to which their toxicity does not interfere with microbial activity. This review discussed the relevance of alkane hydroxylase enzymes that facilitate degradation of hydrocarbons. The central and peripheral metabolic pathways of versatile bacterial metabolism require specialized genes that encode the essential enzymes for the degradation of these aliphatic and polycyclic aromatic hydrocarbons.

Keywords: Alkane, Degradation, Enzymes, Genes, Hydrocarbons

1. Introduction

A crucial and significant commodity that is important and wields so much influence in the world market is crude oil [1]. This product is made up chemically of a complicated mixture of asphaltenes, heterocyclic hydrocarbons, aromatic hydrocarbons, and aliphatic hydrocarbons which are categorized as biodegradable to a degree of 60 to 90% as reported by [2] even though they are toxic and hazardous [3]. Recent improvements in the petroleum industry's methods for extracting, transporting, and storing petroleum products in underground reservoirs, including refining procedures, have resulted in serious environmental contamination and related problems to the environment [4]. One of the major issues facing the world today is soil degradation brought on by petroleum and its products causing environmental impact among which is spillage. Oil exploration, waste disposal, and unintentional spills are just a few examples of human activities that have caused serious environmental problems. These activities have altered the chemical composition of soils, reduced microbial diversity, disrupted ecology with a lack of nutrients available to plants, and caused oxidative stress [5]. Petroleum in the soil environment impairs root growth, hinders seed germination, decreases photosynthetic pigments, slows absorption, results in leaf deformities, and harms cells. Biological membrane disruption, metabolic pathway signaling disturbance, and plant root structure disruption are a few more of the negative effects identified by [6]. Hydrocarbons of low molecular weight can get through plant cells and destroy them as observed by [7]. Although, living bacteria transform organic molecules, such as the aforementioned petroleum pollutants or products, into smaller chemicals, thereby decreasing their visible effect, it is rather difficult to degrade organic petroleum and other aromatic chemicals in the environment [8]. The kind and amount of oil or petroleum products utilized heavily influences both the quantitative and qualitative features of microorganisms [8]. Other factors include the composition of the microbial population, ambient conditions, and the seasonality of the environment [9]. The activity of the microbial population has a

significant impact on the biodegradation of pollutants from petrochemicals and petroleum hydrocarbons, even though growth rates vary [10]. However, studies conducted over the past few decades have also revealed that little is known about how algae and protozoa function in the biodegradation of petroleum compounds [10].

This is primarily an adaptive process, which is frequently governed by external factors, and the microbial conversion of organic pollutants, which typically occurs because the organisms may use the pollutants for their energy demands, growth, and reproduction, gives these microbes relevance in the biodegradation process [11]. Many different compounds, including hydrocarbons, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), radionuclides, and metals, can be converted by some bacteria or produced by them spontaneously. Genetic potentials and environmental elements including oxygen concentration, moisture content, pH, and temperature have been found to have an impact on the rate and efficiency of degradation in soil and water [12].

1.1. Distribution of Hydrocarbon Degrading Microorganisms

The most significant and prevalent group of organisms known to degrade hydrocarbons is bacteria [13]. Yet, as reported by [14,[15], some obligatory hydrocarbonoclastic bacteria (OHCB) is class of bacteria that only consumes hydrocarbons as a substrate. Prior to hydrocarbon pollution, this group is prevalent in some specific environments at negligible or undetectable levels. In non-contaminated environments, they are extremely rare and may only make up 0.1% of the entire microbial population; nonetheless, they expand in number and the activities they perform thereby making up an entire microbial community [16]. In environments contaminated with hydrocarbons, several microorganisms were isolated and identified. However, only a small percentage (1- 2%) of those communities can be grown using conventional approach [17]. The bacterial makeup and organization in various environments

were also studied using a number of molecular methods, such as 16S rRNA gene clone libraries [18], automated ribosomal intergenic spacer analysis (ARISA) [19], terminal restriction fragment length polymorphism (T-RFLP) [20], and denaturing gradient gel electrophoresis [21]. More methodologies based on high throughput DNA sequencing (next generation sequencing technologies) are enhanced in metagenomic research as a result of recent advancements in genomics and sequencing technology [22]. These hydrocarbonoclastic bacteria are crucial since they are part of the essential participants in the cleanup of contaminated locations of oil [23]. The most representative genera of these OHCB are *Alcanivorax*, *Marinobacter*, *Thallassolituus*, *Cycloclasticus*, and *Oleispira*, and they are crucial to the breakdown of hydrocarbons [24]. *Alcanivorax borkumensis* is a common obligate hydrocarbonoclastic bacterium in hydrocarbon-polluted environments. Although this strain can break down linear and branched alkanes, it cannot break down aromatic hydrocarbons [25]. Similarly, *Thallassolituus oleivorans* is a highly specialized organism that can break down aliphatic hydrocarbons with carbon atoms ranging from C7 to C20 [15]. Particularly well-known for degrading aliphatic hydrocarbons are *Oleiphilus* and *Oleispira* [14]. In contrast, it has been reported that *Cycloclasticus* mineralizes a number of PAHs, which includes but not limited to naphthalene, phenanthrene, anthracene, pyrene, and fluorene [27]. Additional non-obligate hydrocarbonoclastic bacteria were isolated from an oil-contaminated environment and have been reported for their capacity to break down several kinds of hydrocarbons [28]. Other researches by [14] and [29] distinguished some classes of *Pseudomonas*, *Marinomonas*, *Halomonas*, and *Micrococcus* within this group that can breakdown PAHs although they reported the decomposition of aliphatic chemicals by *Acinetobacter* and *Sphingomonas* as well. As crude oil is a complicated mixture of hydrocarbons and each individual microbe can only break down a small portion of it, various studies have shown how effectively a consortium of microorganisms can break it down. Most consortia are made up of a mixture of recognized hydrocarbon-degrading microorganisms or native microbial consortia that have been

either enhanced or known degraders of hydrocarbons. The use of consortia of bacteria with a broad enzymatic capacity to break down a variety of hydrocarbons fasten up the bioremediation process as reported by [29-31]. These groups of constituted consortia were made up of a combination of both bacteria and fungus and were successful in achieving an effective breakdown of petroleum due to their complimentary metabolic potentials to digest the distinct components.

1.2. Mechanism of Microbial Bioremediation of Hydrocarbon

Microorganisms are crucial in remediation processes owing to their metabolic capabilities and capacity to adapt to harsh environments. They also have a propensity to break down a variety of organic contaminants. However, as noted by [32], the chemical composition and quantity of the contaminants, their accessibility to bacteria, and the physicochemical properties of the environment all determine how successful they perform. Additionally, some crude oil products with higher fluidity have complex detrimental effects on bacteria that can be broken down, but other substances don't significantly impede the development of microbes [32]. Two of the most important mechanisms through which hydrocarbons are degraded are aerobic and anaerobic degradation pathways.

1.3. Aerobic Degradation of Hydrocarbons

Hydrocarbon degradation in the presence of oxygen (aerobic) is a process that can be performed by bacteria, fungus, and algae [34]. The microorganisms act on alkenes with double bonds and short-chain alkanes, which make up the most easily broken group hydrocarbons. Other branched alkanes with side chains and aromatics constitute a more complex structure that is a little bit difficult to compromise (Figure 4.1) [35]. However, the more complex a hydrocarbon is, the lower the degradation rate and this become more dependent on the environment. These degradation rates are highly variable since the hydrocarbon composition changes depending on the petroleum exploration

source. In contrast, the addition of nitrogen can result in up to 100% degradation. Similarly, the amount of oxygen that microorganisms can absorb quickly within is also an important environmental factor that affects its availability. The addition of oxygen can significantly speed up the deterioration process compared to natural rates. Alkanes with 14 or fewer carbon atoms are more volatile in an oxygenated environment than those with more carbons, which vaporize less [36].

Looking at the schematic presentation of hydrocarbon degradation in Figure 4.2, bacteria are easily able to breakdown alkanes with short chains, typically between 10 and 24 carbon atoms as highlighted by, [37-39] [38]. The addition of oxygen at the terminal methyl group, which results in the production of alcohol, opens the routes for the biodegradation of n-alkanes. The alcohol produced is first converted to aldehyde, then further to carboxylic acid by dehydrogenation. By way of the β-oxidation pathway, carboxylic acid is further metabolized to form fatty acids. When acetyl coenzyme-A is removed as a result of β -oxidation, fatty acids are converted to molecules with only two carbons. Alkane undergoes β -hydroxylation at both of its terminal ends as part of the di-terminal pathway (Figure 4.2), which results in the creation of di-carboxylic acid. Alkanes are oxidized in sub-terminal oxidation (Figure 4.2) to create secondary alcohol, the equivalent of ketone and also ester. The ester is subsequently hydrolyzed further to produce a by-product in form of an alcohol.

Alkanes and alkenes, with the exception of cyclic alkanes, are the hydrocarbons that degrade the most easily; degradation of alkanes by microorganisms with up to 44 carbons has been observed and reported by [36,37][37]. The early step of deterioration and the availability of oxygen are rate-limiting factors. The addition of molecular oxygen to the hydrocarbon molecules by oxygenases, an enzyme class, occurs during the initial phases of aerobic alkane breakdown [40]. As a result, alcohols are produced, which are then further oxidized to become fatty acids, and finally decomposed into acetyl-CoA, CO_2, and water [41]. Numerous mono- and di-oxygenases, along

with other oxidases, have a wide range of substrates and can react quickly with a variety of hydrocarbons [37]. Since they are more poisonous than shorter alkanes and alkenes, aromatic hydrocarbons are frequently more difficult to break down, but many bacteria and fungi are capable of doing so. Due to increase in the hydrophobic nature and sorption capacity of hydrocarbons, their degradability diminishes with increasing molecular size and ring count [36]. The addition of O_2 by mono- and di-oxygenases is the first step in the overall pathway for the breakdown of aromatic compounds [42]. The result is the production of important intermediates such phenol, benzyl-alcohol, catechol, protocatechuate, and gentisate. After ring breaking of these intermediates by a number of oxygenases, carboxylic acids are produced [43]. Acetyl-CoA and succinyl-CoA, which pass into central metabolism, are subsequently subject to further degradation [43,44]. Although many of the processes in fungi are similar to those in bacteria, fungi-facilitated degradation is mainly achieved through non-specific enzymes of extracellular origin capable of producing radical intermediates.

1.4. Anaerobic Degradation of Hydrocarbons

Anaerobic degradation is a process through which organic matter is broken down in the absence of oxygen. Anaerobic circumstances result in slower hydrocarbon breakdown than in aerobic settings because of less favorable reaction dynamics with alternate electron acceptors [45]. Despite this limiting effect, facultative and obligately anaerobic bacteria and archaea have been shown to be capable of degrading hydrocarbons in the absence of oxygen [46]. Anaerobic activities significantly affect how hydrocarbons function in the environment since oxygen is quickly used by these microbes in hydrocarbon-impacted places [46]. Although anaerobic hydrocarbon degraders have doubling periods that range from days to months, adding an additional oxidized functional group to activate the molecule during the earliest stages of anoxic hydrocarbon breakdown is typically restricting as noted by [47]. It was observed that absolute hydrocarbon degradation occurs without oxygen

notwithstanding the slow rate. For instance, [47] found that polycyclic aromatic hydrocarbons, or molecules with many aromatic rings, degrade within 90 days, whereas benzene degrades over a period of 120 weeks. According to [49], linear alkanes appear to degrade under methanogenic conditions in less than 200 days. Basically, nitrate, sulfate, carbon dioxide, oxidized metals, and even some organic compounds are used by anaerobic microbes as terminal electron acceptors during respiration in addition to oxygen. As a function of diminishing reduction potential, bacteria at a contaminated site typically consumes oxygen, nitrate, ferric iron, sulfate, and H_2 in that order [37]. In a few instances, it has been demonstrated that particular species of denitrifying or sulfate-reducing bacteria may totally metabolize some hydrocarbons into CO_2 and water.

Nevertheless, anaerobic hydrocarbon breakdown, more frequently, takes place via syntrophy, where the activity of one microbe is dependent on the activity of another bacterium responsible for maintaining low concentrations of intermediate products like formate and $H_{2\,[51]}$[50,51].

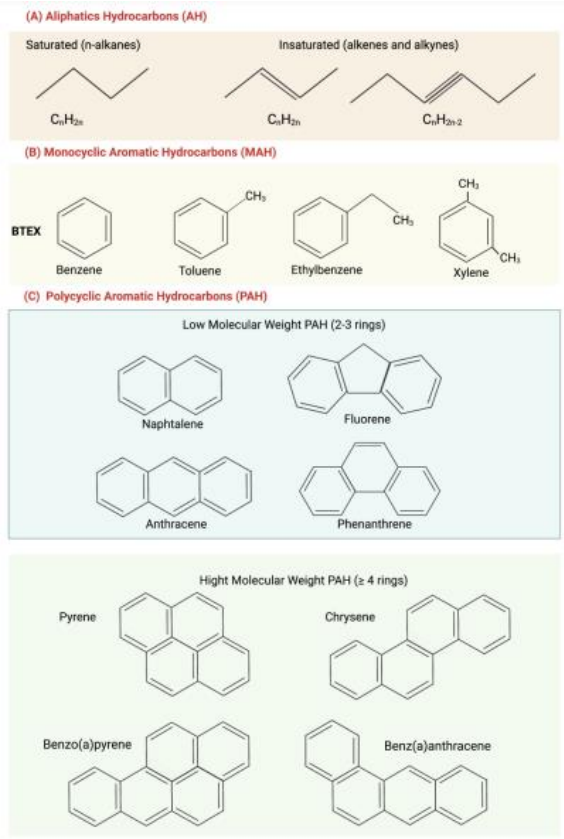

Figure 4.1. Structure of hydrocarbons

Low product concentrations promote reactions that would not otherwise be thermodynamically beneficial. Since using oxygen as a terminal electron acceptor is more advantageous energetically, syntrophy occurs more frequently in anaerobic environments [52]. Although methanogens (archaea that make methane) can only metabolize simple substrates like acetate and hydrogen, syntrophic activities are essential for complete degradation to methane and carbon dioxide [53].

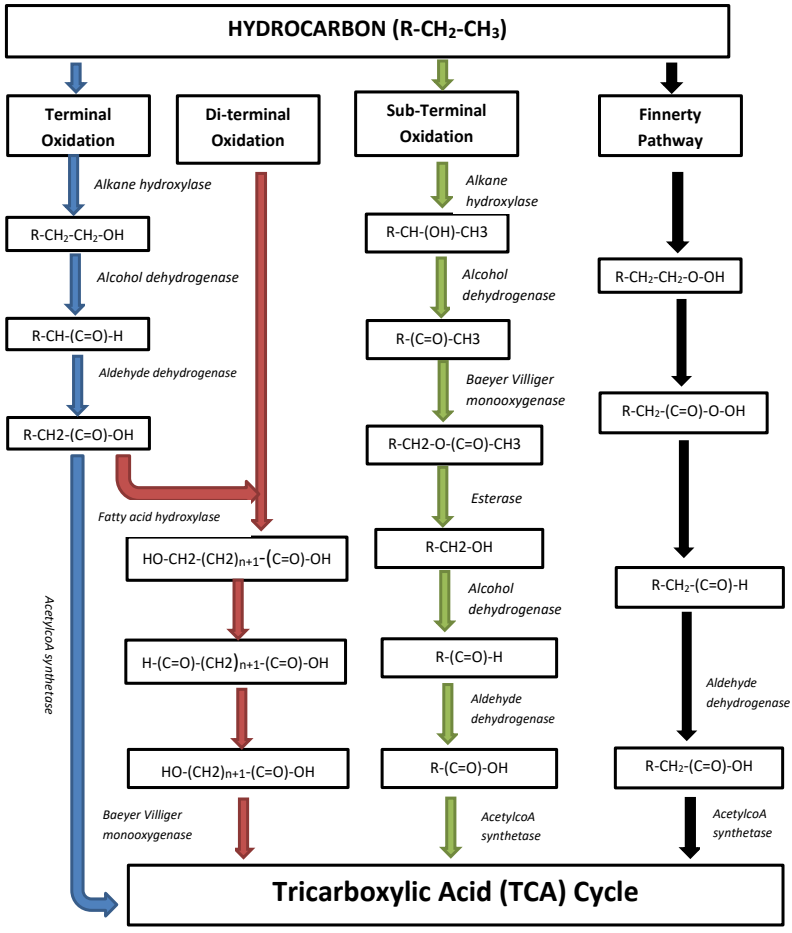

Figure 4.2. Alkane degradation pathways

1.5. Factors Affecting Bioremediation of Hydrocarbons

The rate at which hydrocarbon is broken down is influenced by a number of physical, chemical and other biological factors in the environment coupled with microbial community activity [13]. Other environmental factors of relevance include nutrient availability, temperature, water content or humidity, oxygen, bioavailability of pollutant, and pH.

1.6. Nutrient Availability

While inorganic sources like nitrogen, phosphorus, potassium, hydrogen, or oxygen are necessary for microbial metabolism and have an impact on the growth and activity of microorganisms, micronutrients like zinc, manganese, iron, nickel, cobalt, molybdenum, copper, and chlorine are only slightly necessary. Carbon to nitrogen or carbon to phosphorus ratio is high in hydrocarbon-contaminated environments, which is regarded to be an impediment in biodegradation rates [55].

1.7. Effect of Temperature

The influence of temperature to enhance or influence the ability of microorganisms to degrade hydrocarbons is recognized by several research. High environmental temperatures basically improve solubility, bioavailability, the spread of hydrocarbons, and the velocity of diffusion, all of which support and accelerate microbial biodegradation. On the other hand, extremely high temperatures inhibit aerobic microbial biodegradation and reduce oxygen solubility as reported by [56]. In addition, [57] supported the idea that pollutant degradation at mesophilic temperatures is superior to that at very low or high temperatures. Nonetheless, it has been documented that microbes may break down PAHs even at very high or very low temperatures. For instance, seawater showed a high rate of PAH degradation at both 0°C and 50°C [58].

1.8. Effect of Oxygen Concentration

By using oxygenases, which need molecular oxygen to function, bacteria and fungi first oxidize the substrate before beginning to break down aliphatic, cyclic, and aromatic hydrocarbons [13]. Despite the fact that anaerobic biodegradation has been demonstrated to occur in a variety of habitats, including marine environments, its ecological relevance has generally been viewed as being minimal and the biodegradation rate is quite low [61]. Normal oxygen-limited conditions can be found in aquatic soils and sediments. In the

presence of readily usable substrates that promote microbial oxygen demand, oxygen depletion can happen. In a few of situations, the level of dissolved oxygen might be almost low, almost eliminating aerobic biodegradation rates.

1.9. Effect of pH

In aquatic habitats, pH varies less, and the majority of bacteria and fungi that can break down hydrocarbons need a neutral pH [56]. Generally speaking, extremely low or high pH have an impact on microbial activity [59]. At pH 5.5, *Burkholderia cocovenenans* degraded phenanthrene by 40%, according to [60]. Nevertheless, the deterioration under the identical circumstances at neutral pH was 80%. Moreover, according to [59], the rate of naphthalene microbial breakdown decreased at pH 5.0 compared to the greatest rate seen at pH 7. Moreover, additional studies have shown how effectively some microbes, including *Pseudomonas*, can break down hydrocarbons at an alkaline pH [60].

1.10. Bioavailability of Hydrocarbons

The bioavailability, which refers to the rate at which substrate mass is transferred into microbial cells, plays a crucial role in determining the degradation rate of hydrocarbons. Poor solubility in water results in low bioavailability for polycyclic aromatic hydrocarbons (PAHs), making them resistant to degradation and persistent in the environment [61]. The limited bioavailability of PAHs has been linked to documented cases of unsuccessful cleanup in areas contaminated by these compounds [61]. Over time, the bioavailability of hydrocarbons tends to decrease [61]. However, the process of photo-oxidation has been shown to accelerate the biodegradation of petroleum hydrocarbons by enhancing their bioavailability and stimulating microbial activity. This suggests that photo-oxidation can improve the accessibility of hydrocarbons to microbial degradation processes, overcoming their inherent low bioavailability.

1.11. Some Genetic Markers in Alkane Degradation Pathway

Various families of enzymes can initiate the terminal hydroxylation of n-alkanes (Figure 4.2). According to [62], microorganisms that degrade short-chain alkanes (C2-C4) possess enzymes related to methane monooxygenases. In contrast, strains that break down medium-chain alkanes (C5-C17) often contain integrated membrane non-heme iron monooxygenases like AlkB and soluble cytochrome P450s [63]. Interestingly, the characterized long-chain alkane hydroxylases (>C18) are distinct from the aforementioned hydroxylases [64]. One such hydroxylase is AlmA, a lower chain alkane monooxygenase found in *Acinetobacter*. Another is LadA, a thermophilic soluble lower chain alkane (LC-alkane) monooxygenase from *Geobacillus* sp. [65]. In some *Acinetobacter* sp., the almA gene encodes a putative monooxygenase that likely belongs to the flavin-binding family. This gene represents the first experimentally verified enzyme related to the metabolism of LC n-alkanes (C32 and longer). We found that the almA gene is highly expressed in both *A. hongdengensis* and *A. dieselolei* B-5 strains, facilitating efficient degradation of LC n-alkanes. This provides the initial evidence that AlmA in the genus *Alcanivorax* functions as an LC-alkane hydroxylase [64]. Genes with similarities to almA can also be found in other genera, such as *Oceanobacter* sp., *Ralstonia* spp., *Mycobacterium* spp., *Photorhabdus* sp., *Psychrobacter* spp., and *Nocardia farcinica*, as listed in the GenBank database.

Geobacillus thermodenitrificans, a thermophilic bacterium, possesses a distinct LC-alkane hydroxylase called LadA. LadA is an enzyme that oxidizes alkanes with carbon chain lengths ranging from C15 to C36, generating the corresponding primary alcohols [67]. LadA is a two-component, flavin-dependent oxygenase that belongs to the family of bacterial luciferases. Its crystal structure has been determined [68]. The ability of LadA to recognize and hydroxylate LC-alkanes is likely due to its alkane-binding mechanism, which resembles that of other flavoprotein monooxygenases [68]. Therefore, the hydroxylases

involved in LC-alkane degradation appear to have evolved distinctively compared to other alkane monooxygenases such as AlkB and P450. Interestingly, it is believed that linear alkanes are more easily degraded than branched-chain alkanes, but *Alcanivorax* bacteria effectively degrade branched alkanes [70]. Isoprenoid hydrocarbon (phytane) significantly stimulates P450(a) and alkB2 in *A. borkumensi*. In their study, [72] discovered that both pristane and phytane activate the expression of alkB1 and almA in *A. dieselolei*. Additionally, pristane was found to preferentially increase the expression of alkB1, P450-3, and almA in *A. hongdengensis* A-11-3 [64]. Although the metabolic mechanisms involved in reducing or oxidizing hydrocarbon molecules are not well understood, they likely play a role in these activities [73].

1.12. Importance of Genetic Markers in Microbial Degradation of Hydrocarbons

The first step in analyzing the environmental capacity for degradation is choosing the genes that will be used as biomarkers as observed by [74]. In environmental investigations conducted all around the world, a membrane monooxygenase (AlkB) that facilitates the conversion of the alkane into the appropriate alcohol is the chosen target. Moreover, aliphatic hydrocarbons can be broken down by a number of microbes, including *Pseudomonas putida* GPo1, the model for alkane metabolism [75]. Based on established research results, various microbial populations found in various environmental compartments have demonstrated the ability to use various enzyme-mediated mechanisms to degrade low and high molecular weight hydrocarbons such as and its respective families as an energy source (catabolic genes) [76]. It is of utmost importance to identify the presence of active microorganisms possessing the necessary catabolic genes to effectively facilitate the remediation process prior to commencing bioremediation at a contaminated site [77]. The identification of specific degradative bacteria, along with the type and location of these genes, will provide valuable guidance for enhancing the bioremediation process. Due to the diverse

and intricate nature of hydrocarbons, the breakdown of each component is initiated by a distinct enzyme. For example, the nahAC gene is responsible for the degradation of naphthalene, while alkB is responsible for alkanes and phnAC facilitates the breakdown of phenanthrene.

1.13. Diversity and Distribution of Alkane Hydroxylase Enzymes

According to [62], the breakdown of bacterial n-alkanes typically involves several distinct enzyme systems with different substrate chain lengths and reaction mechanisms. For instance, alkane 1-monooxygenases, also known as particulate or membrane-associated non-heme iron alkane hydroxylases, oxidize substrates ranging from C5 to C16. These hydroxylases, referred to as "alkB," are present in various bacterial species. Additionally, three genes alkB (encoding the active alkane hydroxylase), alkG, and alkT (responsible for rubredoxin and rubredoxin reductase, respectively) are responsible for their production [78]. Despite some variations, the alkB gene sequences exhibit sufficient conservation, allowing the development of broad-spectrum PCR primers that yield amplicons with characteristic histidine motifs [79]. Numerous studies in molecular ecology have utilized the conservation of the alkB sequence to assess the distribution and diversity of alkane-degrading bacteria in hydrocarbon-contaminated soils and sediments [62]. However, only a limited number of investigations have explored alkane degraders in aquatic environments [80].

1.14. Methane Monooxygenases (MMO)

Soluble methane monooxygenase (sMMO) and copper-containing particulate methane monooxygenase (pMMO) are two types of alkane hydroxylases that have a wide global distribution and can oxidize hydrocarbons ranging from C1 to C8 [82]. While the integral membrane-bound AlkB enzyme introduces oxygen to alkanes with chain lengths from C5 to C16, other enzymes such as cytochrome P450, LadA, or AlmA

utilize oxygen for alkanes greater than C20, occasionally even accepting medium-chain alkanes as substrates [83]. AlkB is highly sought after for the synthesis of industrially important chemicals, including secondary metabolites, steroids, polyketides, medicinal compounds, cosmetics, fragrances, and agrochemical intermediates [85]. It also shows promise in bioremediation, biotransformation, and the conversion of petroleum waste into activated intermediates. Depending on specific requirements, AlkB can be used either in whole-cell or partially purified form for biotransformation processes. [85]

Conducted research on AlkB using recombinant production of the enzyme in Escherichia coli for the region-selective synthesis of 2, 2, 3, and 4, 4-difluorooctan-1-ols from readily available starting materials in a whole-cell biotransformation system. However, the use of whole cells for oxidation is challenging due to the delayed uptake of lipophilic substrates, which can lead to the production of hazardous chemicals and poor oxygen transfer rates [87].

Several bacterial strains, including *Burkholderia*, *Pseudomonas*, *Acinetobacter*, *Alcanivorax*, and *Rhodococcus*, as well as a few fungal strains such as *Aspergillus* sp., possess the alkane hydroxylase system [88,89]. Overexpression of genes from gram-positive and gram-negative bacteria in alternative hosts has been employed to achieve the overproduction of AlkB, with rubredoxin and rubredoxin reductase identified as crucial cofactors [90]. Similarly, [89,91] demonstrated increased AlkB specific activity in *Alcanivorax borkumensis* and *Aspergillus* sp. RFC-1 through medium engineering and the selection of appropriate carbon sources.

1.15. Particulate Methane Monooygenases (pMMO)

It was observed that a bacterial strain that express propane or butane monooxygenases (BMOs), which are similar to pMMO or sMMO, degrade other gaseous alkanes. Recently, a newly identified bacterium called *Gordonia* sp. strain reported by [7] has been found to utilize propane and n-alkanes with carbon

chains ranging from C13 to C22 as its sole carbon source for growth. The prmABCD gene cluster encodes the components of a potential dinuclear-iron-containing multicomponent monooxygenase, which has been cloned and sequenced. It has been observed that mutants with disrupted prmABCD genes are unable to grow on propane, indicating the essential role of prmABCD gene products in the bacterium's ability to oxidize propane.

1.16. Butane Monooxygenases (BMO) and Soluble Monooxygenases (SMO)

Prior to the discovery made by [92], there was no evidence indicating that BMO is capable of oxidizing methane, despite its significant similarity to sMMO. It is believed that the release of products during the oxidation process is limited by the geometric constraints within the active site. This constraint was evident when the accumulation of methanol completely hindered methane oxidation in the wild-type strain of *T. butanivorans*. In an attempt to alleviate this limitation, specific amino acid changes were introduced to the BMOH subunit, resulting in a mutated version of the enzyme that partially mitigated this restriction. As a consequence, the modified enzyme exhibited reduced sensitivity to methanol, allowing it to tolerate high concentrations of up to 83M. Additionally, the transcriptional activity of the BMO promoter can be reduced by 10M propionate, indicating its influential role as a regulator of BMO expression [93,94].

1.17. The *AlkB* Family

The alkane degradation system of *Pseudomonas putida* GPo1 has been extensively researched and is known for its ability to convert n-alkanes ranging from C5 to C12 into alkanols, as reported by [98,99]. Subsequently, the alkanol products are sequentially transformed into the corresponding aldehydes, carboxylic acids, and acyl-coenzyme A (CoAs), which enter the oxidation pathway [95]. According to [95,96], the alk system is also capable of catalyzing various reactions, including the

hydroxylation of linear and branched aliphatic, alicyclic, and alkyl aromatic compounds, the demethylation of branched methyl-ethers, and the epoxidation of terminal olefins. Furthermore, this enzyme system has been associated with the degradation of gaseous poisons such as propane and butane [97].

The *Pseudomonas putida* GPo1 alkane hydroxylase system consists of three enzymes: rubredoxin (AlkG), rubredoxin reductase (AlkB), and alkane hydroxylase (AlkT). AlkB, an integral membrane protein without heme iron, is responsible for catalyzing the hydroxylation reaction. It also serves as a supplier of electrons from reduced nicotinamide to the flavoprotein rubredoxin reductase. Rubredoxin, a small red iron-sulfur protein, transfers reducing equivalents to AlkB. Despite the lack of precise structural information and comprehensive metabolic data for AlkB with a wide range of substrates, a topological model has provided some insight into the relationship between its structure and function. AlkB is predicted to consist of six alpha-helical transmembrane segments that likely form a hydrophobic pocket (van Beilen et al., 1992). In the cytoplasm, the N-terminus, two hydrophilic loops, and a significant C-terminal region are located, while the periplasm is only exposed to three small loops adjacent to amino acid sites 62, 112, and 251.

1.18. Cytochrome P450

Cytochrome P450 enzymes, referred to as terminal monooxygenases, have been discovered in various forms of life, encompassing both prokaryotes like *Pseudomonas putida* and eukaryotes like yeasts, as stated by [100]. Their broad range of substrates, which includes fatty acids, steroids, prostaglandins, and numerous exogenous substances such as medications, anesthetics, organic solvents, ethanol, alkyl aryl hydrocarbon compounds, pesticides, and carcinogens, is a result of their ubiquitous presence in nature, as described by [101]. These enzymes can be categorized into two classes based on variations in the components of the P450 monooxygenase systems.

Class I P450s are exemplified by those isolated from bacteria and mitochondria. They rely on an FAD-containing reductase that receives electrons from NAD(P)H, as well as an iron-sulfur protein that facilitates electron transfer from NAD(P)H to the substrate-bound P450 enzyme. In this process, electrons are shuttled from the reductase to the P450 enzyme. On the other hand, class II P450s are found in microsomal drug-metabolizing forms and obtain electrons from NAD(P)H through a cytochrome P450 reductase containing FAD and flavin mononucleotide (FMN), as explained by [95].

Bacterial P450 enzymes are typically soluble, while those from yeast and mammals are usually membrane-bound, making them more challenging to study. Despite the identification of over 4,000 P450 enzymes, the number of P450s capable of degrading n-alkanes remains relatively low, as noted by [95]. Among these enzymes, two notable examples are cytochrome P450 from *Pseudomonas putida*, which requires putidaredoxin and putidaredoxin reductase to transfer electrons from NADH for the oxidation of camphor to 5-exo-hydroxycamphor from *Bacillus megaterium*, which hydroxylates medium-chain saturated fatty acids ranging from C12 to C18 [102].

1.19. Long Chain Alkane Monooxygenase (*LadA*)

Several bacterial species, as reported by [103], possess the ability to utilize n-alkanes with carbon chains longer than 20 atoms. In some thermophilic bacteria such as *Geobacillus thermodenitrificans*, a terminal oxidation pathway involving LadA enzyme converts long-chain n-alkanes into corresponding primary alcohols, as reported by [40]. The well-characterized pathway consists of LadA, the primary initiating enzyme, two alcohol dehydrogenases (ADH1 and ADH2) which are responsible for the conversion of alkyl alcohols to alkyl aldehydes, and an aldehyde dehydrogenase (ALDH) that converts alkyl aldehydes to fatty acids, as described by [105].

Interestingly, LadA was discovered to belong to the SsuD subfamily of the bacterial luciferase family, establishing a

significant structural link, as noted by [3]. The LadA structure exhibits a triosephosphate isomerase (TIM) barrel fold, albeit with five expanded insertion regions (IS1-5) and an elongation at the C-terminus, distinguishing it from the conventional TIM barrel-like structure [3]. Within the LadA structure, a pocket at the C-terminal entry of the TIM barrel accommodates a FMN molecule, oxygen, and a portion of the terminal of a long-chain n-alkane. The enzymatic role of LadA as a flavoprotein monooxygenase, utilizing dioxygen to introduce an oxygen atom into the substrate, has been established, as reported by [40].

1.20. Long Chain Alkane Hydroxylase

The majority of research on the degradation of long-chain alkanes has primarily focused on the genetic aspect, with limited exploration of the associated enzymes. However, some studies have reported the presence of a dioxygenase enzyme in some *Acinetobacter* sp. that exclusively utilizes n-alkanes with 10 to 30 carbon atoms as its carbon and energy source. The activity of this enzyme requires FAD and Cu^{2+} and follows the Finnerty pathway (Figure 4.2)[106]. Another study has shown that some strains of *Acinetobacter haemolyticus* can utilize long-chain n-alkanes ranging from C16 to C35 through a mono-terminal oxidation route, which sets it apart from other known hydrocarbon-degrading *Acinetobacter* spp. [107].

These findings highlight the significance of the alkM gene-encoded non-heme iron integral membrane alkane hydroxylase as the primary enzyme in the mono-terminal oxidation pathway. *Acinetobacter* sp. strain DSM 17874 is capable of utilizing C10-C40 n-alkanes as a carbon source, with long-chain alkanes (>C32) being metabolized by a putative flavin-binding monooxygenase encoded by the almA gene. Interestingly, the same strain employs a different enzyme system for the degradation of shorter alkanes, as an almA-deficient mutant can still thrive using C24 and shorter alkanes as the sole carbon source [95].

In a separate study, [72] successfully amplified the almA gene from the marine alkane degrader *Alcanivorax dieselolei* using highly degenerate primers. The almA gene exhibited a substrate range of n-alkanes from C22 to C36, complementing the strain's alkB and p450 gene repertoires. Studies employing similar techniques have demonstrated that almA plays a crucial role in the degradation of long-chain alkanes in the marine environment and is more commonly found in marine hydrocarbon-degrading bacteria [64].

2. Conclusion

Microbial enzymes, among which are alkane hydroxylase, at optimum environmental conditions can facilitate the transformation of specific pollutants through various enzymatic processes. Similarly, the field of waste management can be enhanced through bioengineered microorganisms and some important metabolic products emanating from them.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Chen, S. and Zhong, M. *Bioremediation of Petroleum-Contaminated Soil. In Environmental Chemistry and Recent Pollution Control Approaches*; Intech Publishers: London, UK, 2019.

[2] Jia, J.; Zong, S.; Hu, L.; Shi, S.; Zhai, X.; Wang, B.; Li, G.; Zhang, D. The dynamic change of microbial communities in crude oil-contaminated soils from oil fields in China, 2017; *Soil Sediment Contam. Int. J.* 26, 171–183.

[3] Li, C, Cui C, Zhang J, Shen J, He B, Long Y. and Ye, J. Biodegradation of petroleum hydrocarbons-based pollutants in contaminated soil by exogenous effective microorganisms and indigenous microbiome. *Ecotoxicology and Environmental Safety*, 2023; 253(15), 114673

[4] Essien, N. E., Ubuoh E. A. and Ogwo P. A. Screening Degrading of Hydrocarbon Bacteria and Fungi from Waste Motor Engine Oil Contaminated Soil, their Distribution Frequency and Hydrocarbon Utilization Potentials, *J. Mater. Environ. Science*, 2023; 14(3), 293-305.
[5] Somee, M.R., Amoozegar, M.A., Dastgheib, S.M.M. *et al.* Genome-resolved analyses show an extensive diversification in key aerobic hydrocarbon-degrading enzymes across bacteria and archaea. *BMC Genomics,* 2022; **23**, 690 https://doi.org/10.1186/s12864-022-08906-w
[6] Zhang, Y. Hsu, H.-H.; Wheeler, J.J., Tang, S. and Jiang, X. Emerging investigator series: Emerging biotechnologies in wastewater treatment: From biomolecular engineering to multiscale integration. *Environ. Sci. Water Res. Technol*, 2020; 6, 1967–1985.
[7] Pandolfo, E. Barra Caracciolo, A. and Rolando, L. Recent Advances in Bacterial Degradation of Hydrocarbons. *Water*, 2023; 15, 375. https://doi.org/10.3390/w15020375
[8] Adedeji, J.A.; Tetteh, E.K.; Opoku Amankwa, M.; Asante-Sackey, D0.; Ofori-Frimpong, S.; Armah, E.K.; Rathilal, S.; Mohammadi, A.H. and Chetty, M. Microbial Bioremediation and Biodegradation of Petroleum Products—A Mini Review. *Appl. Sci*, 2022; 12, 12212. https://doi.org/10.3390/app122312212
[9] Urgun-Demirtaş, M.; Stark, B.; Pagilla, K. Use of Genetically Engineered Microorganisms (GEMs) for the Bioremediation of Contaminants. *Crit. Rev. Biotechnol*, 2006; 26, 145–164.
[10] Varjani, S.J. and Upasani, V.N. A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. *Int. Biodeterior. Biodegrad*ation, 2017; 120, 71–83
[11] Jaiswal, S. and Shukla, P. Alternative Strategies for Microbial Remediation of Pollutants via Synthetic Biology. *Frontiers in Microbiology*, 2020; 11, 808. (CrossRef) (PubMed)
[12] Thacharodi, A., Hassan, S., Singh, T., Mandal, R., Khan, H. A., Hussain, M. A. and Pugazhendhi, A. Bioremediation of polycyclic aromatic hydrocarbons: An updated microbiological review. *Chemosphere*, 2023; 138498.

[13] Smits, T. H. M., Balada, S. B., Witholt, B., and Van Beilen, J.B. Functional analysis of alkane hydroxylases from Gram- negative and Gram-positive bacteria. *Journal of Bacteriology,* 2002; 184, 1733–1742
[14] Harayama, S., Kasai, Y., and Hara, A. Microbial communities in oil-contaminated seawater. *Curr. Opin. Biotechnology,* 2004; 15, 205–214.
[15] Yakimov, M.M., Timmis, KN. And Golysin, PN. Obligate oil degrading bacteria. *Current Opinion in Biotechnology*, 2007; 18(3): 257-266 doi: 10.1016/j.copbio.2007.04.006.epub
[16] Rodriguez-r LM, Overholt WA, Hagan C, Huettel M, Kostka JE, Konstantinidis KT. Microbial community successional patterns in beach sands impacted by the Deepwater Horizon oil spill. The ISME journal. 2015 Sep;9(9):1928-40.
[17] Hatice O., Ferhat Kantar, Burak Alaylar, Yasemin Numanoğlu Çevik and Medine G. Isolation and Characterization of Hydrocarbon and Petroleum Degrading Bacteria from Polluted Soil with Petroleum and Derivatives by MALDI-TOF MS Method, *Geomicrobiology,* 2022; DOI: http://dx.doi.org/10.1080/01490451.2022.2074575
[18] Yang, N., Tian, C., Lv, Y., Hou, J., Yang, Z., Xiao, X. and Zhang, Y. Novel primers for 16S rRNA gene-based archaeal and bacterial community analysis in oceanic trench sediments. *Applied Microbiology and Biotechnology*, 2022; *106*(7), 2795-2809.
[19] Nurye, M. and Wolkero, T. Identification of Lactic Acid Bactria in Dairy Products using Culture-independent Methods: A Review. *Asian Journal of Dairy and Food Research*, 2023; *42*(1), 1-8.
[20] Chen, N. T., Tam, L. M., Wu, J. H., Cheong, N. S., Lin, C. Y., Tseng, C. C. and Su, H. J. Changes in ambient bacterial community in Northern Taiwan during long-range transport: Asian dust storm and frontal pollution. *Atmosphere*, 2022; *13*(5), 841.
[21] Nencioni, A., Pastorelli, R., Bigiotti, G., Cucu, M. A. and Sacchetti, P. Diversity of the Bacterial Community Associated with Hindgut, Malpighian Tubules, and Foam of Nymphs of

Two Spittlebug Species (Hemiptera: Aphrophoridae). *Microorganisms*, 2023; *11*(2), 466.
[22] Kobras, C.M., Fenton, A. K. and Sheppard, S.K. Next-generation microbiology: from comparative genomics to gene function. *Genome Biology*, 2021; **22**: 123 https://doi.org/10.1186/s13059-021-02344-9
[23] Ganesh, Kumar, A.; Mathew, N.C.; Sujitha, K.; Kirubagaran, R. and Dharani, G. Genome analysis of deep sea piezotolerant *Nesiotobacter exalbescens* COD22 and toluene degradation studies under high pressure condition. *Sci. Rep*. 2019; 9, 18724.
[24] Genovase et al. 2014
[25] Schneiker, S., Martins dos Santos, V. A., Bartels, D., Bekel, T., Brecht, M., Buhrmester, J., et al. Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nat. Biotechnology*, 2006; 24, 997–1004.
[26] Sanchez-Salas JL, Gutierrez MR, Bandala ER. Aerobic treatment of petroleum industry effluents. In Current Developments in Biotechnology and Bioengineering, 2017; Jan 1 (pp. 79-102). Elsevier.
[27] Kasai, Y. Kishira, H. and Harayama, S. Bacteria belonging to the genus cyclolasticus play a primary role in degradation of HC released in marine environment. *Applied Env. Microbiology*, 2002; doi.101128/aem.68.11.5625-5633.2002
[28] Benibo N., Obire O, Douglas SI and Nrior RR. Hydrocarbonoclastic Bacteria and Polycyclic Aromatic Hydrocarbon Profile of Surface Water in Borikiri Wetlands, Port Harcourt, Nigeria". *Acta Scientific Microbiology,* 2023; 6.3 (2023): 41-45.
[29] Olajire, A. A., and J. P. Essien. "Aerobic degradation of petroleum components by microbial consortia." *Journal of Petroleum & Environmental Biotechnology,* 2014; 5.5: 1.
[30] Santisi S, Cappello S, Catalfamo M, Mancini G, Hassanshahian M, Genovese L, Giuliano L, Yakimov MM. Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium. *Brazilian Journal of Microbiology,* 2015; 46:377-87.

[31] Adams GO, Fufeyin PT, Okoro SE, Ehinomen I. Bioremediation, biostimulation and bioaugmention: a review. *International Journal of Environmental Bioremediation & Biodegradation*, 2015; 3(1):28-39.
[32] Joutey, N.T.; Bahafid, W.; Sayel, H. and El Ghachtouli, N. *Biodegradation: Involved Microorganisms and Genetically Engineered Microorganisms. In Biodegradation-Life of Science;* Intech Publishers: London, UK, 2013; Volume 14, pp. 289–320. (CrossRef)
[33] Karamalidis, A.K.; Evangelou, A.C.; Karabika, E.; Koukkou, A.I.; Drainas, C. and Voudrias, E.A. Laboratory scale bioremediation of petroleum-contaminated soil by indigenous microorganisms and added *Pseudomonas aeruginosa* strain Spet. *Bioresour. Technology*, 2010; Doi: 101, 6545–6552.
[34] McGenity, T.J. *Taxonomy, Genomics and Ecophysiology of Hydrocarbon-Degrading Microbes* Springer; 2019.
[35] Xue, J., Yu, Y., Bai, Y., Wang, L. and Wu, Y. Marine oil-degrading microorganisms and biodegradation process of petroleum hydrocarbon in marine environments: a review. *Current Microbiology*, 2015; 71(2), 220-228. doi: 10.1007/s00284-015-0825-7
[36] Chikere, C.B., Okpokwasili, G.C. and Chikere, B.O. Monitoring of microbial hydrocarbon remediation in the soil. *3 Biotech*, 2011; 1(3), 117-138. doi: 10.1007/s13205-011-0014-8
[37] Abbasian, F., Lockington, R., Mallavarapu, M. and Naidu, R. A comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. *Applied Biochemistry and Biotechnology*, 2015; 176(3), 670-699. doi: 10.1007/s12010-015-1603-5
[38] Salim, A., Khushboo, K. and Durgeshwer, S. Different strategies and bio-removal mechanisms of petroleum hydrocarbons from contaminated sites, *Arab Gulf Journal of Scientific Research*, 2023*;* https://www.emerald.com/insight/1985-9899.htms
[39] Adams and Philip, 2005
[40] Chunyan, X., Qaria, M. A., Qi, X. and Daochen, Z. (2023). The role of microorganisms in petroleum degradation: Current development and prospects. *Science of Total Environment, 865*, 161112.

[41] Doukani, K., Boukirat, D., Boumezrag, A., Bouhenni, H., & Bounouira, Y. Biodegradation of Pollutants. In *Handbook of Biodegradable Materials*, 2023; (pp. 899-925). Cham: Springer International Publishing.
[42] Baboshin, M.A. and Golovleva, L.A. Aerobic bacterial degradation of polycyclic aromatic hydrocarbons (PAHs) and its kinetic aspects. *Microbiology*, 2012; 81(6), 639-650. doi: 10.1134/S0026261712060021
[43] Fuchs G, Boll M, Heider J. Microbial degradation of aromatic compounds—from one strategy to four. *Nature Reviews Microbiology*. 2011; 9(11):803-16.
[44] Haritash, A.K. and Kaushik, C.P. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. *Journal of Hazardous Materials*, 2009; 169(1), 1-15. http://dx.doi.org/10.1016/j.jhazmat.2009.03.137 https:// doi. org/ 10. 1016/j. ijbio mac. 2018. 01. 177
[45] da Silva Gonçalves, A. H., de Andrade, F. P., De Farias Silva, C. E., Medeiros, J. A., Santos, G. K. S., do Nascimento, M. A. A., ... and Almeida, R. M. R. G. Biological treatment of petroleum produced water ex situ using microorganisms: an overview, main developments and challenges. *Energy, Ecology and Environment*, 2023; 1-15.
[46] Harindintwali, J. D., Xiang, L., Wang, F., Chang, S. X., Zhao, Z., Mei, Z. and Tiedje, J. M. Syntrophy of bacteria and archaea in the anaerobic catabolism of hydrocarbon contaminants. *Critical Reviews in Environmental Science and Technology*, 2023; *53*(13), 1331-1357.
[47] Mackenstock and Moutaki, 2011
[48] Mackenstock et al, 2016
[49] Jiménez N, Richnow HH, Vogt C, Treude T, Krüger M. Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. *Microbial Physiology*, 2016; 10; 26(1-3):227-42.
[50] Zhang et al 2022
[51] Laczi, K. Erdeiné Kis Á, Szilágyi Á, Bounedjoum N, Bodor A, Vincze GE, Kovács T, Rákhely G and Perei K. New Frontiers of Anaerobic Hydrocarbon Biodegradation in the Multi-Omics Era. *Frontiers in Microbiology*, 2020; 11:590049. doi: 10.3389/fmicb.2020.590049

[52] Morris BE, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. *FEMS microbiology reviews*, 2013; 1;37(3):384-406.
[53] Madondo, N. I., Rathilal, S., Bakare, B. F. and Tetteh, E. K. Application of Bioelectrochemical Systems and Anaerobic Additives in Wastewater Treatment: A Conceptual Review. *International Journal of Molecular Sciences*, 2023; *24*(5): 4753.
[54] Mahjoubi M, Cappello S, Souissi Y, Jaouani A, Cherif A. Microbial bioremediation of petroleum hydrocarbon–contaminated marine environments. Recent insights in petroleum science and engineering. 2018 Feb 7; 325:325-50.
[55] Breedveld GD, Sparrevik M. Nutrient-limited biodegradation of PAH in various soil strata at a creosote contaminated site. *Biodegradation*, 2000; 11:391-9.
[56] Margesin R, Schinner F. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied microbiology and biotechnology*, 2001; 56:650-63.
[57] Boopathy R. Factors limiting bioremediation technologies. *Bioresource technology*, 2000; 1;74(1):63-7.
[58] Shallu et al 14
[59] Leahy JG, Colwell RR. Microbial degradation of hydrocarbons in the environment. *Microbiological reviews*, 1990; 54(3):305-15.
[60] Bamforth SM, Singleton I. Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. Journal of Chemical Technology & Biotechnology: *International Research in Process, Environmental & Clean Technology*, 2005; 80(7):723-36.
[61] Ghosal D, Ghosh S, Dutta TK, Ahn Y. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. *Frontiers in microbiology*, 2016; 1369.
[62] Beilen, J. B. van, & Funhoff, E. G. *Expanding the alkane oxygenase toolbox: new enzymes and applications. Current Opinion in Biotechnology*, 2005; *16(3), 308–314.* doi: 10.1016/j.copbio.2005.04.005

[63] Austin RN, Groves JT. Alkane-oxidizing metalloenzymes in the carbon cycle. *Metallomics.* 2011; 3(8):775-87.
[64] Wang, W. and Shao, Z. Genes involved in alkane degradation in the *Alcanivorax hongdengensis* strain A-11-3. *Appl. Microbiol. Biotechnology*, 2012*;* 94:437–448. https:// doi. org/ 10. 1007/ s00253- 011- 3818-x
[65] Mujumdar, S, Joshi P. and Klarve N. Production, characterization, and applications of bioemulsifiers (BE) and biosurfactants (BS) produced by Acinetobacter spp.: a review. *J Basic Microbiology, 2019*;59:277–87 Wiley Online Library.
[66] Wang and Shao 2012a
[67] Feng, L., Wang, W., Cheng, J., Ren, Y., Zhao, G., Gao, C., et al. Genome and proteome of long- chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc. Natl. Acad. Sci. U.S.A*, 2007*;* 104, 5602–5607.
[68] Li, L., Liu, X., Yang, W., Xu, F., Wang, W., Feng, L., et al. Crystal structure of long chain alkane monooxygenase (LadA) in complex with coenzyme FMN: unveiling the long-chain alkane hydroxylase. *Journal of Mol. Biol.* 2008; 376, 453–465.
[69] Pirnik et al 74
[70] Hara, A., Syutsubo, K., and Harayama, S. *Alcanivorax* which prevails in oil-contaminated sea water exhibits broad substrate specificity for alkane degradation. *Environ. Microbiology,* 2003; 5, 746–753.
[71] Schneiker, S., Martins dos Santos, V. A., Bartels, D., Bekel, T., Brecht, M., Buhrmester, J., et al. Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nat. Biotechnology*, 2006; 24, 997–1004
[72] Liu PW, Chang TC, Whang LM, Kao CH, Pan PT, Cheng SS. Bioremediation of petroleum hydrocarbon contaminated soil: effects of strategies and microbial community shift. *International Biodeterioration & Biodegradation*, 2011; 1; 65(8):1119-27.

[73] Watkinson, R.J. and Morgan, P. Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegradation,* 1990; 1: 79–92.
[74] Lanfranconi MP, Alvarez HM. Molecular Markers in Hydrocarbon Degradation: State of the Art and Prospective in South America. Bioremediation in Latin America: *Current Research and Perspectives,* 2014; 20:193-208.
[75] Fenibo, E. O., Selvarajan, R., Abia, A. L. K., and Matambo, T. Medium-chain alkane biodegradation and its link to some unifying attributes of alkB genes diversity. *Science of The Total Environment*, 2023; *877*, 162951.
[76] Onwujekwe, E. C., Ogbonna, J. C., Eze, C. C. and Eze, C. N. Biodegradation and Impact of Polycyclic Aromatic Hydrocarbons in the Environment: A Review. *Journal of Advances in Biology & Biotechnology*, 2022; *25*(9), 35-47.
[77] Ehis-Eriakha, C.B. Stephen Eromosele Akemu, Simon Obgaji Otumala and Chinyere Augusta Ajuzieogu. Biotechnological Potentials of Microbe Assisted Eco-Recovery of Crude Oil Impacted Environment, New Technologies and Recent Approaches, *IntechOpen*, 2021; DOI: 10.5772/intechopen.98808
[78] Cappelleti et al. 2011
[79] Smits, T. H. M., Balada, S. B., Witholt, B., and Van Beilen, J.B. Functional analysis of alkane hydroxylases from Gram- negative and Gram-positive bacteria. *Journal of Bacteriology,* 2002; 184, 1733–1742.
[80] Wasmund, K., Burns, K.A., Kurtböke, I. and Bourne, D.G. Novel alkane hydroxylase gene (*alkB*) diversity in sediments associated with hydrocarbon seeps in the Timor Sea, Australia. *Appl. Environ. Microbiology,* 2009; 75, 7391–7398.doi: 10.1128/AEM.01370-09
[81] Wang, L., Wang, W., Lai, Q., and Shao, Z. Gene diversity of CYP153A and AlkB alkane hydroxylases in oil-degrading bacteria isolated from the Atlantic Ocean. *Environ. Microbiology,* 2010a 12, 1230–1242.doi:10.1111/j.1462-2920.2010.02165.x
[82] Carter, J. H., Dummer, N. F., Chow, Y. K., Williams, C., Nasrallah, A., Willock, D. J., ... and Taylor, S. H. The Selective Oxidation of Methane to Oxygenates Using Heterogeneous

Catalysts. *Heterogeneous Catalysis for Sustainable Energy*, 2022; 183-201.
[83] Aliakbari, E, Tebyanian H, Hassanshahian M. and Kariminik A. Degradation of alkanes in contaminated sites. *Int J Adv Biol Biomed Resource,* 2014; 2:1620–1637
[84] Xu, J., Liu H, Liu J. and Liang, R. Isolation and characterization of *Pseudomonas aeruginosa* strain SJTD-2 for degrading long-chain n-alkanes and crude oil. A*cta Microbiologica Sinica,* 2015; 55:755–763
[85] Ramu, R., Chang, C.W., Chou, H.H., Wu LL, Chiang CH and Yu, S.S.F. (2012). Erratum: Regio-selective hydroxylation of gem-difluorinated octanes by alkane hydroxylase (AlkB) (Tetrahedron Letters (2011) 52(23) 2950-2953). *Tetrahedron Letters*, 2012; 53:5458. https:// doi. org/ 10. 1016/j. tetlet. 2012. 07. 099
[86] Das and Negi 2022
[87] Ayala, M. and Torres, E. Enzymatic activation of alkanes: constraints and prospective. *Appl Catalysis,* 2004*;* A-Gen 272:1–13. https:// doi. org/ 10. 1016/j. apcata. 2004. 05. 046
[88] Nie, Y., Chi CQ, Fang H, Liang JL, Lu SL, Lai GL, Tang Y.Q. and Wu, X.L. Diverse alkane hydroxylase genes in microorganisms and environments. *Scientific Reports*, 2014; 4:1–11. https:// doi. org/ 10. 1038/ srep0 4968
[89] Kadri T, Rouissi T, Magdouli S, Brar SK, Hegde K, Khiari Z, Daghrir R, Lauzon J.M. Production and characterization of novel hydrocarbon degrading enzymes from *Alcanivorax borkumensis*. *Int J Biol Macromolecules,* 2018; 112:230–240.
[90] Luo, Q, He Y, Hou DY, Zhang JG, Shen X.R. GPo1 *alkB* gene expression for improvement of the degradation of diesel oil by a bacterial consortium. *Brazillian Journal of Microbiology*, 2015; 46:649–657. https:// doi. org/ 10. 1590/ S1517- 83824 63201
[91] Al-Hawash, A.B, Zhang J, Li S, Liu J, Ghalib HB, Zhang X. and Ma F. Biodegradation of n-hexadecane by *Aspergillus* sp. RFC-1 and its mechanism. *Ecotoxicol Environ Safet*, 2018; 164:398–408. https:// doi. org/ 10. 1016/j. ecoenv. 2018. 08. 049
[92] Halsey, K.H., Sayavedra-Soto, L.A., Bottomley, P.J., and Arp, D.J. Site-directed amino acid substitutions in the

hydroxylase alpha sub-unit of butane monooxygenase from *Pseudomonasbutanovora*: implications for substrate knocking at the gate. *Journal of Bacteriology*, 2006; 188: 4962– 4969.
[93] Al-Luaibi, Y. Y. *Molecular genetics and microbiology of bioremediation using methane-oxidising bacteria*. Sheffield Hallam University (United Kingdom) 2015.
[94] Ji et al 2013
[95] Throne-Holst, M., Wentzel, A., Ellingsen, T., Kotlar, H., and Zotchev, S. Identification of novel genes involved in long-chain n-alkane degradation by *Acinetobacter* sp. Strain DSM17874. *Appl. Environ. Microbiol*. 2007; 73,3327–3332.
[96] Van Beilen, J.B. and Funhoff, E.G. Alkane hydroxylases involved in microbial alkane degradation. *Appl Microbiol Biotechnology*, 2007; 74:13–21. https:// doi. org/ 10. 1007/ s00253- 006- 0748-0
[97] Johnson, E. L., and Hyman, M. R. Propane and *n*-butane oxidation by *Pseudomonas putida* GPo1. *Applied Environmental Microbiology*, 2006; 72, 950–952.
[98] Ji, Y., Mao, G., Wang, Y., and Bart- lam, M. Crystallization and preliminary X-ray characterization of an NAD (P)-dependent butanol dehydrogenase A from *Geobacillus thermodenitrificans* NG80-2. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 2013;* 69: 184–1
[99] Smits, T. H. M., Balada, S. B., Witholt, B., and Van Beilen, J.B. Functional analysis of alkane hydroxylases from Gram- negative and Gram-positive bacteria. *Journal of Bacteriology*, 2002; 184, 1733–1742
[100] Reineke, W., & Schlömann, M. Microbial Degradation of Pollutants. In *Environmental Microbiology*, 2023; 161-290. Berlin, Heidelberg: Springer Berlin Heidelberg.
[101] Bernhardt, R. Cytochromes P450 as versatile biocatalysts. *J. Biotechnology*, *2006;* 124, 128–145.
[102] Ravichandran, K. G., Boddupalli, S. S., Hasermann, C. A., Peterson, J. A., and Deisenhofer, J. Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450$^{\times}$s. *Science*, 1993; 261, 731–736.
[103] Liu J, Zhang AN, Liu YJ, Liu Z, Liu Y, Wu XJ. Analysis of the mechanism for enhanced pyrene biodegradation based on the interactions between iron-ions and Rhodococcus ruber strain

L9. *Ecotoxicology and Environmental Safety*, 2021; 1;225:112789.
[104] Ju et al 2023
[105] Feng, L., Wang, W., Cheng, J., Ren, Y., Zhao, G., Gao, C., et al. (2007). Genome and proteome of long- chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5602–5607.
[106] Maeng, J. H., Sakai, Y., Tani, Y., and Kato, N. Isolation and characterization of a novel oxyge- nase that catalyzes the first step of *n*-alkane oxidation in *Acinetobacter* sp. strain M-1. *J. Bacteriol,* 1996; 178, 3695– 3700.
[107] Bihari, Z., Szvetnik, A., Szabo, Z., Blastyak, A., Zombori, Z., Balazs, M., et al. Functional analysis of long-chain *n*-alkane degradation by *Dietzia* spp. *FEMS Microbiol. Letters,* 2011; 316, 100–107
[108] Wang, W. and Shao, Z. (2012) Genes involved in alkane degradation in the *Alcanivorax hongdengensis* strain A-11-3. *Appl Microbiol Biotechnol* 94:437–448. https:// doi. org/ 10. 1007/ s00253- 011- 3818

CHAPTER 5

Molecular Diagnosis of Infectious Diseases

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Molecular Diagnosis of Infectious Diseases

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Abstract

Infectious diseases are a major threat in developing countries and have high zoonotic potential and it is estimated that out of every 10 known infectious diseases in people more than 6 spread from animals (CDC) leading to huge financial losses to human population. Traditional methods employed for diagnosis of infectious diseases included studying pathogen morphology, its ability to grown on different media (selective and non-selective) and under different laboratory conditions, and also disease topography. Traditional methods are too slow having no direct influence on clinical management of a particular disease. Therefore, there is a need in the development of rapid diagnostic methods in animal disease diagnostics having speed, simplicity, sensitivity, specificity, reproducibility and low cost. Molecular methods are rapid diagnostic methods applicable for detection of the bacterial or viral or parasitic genome and are increasingly used nowadays. Molecular methods are more specific and standardized for the different microorganisms. Different molecular techniques have been developed over the time, the applicability of which vary for different laboratory set ups and also resources. The development of low-cost diagnostic techniques and their utility for disease diagnosis is of paramount

importance for control and management of infectious diseases. This chapter will provide an insight about the various molecular techniques used for diagnosis of infectious diseases.

Keywords: Infectious diseases, microorganism, diagnosis, molecular techniques, molecular diagnosis

1. Introduction

A quick and precise detection of pathogens responsible for infectious diseases viz. viruses, bacteria, fungi, protozoa, and helminths are vital for rational treatment of infections and their control [1]. Since the introduction of the PCR, several applications have been developed which has revolutionized the infectious disease diagnosis. Moreover, a number of applications with a more user-friendly, economical, and accurate profile have been included into normal diagnostic labs for routine use, benefitting millions of people worldwide [2]. Real time PCR has emerged as a new powerful tool allowing scientific technology to go from reference centers to mainstream field laboratories. Its ability to rapidly detect and quantify bioload of various pathogens has made it a go to test particularly during emergency situations [3]. Nucleic acid testing methods are very specific and highly sensitive and are based on the detection of specific DNA or RNA sequences from organisms and can be either amplified or nonamplified type [4]. The DNA-labeled or RNA-labeled probes bind to the nucleic acid and degenerate a signal in case of nonamplified method while amplified method allows the use of several different types of post amplification technologies for further characterization of the amplified targets [5]. Molecular methods are rapid diagnostic methods applicable for detection of the bacterial or viral or parasitic genome and are increasingly used nowadays. Molecular methods are more specific and standardized for the different microorganisms. Different molecular techniques have been developed over the time, the applicability of which vary for different laboratory set ups and also resources. The development of cheaper diagnostic techniques and effective vaccines has been used for disease control.

1.1. Polymerase Chain Reaction (PCR)

PCR was developed by Kary Banks Mullis in 1985 and was awarded Nobel Prize in chemistry in 1993. It involves the amplification of a single or some copies of a piece of DNA, generating billions copies of a particular DNA sequence. The PCR-technique involves repeated cycling process (heating and cooling) of the reaction mixture for DNA melting and subsequent enzymatic replication of the DNA [6]. A heat-stable DNA polymerase called Taq polymerase, an enzyme purified from the bacterium *Thermus aquaticus*, is used in nearly all PCR applications. DNA analysis using a one-tube technique is quick, accurate, and reproducible with little chance of sample contamination [7, 8].

1.2. PCR Reagents

- 1X Buffer: 10mM Tris-HCl, 50mM KCl
- MgCl2: 1mM - 4mM (1.5mM)
- dNTPs: 200μM
- Primers: 100nM-1μM, 200nm (or less) for real time analysis
- DNA polymerase: Taq DNA polymerase (1-4 Units)
- DNA: 10pg-1μg (20ng)

1.3. PCR Cycling Conditions

Steps	Temperature	Time
Initial denaturation	90-95 ℃	1-3 min
Denaturation	90-95 ℃	0.5 – 1 min
Primer annealing	45-65 ℃	0.5 – 1 min
Primer extension	70-75 ℃	0.5 – 2 min
Final extension	70-75 ℃	0.5 – 10 min
Stop reaction	4 ℃ (or 10 nM EDTA)	Hold

1.4. Other PCR Variants

*** Reverse Transcription PCR (RT-PCR):** Reverse Transcriptase PCR, often known as RT-PCR, is a type of

polymerase chain reaction used to measure RNA expression levels. It is a laboratory technique which uses mRNA as the starting template instead of DNA. The process involves converting mRNA template to complementary DNA (cDNA) using enzyme reverse transcriptase following which the single stranded DNA is converted by DNA polymerase to double-stranded DNA, and these act as templates for PCR amplification [9]. The reverse transcription step is very vital for sensitive and accurate quantification of RNA and any lapse in this step will affect the outcome. The quantity of complementary DNA (cDNA) that the reverse transcriptase generates must properly reflect the quantity of RNA input. RT reactions are typically conducted between 40°C and 50°C with random hexamer or oligodT primers as well as specialised primers as a primer set. RT-PCR is one of the important molecular diagnostic tools with wide applicability in the field of agriculture, medicine and biotechnology owing to high sensitivity for the detection and quantification of mRNA from various types of samples. It is particularly very important for diagnosis of important viral pathogens of both humans and animals as it is sensitive, highly specific with ability of quick detection and quantification of viral RNA's [10, 11].

*** Colony PCR:** Colony PCR is a quick and easy, high-throughput technique to verify whether cloning was successful and whether DNA insert is present in plasmid constructions or not. It is crucial for checking yeast or bacterial clones for the proper ligation or plasmid products.

*** Nested PCR:** Nested PCR is a modification designed to improve sensitivity and specificity of conventional PCR. It involves using two primer sets and two sequential PCR reactions wherein the products of first PCR reaction serve as template for second amplification. Due to the binding of two different sets of primers to the identical target template, the reaction's specificity increases [12, 13].

* **Multiplex PCR:** Multiplex PCR is a modification of the conventional PCR having the ability to amplify two or more

target sequences in a single reaction tube. It has wide applicability in various areas of DNA testing like analysis of gene deletions, mutations and polymorphism analysis, quantitative analysis etc [14].

* **Hot start PCR:** It is a method wherein the primer extension is blocked to prevent primer dimmers or non-specific amplification during the initial set up stages of PCR by withholding DNA polymerase from the reaction mixture. Once the reaction mixture reaches the hot start temperature the DNA polymerase is added for carrying out amplification [13, 15].

*** Long PCR:** It is a method used to amplify DNA over the entire length up to 25-30kb of genomic DNA segments with the help of high-fidelity DNA polymerase [16].

*** Inverse PCR**: Inverse PCR also called as inverted or inside-out PCR is a modification of the conventional PCR which is used to amplify DNA sequences outside of a known sequence or flanking the known DNA sequence [17, 18].

* **Quantitative PCR**: It is a PCR-based technique which allows for the real time measurement of PCR product during amplification process, and which is compared with a standard DNA [19, 20].

* **Asymmetric PCR**: It is a modification of PCR wherein molar concentrations of the one of the primers is higher than other so as to preferentially generate large amount of target ssDNA. It has important role in sequencing and hybridization probing where amplification of only one of the two complementary strands is required [21, 22].

* **Touchdown PCR** (**Step-down PCR):** a variant of PCR that aims to reduce nonspecific background by selecting higher annealing temperature (5°C -10°C higher than Tm of the primers) initially to allow formation of perfect primer-template hybrids and then gradually lowering the annealing temperature as PCR cycling progresses [13].

1.5. Applications

- Diagnosis of diseases caused by bacteria eg, strangles, anthrax, tuberculosis, paratuberculosis, listeriosis, brucellosis, compylobacterioisis, heamorhagic septicaemia.
- Diagnosis of diseases caused by viral pathogens, eg, African horse sickness, avian influenza virus, blue tongue, classical swine fever, foot and mouth disease, malignant catarrhal fever, Newcastle disease virus, rinderpest disease virus etc.
- Diagnosis of diseases caused by parasites eg, babesiosis, echinococcosis, *Leishmania* and *Plasmodium*.
- It is extensively used for detection of bacterial, parasitic and viral DNA in various sample types such as: CSF, blood, feces or nasopharyngeal aspirates.
- Forensics: Identify criminal suspects and paternity cases.

1.6. Real-Time or Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR (qPCR) is one the important techniques that has revolutionized the infectious disease diagnosis. qPCR allows for the detection and quantification of amplified product in real time without the need for gel electrophoresis a feature that makes it an indispensable or go to diagnostic method now-a-days. The method uses primers, probes or dyes that are fluorescently labeled and generate signals which are subsequently detected by instruments like digital cameras and fluorometer that are coupled to the reaction tube. All qPCR systems depend upon the fluorescent reporter detection and quantization whose signal increases in direct proportion to the amount of amplified product in a reaction. The technique uses various fluorescent agents like SYBR Green I or EvaGreen (intercalating fluorescent dye), TaqMan probes, florescent resonance energy transfer (FRET), and Scorpion primers. There are minimal chances of cross contamination in qPCR as it is closed automatized amplification system and once the product is amplified there is no manipulation. qPCR is used to quantify

viral or parasitic nucleic acids from clinical samples as well as to estimate the intensity of infection [23-26].

1.7. Reporters

The detection of PCR products in real-time in qPCR is made possible by a fluorescent molecule called reporter which can be either be fluorescent dye (e.g., SYBR Green) or fluorescently labeled sequence specific primers or probes (TaqMan). The amount of fluorescence generated reflects the amount of amplified product in each cycle and it increases as the product accumulates with every amplification cycle [27]

1.8. SYBR Green

SYBR Green is the simplest and most economical reporter used in real time PCR that intercalates nonspecifically into double strand DNA. The reporter upon excitation emits light and as the amount of double stranded DNA increases with time the fluorescence increases. It has the advantage of being cheap, easy to use and high sensitivity but being non-specific it may lead to overestimation. Probe based detection like TaqMan and Molecular Beacons which depend on hybridization probes target DNA sequences and as a result are more specific and are the better alternatives for SYBR Green [27, 28].

1.9. Molecular Beacons

Molecular beacons are oligonucleotide hybridization probes with hairpin shaped structure consisting of a loop portion (probe sequence) and a stem (complementary arm sequences). The probe sequence in a molecular beacon is complementary to sequence in the target DNA or RNA while the arm sequences are unrelated to target. Molecular Beacons are labeled with fluorescent dyes which emit fluorescence upon hybridization with a complementary RNA or DNA target sequence [29-31].

1.10. Applications

- The important application of real time PCR is the rapid identification, quantification and genetic typing of various microbial pathogens viz., bacteria, virus, fungi and parasites thereby allowing precise and timely administration of appropriate rational therapy.

- Another important application is for the diagnosis of pathogens which are atypical or poorly cultivatable. Real time PCR assays have been developed for various organisms like *Bordetella pertussis, Listeria monocytogenes, Legionella pneumophila, Mycobacterium, Mycoplasma pneumoniae, Chlamydia pneumophila, Avain Newcastle disease virus, herpes viruses, Aspergillus fumigatus* and *Aspergillus flavus* etc.

- Real-time PCR also has the ability to determine the pathogen load i.e., helps in quantification of bacteria, viruses and to a lesser extent also for fungi, parasites and protozoans.

- Real time PCR has also applicability in cancer diagnostics helping in monitoring minimal residues, single nucleotide polymorphisms, detection and quantification of chromosomal translocations, show graft-versus lymphoma effects etc.,

- Assays for Zygomycetes, Aspergillus, Candida sp., *Pneumocystis jiroveci*, and Coccidiodes have recently been developed, and they show promise for resolving some of the usual issues with analysis for these diseases [32, 33].

1.11. Hybridization of Nucleic Acids

Nucleic acid hybridization is a technique in which a short complementary nucleic acid strand (probe) binds with a target sequence in a complex mixture of nucleic acids. The probes used in nucleic acid hybridization are DNA or RNA fragments which are chemically synthesized and labeled with a radioactive isotope or fluorescent dyes. Southern blotting and Northern blotting are the two important nucleic acid hybridization techniques generally used. Southern blotting is used for

identifying specific sequences of DNA while Northern blotting is used to identify specific RNA sequences in a heterogeneous mixture of nucleic acids with help of labeled DNA or RNA probes. Western blotting is also a type of blotting technique which involves hybridization and is used for identifying specific protein molecules using labeled protein probes (antibodies) [34, 35].

1.12. Preparation and Labeling of Nucleic Acid

Probes are single-stranded or double-stranded molecules that may be an oligonucleotide (15-50 nucleotides) genomic DNA fragments, a cDNA fragment or an RNA molecule. A working probe must be a single-stranded molecule labeled with a detectable tracer, which is either isotopic or non-isotopic. The purified oligonucleotides are labeled *in vitro* with the help of a suitable enzyme to a labeled nucleotide. The commonly used labels may be either radioactive (^{32}P and ^{35}S) or nonradioactive (digoxigenin, biotin, fluorescein) substances. After hybridization, the location and the quantity of the hybrid molecules can be determined.

1.13. Applications

- Diagnosis of viral diseases such as, infectious bursal disease, Marek's disease, parapoxvirus, infectious bovine rhinotracheitis
- Diagnosis of parasitic diseases such as, giardiasis, theleriosis

1.14. Loop Mediated Isothermal Amplification (LAMP)

It is a rapid, specific, simple, and cost-effective nucleic acid-based amplification method based on a single step gene amplification at a constant temperature and thereafter its detection. This single step reaction involves incubation of the mixture consisting of samples, DNA polymerase, primers and substrates at a constant temperature (63°C). LAMP is becoming ideal in detection of DNA or RNA of the viral and bacterial

pathogens [10] and it has advantage over PCR of detecting the pathogenic agents earlier. A one step single tube real time accelerated reverse transcription loop mediated isothermal amplification (RT-LAMP) has been also developed that enables rapid detection of some recently emerged viral pathogens such as, West Nile virus [11].

1.15. Advantages of LAMP over PCR

- Thermocycler is not required for LAMP as required in PCR thus reducing the cost of diagnosis and also as the LAMP requires 4/6 oligonucleotide primers it has got extremely high amplification specificity (detection limit of LAMP ≥ PCR) and less detection time.
- Other advantage over PCR is that it doesn't require expensive Jel Documentation system instead visualization of DNA products in LAMP is done either naked eye (turbidity/ colour change) or by using Real Time Turbidimeter or by Electrophoresis
- LAMP resists contamination with hemoglobin, Ig and Heparin however PCR is susceptible to these impurities.
- LAMP is feasible for crude DNA extracted by ordinary physical methods and also can amplify parasitic DNA extracted from fresh infected blood.

1.16. Applications

- Detection of West Nile virus, H5N1 highly pathogenic avian influenza, Coronavirus, Dengue, Japanese encephalitis, FMD, various pox viruses such as camel pox virus, capripox viruses, Porcine circo virus 2, Chicken anaemia virus.

- Detection of parasites such as *Echinococcus multilocularis, Eimeria* spp etc.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Boza-Moran G. 2021. Molecular diagnosis of infectious diseases. The fifth eBook of the Hamilton series.
[2] Mullis K, Faloona F, Scharf S, Saiki R, Horn G and Erlich H. 1992. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Biotechnology Series, pp.17-17.
[3] Espy M J, Uhl J R, Sloan L M, Buckwalter S P, Jones M F, Vetter E A, Yao J D C, Wengenack N L, Rosenblatt J E, Cockerill III F R and Smith T. 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clinical Microbiology Reviews 19(1): 165-256.
[4] Vazquez-Pertejo M T. 2022. Nucleic Acid–Based Identification Methods for Infectious Disease. MSD Manual.
[5] Muldrew, K.L. 2009. Molecular diagnostics of infectious diseases. *Current opinion In Pediatrics* 21(1): 102-111.
[6] Giasuddin A S M. 1995. Polymerase chain reaction technique: fundamental aspects and applications in clinical diagnostics. *Journal of Islamic Academy of Sciences* 8(1): 29-32.
[7] Salipante SJ, Jerome KR. 2020. Digital PCR-An emerging technology with broad applications in microbiology. Clinical Chemistry 66: 117-123.
[8] Huang H S, Tsai C L, Chang J, Hsu T C, Lin S, Lee C C. 2018. Multiplex PCR system for the rapid diagnosis of respiratory virus infection: Systematic review and meta-analysis. Clinical Microbiology and Infections 24: 1055-1063.
[9] Carter M and Shieh J. 2015. Molecular cloning and recombinant DNA technology. In Book: Guide to Research Techniques in Neuroscience, pp. 219-237.
[10] Malewski T, Malewska A and Rutkowski R. 2003. RT-PCR technique and its applications. State-of the-art. *Journal of Animal and Feed Sciences* 12(3): 403-416.
[11] Hoffmann B, Beer M, Reid S M, Mertens P, Oura C A, van Rijn P A, Slomka M J, Banks J, Brown I H, Alexander D J and King D P. 2009. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health.

[12] Carr, J., Williams, D.G. and Hayden, R.T., 2010. Molecular detection of multiple respiratory viruses. In Molecular diagnostics (pp. 289-300). Academic Press.
[13] Green, M.R. and Sambrook, J., 2019. Nested polymerase chain reaction (PCR). Cold Spring Harbor Protocols.
[14] Henegariu O, Heerema N A, Dlouhy S R, Vance G H and Vogt P H. 1997. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques *23*(3): 504-511.
[15] Lebedev A V, Paul N, Yee J, Timoshchuk V A, Shum J, Miyagi K, Kellum J, Hogrefe R I and Zon G. 2008. Hot Start PCR with heat-activatable primers: a novel approach for improved PCR performance. Nucleic acids research, 36(20): e131-e131.
[16] Waggott W. 1998. Long range PCR. Clinical Applications of PCR, pp.81-91.
[17] Ochman H, Gerber A S and Hartl D L. 1988. Genetic applications of an inverse polymerase chain reaction. Genetics, 120(3): 621-623.
[18] Clark D P and Pazdernik N J. 2012. Polymerase chain reaction. In: Molecular biology. Elsevier.
[19] Dymond J S. 2013. Explanatory chapter: quantitative PCR. In: Methods in enzymology, 529: 279-289.
[20] Maddocks, S. and Jenkins, R., 2016. Understanding PCR: A practical bench-top guide. Chapter 4-Quantitative PCR: Things to Consider Author links open overlay panel**.**
[21] Shen H, Zhou H, Wang Y, Yang Z and Wang C. 2005. Asymmetric PCR using the primers anchored on the surface of magnetic nanoparticles. Bulletin of the Chemical Society of Japan, 78(9): 1649-1653.
[22] Citartan M, Tang T H, Tan S C, Hoe C H, Saini R, Tominaga J and Gopinath S C. 2012. Asymmetric PCR for good quality ssDNA generation towards DNA aptamer production. Sonklanakarin Journal of Science and Technology, 34(2): 125.
[23] Mackay I M, Arden K E and Nitsche A. 2002. Real-time PCR in virology. Nucleic acids research, 30(6): 1292-1305.
[24] Bennett S and Gunson R N. 2017. The development of a multiplex real-time RT-PCR for the detection of adenovirus,

astrovirus, rotavirus and sapovirus from stool samples. Journal of Virological Methods 242: 30-34.
[25] Castelli G, Bruno F, Reale S, Catanzaro S, Valenza V and Vitale F. 2021. Molecular diagnosis of leishmaniasis: quantification of parasite load by a real-time PCR assay with high sensitivity. Pathogens, 10(7): 865.
[26] Tang, J.Y., 2023. Detection of Microbiological Hazards. In Food Safety Management (pp. 835-850). Academic Press.
[27] Marmiroli N, Maestri E. 2007. Polymerase chain reaction (PCR). SYBR Green I chemistry. In: Food Toxicants Analysis, (Ed) Picó, Y. Food toxicants analysis: techniques, strategies and developments. Elsevier.
[28] Biorad 2006. Real-Time PCR Applications Guide. https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf.
[29] Sigma life sciences. 2008. qPCR Technical Guide. https://www.gene-quantification.de/SIAL-qPCR-Technical-Guide.pdf
[30] Tyagi, S. and Kramer, F.R., 2012. Molecular beacons in diagnostics. F1000 medicine reports, 4.
[31] Tyagi S and Kramer F R. 1996. Molecular beacons: probes that fluoresce upon hybridization. Nature Biotechnology, 14(3): 303-308.
[32] Kaltenboeck B and Wang C. 2005. Advances in real-time PCR: Application to clinical laboratory diagnostics. Advances in Clinical Chemistry 40: 219.
[33] Saeed K. and Ahmad N S. 2013. Real-time polymerase chain reaction: applications in diagnostic microbiology. International Journal of Medical Students 1(1): 28-36.
[34] Vasilica B. 2007. Molecular hybridization techniques of nucleic acids. Innovative Romanian Food Biotechnology 1: 1-11.
[35] Ha C E and Bhagavan N V. 2011. *Essentials of medical biochemistry: with clinical cases*. Academic Press.

CHAPTER 6

Exosome: Role in Cancer Invasion and Use as Drug Delivery Agent

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Exosome: Role in Cancer Invasion and Use as Drug Delivery Agent

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Abstract

Various tumor cells secrete exosomes. Exosome has a spheroid shape (40-100 nm) with the ability to carry different bioactive materials (proteins, RNA, DNA, and lipids) not only in the inner section but also in the surface area. Exosomes can recruit white blood cells to premetastatic tumor tissue, remodeling the extracellular matrix (ECM) to promote tumor metastases and cell growth. Exosomes can affect target cells and provide cellular communication. The demonstration of different surface markers of exosomes has made exosomes suitable candidates for drug delivery systems such as the trojan horse. Exosomes can easily penetrate the cell membrane and release the target drug into the cancer cell cytoplasmic region more specifically than conventional drugs. Exosomes can carry chemotherapy, genetic materials (miRNA, mRNA, DNA, siRNA, …) different proteins, and peptides. Different cancer drugs like 5-flu and paclitaxel can easily transport to the cancer cells. It increased cytotoxicity and decreased side effects related to cancer cells.

Keywords: Exosome, cancer, Drug delivery system, cancer ECM.

1. Introduction

Exosomes are secreted by a variety of cell types, including immunological and malignant cells. The multivesicular endosomes that create these cysts release them through the

plasma membrane [1]. Content of released exosomes includes proteins (such as oncoproteins, transcriptional regulators, and tumor suppressor proteins) [2], lipids (such as phosphatidylcholines, phosphatidylserine, and phosphatidylethanolamines) [3, 4], DNA (such as single-stranded DNA, genomic DNA, XX DNA, and retrotransposon elements), and RNAs (messenger RNAs [mRNA] 's][4] carry a variety of bioactive molecules such as long non-coding RNAs [1nRNAs], microRNAs [including miRNAs (including resistance genes)] and other non-coding RNAs [ncRNAs]) [5].

Exosomes, like cytokines, can draw white blood cells to premetastatic tumor tissue and alter the extracellular matrix (ECM) to encourage the spread of the tumor and the proliferation of cancerous cells. Consequently, matrix metalloproteinase-9 (MMP9) plays a vital role in the breakdown of the extracellular matrix (ECM) by activating the transformation growth factor β (TGF-β)/Smad signaling pathway. Exosomes generated by tumors also contribute to the immune response [6, 7].

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Nanoscale drug delivery systems are necessary in modern medicine to increase the therapeutic effectiveness of chemical and biomolecular drugs. These transportation systems do face some challenges, though. These include a substance's cytotoxicity and how quickly the reticuloendothelial system (RES) or the mononuclear phagocyte system (MPS) removes it from the environment. According to recent studies, these cysts can be modified as a drug delivery system. The drug delivery system created using this way will be more favorable than systems designed using other methods [10].

1.1. Cancer

Cancer is a type of disease in which certain cells in the body grow uncontrollably and spread to other areas. It can start almost anywhere in the human body, which is made up of trillions of cells. Normally, human cells grow and multiply to form new cells in line with the body's needs. Aged or damaged cells die and are replaced by new cells [11].

Sometimes this orderly progression is disrupted and abnormal or damaged cells that should not grow are observed to multiply. Normal cells become cancerous when too many mutations cause the cell to grow and divide uncontrollably. In a way, this shows that the cancer cell is the cell that has achieved immortality. Cancer cells are not cells that reproduce where they originate, like normal cells. They have the characteristics of both invading nearby tissues and spreading to distant areas with their features called metastasis. Cancer cells differ from normal cells in many ways [12].

Briefly; 1. Normal cells need growth factors to grow, while cancerous cells can grow without these factors. 2. They ignore signals (a process known as programmed cell death or apoptosis) that stop cells from dividing or cause them to die.

3. Unlike normal cells, they do not stop growing when they encounter other cells, and they can spread throughout the body. 4. The immune system can normally eliminate damaged or

abnormal cells, while tumor cells can be hidden. 5. Some cells of the immune system can protect cancer cells, ensuring their survival and growth [13].

1.2. Types of Cancer

Cancer types are also named according to the organs or tissues where cancers occur, and there are more than 100 types of cancer. Basically, they are collected into 4 main groups. These are carcinoma, sarcoma, leukemia, and lymphoma types of cancer. It is seen that worldwide cancer cases are nineteen million and cancer-related deaths are ten million. The top 5 most common cancer types worldwide are breast, lung, colorectal, prostate, and stomach cancers. 23 In Turkey, according to 2020 statistics, the number of cancer cases is two hundred thirty-three thousand, while the number of cancer-related deaths is one hundred twenty-six thousand. According to the data of 2020, the top 5 most common cancer types are lung, breast, colorectal, prostate, and thyroid cancers. While the incidence of colorectal cancer in our country is 9% in men, this rate is 9.1% in women [12].

1.3. Exosome

1.4. Exosome Biogenesis

Exosomes are membrane microvesicles of small endosomal origin in the size range of 30-100 nm (Figure 6.1). The presence of exosomes was identified in the extracellular space towards the end of the 1980s, and interest in these vesicles has increased over the last decade. After its discovery, it was first thought that exosomes were secreted from cells due to cell damage or byproducts of cell homeostasis. However, as a result of various studies, it has been revealed that these extracellular vesicles have a complex structure that carries lipid, protein, and nucleic acid substances. After they were defined as functional vehicles, important properties such as carrying cargo to target cells and releasing recipient cells were also determined. Thus, exosomes have begun to be defined as a new intercellular communication

tool that can play a role in different cellular processes such as signal transduction, immune responses, and antigen presentation [14].

Exosomes can be secreted by almost all eukaryotic cells. Therefore, the structures and carrier properties of exosomes vary according to the characteristics of cell types. For example, it has been determined that cancer-derived exosomes are very important in cancer and can even be used as cancer markers, therapeutic targets, or anticancer drug carriers with their structures and cargoes [15].

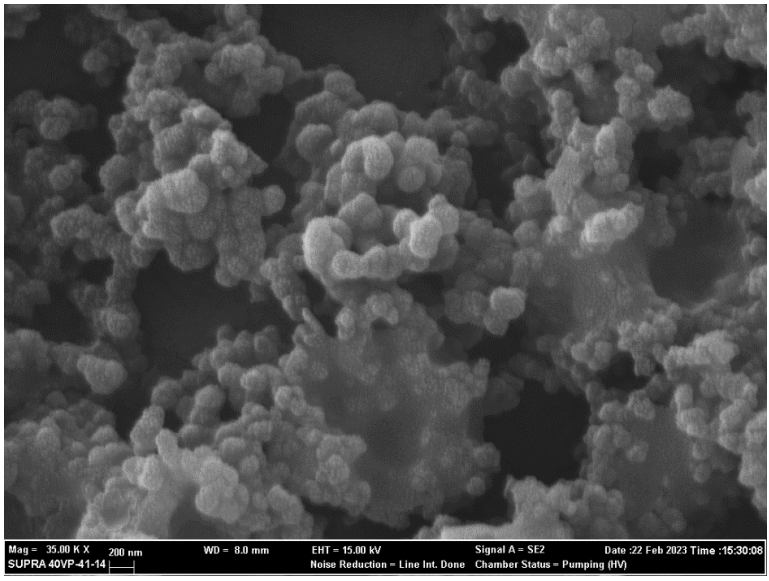

Figure 6.1. SEM image of exosomes. The obtained exosomes range in size from 15 to 100 nm.

The first endosomes begin with the accumulation of proteins in the cell membrane and bioactive substances in the early endosomes as a result of endocytosis. Early endosomes then become late endosome structures with the aid of the endocytosis sorting complex and proteins required for transport. Late endosomes also form multivesicular bodies (MVB) after a second recess. With the fusion of MVBs with the cell

membrane, the substances inside the vesicle are released in the form of vesicle structures (Figure 6.2)[16].

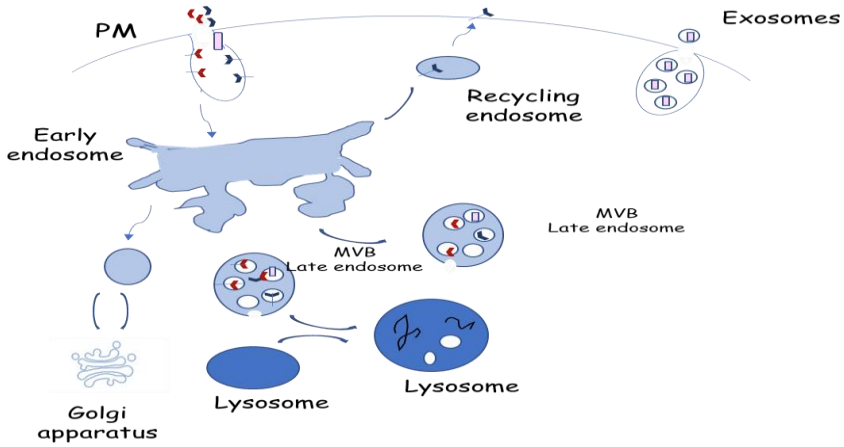

Figure 6.2. Diagram of the exosome mechanism. Endocytosis sorts of cargo to the early endosomes. The endosomal maturation route is committed by early endosomes, which causes late endosomes (MVB) to appear multivesicular. Exosomes are eventually released when MVBs fuse with the plasma membrane (PM).

1.5. Exosome Components

Various structures within and on the surface of exosomes, including transcription factors, enzymes, receptors, extracellular matrix proteins, lipids, and nucleic acids (DNA, RNA, mRNA, and microRNA (miRNA)) make up the content of exosomes. Specific content and surface receptors in exosomes of some cellular structures There are also components found in all exosomes in common [14].

Examples of specific exosome contents are surface proteins such as cell adhesion molecules (CAM), tetraspanins (CD9, CD63, CD81, CD82, CD106), MHC class I-II adhesion molecules, and integrins found in lymphocytes and dendritic

cells. Likewise, cytoskeletal proteins such as Rab2, Rab7, annexin proteins, Hsp70 and Hsp90 heat shock proteins, actin, and myosin are among the nonspecific protein types of exosomes. For this reason, these proteins are also called "exosomal marker proteins". These mentioned proteins have very important functions in exosomes. Tetraspanins facilitate the entry of certain transporters into exosomes, and the metalloproteinase of CD9 binds CD10 to the exosome [1].

Heat shock proteins facilitate protein folding balance, which acts as molecular chaperones (companion proteins involved in the folding of proteins into three dimensions) and plays anti-apoptotic roles in tumors. Hsp90 is the principal intercellular chaperone that interacts with various intracellular proteins and ensures proper protein folding. Tetraspanins, on the other hand, are protein structures with short cytoplasmic N and C terminal ends and a cysteine motif. They are also effective in the assembly of exosomes beyond assembling potential ligands for receptors on selective targets. They are predominantly expressed on the cell surface and/or intracellular vesicles. They are protein structures involved in cell motility, morphogenesis, adhesion, cell, and vesicular membrane fusion. In addition, they form a communication network by regulating signal transmission pathways and the formation of premetastatic regions in the cancer microenvironment. Some of the major tetraspanins associated with exosomes and promoting cancer cell motility, metastasis, and angiogenesis are CD9, CD8, CD81, and CD151 [17].

1.6. Exosome Mediated Cellular Communication

Exosomes can affect target cells and provide cellular communication by basically four different mechanisms. These; 1. Direct contact between the proteins on the exosome membrane surface and the proteins on the plasma membrane of the recipient cell, subsequently triggering intracellular signal cascades, 2. Division of proteins in the exosome membrane, the interaction between the receptors on the cell surface and protein fragments, 3. Fusion of cell membrane and exosomes As a

result, the exosome loads are released into the cell contents, 4. Finally, the entire exosome is taken into the cell by phagocytosis [18].

In addition, exosomes derived from various cell types have also been found to have the potential to transport content by inducing pro- or antiangiogenic signaling in endothelial cells. This provides evidence that exosomes stimulate their compounds in response to changes in their immediate environment and regulate angiogenesis. Important functions of exosomes derived from mesenchymal stem cells (MSCs) such as anti-inflammatory, neuroprotective, immunomodulation, and tissue repair have been identified. It has even been shown that exosomes derived from MSCs are more stable than MSCs per se, and exosomes injected in vivo are not rejected by immunity. To date, it has been shown that cells such as cultured glioblastoma, brain microvascular endothelium, primary cultured rat microglia, and astrocytes, as well as brain cells (neurons, astrocytes, oligodendrocytes, microglia, and others), can secrete and synthesize exosomes. Exosome-mediated cellular communication is very important in brain remodeling and vascular components. The effects of exosomes on the immune system are manifested as antigen presentation, suppression or enhancement of the immune system, and immune tolerance [19]. For example, macrophage-mediated exosomes can transfer surface antigens to dendritic cells and enhance the function of CD4+ T cells. Studies on the immunosuppressive functions of exosomes have also shown that exosomes derived from tumor cells can impair T and B cells, monocytes, NK cells, and dendritic cell functions.

1.7. Tumor-Mediated Exosomes

Tumor-mediated exosomes (TAE) can regulate cancer progression through the regulation or suppression of immune response and therapy resistance. Functions such as tumor progression, promoting angiogenesis, and stimulating the migration of tumor cells during metastasis play a role in their acceptance as versatile regulators of cancer development.

Studies have shown that TAEs can have immunosuppressive effects by suppressing potential anti-cancer effects.

These immunosuppressive effects include suppression of the immune system to allow the tumor to spread more aggressively. For this reason, TAE has various mechanisms that suppress the immune system. One of these is the deactivation of T cells via the programmed death ligand-1 (PD-L1)/programmed death-1 receptor (PD-1) checkpoint. TAE containing PD-L1 on its surface can act by suppressing activated T cells that express the PD-1 receptor. It has also been shown that TAEs induce and increase the formation of immunosuppressive regulatory T cells (Treg) through the effects of TGF-β and IL-10. Cancer cells tend to express and use immunosuppressive molecules to suppress antitumor immune responses. Exosomes secreted from these cells also contain death receptor ligands such as FasL or TRAIL (TNF-related apoptosis-inducing ligand); checkpoint receptor ligands such as PD-L1; In terms of inhibitory cytokines such as IL-10 and TGF-β1 is quite rich [20].

1.8. Exosomes and Application Areas

Various properties are effective in the use of exosomes in applications. First of all, it can be said that it can be isolated not only from eukaryotic cells but also from prokaryotic cells. Second, exosomes are capable of participating in biological processes such as tissue repair, blood coagulation, and immune responses. Third, they are effective in the pathobiological mechanisms of common diseases such as tumorigenesis and neurodegenerative disorders. Fourth, these vesicles are highly stable both in vivo (in the systemic circulation) and in vitro. The presence of structures such as specific proteins or miRNAs (such as miR-143, miR375, miR221) belonging to the cells they secrete makes them distinctive. The presence of mRNA in exosomes is important in terms of allowing the transfer of genetic information by allowing protein expression to occur at a certain distance. The fact that they can be obtained from fluids such as urine, saliva, joint fluid, breast milk, and blood is also effective in the prognosis of diseases and their use as a

biomarker. Cancer diagnosis today is based on biopsies, while the use of exosomes allows tumors to be diagnosed at an early stage. The relationship of exosomes isolated from urine with renal pathologies such as renal ischemia/reperfusion and acute kidney injury has been defined by studies. Again, since the level of exosomal microRNAs associated with cardiovascular diseases such as miR-499, miR-133, miR-208, miR-192, and miR-194 are increased in patients with heart failure, they can be used as diagnostic markers [21].

Any structure used for drug transport must have certain properties. These are: 1) It must be able to take the drug in sufficient amounts for the therapeutic effect to occur, 2) The therapeutic agent must be able to maintain its size, structure, and bioactivity in the bloodstream stably until the target organ is reached, 3) They must be biocompatible with the immune system, have no toxic effect or have a very low structure. Exosomes also meet these criteria due to their structure and have succeeded in becoming a sought-after agent in biomedical applications. It has been shown that exosomes derived from brain cells can cross the blood-brain barrier and be used for drug delivery by expression of brain-specific surface proteins. Again, bone marrow mesenchymal stem cell (MSC) mediated exosomes were tested in drug distribution by providing the transport of functional anti-miRNA-9 to brain tumor cells [22]. As a result, it was determined that exosomes provide communication between MSC and glioblastoma cells. When exosomes obtained from HT1080 (fibrosarcoma) and HeLa (cervical cancer) cancer cell lines were compared, it was observed that the amount of drug loaded on HT1080 exosomes was twice that of HeLa exosomes. In addition, drug loaded HT1080 exosomes were found to accumulate more at the cancer site compared to HeLa exosomes. One of the chemotherapeutics used in CRC patients is 5-Fluorouracil (5-FU). Some studies have shown that overexpression of miR-196b-5 isolated from serum exosomes reduces the survival rate in patients with CRC. It was also found that overexpression of exosomal miR-196b-5 increased the resistance of CRK cells to 5-FU by activating the

signal transducer and activator of the transcription 3 (STAT3) pathway [23, 24].

Proteins and nucleic acids can be transported and distributed intracellularly via exosomes and microparticles. They are, therefore, very appealing for delivering therapeutic proteins and nucleic acids like short-interfering RNA (siRNA). As a result of naked siRNAs' quick degradation in the bloodstream, their large size and negative charge restrict transmembrane migration and cellular uptake, some siRNA sequence motifs can cause unfavorable immune reactions, and they specifically target specific tissues and cells, delivering siRNA inside cells is a challenging task. Therefore, it's crucial to lessen the harm that off-target silence does. To address several of these delivery issues, encapsulating nucleic acid-based medicines in endogenously carried vesicles is a promising new approach. Given that they are small (40–100 nm), generally homogenous in size, and well-researched, exosomes may be the best choice for such tactics. They are excellent for usage as drug delivery systems due to their length of 100 nm or less. The mononuclear phagocyte system can promptly eliminate them because they are nanosized. Due to their small size, they can be immediately eliminated by the mononuclear phagocyte system [25].

1.9. Exosome-Based Drug Delivery Systems

Nanoscale medication delivery methods have become highly important in recent years. Chemical and biomolecular medications' therapeutic potency has been increased by using a variety of nano-based medicinal compositions. Exosomes have captured researchers' interest ever since it was discovered that they serve as intercellular messengers that deliver their contents to destination cells. The optimal drug delivery system should be able to transport combination treatments to specified sites, prevent immune system detection and early breakdown, and allow the regulated release of cargo molecules in response to targeted stimuli. Exosomes are small extracellular vesicles that have gained significant attention in the field of drug delivery. They are secreted by cells and are involved in cell-to-cell

communication by transferring biomolecules, such as proteins, RNA, and lipids, between cells. Due to their unique properties, exosomes have emerged as a promising drug delivery system with potential therapeutic applications [26].

Exosomes have several advantages over other drug delivery systems, such as liposomes and nanoparticles. They are biocompatible and biodegradable, which reduces the risk of toxicity and immunogenicity. Exosomes can also be targeted to specific cells or tissues by modifying their surface proteins. Moreover, exosomes can cross biological barriers, such as the blood-brain barrier, and deliver drugs to the desired site of action. Exosomes can be loaded with various types of drugs, including small molecules, proteins, and nucleic acids. The loading efficiency of exosomes depends on the type of drug and the method of loading. Exosomes can be loaded with drugs by various methods, such as electroporation, sonication, and extrusion. The choice of loading method depends on the type of drug and the desired loading efficiency [27].

Several studies have demonstrated the potential of exosomes as a drug delivery system for various diseases, such as cancer, neurological disorders, and cardiovascular diseases. For example, exosomes can deliver anticancer drugs to tumor cells and enhance their therapeutic efficacy. Exosomes can also deliver small interfering RNA (siRNA) to target cells and reduce the expression of disease-causing genes. A study by Zhang et al. (2018) demonstrated the therapeutic potential of exosome-mediated siRNA delivery for cancer therapy [28]. The researchers loaded the exosomes with siRNA targeting the oncogene KRAS and delivered them to lung cancer cells. The results showed that exosome-delivered siRNA effectively inhibited the growth of lung cancer cells in vitro and in vivo. Alvarez-Erviti et al (2011) investigated the use of exosomes to deliver miRNA to the brain for the treatment of neurodegenerative disorders. They loaded exosomes with miRNA targeting the β-secretase enzyme involved in the formation of amyloid plaques in Alzheimer's disease [29]. The results showed that exosome-mediated delivery of miRNA

effectively reduced the expression of β-secretase in the brains of mice and is a potential therapeutic strategy for Alzheimer's disease. Exosomes were loaded with the chemotherapy medication doxorubicin as part of Munoz et al. (2020) investigation into the efficacy of exosomes as a delivery mechanism for doxorubicin. They delivered the drug-loaded exosomes to breast cancer cells. It has been shown that exosome-transported doxorubicin effectively inhibits the growth of breast cancer cells in vitro and in vivo [30]. Also, a study investigated the use of exosomes to provide growth factors to support bone regeneration. The researchers loaded the exosomes with bone morphogenetic protein-2 (BMP-2) and delivered them to bone marrow stem cells. The results demonstrated that exosome-mediated delivery of BMP-2 effectively promotes bone regeneration in a rat model, suggesting a potential therapeutic strategy for bone-related disorders [31]. Exosome-mediated CRISPR-Cas9 delivery for genetic disorders: Yang et al. (2020) explored the use of exosomes to provide the CRISPR-Cas9 gene editing system to treat genetic disorders. The researchers loaded the exosomes with CRISPR-Cas9 and delivered them to cells with a mutation in the Duchenne muscular dystrophy (DMD) gene. The results showed that exosome-mediated delivery of CRISPR-Cas9 effectively corrected the mutation and restored dystrophin expression in cells, suggesting a potential therapeutic strategy for DMD and other genetic disorders [32].

It has also been shown that the delivery capabilities of exosomes can be enhanced through modification of their surface. Tian et al. modified exosomes with DBCO, c(RGDyK), Cy5,5 to deliver curcumin to ischemic regions in the brain [33]. Because exosomes have low cytosolic release efficiency of the encapsulated molecule inside the cell, in 2014 Nakase and Futaki combined exosomes with GALA peptides and cationic lipids to increase cellular uptake. Another innovation is the fusion of liposomes and exosomes. Both exosomes and liposomes have beneficial dispersion characteristics. Both of these particles were combined by Sato et al. in order to decrease immunogenicity and enhance colloidal stability [34].

Overall, these studies demonstrate the potential for exosome-based drug delivery for a variety of therapeutic applications. More research is needed to optimize exosome loading and delivery methods and to evaluate their safety and efficacy in clinical trials.

1.10. Limitations of Exosome-Based Drug Delivery System

Notwithstanding encouraging experimental advantages, the variety in origin, structure, and function of exosome-based drug delivery presents limitations. The main drawback among all these restrictions is that exosome biodistribution is non-specific, and they can be identified in a range of biological fluids in the human body. In the last few decades, the use of exosomes has greatly expanded and turned into therapeutic tools. Recently, there has been a steady increase in the number of research papers and review articles published on exosomes. In this book chapter, we bring together a well-established understanding of exosome machinery and an overview of discoveries over the past two years, focusing on exosome biogenesis, release, and uptake. In addition, we aim to emphasize that exosomes will have an important place in the drug transport system. With the development of new techniques, it is crucial to advance the use of exosomes in line with clinical applications [18]. Exosomes' biocompatibility and the very specialized interactions they have with biological systems have sparked the creation of cutting-edge therapeutic and medication delivery methods based on exosomes. When compared to traditional cancer treatments, genetically modified exosomes loaded with certain medications that target cancer cells show superior results. This novel methodology nevertheless comes with certain drawbacks in terms of scalability, purity, and challenges with isolation techniques. In-depth research is required in this field. The quick elimination of exosomes from the bloodstream following in vivo treatment is an essential problem with their usage as drug delivery vehicles. Exosomes are distinctive protein-lipid complexes [35]. However, it has been presented by studies that the rapid clearance of exosomes from the bloodstream is

removed by macrophages. Experimental results clearly showed that exosomes derived from B16-B16 cells are rapidly cleared by liver and spleen macrophages after intravenous injection. It was thought that this problem could be solved to some extent by including polyethylene glycol (PEG) in the structures of exosomes. Exosomes with PEG can be detected even 60 minutes after injection. However, experimental evidence shows that exosomes without PEG can only be seen for 10 minutes[36]. Another drawback is the low exosome yield, which results in less than 1 g of protein being produced per ml of cell culture. Therefore, several cells must be grown in an experiment or clinical investigation; several-like nanovesicles can overcome this restriction. Serial filtering of extruded cells can result in the production of exosome-mimetic nanovesicles (EMNV). It is said that using these methods can raise the yield by up to 100 times [37].

2. Conclusion

In conclusion, exosomes are one of the most effective and competitive nanocarriers for drug delivery, whether they have been changed or not. Exosomes that are produced naturally have unique roles in cell-cell communication, providing them advantageous dispersion properties. However, the study of medication delivery holds great promise for the creation of customized exosomes. Nevertheless, further investigation must be done to enhance characterization procedures and standardize manufacturing for exosomes that have been modified or derivated for use in drug delivery systems.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Raposo, G. and W. Stoorvogel, Extracellular vesicles: Exosomes, microvesicles, and friends. Journal of Cell Biology, 2013. 200(4): p. 373-383.

[2] Al-Nedawi, K., et al., Intercellular transfer of the oncogenic receptor EGFrvIII by microvesicles derived from tumour cells (vol 10, pg 619, 2008). Nature Cell Biology, 2008. 10(6): p. 752-752.
[3] Chen, Y.Y., et al., Protein content and functional characteristics of serum-purified exosomes from patients with colorectal cancer revealed by quantitative proteomics. International Journal of Cancer, 2017. 140(4): p. 900-913.
[4] Balaj, L., et al., Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. Nature Communications, 2011. 2.
[5] Valadi, H., et al., Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology, 2007. 9(6): p. 654-U72.
[6] Sato-Kuwabara, Y., et al., The fusion of two worlds: Non-coding RNAs and extracellular vesicles - diagnostic and therapeutic implications. International Journal of Oncology, 2015. 46(1): p. 17-27.
[7] Taylor, D.D. and C. Gercel-Taylor, MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer (vol 110, pg 13, 2008). Gynecologic Oncology, 2010. 116(1): p. 153-153.
[8] Kim, J., et al., Replication study: Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Elife, 2018. 7.
[9] Peinado, H., et al., Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET (vol 18, pg 883, 2012). Nature Medicine, 2016. 22(12): p. 1502-1502.
[10] Sousa, D., R.T. Lima, and M.H. Vasconcelos, Intercellular Transfer of Cancer Drug Resistance Traits by Extracellular Vesicles. Trends in Molecular Medicine, 2015. 21(10): p. 595-608.
[11] Kar, R., et al., Exosome-Based Smart Drug Delivery Tool for Cancer Theranostics. ACS Biomater Sci Eng, 2023. 9(2): p. 577-594.
[12] Genc, S., et al., Fe3O4 Nanoparticles in Combination with 5-FU Exert Antitumor Effects Superior to Those of the

Active Drug in a Colon Cancer Cell Model. Pharmaceutics, 2023. 15(1).
[13] Helmink, B.A., et al., The microbiome, cancer, and cancer therapy. Nature medicine, 2019. 25(3): p. 377-388.
[14] Genc, S., et al., Exosomal MicroRNA-223, MicroRNA-146, and MicroRNA-21 Profiles and Biochemical Changes in Laryngeal Cancer. ACS Pharmacology & Translational Science, 2023.
[15] Yeni, Y., et al., Glioblastoma cell-derived exosomes induce cell death and oxidative stress in primary cultures of olfactory neurons. Role of redox stress. Molecular Biology Reports, 2023.
[16] Bunggulawa, E.J., et al., Recent advancements in the use of exosomes as drug delivery systems. Journal of Nanobiotechnology, 2018. 16.
[17] Lai, R.C., T.S. Chen, and S.K. Lim, Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. Regenerative Medicine, 2011. 6(4): p. 481-492.
[18] Kalluri, R. and V.S. LeBleu, The biology, function, and biomedical applications of exosomes. Science, 2020. 367(6478): p. 640-+.
[19] Whiteside, T.L., Exosomes and tumor-mediated immune suppression. Journal of Clinical Investigation, 2016. 126(4): p. 1216-1223.
[20] Yoon, Y.J., O.Y. Kim, and Y.S. Gho, Extracellular vesicles as emerging intercellular communicasomes. Bmb Reports, 2014. 47(10): p. 531-539.
[21] Tian, Y.H., et al., A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. Biomaterials, 2014. 35(7): p. 2383-2390.
[22] Barile, L., et al., Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. Cardiovascular Research, 2014. 103(4): p. 530-541.
[23] Wang, X., et al., Mesenchymal stem cell-derived exosomes have altered microRNA profiles and induce osteogenic differentiation depending on the stage of differentiation. PLoS One, 2018. 13(2): p. e0193059.

[24] Zhuang, X.Y. and H.G. Zhang, Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. Cancer Research, 2012. 72.
[25] Genc, S., et al., Potential Neurotoxic Effects of Glioblastoma-Derived Exosomes in Primary Cultures of Cerebellar Neurons via Oxidant Stress and Glutathione Depletion. Antioxidants, 2022. 11(7).
[26] Aryani, A. and B. Denecke, Exosomes as a nanodelivery system: a key to the future of neuromedicine? Molecular neurobiology, 2016. 53: p. 818-834.
[27] Ha, D., N. Yang, and V. Nadithe, Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. Acta Pharmaceutica Sinica B, 2016. 6(4): p. 287-296.
[28] Zhang, X., et al., Exosomes in cancer: small particle, big player. Journal of Hematology & Oncology, 2015. 8.
[29] Alvarez-Erviti, L., et al., Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nature Biotechnology, 2011. 29(4): p. 341-U179.
[30] Munoz, J.L., et al., Delivery of Functional Anti-miR-9 by Mesenchymal Stem Cell-derived Exosomes to Glioblastoma Multiforme Cells Conferred Chemosensitivity. Molecular Therapy-Nucleic Acids, 2013. 2.
[31] Zhang, J.Y., et al., Exosomes/tricalcium phosphate combination scaffolds can enhance bone regeneration by activating the PI3K/Akt signaling pathway. Stem Cell Research & Therapy, 2016. 7.
[32] Mirgayazova, R., et al., Therapeutic Editing of the TP53 Gene: Is CRISPR/Cas9 an Option? Genes (Basel), 2020. 11(6).
[33] Tian, T., et al., Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. Biomaterials, 2018. 150: p. 137-149.
[34] Nakase, I. and S. Futaki, Combined treatment with a pH-sensitive fusogenic peptide and cationic lipids achieves enhanced cytosolic delivery of exosomes. Scientific Reports, 2015. 5.
[35] Patil, S.M., S.S. Sawant, and N.K. Kunda, Exosomes as drug delivery systems: A brief overview and progress update.

European Journal of Pharmaceutics and Biopharmaceutics, 2020. 154: p. 259-269.
[36] Kooijmans, S.A.A., et al., Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles. Journal of Controlled Release, 2013. 172(1): p. 229-238.
[37] Chen, L., et al., Exosomes as drug carriers in anti-cancer therapy. Frontiers in Cell and Developmental Biology, 2022. 10: p. 34.

CHAPTER 7

Bioanalysis of Molecular Based Biomonitoring and Characterization of Toxicants in Aquatic Ecosystems

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Bioanalysis of Molecular Based Biomonitoring and Characterization of Toxicants in Aquatic Ecosystems

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Abstract

The mixtures of toxicants increasing day by day in the aquatic ecosystem have become one of the biggest environmental problems, and therefore various biomonitoring studies are carried out to keep toxicants under control. Pollution biomonitoring studies in recent years have provided a strong prediction by tracking protein biomarkers in an organism. Toxicity tests including standard mortality and reproductive cycle of the organism provided disadvantages in terms of sensitivity of the organism, duration of the experiment and cost. On the other hand, changes at the molecular and biochemical levels usually appear as the first reactions to environmental changes and offer us advantages in terms of the cost. This study focuses on the methods used to monitor stress parameters of environmental pollutants in organisms in aquatic ecosystems and thusly, aims to address various methodologies in the analysis of molecular-based biomarkers. This study also presents a brief discussion and comparison of various methods involving molecular-oriented applications that can support protein-level analyzes in the organism.

Keywords: Aquatic ecosystem, Biomonitoring, Molecular, Pollution, Toxicant.

1. Introduction

Anthropogenic effects, natural dissolution processes and environmental pollutants cause unknown consequences for

aquatic ecosystems and organisms day by day [1, 2]. Providing specific information about types of pollutants in the form of mixtures and biologically based characterizations of the dynamically driven impacts on organisms has now become an important component of programs followed around the world. In addition, it is still not fully understood whether the interactions of combinations of pollutants are greater than the sum of the effects of individual pollutants and their characterizations [3]. The biological monitoring method is based on the detection of molecular and biochemical changes depending on the amount of accumulation of the toxicant in the tissues of the organism exposed to the pollutant (Figure 7.1). Other conditions that will help to determine the source and toxic effect of the toxicant are changes in the health status index of the organism, oxidative stress defense, energy metabolism, the activity of the specific enzyme in xenobiotic detoxification, and sudden state changes of biomolecules [4, 5]. Fluctuations in enzyme activities and protein levels in the organism against environmental changes have often been the first and most important limitations in many studies [6]. Proteomic approaches have been used as a biomonitoring scale in laboratory and field studies to evaluate the mechanism of action of toxicants such as metals, polyaromatic hydrocarbons, diallyl phthalate, and polybrominated diphenyl ethers [7-14]. In biological monitoring studies, certain methods such as mass spectrometry (MS), two-dimensional gel electrophoresis of proteins (2DE), quantitative content studies, spectrophotometric methods, and elemental analyzes have become popular recently; accordingly, the structural changes of proteins and the keystone proteins required for oxidative stress, cell signaling, metabolism of xenobiotics, energy metabolism, protein stabilization, and metabolism of lipids and levels of amino acids are determined more easily and reliably [10, 15, 16]. Furthermore, advanced studies such as labeling of proteins with isotope encoded affinity tag (ICAT), fluorescent dyes (fluorescence difference gel electrophoresis, DIGE), isobaric tag for relative and absolute quantitation (iTRAQ) or 16O/18O tag have been important in follow-up studies [17]. In addition, in terms of public health protein consumption, proteins obtained from organisms living in aquatic

ecosystems meet approximately 14% of the world's terrestrial animal protein needs and 4-5% of total protein needs [18]. This situation is beneficial in terms of eliminating the protein need of the human population and makes the biological analysis of protein quality controls crucial [19]. The effects of clean water ecosystems on human health and animal welfare are becoming increasingly important and accordingly, biological monitoring studies to protect and restore clean water across Europe are among the leading projects carried out with the European Water Framework Directive [20]. In addition, similar directives on water quality control and management are provided for the well-being of organisms in the ecosystem and human populations [21]. Likewise, studies that point out the importance of clean aquatic environments are gaining momentum. With this review, it is aimed to address and summarize main methodologies implemented so far in the biomonitoring of molecular-based biomarkers of the model organisms under the influence of environmental pollutants in the aquatic ecosystem.

1.1. Determination of Total Content of Proteins

It is critical to perform tissue protein analyzes regularly. Elemental analyzes of proteins such as C/N are generally obtained by the Kjeldahl or Dumas method [23]. The sample should be kept to a minimum in order to reduce both analytical error and cost in either method. Although the Kjeldah method was invented in 1883, it has been revised over time and used as the analytical method of many national and international institutions. The Kjeldah method ensures clarity in the results as it gives accurate results for each sample.

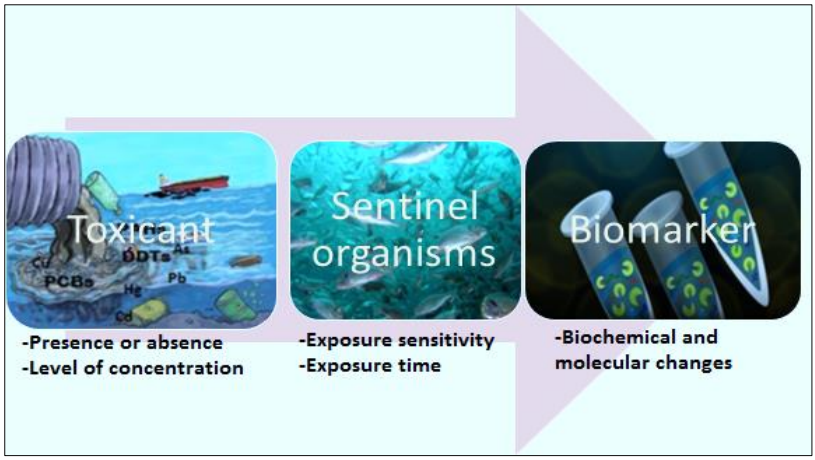

Figure 7.1. Biological monitoring elements of environmental pollutants [22]

It consists of fragmentation of the sample, neutralization, distillation, ammonia capture and titration steps. Despite the advantages of the method, there are also disadvantages. To clarify, the experiment contains hazardous and toxic chemicals for the analysts and the environment. On the other hand, the Dumas method, which is parallel to the Kjeldah method, has recently been preferred because it is faster and cheaper [24]. In the sample catalysis phase, sulfuric acid is used initially, and chemicals such as potassium sulfate and mercury oxide are used later. Hence, ammonium sulphate is obtained from the nitrogen in the catalyzed sample. Then, it is distilled and converted into ammonia held with boric acid, and the amount of nitrogen is recorded by titration. It allows the calculation of the nitrogen content and protein content of structural molecules such as nucleotides, free amino acids, urea and amines. In addition, the protein content is calculated by multiplying the Kjeldahl factor. This factor may vary according to the structural tissues of the organism and the species. For aquatic organisms, this value ranges between 5.43 and 5.82 [24-25]. While the Kjel-Foss® device, one of the devices used in the Kjeldahl method, allows the implementation of the entire micro-Kjeldahl procedure, it uses the Kjel-Tec® device as a lysis block with an apparatus for

automatic distillation and titration. Technicon auto analyzer is used to provide continuous flow analysis [24]. Indeed, the Dumas method is older than the Kjeldah method as it was first referenced in 1831. But it has been noted that the Dumas method has difficulties in use. Basically, in the Dumas method, the sample is burned at 950°-1050°C and purged of atmospheric gas. H_2O, CO_2, SO_2, and O_2 are removed as combustion gases; NO_2 is reduced to N_2 and measured with a thermal conductivity meter [24, 26]. Therefore, all gases except NO_2 and N_2 are eliminated. This method has been calibrated with the Kjeldahl method and approved by the Association of Analytical Chemists (AOAC), the American Petroleum Chemists Association (AOCS) and some other organizations [24]. In the third method, which is called the quantitative amino acid method, the protein content is determined precisely and reliably. In this method, amino acid characterization is achieved by catalyzing the sample with concentrated hydrochloric acid. The principle of quantitative amino acids was explained according to Owusu-Apentene [24]. The fish tissue proteins to be examined consist of three groups according to their dissolution status, the first of which is sarcoplasmic, the second is myofibrillar, while the third is connective proteins [27, 28]. The sarcoplasmic proteins are separated by low ionic buffers such as 50 mM phosphate buffer and are generally in an enzyme structure. The myofibrillar proteins are dissolved with > 0.3 mM saline buffers. In addition, the connective tissue proteins are dissolved by alkaline or acid buffers.

The protein production of organisms in the aquatic environment shows a faster deterioration than mammals. After the tissue dissolution process, the concentrations of soluble proteins can be analyzed by a variety of methods. The absorbance of all proteins is measured in the UV range as one of the simplest methods. The Lambert-Beer law, which is given by the formula: $A = \sum cl$, where A stands for the absorption at a particular wavelength, $\sum$ symbolizes the molar absorption or damping coefficient ($M^{-1}cm^{-1}$), c denotes the molar protein concentration, and l stands for the path length for light (cm),

provides a computation of the protein concentration. Measuring UV absorption at 280 nm is a simple and popular method for determining the protein concentration. Also, the presence of non-protein molecules such as nucleic acids and nucleotides that strongly absorb the UV lights at 260 nm may adversely affect the results. There are a certain set of methods to reduce the effect of uneven scattering of light and nucleic acids/nucleotides in the spectrophotometer [29].

1.2. Analysis of Proteins

Protein content is determined by various calorimetric methods. The first of these is the Biuret method. In this method, a complex is formed between peptide bonds and copper salts in tissue homogenates. The stabilized purple complex is measured at absorption at 540-560 nm. The standard curve is prepared. Though it measures concentrations with a measuring range of 1-10 mg/mL, it has the disadvantage that it is a simple but not very sensitive measurement [29]. Another method is the Lowry method. In this method, as a result of the reaction formed by copper (II) ions of the protein in the alkaline medium, the complexes respond with the Folin-phenol reagent, which is a mixture of phosphomolybdic acid and phosphotungstic acid in phenol, and form molybdenum/tungsten blue. The complex is measured at 750 nm [30]. While the Lowry method provides an advantage in terms of simplicity, precision and accuracy, inconsistencies in the used mixed chemicals and temporal dye bonding methods sometimes cause a disadvantage. The third method is the Bicinchonic acid (BCA) method. Also known as the Smith analysis, the BCA method uses only one reagent. In this method, the total protein concentration reflects a color change in the sample solution from green to purple. The color change shifts with protein concentration, and the protein concentration is determined by using colorimetric techniques [24]. Obviously, the BCA assay is based on two reactions. The first is the reduction of Cu^{2+} ions to Cu^{+} by peptide bonds in the protein, and this step is a temperature-dependent reaction. Cu^{2+} is reduced in parallel with the number of proteins in the solution. In the second reaction, 2 molecules of bicinchoninic acid and

Cu^{+} ion combine to form a purple complex that absorbs light strongly at a wavelength of 562 nm [31]. The fourth method is called the Biorad method, which is based on the binding of Coomassie Brilliant Blue G (CBBG) and is similarly one of the different staining methods [32]. This method is based on the color change that occurs when the CBBG binds to proteins under acidic medium. It yields results and stable reagents in 5 minutes compared to 30-45 minutes of the Lowry method. Furthermore, the potential of proteins binding silver has been used as a very sensitive method to visualize proteins in gel electrophoresis, as well. Silver staining analysis is 100 times more accurate than the CBBG staining method [29].

All the methods mentioned above are highly protein dependent. Although it is best to use the studied protein as a standard protein, this is often not practical or possible. The Lowry method identifies proteins, micropeptides and free amino acids whereas other methods such as Biuret and Biorad only work for analyzing peptide chains above a certain length. Since different amino acids and peptides give different colors in the Lowry method, the method is highly protein-dependent and successful. Some methods and comparisons are shown to determine protein concentration and level (Table 7.1).

Table 7.1. Various methods for determining protein concentration

Method	**Range (µg)**	**References**
Kjeldahl	500-30,000	23
Biuret	1,000-10,000	29
Lowry	10-300	30
Biorad (Coomassie Brilliant Blue)	20-140	32
Biorad (Coomassie Brilliant Blue)-micro	1-20	32
Bicinchonic acid (BCA)	1-50	24

1.3. Immunoassays (IA)

The immunoassay, which was first discovered by Rosalyn Sussman Yalow and Soloman Berson in the 1950s, was used to measure the duration of insulin in the body using radioactive insulin. By using this method, antibodies and hormone levels were determined. Later, this situation was analyzed in reverse, and they developed the immunoassay. It is a biochemical and sensitive test used to determine the presence or concentration of macro or micro molecules (analytes such as proteins, hormones, drugs, etc.) in the blood and tissues, depending on the basis of an antibody or an antigen reaction [33]. The amount of a protein in the tissue homogenate can be determined by enzyme-linked immunosorbent assays (ELISA). The analyzed molecule is bound to a film with a polyclonal or monoclonal antibody via the Fc region of the antibody. Bovine serum albumin (BSA) is then added to block non-specific binding sites. After washing, a second antibody binds to the protein bound to the primary antibody. The amount of secondary antibody bound is proportional to the amount of specific protein in the sample. The secondary antibody is usually associated with peroxidase or alkaline phosphatase. The method should be specific to the antibodies present, in which case the cost may be high [33].

1.4. Protein Electophoresis

Over the past 40 years, the field of molecular genetics has made significant contributions to many areas of biology including systematics, population genetics, and conservation biology. Populations or species researchers have obtained genetic differences between individuals using techniques that would otherwise not be possible. Perhaps the most important of these techniques was the electrophoresis. With the advent of protein electrophoresis in the early 1960s, it helped to identify important variations in wild species and population genetics. Although the electrophoresis technique provides benefits in detecting population variations, it also has limitations such as detecting only 1/3 of the genetic code due to its redundancy and resolving power [34]. Despite these disadvantages, the search

for higher levels of variation at the nucleotide level continued. These included determining the true base pair sequence of the genetic code by DNA sequencing [35, 36].

Also, sequence-based studies were developed after the development of the polymerase chain reaction (PCR) in the mid-1980s [37, 38]. New insights into the determination of phylogenetic relationships between species by polymer chain reaction and the relationships between different alleles at a locus were provided. Therefore, it was possible to examine the mutation and persistence of alleles over time with allozyme-based approaches in the population genetics. Various molecular methods have been used for the genetic variability status of aquatic ecosystem organisms. The majority of data obtained from the implementation these methods consisted of allozymes and DNA sequence data. There are many studies on *Astacus fluviatilis*, which has been accepted as an important study organism in biology since ancient times [39]. These macroinvertebrates are essential for aquatic ecosystems and form a very important model in neurotoxic studies considering their large abdominal neurons, which are among the target systems of toxicants [40]. It has been shown that 50 to 60% of the species identified during extinction caused by environmental variables are of conservation concern [41-42]. The earliest crayfish studies used protein electrophoresis to examine levels of variation within and between species [43-44].

The molecular weight of peptides and proteins in aquatic organisms has often been of great interest. The molecular weights of peptides and proteins are obtained by several methods. In the native gel filtration chromatography, proteins are separated according to their size and shape, called Stokes radius. Beads for low and medium pressure chromatography are provided from dextrans, agarose, polyacrylamide, cellulose, and combinations thereof [24]. As the protein solution moves down the column, the larger proteins will come out of the column first and the smaller proteins spend more time inside the beads. The passage of the protein in the gel filtration column can be explained by the Kav coefficient, which defines the proportion

of pores accessible to that molecule. It is expressed as Kav = $(V_e-V_0)/(V_t-V_0)$, where V_e symbolizes the elution volume of the molecule, V_t is the total volume of the column, and V_0 stands for the void volume of the column. Using known molecular weight proteins, a standard curve is prepared that allows the molecular weight distribution in the protein mixture to be determined.

The molecular weight can be obtained by electrophoresis technique, as well. One of the most widely used methods is SDS-PAGE, which uses polyacrylamide gels and denatures samples by boiling them in a solution of sodium dodecyl sulfate (SDS). SDS binds to proteins in a weight ratio of 1:1.4. Dithiothreitol (DTT) or mercaptoethanol is usually added to reduce disulfide bonds [45]. Denatured proteins are applied to the gel, and an electrical current is applied causing the negatively charged proteins to move across the gel towards the anode. Proteins will move according to their size; larger ones travel a shorter distance, whereas smaller proteins travel farther in the gel [45]. The diagrammatic summary of the electrophoresis analysis procedures belonging to a fish population is shown in Figure 7.2.

1.5. Peptide Characterization

It has been pertinent to examine the composition and structural properties of peptides in aquatic organisms as a result of toxicological exposure. Many peptide proteins have physiological states such as being bioactive, immunostimulating, and antihypertensive. Especially in the characterization of peptide complexes after enzymatic degradation, it is important to determine to the extent to which the protein peptide structures are affected by the enzymatic degradation reaction.

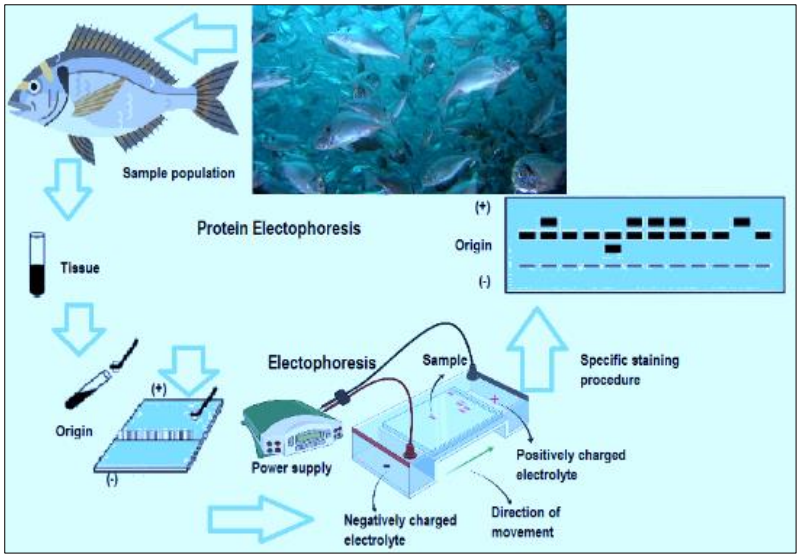

Figure 7.2. Simple schematic showing how electrophoresis is achieved in a sample population

In this analysis, the measurement is based on a percentage of the total number of unhydrolyzed protein peptide bonds to those that have been hydrolyzed. One of the methods used to determine this value is trinitrobenzene-sulfonic acid (TNBS) treatment, which is based on the determination of free amino groups related to the reaction [46]. In the TNBS treatment, the amount of chromophore formed as a result of the reaction with primary amines is determined by spectrophotometric method. The experimental reaction is performed in a low alkaline medium and is terminated by lowering the pH in the medium. Another method used is the characterization of amino groups as a result of titration with formaldehyde [47]. The break in the peptide binds at the oxidative stress levels due to exposure to different ecotoxicants make it possible to report various information about the types of toxicants and their effect mechanisms. It can also provide valuable information about the physiological state of the organism tissue protein and the enzymes that are active. In addition, specific precipitation chemicals such as ethanol, methanol or trichloroacetic acid have been used to determine the number of peptides with a chain

length of less than 1 [48]. Hence, alternative chromatographic methods such as LC-MS and electrophoretic methods were used with the precipitation of simple chain peptide groups. In addition, detailed information is provided about the molecular mass of peptides by mass spectroscopy and the structure of peptides by tandem mass spectroscopy. Human health and the general profile of the ecosystem are affected due to the proliferation of toxins such as cylindrospermopsin in the aquatic environment. Therefore, a series of biological studies have been performed by LC-MS in the liver and muscle tissue of *Oreochromis niloticus*. Various chromatographic methods are now applied in many follow-up studies like this [49].

2. Conclusion

All in all, there are many technical materials (protein, gene), tools, chemical and technical procedures waiting to be investigated for biomonitoring studies in the aquatic ecosystems. Constant updating of new laboratory techniques helps us examine the variation created by environmental variables in organisms from different perspectives and choose the most appropriate and cheapest method for our own study. Although certain shortcomings have been reported about some of the methods examined above, in fact, these studies have upgraded one another or formed a parallel analysis for another study over the course of time.

Conflict of Interest

The author declares no conflict of interest.

References

[1] Szyczewski P, Siepak J, Niedzielski P, Sobczyński T. Research on heavy metals in Poland. Pol. J. Environ. Stud. 2009; 18(5): 755-768.
[2] Norgate TE, Jahanshahi S, Rankin WJ. Assessing the environmental impact of metal production processes. Journal of Cleaner Production. 2007;15(8-9): 838-848.

[3] Mi J, Apraiz I, Cristobal S. Peroxisomal proteomic approach for protein profiling in blue mussels (Mytilus edulis) exposed to crude oil. Biomarkers. 2007; 12(1): 47-60.
[4] Lima I, Moreira SM, Rendón-Von Osten J, Soares AM, Guilhermino L. Biochemical responses of the marine mussel Mytilus galloprovincialis to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere. 2007; 66(7): 1230-1242.
[5] Sarkar A, Ray D, Shrivastava AN, Sarker S. Molecular biomarkers: their significance and application in marine pollution monitoring. Ecotoxicology. 2006; 15: 333-340.
[6] Hagger JA, Lowe D, Dissanayake A, Jones MB, Galloway TS. The influence of seasonality on biomarker responses in Mytilus edulis. Ecotoxicology. 2010; 19: 953-962.
[7] Knigge T, Monsinjon T, Andersen OK. Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels (Mytilus edulis) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. Proteomics. 2004; 4(9): 2722-2727.
[8] Dowling V, Hoarau PC, Romeo M, O'Halloran J, Van Pelt F, O'Brien N, Sheehan D. Protein carbonylation and heat shock response in Ruditapes decussatus following p, p′-dichlorodiphenyldichloroethylene (DDE) exposure: a proteomic approach reveals that DDE causes oxidative stress. Aquatic Toxicology. 2006; 77(1): 11-18.
[9] Chora S, Starita-Geribaldi M, Guigonis JM, Samson M, Roméo M, Bebianno MJ. Effect of cadmium in the clam Ruditapes decussatus assessed by proteomic analysis. Aquatic toxicology. 2009; 94(4): 300-308.
[10] Riva C, Binelli A, Rusconi F, Colombo G, Pedriali A, Zippel R, Provini A. A proteomic study using zebra mussels (D. polymorpha) exposed to benzo (α) pyrene: the role of gender and exposure concentrations. Aquatic toxicology. 2011; 104(1-2): 14-22.
[11] Martins JC, Leao PN, Vasconcelos V. Differential protein expression in Corbicula fluminea upon exposure to a Microcystis aeruginosa toxic strain. Toxicon. 2009; 53(4): 409-416.

[12] Puerto M, Campos A, Prieto A, Cameán A, de Almeida AM, Coelho AV, Vasconcelos V. Differential protein expression in two bivalve species; Mytilus galloprovincialis and Corbicula fluminea; exposed to Cylindrospermopsis raciborskii cells. Aquatic toxicology. 2011; 101(1): 109-116.
[13] Nzoughet KJ, Hamilton JTG, Floyd SD, Douglas A, Nelson J, Devine L, Elliott CT. Azaspiracid: First evidence of protein binding in shellfish. Toxicon. 2008; 51(7): 1255-1263.
[14] Nzoughet JK, Hamilton JT, Botting CH, Douglas A, Devine L, Nelson J, Elliott CT. Proteomics identification of azaspiracid toxin biomarkers in blue mussels, Mytilus edulis. Molecular & Cellular Proteomics. 2009; 8(8): 1811-1822.
[15] Leung PT, Wang Y, Mak SS, Ng WC, Leung KM. Differential proteomic responses in hepatopancreas and adductor muscles of the green-lipped mussel Perna viridis to stresses induced by cadmium and hydrogen peroxide. Aquatic Toxicology. 2011; 105(1-2): 49-61.
[16] Thompson EL, Taylor DA, Nair SV, Birch G, Haynes PA, Raftos DA. A proteomic analysis of the effects of metal contamination on Sydney Rock Oyster (Saccostrea glomerata) haemolymph. Aquatic toxicology. 2011; 103(3-4): 241-249.
[17] Meyfour A, Tavirani MR, Sadeghi MR. Common proteomic technologies, applications, and their limitations. Archives of Advances in Biosciences. 2013; 4: 1-11.
[18] Venugopal V. Methods for processing and utilization of low-cost fishes: a critical appraisal. Journal of Food Science and Technology (Mysore). 1995; 32(1): 1-12.
[19] Julian McClements. Analysis of food products, Analysis of Proteins, Food Science, University of Massachusetts, Chenoweth Lab: Amherst; 2003. 581 p.
[20] Directive, SF. Directive 2008/56/EC of the European Parliament and of the Council. Journal). Council Decision of 23 October 2000 establishing a framework for Commun action in the field of water policy; 2008. Off J Eur Commun 2000; L327:1-72.
[21] UNESCO. United nations world water development report. 3rd ed. Water in a changing world. UNESCO; 2009
[22] López-Pedrouso M, Varela Z, Franco D, Fernández JA, Aboal JR. Can proteomics contribute to biomonitoring of

aquatic pollution? A critical review. Environmental Pollution. 2020; 267: 115473.
[23] Kirsten WJ. Automatic methods for the simultaneous determination of carbon, hydrogen, nitrogen, and sulfur, and for sulfur alone in organic and inorganic materials. Analytical Chemistry. 1979; 51(8): 1173-1179.
[24] Owusu-Apenten RK. Food Protein Analysis: Quantitative Eff ects on Processing. New York: Marcel Dekker; 2002. p. 463.
[25] Mariotti F, Tomé D, Mirand PP. Converting nitrogen into protein-beyond 6.25 and Jones' factors. Critical Reviews in Food Science and Nutrition. 2008; 48(2): 177-184.
[26] Saint-Denis T, Goupy J. (). Optimization of a nitrogen analyser based on the Dumas method. Analytica Chimica Acta. 2004; 515(1): 191-198.
[27] Foegeding EA, Lanier TC, Hultin H. (). Characteristics of edible muscle tissue. In Fennema OR, editor. Food Chemistry. Marcel Dekker: New York; 1996. p. 879-942.
[28] Haard NF. Control of chemical composition and food quality attributes of cultured fish. Food Research International. 1992; 25: 289-307.
[29] Yada, RY. Analysis: Quantitation and physical characterization. In Nakai S, Modler HW, editors. Food Proteins: Properties and Characterization. VCH: New York; 1996.
[30] Lowry OH. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951; 193: 265-275.
[31] Olson BJ, Markwell J. Assays for determination of protein concentration. Current Protocols in Pharmacology. 2007; 38(1): 14-17.
[32] Bradford MM. A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976; 72: 248-254.
[33] Yetisen AK. Paper-based microfluidic point-of-care diagnostic devices. Lab on a Chip. 2013; 13 (12): 2210–2251. doi:10.1039/C3LC50169H
[34] Ramshaw JAM, Coyne JA, Lewontin RC. The sensitivity of gel electrophoresis as a detector of genetic variation. Genetics. 1979; 93: 1019-37.

[35] Maxam AM, Gilbert W. A new method for sequencing DNA. Proceedings of the National Academy of Science, USA. 1977; 74: 560-4.
[36] Sanger E, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Science, USA. 1977; 74, 5463-7.
[37] Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostabile DNA polymerase. Science. 1988; 239: 487-91.
[38] Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985; 230: 1350-4.
[39] Huxley TH. The Crayfish: An Introduction to the Study of Zoology. v. XXVIII of the Inter-national Scientific Series. D. Appleton: New York; 1879.
[40] Narahashi T. Nerve membrane ion channels as the target site of environmental toxicants. Environmental Health Perspectives. 1987; 71: 25-29.
[41] Taylor CA, Warren ML, Jr Fitzpatrick JF, et al. Conservation status of crayfishes of the United States and Canada. Fisheries. 1996; 21: 25-38.
[42] Stein BA, Flack SR. Species Report Card: the state of US plants and animals. The Nature Conservancy;1997. Available from: http://consci.tnc.org/library/pubs/rptcard/
[43] Nemeth ST, Tracey ML. Allozyme variability and relatedness in six crayfish species. The Journal of Heredity. 1979; 70: 37-43.
[44] Brown K. Low genetic variability and high similarities in the crayfish genera Cambarus and Procambarus. The American Midland Naturalist. 1981; 105: 225-32.
[45] Laemmli UK. Cleavage and structural proteins during assembly of the head of bacteriophage T4. Nature. 1970; 227: 680-685.
[46] Adler-Nissen J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. Journal of Agricultural and Food Chemistry. 1979; 27(6): 1256-1262.

[47] Taylor WH. Formol titration: An evaluation of its various modifications. Analyst. 1957; 82: 488-498.
[48] Rohm H, et al. Comparison of ethanol and trichloracetic acid fractionation for measurement of proteolysis in Emmental cheese. International Dairy Journal. 1996; 6: 1069-1077.
[49] Guzmán-Guillén R, Moreno I, Ortega AIP, Soria-Díaz ME, Vasconcelos V, Cameán AM. CYN determination in tissues from freshwater fish by LC-MS/MS: Validation and application in tissues from subchronically exposed tilapia (*Oreochromis niloticus*). Talanta. 2015; 131: 452-459.

CHAPTER 8

Pharmacological and Therapeutic Potential of Adaptogenic Plants

Ilkay Yilmaz, Berrak Delikanli Kiyak

Pharmacological and Therapeutic Potential of Adaptogenic Plants

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Abstract

Compared to past periods, the radical change in living conditions together with the mobility and speed in recent years causes people to push their abilities and limits by being exposed to stress. Although the symptoms seen in the clinic due to the stress exposure vary depending on various factors, it generally triggers the emergence of physical, behavioral, emotional and psychological problems in humans and can lead to cardiovascular diseases, hypertension, diabetes, anxiety, depression, etc. In this case, the term adaptogenic activity occurs and the interest in reliable alternative natural products is increasing. Pharmacologically, adaptogens are defined as substances that increase nonspecific resistance in order to adapt an organism to extraordinary situations by eliminating the causes that cause stress. Natural adaptogens have a very rich phytochemical composition. Extracts from plants such as *Panax ginseng, Eleutherococcus senticosus, Rhaponticum carthamoides, Rhodiola rosea, Schisandra chinensis* are considered as naturally occurring adaptogens. Adaptogens plants show adaptogenic properties with phytochemical compositions of their structure such as triterpenoid saponins, phytosterols, lignans, alkaloids, flavonoids and vitamins. In this section, the importance of adaptogen plants in human nutrition and their pharmacological activities are aimed to be a reference source for potential studies on health promotion.

Keywords: Adaptogen Plants, *Panax ginseng, Eleutherococcus senticosus, Rhaponticum carthamoides, Rhodiola rosea, Schisandra chinensis*

1. Introduction

The term “adaptogen” was first coined in 1947 by the discovery of the adaptogenic activity of Dibazol (2-Benzyl-benzimidazole), which was designed by Russian scientist Lazarev to induce non-specific resistance in humans [1–4]. Pharmacologically, adaptogens are defined as substances that increase non-specific resistance to adapt an organism to extraordinary situations by eliminating the causes that cause stress [1, 2].

The ability of each organism to adapt to its environment differs. In this case, any external or internal stimulus from this environment is responded to with either a specific or non-specific response. This non-specific response is defined as “stress” [2, 4]. In studies on healthy organisms, these internal and external stimuli from the environment are defined as “stress factors” (Table 8.1) [1].

1.1 Mechanism of Action of Adaptogens

It has been observed that with the consumption of adaptogens, changes occur in the endocrine functions of the hypothalamus-adrenal gland axis. In animal studies, it was determined that the adrenocorticotropic hormone (ACTH) serum levels and corticosteroids increased with a single application of adaptogen. It has been determined that stress hormone levels return to normal with subchronic application and the tendency to stress in the behavioral dimension decreases in general [1]. If the stress situation continues; a state of “adaptation” to stress occurs without observing exhaustion in the organism [1, 5].

Table 8.1. Factors Causing Stress

Enfection	Inactivity	Drugs	Conflict
	Restraint	Ethanol	Grief
	Extreme heat and cold		

In addition, non-specific reactions that occur in the organism against stress factors of various origins are defined under the name of "General Adaptation Syndrome." This syndrome occurs regardless of the nature of the stress factor and is completed in 3 phases: alarm, resistance and exhaustion (Figure 8.1) [1–3].

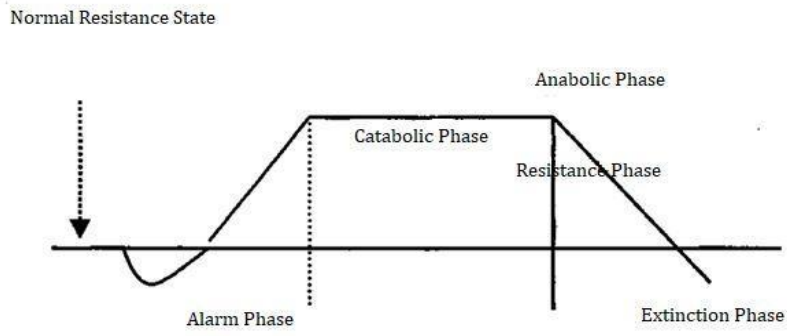

Figure 8.1. General adaptation syndrome phases

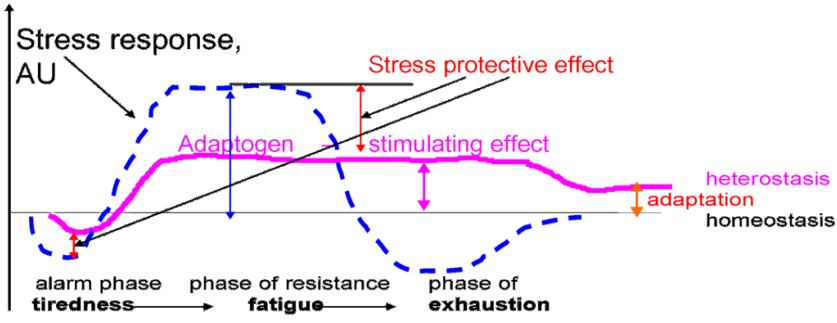

Figure 8.2. The Specific effect of adaptogens against stress in general adaptation syndrome phases

Adaptogens have been used around the world for years. It is studied in two main categories: synthetic adaptogens, also called actoprotectors, and plant adaptogens. [6]. Data on the use of bemethyl, the first synthetic adaptogen, date back to 1970. Since then, many synthetic adaptogens such as bromantane, levamisole, aphobazol, chlodantane, and trecrezan have been developed [7]. However, due to the interest in reliable alternative natural products, especially in recent years, plant adaptogens are widely considered as the preferred group. Extracts from plants such as "*Panax ginseng, Eleutherococcus senticosus, Rhaponticum carthamoides, Rhodiola rosea, Schisandra chinensis"* are considered naturally occurring adaptogens [6, 8]. Triterpenoidsaponins, phytosterols, lignans found in adaptogen plants; alkaloids, flavonoids, vitamins, etc. show adaptogenic properties with their phytochemical compositions [6, 9, 10]. The importance and pharmacological effects of plant adaptogens in human nutrition are still being studied. It also seems to have a promising potential for current studies on health promotion.

1.2. Adaptogen Plants

1.3. *Panax ginseng*

Panax ginseng, which has been called the king of medicinal herbs for thousands of years in East Asian countries, is used as a traditional medicine in the treatment of diseases. [11, 12]. The genus *Panax* from the *Araliaceae* family is mainly found in the northern hemisphere but is cultivated in 35 countries around the world [11]. "*Panax ginseng* (Korean ginseng), *Panax notoginseng* (Chinese ginseng), *Panax japonicum* (Japanese ginseng) and *Panax quinquefolius* (American ginseng)" are among the most common types of ginsengs [13].

The active ingredients of *Panax ginseng* include triterpenes, saponins (ginsenosides), polyacetylenes (falcarinol, panaxinol), free phenolic carboxylic acids, peptidoglycans (panaxans) and polysaccharides, fatty acids, amino acids, lactams, inorganic elements etc. [14].

It is known that *Panax ginseng* has therapeutic effects against inflammation, allergies, diabetes, cardiovascular diseases and cancer with its active components, and it is important in herbal medicine with these effects [15]. In addition to these effects, studies have shown that it has effects such as improvement of learning ability, antidepressant effect, correction of neurological function, reduction of DNA, protein and lipid damage, antioxidant, regulation of blood pressure and increasing circulation, protection of the liver from diseases, and curative effect on erectile dysfunction [11, 13].

It is known that ginsenosides, one of the active compounds of *Panax ginseng*, are an adaptogen substance that increases physical performance, creates resistance against stress and aging, and thus increases the quality of life [15–20]. Ginseng is thought to restore adrenal and thyroid functions, especially under stressful situations [21]. Brekhan and Dardymoy [22] state that ginseng increases the synthesis of glycogen and high-energy phosphate compounds, emphasizing that these functions of ginseng are more prominent, especially under physical stress conditions. It is noted that during exercise, ginseng increases the amount of cortisol, which is a stress hormone due to adrenocorticotropic hormone, through the hypothalamus-pituitary system, and increases physical strength by triggering adrenaline secretion in the adrenal medulla. In addition, Humphreys reported that ginseng as an adaptogen is more prominent in individuals under exercise stress, which causes maximal oxygen consumption [23].

1.4. *Eleutherococcus senticosus* (*Siberian ginseng*)

Eleutherococcus senticosus L., commonly known as "Siberian Ginseng", grows in the Taiga region of Southeast Asia, northern China, Korea, Japan, and southeast Russia [24, 25]. *E. senticosus* is used as anti-fatigue and anti-fatigue, improving sleep quality, strengthening memory, nourishing the heart and calming. [26]. However, production of *Eleutherococcus* by biotechnological methods; it can be a good alternative to its rarity and gradual decrease. *E. senticosus* is also

seen as an inexpensive alternative to Panax ginseng. However, since E. senticosus does not contain ginsenosides, the active ingredient of *Panax* species, there is no close resemblance to these species [27]. *E. senticosus* has active chemical components such as eleutherosides, chiisanoside, acanthosides, daucosterin, β-sitosterol, sesamin, lignans, triterpenoid saponins, flavonoids, polysaccharides etc. [28, 29]. With its current chemical structure, *E. senticosus* has various effects as anti-inflammatory, hypoglycemic, hypnotic, anti-cancer and antioxidant effects [30–34]. In addition, mainly *E. senticosus* extracts have been used globally as an adaptogenic agent. Oxidative stress is generally inhibited by superoxide dismutase and glutathione peroxidase [35]. Studies have shown that *E. senticosus* can increase superoxide dismutase and glutathione peroxidase activities [6]. It is known that the active compounds responsible for this adaptogenic activity are eleutheroside B and E [36–38]. All these properties create a potential environment for the development of medicinal products containing *E. senticosus* extract [6]. When the studies on *E. senticosus* are examined, it is seen that the intake of E. *senticosus* extract supports physical activity, weight loss, mental health and fatigue by helping to increase cognitive function. When people work overtime or are exposed to physical stimulation for a long time, 5-HT is secreted excessively, making people feel tired [39]. In a study examining the effect of *E. senticosus* trunk bark extract on the fatigue duration of mice; it has been determined that the rhizomes of *E. senticosus* reduce 5-HT synthesis and prevent fatigue by this mechanism [40].

In another study on the prevention of fatigue by *E. senticosus*, when the treatment group using *E. senticosus* extracts was compared with the control group, it was found that the swimming time and tissue glycogen content of the treatment group increased, and the blood lactate and serum urea nitrogen content of the treatment group decreased. This result revealed that the aqueous extract of *E. senticosus* has anti-fatigue activity to improve exercise tolerance [41-42- 43].

The effects of *E. senticosus* on bipolar disorder, were examined over 6-weeks period. As a result, it has been found that the crude extract of *E. senticosus* can be used as an effective and safe alternative to treat biopolar disorders [44].

Dementia, a degenerative brain disease, is the most common cause of dementia. In one study, oral administration of a water extract of *E. senticosus* roots to dementia model mice has been found to have neuroprotective effects [45].

Studies have found that in addition to the roots of *E. senticosus*, its leaves and small molecules can also show neural benefits to mice [46].

In a clinical study, *E. senticosus* leaves and drynaria wealth rhizomes were combined and administered to healthy volunteers over a 12-week period. It has been found that a water extract of the leaves of *E. senticosus* can improve cognitive function in healthy adults without adverse effects, and these extracts improved the volunteers' language and memory abilities as well as their anxiety levels and no adverse effects were observed [47].

Stroke is a clinical manifestation of CI (Cardioembolic cerebral infarction) [48]. In a clinical study, injection of 100 mg/kg of *E. senticosus* was found to reduce infarct volume in mice with transient cerebral artery occlusion occurring in ischemic stroke [49]. Many studies have shown that extracts of *E. senticosus* have a certain therapeutic effect on CI; however, studies on the compounds found in the structure of *E. senticosus* are not sufficient [50].

1.5. *Rhaponticum carthamoides*

Rhaponticum carthamoides (Wild.), a perennial herb that grows in the Altai and Saian Mountains, is also known as maral root. For centuries, it has been used in Siberian traditional medicine for post-illness fatigue and general malaise. In the present case, *R. carthamoides* has spread to various parts of

Central and Eastern Europe, where extracts are used in various dietary supplements and nutraceutical preparations because of their adaptogenic properties [51–53]. In addition to its adaptogenic effect, it also has antioxidant, immunomodulatory, anticarcinogenic, and antimicrobial properties with tecdysteroids, triterpenoids, polyacetylenes, flavonoids, caffeoylquinic acid derivatives and phenolic acids in its composition [52, 54, 55]. In a study examining the effects of *R. carthamoides* extracts on athletes, it was determined that plant extracts showed beneficial effects on athletes by increasing the body's adaptation to various factors that can be defined as stress for the body, and that it had no significant side effects [56-57]. However, despite the benefits of using *R. carhamoides* extracts and the absence of serious side effects, human studies are limited and insufficient for a more complete and comprehensive evaluation [58–60–61].

1.6. *Rhodiola rosea*

Rhodiola rosea (Golden Root) is traditionally used for different medical purposes such as stimulating the nervous system, improve physical and mental performance, treat asthenia due to stress and depression, relieving fatigue and helping protect the brain against oxygen deprivation in various countries around the world, especially in Sweden, Norway, Iceland and Russia [62–64]. *R. rosea* L. plant is defined as an adaptive agent that increases physical endurance, affects fatigue, depression and nervous system disorders. The adaptogenic effect of *R. rosea* is thought to be since it supports the activation of the cerebral cortex by increasing the levels of norepinephrine and serotonin [6]. Moreover, *R. rosea* increases the body's resistance to stress by affecting the hypothalamic-pituitary-adrenal axis and protects against mental and physical fatigue caused by stress. For this reason, especially soldiers and athletes use *R. rosea* to increase their stress resistance and fight fatigue [6, 65]. In addition to the positive effect of *R. rosea* on fatigue; it is also known to improve physiological properties such as cardioprotective effects, central nervous system simulation, and cognitive abilities such as memory, learning, and attention [66].

The therapeutic activities of *R. rosea* are related to the presence of active ingredients in its structure and their relative composition [66]. The structure of *R. rosea* contains flavonoids, tyrosol, proanthocyanidins, cinnamyl alcohol, etc. polyphenols (about 41.4 ± 3.41%). Today, *R. rosea* extracts form the active ingredient in many popular commercial preparations sold worldwide as food additives, dietary supplements, pharmaceutical drugs, and beverages [67]. *R. rosea* is widely used in the United States and Europe as a safe non-toxic natural health product. In addition, studies show that there is no documented serious side effect with the consumption of the plant [6, 60, 66].

The synergistic effects of *R. carthamoides* and *R. rosea* were also investigated; however, the synergistic effect of adaptogenic herbs could not be confirmed. Therefore, more studies are needed in this area [6].

1.7. *Schisandra chinensis*

Schisandra chinensis, also known as bitter melon, is an herb that grows in northeast Asia. It is used in both traditional Chinese medicine and modern medicine for its positive effects on health. *S. chinensis* berries and seeds were originally used to improve cough and asthma, as well as increase night vision, reduce hunger, thirst, and fatigue [68]. In 1960, *S. chinensis* was proven to have adaptogenic properties in Russia [69]. It is known that the adaptogenic property of the plant is related to its effect on the hypothalamic-pituitary-adrenal axis by lowering the level of corticotropin-releasing hormone. And all of the phytochemicals in its structure have cytotoxic, antioxidant, neuroprotective, hepatoprotective, physical strength-enhancing, stress-protecting and anti-inflammatory effects [70]. In addition, it has been reported in studies that lignans such as gomycin A, gomycin G, shizandrin and schisanhenol in the plant have antitumor activity [71–73].

2. Conclusion

Natural adaptogens can increase the body's resistance to stress changes caused by different types of stressors. Its adaptogenic properties are not due to a single molecule but to a combination of different substances. There are a limited number of studies examining the benefits of using extracts of "*Panax ginseng, Eleutherococcus senticosus and Rhaponticum carthamoides, Rhodiola rosea, Eleutherococcus senticosus* and *Schisandra chinensis"*, some of the most widely used plant adaptogens today. However, there is potential for incorporation of extracts of these plants into medicinal products aimed at treating chronic fatigue, cognitive impairment, as well as boosting immune defense. For nearly 50 years, plant adaptogens have been used by professional athletes because of their high potential to increase the body's resistance and increase physical endurance. However, since 2020, some adaptogens have been included in WADA's monitoring program and are likely to be included in the banned list. Therefore, there is a need for multicenter, randomized, double-blind studies to better investigate the benefits and future applications of adaptogen extracts.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Wagner H, Nörr H, Winterhoff H. Plant adaptogens. Phytomedicine. 1994; 1:63-76.
[2] Rege NN, Thatte UM, Dahanukar SA. Adaptogenic properties of six rasayana herbs used in ayurvedic medicine. Phytotherapy Research. 1999; 13:275-291.
[3] Arnason JT, Mata R, Remoe JT, Phytochemistry of Medicinal Plants. New York: Plenum Press; 1995. 1 p.
[4] Panossian AG, Efferth T, Shikov AN, Pozharitskaya ON, Kuchta K, Mukherjee PK, Banerjee S, Heinrich M, Wu W, Guo D, et al. Evolution of the adaptogenic concept from traditional

use to medical systems: Pharmacology of stress and agingrelated diseases. Medicinal Research Reviews. 2020; 41: 630-703.
[5] Panossian A, Wikman G. Effects of adaptogens on the central nervous system and the molecular mechanisms associated with their stress protective activity. Pharmaceuticals. 2010; 3: 188-224.
[6] Todorova V, Ivanov K, Delattre C, Nalbantova V, Karcheva-Bahchevanska D, Ivanova S. Plant adaptogens history and future perspectives. Nutrients. 2021; 13: 2861.
[7] Oliynyk S, Oh SK. The pharmacology of Actoprotectors: Practical application for improvement of mental and physical performance. Biomolecules and Therapeutics. 2012; 20: 446-456.
[8] Brekhman AI, Dardymov IV. New substances of plant origin which increase nonspecific resistance. Annual Review of Pharmacology. 1969; 9: 419-430.
[9] Kamal M, Arif M, Jawaid T. Adaptogenic medicinal plants utilized for strengthening the power of resistance during chemotherapy. Oriental Pharmacy and Experimental Medicine. 2017; 17: 1-18.
[10] Kelly GS. Rhodiola rosea: A possible plant adaptogen. Alternative Medicine Review. 2001; 6: 293-302.
[11] Ratan ZA, Haidere MF, Hong YH, Park SH, Lee JO, Lee J, Cho JY. Pharmacological potential of ginseng and its major component ginsenosides. Journal of Ginseng Research. 2021; 45: 199-210.
[12] Chao an 1,2, Saijian Ma 1,2, Xinwei Shi 2,3, Chen Liu 1,2, Hao Ding 1,2 and Wenjiao Xue, Diversity and Ginsenoside Biotransformation Potential of Cultivable Endophytic Fungi Associated with Panax bipinnatifidus var. bipinnatifidus in Qinling Mountains, China. Frontiers in Pharmacology. 2022. 13; 762862.
[13] Kim JH. Pharmacological and medical applications of Panax ginseng and ginsenosides: a review for use in cardiovascular diseases. Journal of Ginseng Research. 2018; 42: 264-269.
[14] Liu L, Xu FR, Wang YZ. Traditional uses, chemical diversity and biological activities of Panax L. (Araliaceae): A review. Journal of Ethnopharmacology. 2020; 5263: 112792.

[15] Irfan M, Kwak YS, Han CK, Hyun SH, Rhee MH. Adaptogenic effects of Panax ginseng on modulation of cardiovascular functions. Journal of Ginseng Research. 2020; 44: 538-543.
[16] O'Hara M, Kiefer D, Farrell K, Kemper K. A review of 12 commonly used medicinal herbs. Archives of Family Medicine. 1998; 7: 523.
[17] Kim YJ, Zhang D, Yang DC. Biosynthesis and biotechnological production of ginsenosides. Biotechnology Advances. 2015; 33: 717-735.
[18] Ma GD, Chiu CH, Hsu YJ, Hou CW, Chen YM, Huang CC. Changbai mountain ginseng (Panax ginseng C.A. Mey) extract supplementation improves exercise performance and energy utilizationand decreases fatigue-associated parameters. Molecules. 2017; 22: 237-250.
[19] Arring NM, Millstine D, Marks LA, Nail LM. Ginseng as a treatment for fatigue. Journal of Alternative and Complementary Medicine. 2018; 24.
[20] Huang X, Li N, Pu Y, Zhang T, Wang B. Neuroprotective effects of ginseng phytochemicals: Recent perspectives. Molecules. 2019; 24: 2939.
[21] Siegel RK. Ginseng abuse syndrome, problems with the panacea. Jama. 1979; 241: 1614-1615.
[22] Brekhman II, Dardymov IV. Pharmacological investigation of glycosides from Ginseng and Eleutherococcus. Lloydia. 1969; 32: 46-51.
[23] Humphreys DJ. Nort American Ginseng and The Stress Response During Acute Exercise. Alberta: Edmonton. 2001.
[24] Guo HY, Zhang J, Lin LM, Song X, Zhang DD, Cui MH, Long CW, Long YH, Xing ZB. Metabolome and transcriptome analysis of eleutheroside B biosynthesis pathway in Eleutherococcus senticosus. Heliyon. 2022; 8: 09665.
[25] Alexander SN, Narkevich IA, Flisyuk EV, Luzhanin VG, Pozharitskaya ON. Medicinal plants from the 14th edition of the Russian Pharmacopoeia, recent updates. Journal of Ethnopharmacology. 2021; 268: 113685.
[26] Li XT, Zhou JC, Zhou Y, Ren YS, Huang YH, Wang SM, Ge YW. Pharmacological effects of *Eleutherococcus senticosus*

on the neurological disorders. Phytotherapy Research. 2022; 36: 3490-3504.
[27] Yun TK. Brief introduction of Panax ginseng C.A. Meyer. Journal of Korean Medical Science. 2001; 16: 3-5.
[28] Davydov M, Krikorian AD. *Eleutherococcus senticosus* (Rupr. & Maxim.) maxim. (Araliaceae) as an adaptogen: a closer look. Journal of Ethnopharmacology. 2000; 72: 345-393
[29] Wang ZB, Guo JT, Jiang H, Yang BY, Kuang HX. Study on the chemical constituents and pharmacological effects of *Acanthopanax senticosus* leaves. Traditional Chinese Medicine. 2013; 30: 29-32.
[30] Lee CH, Huang CW, Chang PC, Shiau JP, Lin IP, Lin MY, et al. Reactive oxygen species mediate the chemopreventive effects of syringin in breast cancer cells. Phytomedicine. 2019; 61.
[31] Shen Z, Yang C, Zhu P, Tian C, Liang AF. Protective effects of syringin against oxidative stress and inflammation in diabetic pregnant rats via TLR4/MyD88/NF-κB signaling pathway. Biomedicine and Pharmacotherapy. 2020; 131.
[32] Wang CY, Zhang Q, Xun Z, Yuan L, Li R, Li X, et al. Increases of iASPP-Keap1 interaction mediated by syringin enhance synaptic plasticity and rescue cognitive impairments via stabilizing Nrf2 in Alzheimer's models. Redox Biology. 2020; 36.
[33] Zhang H, Gu H, Jia Q, Zhao Y, Li H, Shen S, et al. Syringin protects against colitis by ameliorating inflammation. Archives of Biochemistry and Biophysics. 2020; 680.
[34] Dai R, Niu M, Wang N, Wang Y. Syringin alleviates ovalbumin-induced lung inflammation in BALB/c mice asthma model via NF-κB signaling pathway. Environmental Toxicology. 2021; 36: 433-444.
[35] Khoshnam SE, Winlow W, Farzaneh M, Farbood Y, Moghaddam HF. Pathogenic mechanisms following ischemic stroke. Neurological Sciences. 2017; 38: 1167-1186.
[36] Zhang C, Zhao X, Mao X, Liu A, Liu Z, Li X. Pharmacological evaluation of sedative and hypnotic effects of schizandrin through the modification of pentobarbital-induced

sleep behaviors in mice. European Journal of Pharmacology. 2014; 744: 157-163.
[37] Załuski D, Kuzniewski R, Janeczko Z. HPTLC-profiling of eleutherosides, mechanism of antioxidative action of eleutheroside E1, the PAMPA test with LC/MS detection and the structure–activity relationship. Saudi Journal of Biological Sciences. 2018; 25: 520-528.
[38] Graczyk F, Gebalski J, Makuch-Kocka A, Gawenda-Kempczynska D, Ptaszynska AA, Grzyb S, Bogucka-Kocka A, Załuski D. Phenolic profile, antioxidant, anti-enzymatic and cytotoxic activity of the fruits and roots of *Eleutherococcus senticosus* (Rupr. et Maxim.) maxim. Molecules. 2022; 27: 5579.
[39] Kavanagh JJ, McFarland AJ, Taylor JL. Enhanced availability of serotonin increases activation of unfatigued muscle but exacerbates central fatigue during prolonged sustained contractions. The Journal of Physiology. 2019; 597: 319-332.
[40] Rhim YT, Kim H, Yoon SJ, Kim SS, Chang HK, Lee TH, Kim CJ. Effect of Acanthopanax senticosus on 5-hydroxytryptamine synthesis and tryptophan hydroxylase expression in the dorsal raphe of exercised rats. Journal of Ethnopharmacology. 2007; 114: 38-43.
[41] Zhang XL, Ren F, Huang W, Ding RT, Zhou QS, Liu XW. Anti-fatigue activity of extracts of stem bark from Acanthopanax senticosus. Molecules. 2010; 16: 28-37.
[42] Jin ML, Park SY, Kim YH, Park G, Lee SJ. Acanthopanax senticosus exerts neuroprotective effects through HO-1 signaling in hippocampal and microglial cells. Environmental Toxicology and Pharmacology. 2013; 35: 335-346.
[43] Bamalan OA, Al-Khalili Y. Physiology, serotonin. StatPearls: StatPearls Publishing. 2022.
[44] Weng S, Tang J, Wang G, Wang X, Wang H. Comparison of the addition of Siberian ginseng (Acanthopanax senticosus) versus fluoxetine to lithium for the treatment of bipolar disorder in adolescents: A randomized, double-blind trial. Current Therapeutic Research. 2007; 68: 280-290.
[45] Tohda C, Ichimura M, Bai Y, Tanaka K, Zhu S, Komatsu K. Inhibitory effects of *Eleutherococcus senticosus* extracts on

amyloid beta (25-35)-induced neuritic atrophy and synaptic loss. Journal of Pharmacological Sciences. 2008; 107: 329-339.
[46] Yamauchi Y, Ge YW, Yoshimatsu K, Komastu K, Kuboyama T, Yang X, Tohda C. Memory enhancement by oral administration of extract of Eleutherococcus senticosus leaves and active compounds transferred in the brain. Nutrients. 2019; 11: 1142.
[47] Tohda C, Matsui M, Inada Y, Yang X, Kuboyama T, Kimbara Y, Watari H. Combined treatment with two water extracts of *Eleutherococcus senticosus* leaf and rhizome of Drynaria fortuneienhances cognitive function: A placebo-controlled, randomized, double-blind study in healthy adults. Nutrients. 2020; 12: 303.
[48] Virani SS, Alonso A, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, Tsao CW. Heart disease and stroke statistics-2020 update: A report from the American heart association. Circulation. 2020; 141: 139-596.
[49] Bu Y, Jin ZH, Park SY, Baek S, Rho S, Ha N, Kim H. Siberian ginseng reduces infarct volume in transient focal cerebral ischaemia in Sprague-Dawley rats. Phytotherapy Research. 2005; 19: 167-169.
[50] Tan J, Luo J, Meng C, Jiang N, Cao J, Zhao J. Syringin exerts neuroprotective effects in a rat model of cerebral ischemia through the FOXO3a/NF-κB pathway. International Immunopharmacology. 2021; 90: 107268.
[51] Petkov V, Roussinov K, Todorov S, Lazarova M, Yonkov D, Draganova S. Pharmacological investigations on Rhaponticum carthamoides. Planta Medica. 1984; 50: 205-209.
[52] Yance D. *Rhaponticum carthamoides*: Monograph and review of the literature. American Herbalists Guide. 2004; 5: 9-17.
[53] Kokoska L, Janovska D. Chemistry and pharmacology of Rhaponticum carthamoides: a review. Phytochemistry. 2009; 70: 842-855.
[54] Skała E, Kicel A, Olszewska MA, Kiss AK, Wysokinska H. Establishment of hairy root cultures of Rhaponticum carthamoides (Willd.) Iljin for the production of biomass and caffeic acid derivatives. BioMed Research International. 2015;11.

[55] Biskup E, Lojkowska E. Evaluation of biological activities of Rhaponticum carthamoides extracts. Journal of Medicinal Plant Research. 2009; 31: 1092-1098.
[56] Timofeev NP. Leuzea carthamoides DC.: Application prospects as pharmpreparations and biologically active component. In: Martirosyan DM, editors. Functional Foods for Chronic Diseases. Texas, USA: D&A Incorporated. 2006. 105120.
[57] Biskup E, Szynklarz B, Golebiowski M, Borsuk K, Stepnowski P, Lojkowska E. Composition and biological activity of Rhaponticum carthamoides extracts obtained from plants collected in Poland and Russia. Journal of Medicinal Plants Research. 2013; 7: 687-695.
[58] Brown RP, Gerbarg PL, Ramazanov Z. Rhodiola rosea: A phyto-medicinal overview. Herbal gram. Journal of the American Botanical Council. 2002; 56: 40-52.
[59] Xia X, Zhang Q, Liu R, Wang Z, Tang N, Liu F, Sun X. Effects of 20-hydroxyecdysone on improving memory deficits in streptozotocin-induced type 1 diabetes mellitus in rat. European Journal of Pharmacology. 2014; 740: 45-52.
[60] Pu WL, Zhang MY, Bai RY, Sun LK, Li WH, Yu YL, Li TX. Anti-inflammatory effects of *Rhodiola rosea* L. Biomedicine and Pharmacotherapy, 2020; 121: 109552.
[61] The World Anti-Doping Agency (WADA). The Monitoring Program. 2020. Availablefrom: https://www.wadaama.org/sites/default/files/resources/files/wada_2020_english_monitoring_program_pdf [Accessed: 24.05.2023]
[62] Panossian A, Wikman G, Sarris J. Rosenroot (*Rhodiola rosea*): Traditional use, chemical composition, pharmacology and clinical efficacy. Phytomedicine. 2010; 17: 481-493.
[63] Edwards D, Heufelder A, Zimmermann A. Therapeutic effects and safety of *Rhodiola rosea* extract WS® 1375 in subjects with life-stress symptoms results of an open-label study. Phytotherapy Research. 2012; 26: 1220-1225.
[64] Limanaqi F, Biagioni F, Busceti CL, Polzella M, Fabrizi C, Fornai F. Potential antidepressant effects of *Scutellaria baicalensis*, *Hericium erinaceus* and *Rhodiola rosea*. Antioxidants. 2020; 9: 234.

[65] Ankita W, Pranay W, Awani KR, Ruchi T, Sunil KP. Adaptogens with a special emphasis on Withania somnifera and Rhodiola rosea. Debasis B, Sreejayan N, Chandan KS, editors. In: Nutrition and enhanced sports performance. 2nd ed. Chapter 34, Oxford, United Kingdom: Academic Press; 2019. P. 407-418.
[66] Sist P, Tramer F, Lorenzon P, Urbani R, Vrhovsek U, Bernareggi A, Sciancalepore M. Rhodiola rosea, a protective antioxidant for intense physical exercise: An in vitro study. Journal of Functional Foods. 2018; 48: 27-36.
[67] Evstatieva L, Todorova M, Antonova D, Staneva J. Chemical composition of the essential oils of Rhodiola rosea L. of three different origins. Pharmacognosy Magazine. 2010; 6: 256-258.
[68] Hancke JL, Burgos RA, Ahumada F. Schisandra chinensis (Turcz.) baill. Fitoterapia. 1999; 70: 451-471.
[69] Lu Y, Chen DF. Analysis of Schisandra chinensis and Schisandra sphenanthera. Journal of Chromatography. 2009; 1216: 1980-1990.
[70] Yang BY, Guo JT, Li ZY, Wang CF, Wang ZB, Wang QH, Kuang HX. (). New thymoquinol glycosides and neuroprotective dibenzocyclooctane lignans from the rattan stems of Schisandra chinensis. Chemistry and Biodiversity. 2016; 13: 1118-1125.
[71] Chen M, Kilgore N, Lee KH, Chen DF. Rubrisandrins A and B, lignans and related anti-HIV compounds from Schisandra rubriflora. Journal of Natural Products. 2006; 69:1697-1701.
[72] Fong WF, Wan CK, Zhu GY, Chattopadhyay A, Dey S, Zhao Z, Shen XL. Schisandrol A from Schisandra chinensis reverses P-glycoprotein-mediated multidrug resistance by affecting Pgp-substrate complexes. Planta Medica. 2007; 73: 212-220.
[73] Yoo HH, Lee M, Lee MW, Lim SY, Shin J, Kim DH. Effects of Schisandra lignans on P-glycoprotein-mediated drug efflux in human intestinal Caco-2 cells. Planta Medica. 2007; 53: 444-450.

CHAPTER 9

MXene Nanomaterials for Cancer Therapy

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MXene Nanomaterials for Cancer Therapy

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Abstract

MXenes, known for their unique physicochemical properties and excellent compatibility with living organisms, have generated significant interest in diverse fields of biology and medicine. Modifying their external characteristics while overcoming preparative limitations allows for customization in cancer treatment and diagnosis. MXene-based micro and nano systems show promise in delivering anticancer drugs and therapeutic agents with low toxicity and high biocompatibility. However, there is a need to comprehensively address the enhancement of biocompatibility, pharmacokinetics, and biodegradability of these structures. This entails the exploration of simple, cost-effective, and environmentally friendly approaches for synthesis and functionalization, optimization of reaction conditions, and the efficient combination of biocompatible and biodegradable agents to improve the properties and efficacy of MXene-based systems. A major challenge lies in confirming the successful absorption of therapeutic substances from the bloodstream to the desired target site, with the vascular endothelial cell acting as a crucial barrier. The acceptance of manmade nanosystems by endothelial cells is therefore vital for their biological and therapeutic applications. Functionalizing or modifying MXene-based composites with appropriate agents or functional groups can enhance their capacity for drug loading, biocompatibility, and bioavailability. Furthermore, this approach can help mitigate undesirable side effects and potential immune reactions. The rapid progress of MXene-based materials for targeted drug delivery in cancer therapy is expected to continue in the future. The insights and

future directions presented in this review aim to guide scientists and engineers in the development of MXenes and their derivatives for targeted drug delivery and cancer therapy.

Keywords: MXenes, cancer therapy, drug delivery, photothermal therapy, photodynamic therapy, nanocarriers, biocompatibility and safety considerations.

1. Introduction: An Overview of MXenes

1.1. Rationale for MXene Application in Cancer Therapy

Cancer, a leading cause of death worldwide, necessitates the development of advanced and targeted strategies for early diagnosis and treatment. Graphene and the more recent discovery, MXene, belong to the family of two-dimensional (2D) materials and have garnered significant attention in cancer research as multimodal nanoplatforms for diagnostics and treatment, particularly in the realm of photodynamic therapy (PDT). Leveraging their unique physicochemical properties, these materials demonstrate promising potential as therapeutic agents for PDT, as well as for bioimaging, photothermal therapy, and drug/gene delivery. This comprehensive review delves into the rapidly progressing literature surrounding the use of these exceptional 2D materials in cancer theranostics, highlighting their future advancements in PDT. Nanotechnology has played a pivotal role in advancing PDT by utilizing two-dimensional nanomaterials (NMs) for theranostic purposes. By combining therapeutic strategies with imaging modalities, these NMs enable the monitoring of therapeutic agent biodistribution and facilitate the identification and localization of tumor masses and their growth [1].

1.2. MXenes as Versatile Substrate for Advanced Sensors in Cancer Treatments

MXenes have found applications in electrochemical (bio) sensors due to their conductivity, high surface area, and thin-flake morphology. They can act as charge-transfer facilitators and catalysts in electrochemical oxidation or reduction reactions. For example, MXenes have been used as redox transducers and electro-catalysts for direct electrochemical sensing of carbendazim pesticide. MXenes, such as $Ti_3C_2T_x$, have also been utilized as immobilization matrices and redox mediators for the detection of hydrogen peroxide (H_2O_2) using enzymes like hemoglobin (Hb). In the future, MXenes show promise in biotechnology, nanomedicine, and sensors. They can be used in anticancer therapies, particularly in photothermal therapy (PTT), where MXenes with efficient light-to-heat energy conversion, biocompatibility, and simple surface functionalization can selectively kill tumor cells when exposed to near-infrared (NIR) irradiation. MXenes have also demonstrated potential as photothermal agents for bioimaging and have shown high photothermal conversion efficiencies in killing cancer cells. Additionally, MXenes, such as Ti-based MXenes, have been evaluated as photothermal agents against malignant cells, showing selectivity towards cancerous cells while negligibly affecting the viability of normal cells. These MXenes have shown promise in enhancing the selectivity and effectiveness of cancer treatment methods [2].

1.3. Biomedical Applications of MXene

1.4. Regenerative Effect of MXenes in Cardiac Tissue Engineering

Cardiac failure is a prevalent condition affecting millions of people worldwide. To improve heart tissue regeneration, the development of conductive cardiac patches that facilitate electrophysiological coupling with the host tissue has emerged as a promising strategy. Various materials, including conductive polymers, carbon nanotubes, graphene, gold nanorods, and

MXenes, have been integrated into hydrogels or cryogels to enhance the electrophysiological properties for cardiac tissue engineering. The alignment of cardiomyocytes (CMs) plays a crucial role in achieving functionality in cardiac tissue. Integration of $Ti_3C_2T_x$ MXene into nonconductive hydrogels has been shown to improve the synchronous beating rate of induced cardiomyocytes (iCMs). Additionally, Ti_3C_2 MXene quantum dots incorporated into chitosan-based hydrogels have demonstrated their potential for in vitro applications by supporting cell survival, proliferation, and electrical signal transfer among cells. Chitosan-MXene quantum dots composites have shown compatibility with various types of stem cells and possess anti-inflammatory and antibacterial properties, making them suitable for tissue engineering demands. These advancements pave the way for the utilization of MXenes in cardiac tissue engineering applications [3,4].

1.5. MXenes Infection Therapy Functions

The extensive use of antibiotics has led to the emergence of drug-resistant bacteria, necessitating the development of alternative antibacterial materials. MXene nanosheets have demonstrated bactericidal properties by disrupting bacterial membranes and inducing oxidative stress, resulting in bacteria's elimination. Synergistic antimicrobial agents have been developed by conjugating Au nanoparticles (AuNCs) with $Ti_3C_2T_x$ MXene nanosheets. The antibacterial efficacy of MXenes was significantly enhanced after the conjugation, with MXene nanosheets physically damaging bacterial membranes, while Au nanostructures induced oxidative stress by generating reactive oxygen species (ROS). The ROS reservoirs contributed to increased bacterial membrane damage and the oxidation of bacterial DNA, ultimately leading to bacterial killing. Crumpled MXene-Au nanocomposites effectively inhibited biofilm formation due to their hydrophobic surface and higher density of bactericides compared to planar structures. Although in vivo studies are lacking, the synergistic effect of Au-conjugated MXenes holds great promise for designing novel and effective MXene-based nanocomposites against bacterial infections.

MXene exhibits antibacterial properties through both chemical and physical mechanisms, primarily by inducing oxidative stress. However, further research is needed to address the limitations of this mechanism against certain bacteria [5].

1.6. MXene in Breast Cancer Diagnosis and Treatment

Breast cancer is the second most common cancer worldwide, and the use of nanomaterials has shown promise in timely diagnosis and effective treatment. MXenes, a 2D material with unique attributes such as electrical conductance, optical characteristics, mechanical consistency, and excellent biocompatibility, have emerged as promising tools in biotechnology applications. MXenes have various applications in biotechnology and biomedicine, including drug delivery/loading, biosensors, cancer treatment, and bioimaging programs, due to their high surface area and physicochemical properties. Surface modifications of MXenes enable them to have multifunctional properties, such as preferential agglomeration at tumor sites for photothermal treatment. Studies have demonstrated that MXene-based nanostructures are more effective in breast cancer detection and therapy, both in vivo and in vitro, compared to current nanosystems. This review article aims to investigate the structure of MXene, various synthesis methods, its application in cancer diagnosis, cytotoxicity, biodegradability, and cancer treatment through the photothermal process [6,7].

1.7. MXene-Based Biosensors

MXenes have been utilized in the development of biosensors for the diagnosis of various cancers. For instance, Ti_3C_2 nanosheets functionalized with APTES were used to design an electrochemical biosensor for carcinoembryonic antigen (CEA) diagnosis. This biosensor exhibited high sensitivity, wide detection range, and a low limit of detection (LOD). Another study focused on detecting MUC1, a breast cancer marker, using a competitive electrochemical biosensor based on cDNA-Fc/MXene probes. The biosensor showed a large linear range

and a low LOD, making it suitable for clinical detection. These MXene-based biosensors offer significant advancements in cancer diagnosis and show promise in improving detection sensitivity[8].

1.8. Cancer Theranostic and Nanotheranostics Applications of MXene

MXenes have emerged as versatile nanomaterials with exciting physical and chemical properties. Their biocompatibility and tunable electronic and optical properties make them promise for biomedical applications, particularly in cancer theranostics. MXenes have been utilized in in vitro and in vivo cancer models for drug delivery systems, sensoring probes, and auxiliary agents in photothermal therapy and hyperthermia. This mini review summarizes the use of MXenes, such as Ti_3C_2, Nb_2C, and Ti_2C, in conjunction with drug molecules, metallic nanoparticles, and macromolecules for cancer theranostic applications. The preparation methods for MXenes include top-down and bottom-up approaches, with hydrofluoric acid-based exfoliation and chemical vapor deposition being commonly used. MXenes possess layered structures that provide unique properties, including metallic, semi-metallic, and insulating behavior, flexibility, stiffness, and heat resistance. They also exhibit high surface reactivity, hydrophilicity, and adsorption ability due to their large surface area. MXenes have found applications in various fields, including energy storage, optoelectronic devices, wastewater treatment, gas sensors, electromagnetic interference shielding, and medicine. Recent studies have focused on surface-engineered MXenes, such as Nb_2C, for phototherapeutic applications in the near-infrared II (NIR-II) bio window, as radio protectants in chemotherapy, and as bone scaffolds for bone cancer treatment. Surface-engineered MXenes with anticancer drugs and tumor-targeting agents show high drug loading capacity, making them promising nanoplatforms for anti-cancer treatment [9].

MXenes have garnered attention in cancer nanotheranostics due to their unique features, including mechanical, optical,

electronic, and thermal properties. Their hydrophilicity and high surface area make them suitable for functionalization and modification, enabling targeted cancer nanotherapy and specific imaging/diagnosis of cancer cells and tumor sites. MXene-based systems have shown potential for targeted anticancer drug delivery, as well as photothermal, photodynamic, and chemodynamic therapies, along with magnetic resonance and computed tomography imaging. The combination of MXenes with other nanomaterials, such as gold nanorods, has shown excellent photothermal conversion efficiency for cancer therapy. MXenes can also load anticancer drugs and exhibit pH/NIR-responsive drug release behaviors, allowing for on-demand drug release and synergistic photothermal tumor ablation and chemotherapy. However, the long-term toxicity of pristine and surface-modified MXenes needs to be evaluated for their human and environmental impact[4,7].

1.9. MXenes as Drug Delivery Vehicles

MXenes, have shown great potential in various applications. However, their specific performance and applications in theranostic nanomedicine have been less explored. This work focuses on the drug-delivery performance and synergistic therapeutic efficiency of Ti_3C_2 MXenes for tumor eradication. Ti_3C_2 MXenes exhibit high drug-loading capability and demonstrate pH-responsive and near infrared laser-triggered on-demand drug release. Additionally, their high photothermal-conversion capability enables efficient tumor eradication through synergistic photothermal ablation and chemotherapy, both in vitro and in vivo. Ti_3C_2 MXenes also serve as desirable contrast agents for photoacoustic imaging, enabling diagnostic imaging guidance and therapy monitoring. The high in vivo histocompatibility and easy excretion of Ti_3C_2 MXenes highlight their potential biosafety for clinical translation. This work opens up new avenues for MXenes in nanomedicine, showcasing their performance and functionality for synergistic cancer therapy [10].

1.10. Synthesis and Functionalization of Mxenes

1.11. Synthesis Methods of MXenes

MXenes can be synthesized through either a top-down or bottom-up approach. The top-down approach involves acid exfoliation of layered MAX phase materials, which are then delaminated to obtain MXene layers. The MAX phase is typically synthesized by mixing powders of M, A, and X elements and heating them at high temperatures. The etching of the MAX phase, usually using HF, selectively removes the A element to form MXene layers. On the other hand, the bottom-up approach involves atomic-scale control and crystal growth of organic/inorganic molecules or atoms to form ordered MXene layers. Common methods for bottom-up synthesis include chemical vapor deposition (CVD), template method, and plasma-enhanced pulsed laser deposition (PELPD). After synthesis, the MXene surface can be further modified to enhance biocompatibility and reduce cytotoxicity for biomedical applications [11].

One such discovery is MXene, which was initially introduced by Gogotsi et al. in 2011. Since then, over 20 species of MXenes have been successfully synthesized, and more than 70 have had their structure and properties predicted through computational modeling. MXenes consist of early-transition-metal carbides, carbonitrides, and nitrides with the structural formula $M_{n+1}X_n$, where M represents an early transition metal, X represents carbon, nitrogen, or both, and n ranges from 1 to 3. The synthesis of MXenes involves selective etching of the A-group element from precursor ternary-layered carbides of MAX phases, where A corresponds to a group of 12-16 elements from the periodic table. This selective etching process, using etchants containing fluorine (such as HF), results in MXenes with abundant surface-terminating functional groups such as hydroxyl (-OH), oxygen (-O), or fluorine (-F). These functional groups impart MXenes with hydrophilicity, allowing for flexible surface modification and functionalization. MXenes have gained

increasing attention due to their scalability in production, versatile surface chemistry, metallic conductivity, excellent mechanical/thermal properties, and ease of processing. Their high surface-to-volume ratio enables superior biofunctionalization, facilitating the addition of various drugs and molecules, including fluorescent probes, genes, and targeting moieties that specifically recognize cancer cells. This allows for guided and controlled release of these agents to the targeted cells [1].

1.12. Surface Functionalization Techniques

While MXene types, especially Ti_3C_2, have received significant attention in biomedical applications due to their unique chemical and physical properties, future research in cancer theranostics should also explore other types of MXenes. Optimal synthesis and functionalization techniques need to be carefully considered for these alternative MXenes. In the context of photothermal cancer nanotherapy, improving the cellular internalization of MXenes can be achieved by coating their surfaces with ligands that have high specificity towards cancer cells. Additionally, MXene-based structures with responsiveness to biological triggers such as pH, temperature, and enzymes should be innovatively designed to enhance therapeutic outcomes [12].

1.13. Surface Modification for Improved Biocompatibility

Two-dimensional (2D) materials have garnered significant attention due to their ultrathin planar nanostructure and unique physicochemical properties. In addition to graphene, there are various 2D functional materials, such as metals, metal oxides, transition metal dichalcogenides (TMDCs), and MXenes. MXenes are obtained by extracting the A-element from layer-structured MAX-phase ceramics. While MXenes have been primarily explored in energy storage and conversion, this work focuses on their biomedical applications, particularly in cancer treatment. The large surface area of 2D MXene nanosheets enables them to serve as drug-delivery systems for efficient

intracellular drug delivery. Additionally, Ti_3C_2 MXene nanosheets possess inherent photothermal-conversion capability, making them suitable for photothermal therapy (PTT) in biomedicine. By combining the structural features of 2D planar nanosheets for drug delivery with the photothermal property of Ti_3C_2 MXenes, synergistic chemotherapy and photothermal ablation of cancer can be achieved, leading to improved therapeutic outcomes [13].

1.14. MXenes Adaptive Properties for Selective Tumor Ablation

1.15. Light Absorption:

MXenes exhibit strong light absorption across a wide range of spectra, including the ultraviolet (UV), visible, and near-infrared (NIR) regions. This makes them efficient photothermal agents for photothermal therapy (PTT) and enables applications such as photoacoustic imaging (PAI). Some MXenes, like Nb_2C, have shown strong optical absorption in the NIR-II region, allowing for deep-tissue PTT and PAI [14,12].

1.16. Photothermal Conversion Efficiency (PCE):

MXenes have a high PCE, with values reaching up to 50%. This means they can effectively convert light energy into heat for therapeutic applications, making them valuable in PTT [14,12].

1.17. Electronic Properties:

MXenes exhibit a wide range of electronic properties depending on their metal composition and surface terminations. Metallic MXenes, such as Ti_2C and Ti_3C_2, have good conductivity, while others, like Cr and Mo-containing MXenes, can exhibit conductive insulation. Surface modifications and defects can further tailor the electronic conductivity of MXenes for specific applications, such as electrochemical sensors or catalysts. MXenes can also generate active electrons and

vacancies under external stimuli, which can be utilized for reactive oxygen species (ROS) generation or catalytic reactions. Magnetic properties: While the magnetic properties of MXenes are still primarily studied theoretically, certain MXenes, such as Cr_2C and Cr_2N, are predicted to possess magnetic properties without surface termination. However, the experimental preparation of termination-free MXenes has not been reported. Recent research has focused on integrating MXenes with inorganic magnetic nanomaterials, such as MnOx and Fe_3O_4, to create multifunctional materials with applications in MRI-guided diagnosis [14,12].

1.18. MXenes in Photothermal Therapy (PTT)

1.19. Photothermal Conversion Properties of MXenes

The photothermal conversion performance of MXene nanosheets is highly efficient, making them suitable for acquiring photothermal conversion capacity. MXene nanosheets exhibit significant light absorbance in the near-infrared region, as observed in the optical absorption studies of MAP nanofibrous membranes. MXenes, such as titanium carbide (Ti_3C_2), have attracted attention in biomedical and nanobiotechnology applications. MXenes are 2D materials produced by selectively etching A layers from MAX parent phases, resulting in MXene nanosheets with high specific surface area. These nanosheets possess attractive properties, including chemical, electronic, optical, and magnetic properties, making them potential candidates for various applications, including catalysis, Li-ion batteries, supercapacitors, magnetics, chemical adsorption, and sensing. MXene nanosheets also exhibit photothermal stability, antibacterial activity, hydrophilicity, flexible functionalization, and intrinsic biocompatibility, making them suitable for biomedical applications such as biosensors, antibacterial materials, bioimaging probes, and photothermal therapy of tumors. MXenes, particularly $Ti_3C_2T_x$, have shown good biocompatibility and potential osteoinductivity in bone regeneration studies. However, MXene films may exhibit

chemical instability and oxidation when exposed to an atmosphere containing oxygen for a long time. Hydroxyapatite (HAP), a bioceramic material with favorable bioactivity and osteoconductivity, has been extensively used in clinical bone substitutes, implant surface coatings, and drug delivery systems. Combining HAP with other materials, such as polymers, can enhance the properties and functions of composites. Incorporating ultralong hydroxyapatite nanowires (UHAPNWs) into MXene nanosheets has been achieved using a solution co-blending self-assembly method and vacuum-assisted filtration, resulting in nanocomposite membranes with stacked MXene flakes and uniformly distributed UHAPNWs [15].

1.20. Synergistic Therapy Approaches with PTT and Chemotherapy

Nanosized materials, including MXenes, have been investigated for cancer therapy using photothermal therapy (PTT), which involves targeted heating of photothermally active nanomaterials at the site of cancer without damaging surrounding healthy tissue. MXenes, such as Ti-MXene quantum dots (MQDs) and manganese oxide nanoparticles modified with soybean phospholipid (MnO_x/Ti_3C_2-SP), have been shown to have high biocompatibility and can effectively ablate tumor tissue through NIR-mediated PTT. MXenes have also been explored for concurrent diagnostic imaging and therapy by functionalizing their surface with polyoxometalates (POMs) and GdW10 nanoclusters. Moreover, MXenes, such as ultrathin niobium carbidepolyvinylpyrrolidone (Nb_2C-PVP) MXenes, have demonstrated deep tissue PTT and high photothermal conversion efficiency, making them promising agents for in vivo tumor ablation. MXenes have shown potential as drug carriers for cancer therapy as well. SP-modified Ti_3C_2 nanosheets have been used to efficiently deliver chemotherapeutic drugs, such as doxorubicin (Dox), to tumor cells. These nanosheets exhibit pH-responsive and NIR laser-triggered drug release, allowing for controlled release of the drug at the tumor site [16].

1.21. MXenes in Photodynamic Therapy (PDT)

PDT is a non-invasive anticancer therapy that involves the administration and accumulation of a PS in tumor tissues, followed by light irradiation to generate reactive oxygen species and induce cell death. The therapeutic properties of light have been recognized since ancient times, but the concept of photodynamic action gained prominence in the early 20th century. The selective localization of PSs in tumors is a complex process influenced by various factors, including pharmacokinetics, tumor neovasculature permeability, distinctive properties of tumor cells, and tumor-associated macrophages. Laser light sources are commonly used in PDT due to their coherent nature. The penetration of light through tissue is influenced by scattering and absorption, with longer wavelengths penetrating more efficiently. The "optical window" refers to the wavelength range (approximately 650-850 nm) where tissue permeability is maximized. PSs with strong electronic transition intensities within this range are preferred for PDT. PDT is an effective treatment modality utilizing light, oxygen, and a photosensitizer to selectively destroy target tissues. BODIPY-based PSs have shown promise in recent advancements for oncologic and antimicrobial PDT[17].

MXene are being extensively studied for their potential in cancer research. They can serve as multimodal nanoplatforms for cancer diagnostics and treatment, particularly in photodynamic therapy (PDT). PDT involves using a photosensitizer (PS) that generates reactive oxygen species (ROS) upon light excitation to selectively destroy cancer cells. Several studies have explored the use of graphene-based materials (GBMs) for PDT. For example, polyethylene glycol-functionalized graphene oxide (GO) loaded with PS Ce6 demonstrated enhanced anti-cancer activity compared to free Ce_6. GO functionalized with folic acid and loaded with Ce_6 also showed effective cancer cell killing. Another study loaded GO with PS hypocrellin B (HB) and demonstrated its ability to generate ROS upon irradiation[1].

1.22. Combination Therapies Incorporating PDT and PTT for Cancer Treatment

In recent research, Mo_2C MXene synthesized in a nanosphere topology showed promise as a theranostic nanoagent for combined cancer therapy. It was capable of generating ROS and exhibited synergistic photothermal therapy (PTT) and PDT effects on liver carcinoma cells. In vivo experiments on tumor-bearing mice showed complete tumor ablation and lack of regrowth after NIR exposure in the presence of Mo_2C. Some studies have explored the combination of PDT with drug delivery and PTT. For example, a graphene-based system loaded with PS and CuS nanoparticles exhibited efficient controlled theranostic applications, including bimodal PDT and PTT therapy, as well as NIR imaging for deep-seated cancer tissues. Another study developed a multimodal system incorporating photodynamic toxicity, photothermal damage, chemotherapy, and mechanical stimulation mediated by a magnetic field [18,1].

1.23. Biocompatibility and Safety Considerations

In terms of biomedical applications, MXenes have shown promise in various areas. They possess high electrical conductivity, good biocompatibility, high surface area to volume ratio, and can be easily functionalized. MXenes have been used in enzymatic biosensors for the detection of small molecules such as hydrogen peroxide and nitrite, as well as glucose and phenol. These biosensors utilize immobilized enzymes on MXene nanosheets for catalysis and detection. MXenes have also been employed in the detection of cancer biomarkers, including carcinoembryonic antigen (CEA) and osteopontin (OPN), using different surface functionalizations and sensing platforms. Additionally, MXenes have been explored for cancer theranostics, combining therapeutic and diagnostic functions. MXene composites have been developed for imaging techniques such as photoacoustic imaging and photothermal therapy, enabling precise diagnosis and treatment of cancer [11].

In the field of medicine, MXenes have shown potential for applications in sensors, targeted drug delivery, and photo/chemotherapy of cancer. MXenes have been used as the basis for developing highly sensitive gas sensors and biosensors, enabling the detection of various analytes. They have also been explored for targeted drug delivery, where their hydrophilicity and ability to be functionalized with therapeutic molecules make them suitable platforms. MXenes have demonstrated efficient photothermal cancer therapy, selectively ablating cancer cells while sparing non-malignant cells. They have also been investigated for their potential in photoacoustic and optical imaging, diagnostic imaging guidance, and monitoring during therapy. MXenes, such as Ti_3C_2, have shown high drug-loading capacity, pH-responsive and laser-triggered on-demand drug release, and excellent biocompatibility for cancer treatment. Furthermore, MXenes have been integrated into 3D scaffolds for bone tumor treatment and bone tissue regeneration. While the scientific interest in MXenes is steadily increasing, there is still a need for systematic study of their biological effects, including toxicity and biocompatibility. Factors such as synthesis methods, composition, structure, functionalization, test concentrations, duration of exposure, and individual characteristics of biological test objects contribute to the complexity of understanding MXene toxicity. Further research is necessary to overcome these barriers and develop predictive computational methods for nanotoxicology. The ultimate goal is to ensure widespread industrial production and safe use of MXene-based products in various applications, including biomedicine and environmental technologies[19].

1.24. Cytotoxicity Assessments of MXene Nanomaterials

Biosafety investigations are particularly important to comprehensively address critical issues such as biocompatibility, biodegradability, blood circulation, and excretion behaviors of MXenes. The long-term presence of nanomaterials in the body can lead to problems such as inflammation, oxidative damage, and fibrosis, so it is essential to

understand their degradation rate, degree, and overall impact on the body [12].

1.25. In vivo Studies and Biocompatibility Evaluation

MXenes are a promising group of 2D materials with potential applications in biomedicine and environmental fields. They possess properties such as high surface area, conductivity, and near-infrared absorption, making them suitable for bioimaging, drug delivery, tissue engineering, and antibacterial applications. Limited experimental data suggests MXenes exhibit biocompatibility in most cases, with some exceptions showing toxicity against specific cancer cell lines. The toxicity of MXenes can be influenced by surface modifications. MXenes have shown no toxicity in in vivo experiments on lab mice and are excreted without accumulation in major organs. However, chronic experiments and studies on other mammals are lacking. MXenes have demonstrated selective toxicity towards cancer cells, possibly due to enhanced permeability and retention effect. Standardized synthesis methods, physicochemical properties, and test objects vary among studies, making it challenging to compare results. MXenes may pose potential ecological hazards based on their toxicity towards zebrafish embryos. The safety and environmental impact of MXenes require further investigation. MXene-based adsorbents are effective for heavy metal removal, but uncontrolled release into the environment may affect aquatic organisms. MXenes' long-term safety is yet to be fully understood, and vulnerable systems could include the respiratory system, digestive tract, and skin. MXenes may have implications for humans through various consumer products and occupational exposure. Standardized test particles and protocols are needed to ensure reliable toxicological evaluation. Modern nanotoxicology methods and computational approaches can enhance safety assessment. MXenes show great potential but require deeper understanding of their toxicity mechanisms, including long-term effects and behavior in the environment [20].

1.26. Potential Long-Term Effects and Toxicity Concerns

MXenes and their composites have shown promising potential in cancer nanotheranostics, combining diagnostic and therapeutic capabilities. These materials possess unique properties such as high electrical conductivity, hydrophilicity, optical/thermal characteristics, and biocompatibility, making them suitable for cancer applications. MXenes have been utilized in the design of biosensors for cancer diagnosis, offering high sensitivity and detection range. In cancer nanotheranostics, MXenes have been employed for targeted drug delivery, photothermal therapy, and imaging modalities. They exhibit excellent photothermal conversion efficiency and can be combined with other nanomaterials for synergistic treatment. Surface modifications of MXenes enhance their stability, biocompatibility, and targeting capabilities. However, the toxicity and biosafety aspects of MXenes require further investigation, and comprehensive preclinical studies are necessary for clinical translation. Biodegradability is an important factor for evaluating their biosafety, and MXenes have demonstrated enzymatic and pH-dependent degradation behaviors. Future research should focus on understanding the biodegradability mechanisms and optimizing synthesis conditions. The rational design of MXenes for cancer nanotheranostics remains a challenge, and additional studies should explore different types of MXenes, optimization techniques, clinical translation, long-term toxicity, targeting properties, and stimuli-responsive behavior. Improvements in cellular internalization and the development of MXene-based structures with responsiveness to biological triggers can lead to enhanced therapeutic outcomes in cancer treatment[7].

2. Conclusion: Challenges and Future Perspectives

Looking ahead there are promising prospects for the further development and application of MXenes in biomedicine. The synthesis of various types of MXenes and their potential for biomedical use should be explored. MXene-based nanoplatforms that respond to biomolecular cues, such as

temperature and pH, can be developed for targeted drug delivery and probes. This could lead to improved diagnosis and therapy with reduced side effects. MXenes also show potential in the theragnostic approach for cancer treatment, drug delivery, biosensors, and antimicrobial applications. Continued research on MXenes can contribute to advancements in breast cancer management and overall biomedical research [7].

Conflict of Interest

The author declares no conflict of interest.

References

[1] A. Gazzi et al., "Photodynamic therapy based on graphene and MXene in cancer theranostics," Front. Bioeng. Biotechnol., vol. 7, no. OCT 2019.
[2] R. A. Soomro, S. Jawaid, Q. Zhu, Z. Abbas, and B. Xu, "A mini-review on MXenes as versatile substrate for advanced sensors," Chinese Chem. Lett., vol. 31, no. 4, pp. 922–930, 2020.
[3] S. Iravani and R. S. Varma, "MXenes and MXene-based materials for tissue engineering and regenerative medicine: Recent advances," Mater. Adv., vol. 2, no. 9, pp. 2906–2917, 2021.
[4] N. H. Solangi, S. A. Mazari, N. M. Mubarak, R. R. Karri, N. Rajamohan, and D. V. N. Vo, "Recent trends in MXene-based material for biomedical applications," Environ. Res., vol. 222, no. October 2022, p. 115337, 2023.
[5] A. Maleki et al., "Biomedical Applications of MXene-Integrated Composites: Regenerative Medicine, Infection Therapy, Cancer Treatment, and Biosensing," Adv. Funct. Mater., vol. 32, no. 34, 2022.
[6] S. Shurbaji, N. P. A. Manaph, S. M. Ltaief, A. R. Al-Shammari, A. Elzatahry, and H. C. Yalcin, "Characterization of MXene as a Cancer Photothermal Agent Under Physiological Conditions," Front. Nanotechnol., vol. 3, no. September, pp. 1–12, 2021.

[7] S. Ranjbari et al., “Application of MXene in the diagnosis and treatment of breast cancer: A critical overview,” Front. Bioeng. Biotechnol., vol. 10, no. August, pp. 1–15, 2022.
[8] A. Schwaighofer, C. K. Akhgar, and B. Lendl, “Broadband laser-based mid-IR spectroscopy for analysis of proteins and monitoring of enzyme activity,” Spectrochim. Acta - Part A Mol. Biomol. Spectrosc., vol. 253, p. 119563, 2021.
[9] V. S. Sivasankarapillai, A. K. Somakumar, J. Joseph, S. Nikazar, A. Rahdar, and G. Z. Kyzas, “Cancer theranostic applications of MXene nanomaterials: Recent updates,” Nanostructures and Nano-Objects, vol. 22, p. 100457, 2020.
[10] F. Mohajer, G. M. Ziarani, A. Badiei, S. Iravani, and R. S. Varma, “Advanced MXene-Based Micro- and Nanosystems for Targeted Drug Delivery in Cancer Therapy,” Micromachines, vol. 13, no. 10, 2022.
[11] A. Zamhuri, G. P. Lim, N. L. Ma, K. S. Tee, and C. F. Soon, “MXene in the lens of biomedical engineering: synthesis, applications and future outlook,” Biomed. Eng. Online, vol. 20, no. 1, pp. 1–24, 2021.
[12] A. Szuplewska et al., “2D Ti 2 C (MXene) as a novel highly efficient and selective agent for photothermal therapy,” Mater. Sci. Eng. C, vol. 98, no. December 2018, pp. 874–886, 2019.
[13] X. Han, J. Huang, H. Lin, Z. Wang, P. Li, and Y. Chen, “2D Ultrathin MXene-Based Drug-Delivery Nanoplatform for Synergistic Photothermal Ablation and Chemotherapy of Cancer,” Adv. Healthc. Mater., vol. 7, no. 9, pp. 1–13, 2018.
[14] B. Akgenç, “Two-dimensional Ti2C monolayer (MXene): Surface functionalization, induced metal, semiconductor transition,” Turkish J. Phys., vol. 43, no. 5, pp. 531–539, 2019.
[15] A. Rafieerad, G. L. Sequiera, W. Yan, P. Kaur, A. Amiri, and S. Dhingra, “Sweet-MXene hydrogel with mixed-dimensional components for biomedical applications,” J. Mech. Behav. Biomed. Mater., vol. 101, no. September 2019, 2020.
[16] M. Soleymaniha, M. A. Shahbazi, A. R. Rafieerad, A. Maleki, and A. Amiri, “Promoting Role of MXene Nanosheets in Biomedical Sciences: Therapeutic and Biosensing

Innovations," Adv. Healthc. Mater., vol. 8, no. 1, pp. 1–26, 2019.
[17] S. G. Awuah and Y. You, "Boron dipyrromethene (BODIPY)-based photosensitizers for photodynamic therapy," RSC Adv., vol. 2, no. 30, pp. 11169–11183, 2012.
[18] Y. Liu et al., "Two-dimensional MXene/cobalt nanowire heterojunction for controlled drug delivery and chemo-photothermal therapy," Mater. Sci. Eng. C, vol. 116, no. June, p. 111212, 2020.
[19] M. Carey and M. W. Barsoum, "MXene polymer nanocomposites: a review," Mater. Today Adv., vol. 9, p. 100120, 2021.
[20] I. A. Vasyukova, O. V. Zakharova, D. V. Kuznetsov, and A. A. Gusev, "Synthesis, Toxicity Assessment, Environmental and Biomedical Applications of MXenes: A Review," Nanomaterials, vol. 12, no. 11, 2022.

CHAPTER 10

Principles, Designs and Applications of Lateral Flow Assay

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Principles, Designs and Applications of Lateral Flow Assay

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Abstract

Lateral flow assay (LFA) has become popular in recent years, especially with the COVID-19 outbreak. On-site and rapid detection of epidemic diseases is very important. There are alternative methods for detection such as PCR and ELISA that require time, equipment and expertise. There are many reasons for preference of LFA such as portable, low cost, no need for equipment and specialists. Thanks to its long shelf life and no need for a cold chain, it is suitable for use in everywhere. The lateral flow assay consists of 4 main components. That are sample, conjugate, absorbance pads and membrane. The sample pad is the part where sample is dripped. The conjugate pad is labeled biorecognition element. The visibility of the control and test lines on the membrane determines the result of the test. The sample flows across the membrane and reaches the absorbent pad, which facilitates flow. The results can be seen with naked eye or device thanks to labels. A wide variety of labels are used, such as magnetic nanoparticles, colored latex beads, quantum dots, textile dyes, enzymes, liposomes, organic fluorophores, upconverting phosphorus, gold, carbon, selenium and silver nanoparticles. Gold nanoparticles are the most preferred labels. Antibodies and nucleic acids are used as biorecognition elements. While antibodies are structures that bind to the target antigen with high affinity, aptamers are artificially synthesized, short, single-stranded oligonucleotides composed of nucleic acid-based systems. Detection can be made from urine, saliva, sweat, serum, plasma, blood, water and soil samples. LFA can be used in the detection of pesticides, biotoxins, heavy metals,

chemical contamination, drug, pathogenic agents and toxic components.

Keywords: Lateral flow assay, Detection, Strip tests, Biosensor, Rapid tests

1. Introduction

Identification of disease-causing and harmful target molecules is important for humans, animals and plants. For early diagnosis and treatment, there are rapid diagnostic kits that are highly preferred instead of traditional methods in recently. Polymerase chain reaction (PCR), gas chromatography mass spectrometry (GC-MS), ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), high pressure liquid chromatography (HPLC), enzyme-linked immunoabsorbent assay (ELISA) and biosensors have been used for diagnosis. The use of these methods takes time and expensive devices are needed [1, 2] and also, these devices must be transported to the area to be used [3]. On the other hand, sample preparation requires additional procedures, and results interpretation requires qualified personnel [1]. Lateral flow assay (LFA) systems have seen as an alternative to these traditional methods. In recent years, LFA systems have come to the fore with their rapid results in 5-10 minutes [4]. Qualitative, semi-quantitative or quantitative result can be determined by the naked eye or instrumentation [5]. The optical properties of the labels used allow to see the result with the naked eye. This eliminates the need for an expert to interpret the result and a device to display it [1, 4]. LFAs, thanks to its long shelf life and no need for cold chain for storage it is suitable for use in rural, contaminated and battlefields away from the hospital. They are simple, inexpensive to manufacture, and easy to use [6]. Urine, saliva, sweat, serum, plasma, whole blood and other fluids can be tested using LFAs [4]. Apart from biological samples, water, soil and food samples can also be used. There is no need to move them somewhere to test. Thus, the risks during the deterioration and storage of the samples are eliminated. After the

sample is taken, this test is applied on it, no additional process is required.

Thanks to its many advantages, it has a large market share. This market share is mostly in veterinary diagnosis, water and food safety, drug development and quality control of point-of-care (POC) tests [7]. The demand for POC testing has increased with the COVID-19 pandemic [4]. Thanks to its advantageous features, it has been seen as a good alternative to traditional methods such as PCR and ELISA used for diagnosis in the outbreak. At that time, people could learn COVID-19 result of themselves at home without the need for a device and an expert. This have reduced the risk of transmission through contact.

WHO (Word Health Organization) has determined the features that should be in on-site detection tests for developing countries, namely the ASSURED criteria. According to these criteria, it should low-cost, user friendly, easy to use, rapid, specific, stable and should not require a device [7, 8]. LFA has become one of the most widely used POC diagnostic technologies, meeting ASSURED's demand in the last two decades [9].

1.1. LFA Principles and Formats

1.2. Components

The test consists of four components: sample, conjugate, absorbent pads and membrane. (Figure 10.1). All these parts are mounted on a plastic board to ensure flow and stability [5].

1.3. Sample Pad

The pad is usually made of cellulose fiber. Glass fiber and cross-linked silica are also used. These structures prevent analyte accumulation. The horizontal flow test is located at the tip and the test is started by dripping the sample. The task of the sample pad; to carry the sample to the conjugate pad smoothly, continuously and homogeneously. Parasites can be removed,

and its pH can be adjusted by pretreatment on the sample pad. Depending on its biological matrix, pretreatment may be required before dropping the sample onto the pad. This pretreatment is done using certain proteins such as bovine serum albumin (BSA), buffer salts or other liquids to ensure flow rate control and prevent non-specific binding. It is required to optimize pH, sample viscosity and to control of flow rate [2].

1.4. Conjugate Pad

The conjugate pad can be made from cross-linked silica glass fiber, cellulose and polyesters. The analyte or recognition element conjugated with a label is adsorbed onto the conjugate pad and dried there. The function of the conjugate pad is to trap the conjugates and keep them functionally stable. It is extremely important to keep the conjugate stable until the lateral flow test is performed. Because any variation in the dispersion, drying or release of the conjugate may alter the test result. This stabilization is achieved with a conjugate buffer containing borate and carbohydrates such as sucrose. When the conjugate is dried in the presence of sugar, the sugar molecules form a layer around them that stabilizes the biological structures. Carbohydrates act role as a preservative and a solvent. When the sample conjugate comes to the pad, the sugar molecule particles dissolve rapidly and interact with the liquid flow. Inadequately prepared conjugate adversely affects the sensitivity of the test [4, 10, 11]. To increase sensitivity, it can be pretreated to minimize non-specific binding and at the same time the pH should be checked [2].

1.5. Nitrocellulose Membrane

Nitrocellulose (NC) membrane is the most critical element for the reading and interpretation of the test result [2]. Membranes are produced from nylon, polyethersulfone, polyethylene or fused silica [10]. Membranes with nitrocellulose structure are the most widely used [6]. NC membranes are preferred because they are cheap, easy to use, and have high affinity for proteins and other biomolecules [11]. It has a

hydrophilic and porous structure to carry the analyte-containing liquid [12]. It is usually thin and fragile, so the backing is glued onto the card [10]. The chemistry and physical arrangement of the fibers in the membrane controls the trial time and also helps filter out unwanted components from the sample. The filtration rate is one of the important factors that can affect the sensitivity of the test [8]. The membrane has a pore size between 0.05 and 2 μm [4]. As the pore size increases, the amount of membrane-bound protein decreases [1]. Capture molecules, also known as biological recognition elements, such as antibodies, analytes, and nucleic acids, are fixed to the NC membrane by forming at least two lines (the control and test) thanks to electrostatic interaction, hydrogen bonds and/or hydrophobic forces. According to the number of analytes to be detected, the number of test lines can be increased [1, 9, 10].

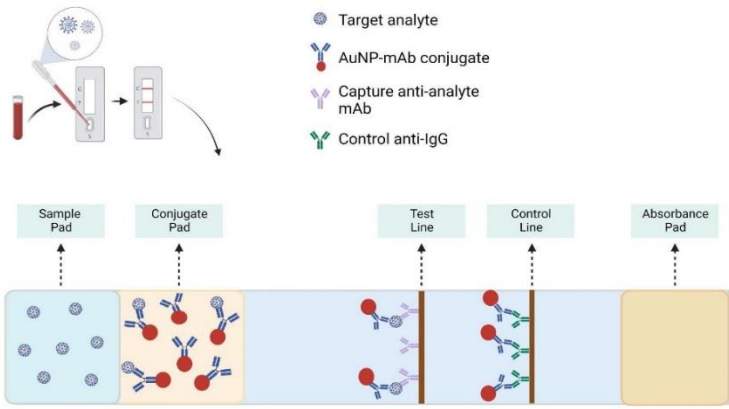

Figure 10.1. Lateral flow assay components (Figure was created with BioRender.com, licence to publication number: JZ25E0WC42).

1.6. Absorbent Mad

During the horizontal flow test, absorbent pad protects the liquid flow moving with capillary force, absorbs the excess

liquid coming to the end of the NC membrane and prevents the flow from going in the opposite direction [4, 10, 11]. The absorbent pad allows larger sample volumes to be used, resulting in increased test sensitivity. Mostly cellulose fibers are used [2, 4].

1.7. Recognition Elements

Recognition elements significantly affect sensitivity and specificity, as interactions between target antigens and capture reagents occur within a few seconds. Antibodies and aptamers are frequently used.

1.8. Antibodies

Antibodies (Ab) contain components such as antigen-binding fragment (Fab) and crystallizable fragment (Fc). It consists of 2 heavy and 2 light chains, which are linked with disulfide bonds. The heavy chain has one variable (variable heavy or VH) and three constant regions (CH1, CH2 and CH3). The light chain has a variable (variable light or VL) and a constant region (CL). The Fab component contains both variable domains and, through its complementarity determining regions (CDRs), enables recognition of the specific antigen by the antibody, similar to key lock congruence. The Fc component is involved in functions such as Ab-dependent cell-mediated cytotoxicity (ADCC), Ab-dependent cellular phagocytosis, antigen presentation to the immune system, degranulation, complement-mediated lysis, regulation of cell activation and proliferation [13].

Antibodies have been used as recognition elements for years because of their high affinity for their targets and their ability to be produced against a variety of molecules [14]. Abs are classified into two types, monoclonal and polyclonal, and widely used in diagnostic tools because of their high specificity against antigens. Ab-based immunoassays are often preferred diagnostic techniques and they are fastest developing assays in biomolecule analysis [13].

1.9. Monoclonal Antibodies

Monoclonal antibodies (MAbs) are monospecific in nature. These antibodies are produced by B cells with high specificity for a single epitope of an antigen. MAbs can be produced against any epitope present on an antigen or immunogen. Monoclonal antibodies are produced using methods such as hybridoma technology, phage display and recombinant production. Hybridoma technology is a primitive, basic and accomplished method for mAbs isolation. It is the most preferred technology to develop mAbs for in vivo applications [15].

1.10. Polyclonal Antibodies

Polyclonal antibodies (pAbs) are secreted by different B cell lines. they are mix of immunoglobulins that react against multiple epitopes of a particular antigen. pAbs are produced by injecting an immunogen into an animal to produce a high titer of antibodies against the particular antigen. The affinity of the antibody specifically synthesized for the analyte plays a major role in the sensitivity of LFA. pAbs are used in LFA conjugate pad, test and control lines [10,11].

1.11. Aptamers

Aptamers are artificially synthesized, short, single-stranded oligonucleotides composed of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) [1, 11]. Aptamers have a length ranging from 15-20 bases. Shorter aptamers generally do not show high affinity, while longer aptamers can cause misfolding and aggregation. Aptamers for various targets such as metal ions, vitamins, organic dyes, toxins, antibiotics, proteins, viruses, prokaryotic and eukaryotic cells have been identified [14, 16]. They are preferred over antibodies for many reasons such as their stability, post-selection amplification, simple structure modifications, versatile application, and ease of production and labeling [11]. Aptamers are isolated from randomly sequenced nucleic acid pools by an in vitro process called systematic

evolution of ligands by exponential enrichment (SELEX). In recent years, different selection methods have been developed apart from the traditional SELEX method. These are capillary electrophoresis-SELEX (CE-SELEX), magnetic bead-based SELEX and cell-SELEX [16].

1.12. Labels

A wide variety of labels are used in LFA tests, such as magnetic nanoparticles, colored latex beads, quantum dots, textile dyes, enzymes, liposomes, organic fluorophores, upconverting phosphorus, gold, carbon, selenium and silver nanoparticles (NPs). An ideal label should be detected at very low concentrations, maintain its stability for a long time and under different conditions, be low in commercial cost, be easy to conjugate with a biomolecule, and should not change its own and biorecognition elements' properties after conjugation [11, 17].

Gold nanoparticles (AuNP) are the most preferred labels in LFA tests due to their good optical properties. It is suitable for use outside the laboratory for rapid detection [1]. It has high stability in liquid and dried form, easy to synthesize, low cost, easily conjugated to biorecognition molecules and binds to biorecognition molecules with great affinity [11, 17].

AuNPs can be used in different biomolecules such as oligonucleotides and proteins. Antibodies to the AuNP surface form an immunocomplex by non-covalent interactions such as the London-Van der Waals force and hydrophobic interactions. It moves smoothly with the sample through the nitrocellulose membrane. As the AuNP/sample ratio increases, a more intense signal occurs on the test line [1]. AuNP shape, size, and stability affect testing. AuNP size has an important role for sensitivity of LFA. The size and color of AuNPs can be determined by varying the amount of sodium citrate. It was reported that AuNPs with a diameter of 30-100 nm performed best [1, 18]. BSA is used to stabilize the AuNP-antibody conjugate and counteract the matrix effect of various biomolecules. The

stability of AuNPs in a solution depends on the electrostatic repulsion between the particles. The pH of an AuNP solution is mostly adjusted before antibodies are add. Because The immobilization of the antibody on an AuNP surface is based on the electrostatic effect [1].

After AuNPs, the most widely used latex NPs that are magnetic nanoparticles. It can be applied in multiple tests, its production is cheap, and it can be produced in different colors [1, 4]. There are various magnetic NPs, commonly used NP in black or brown color, mainly Fe_3O_4 (magnetite) and c-Fe_2O_3 (magnhemite). Magnetic NPs size and shape can be easily adjusted with added salt types such as chlorides, sulfates, perchlorates [18]. It has been reported that magnetic signals are stable for a longer time compared to optical signal. It increases the sensitivity of LFA by decuple or thousandfold [11, 19].

Surface-enhanced Raman spectroscopy (SERS) nanotags modified with AuNPs as signal reporters are part of the SERS-based LFA system. SERS nanotags consist charged Raman dye molecules, (ii) core based on gold or silver material as enhanced substrate, and (iii) surface modified antibodies to provide a strong and specific SERS signal. In addition, the SERS-based LFA provide outstanding multi-channel detection ability in a single assay [20].

Carbon-based materials readily conjugate to biological recognition elements. Generally, carbon nanotubes, carbon NPs, carbon nanostrings are the preferred materials. It is an alternative to AuNPs due to its easy preparation, high stability and lack of toxicity. Thanks to its large surface area, good electrical and optical properties; it is used as convenient support material in biosensors and as a signal material in immunochromatographic LFA. Carbon NPs have been used successfully in medical diagnosis, microbiology, food safety and environmental monitoring [1, 11, 19].

In recent studies, lower detection limits have been obtained by using fluorescent nanoparticles instead of colorimetric

markers [9]. Fluorescent molecules are commonly used as labels in LFA, and the amount of fluorescence is used to learn the analyte concentration in the sample [8]. Quantum dots (QDs) are proper fluorescent labels and fluorescent molecules that are widely used to improve the detection sensitivity of LFA due to their narrow emission spectra, wide excitation range, and high fluorescent quantum yields. The perfect brightness and outstanding stability of the QDs increase sensitivity [1]. These semiconductor nanocrystals are water soluble and can be easily conjugated with biomolecules due to their close size [11]. Their diameters vary between 2-6 nm. Cadmium is the most widely used material for the synthesis of QDs due to its excellent optical and electrochemical properties, but its application in the biological field is quite limited due to its toxicity [18]. It has been used in drug, toxin or pathogen detection and analysis [39]. Another used fluorescent molecule upconverting phosphorus nanoparticles (UCPs). UCPs are submicron size particles composed of rare earth elements embedded in a crystal [18]. It is characterized by excitations in the infrared region and emissions in the high-energy visible region. Compared with other fluorescent materials, they have the unique advantage of not showing any autofluorescence [11]. Recently, UCPs have attracted great attention as they have high chemical stability, regulatable emission colors, high photo stability and low cytotoxicity [19].

Apart from NP and fluorescent molecules, enzymes and liposomes are also used as labels. In LFAs where enzymes are used, an additional step is required because the substrate is applied. Detection sensitivity depends on the enzyme substrate combination. It was observed that the sensitivity increased when enzyme-loaded gold nanoparticles were used as labels. It has been used as a label to produce chemiluminescence as a result of reaction with the appropriate substrate in the detection of explosives [11]. Liposomes are spherical artificial vesicles composed of one or more phospholipid bilayers. The properties of the liposome vary considerably according to its lipid composition, surface charge, size and method of preparation. It is very stable, and its large internal volume allows interaction

with various biological recognition elements such as peptides, hormones, antibodies, sugars and nucleic acids. Liposomes have disadvantages with pH changes and osmotic pressure [1].

1.13. Types of Lateral Flow Assay Formats

According to the recognition elements used in the lateral flow assay, it can be categorized as lateral flow immunoassay and nucleic acid dependent lateral flow test [4].

1.14. Antibody Dependent LFA

The lateral flow test using antibodies as the biorecognition element is called the immunolateral flow test. Antibodies are biorecognition molecules that form a complex with antigen. Antibody is used as both capture and detection molecule. An antibody that specifically binds to the particular antigen is called a primary antibody, and an antibody that binds to an antigen-containing antibody or other antibody is called a secondary antibody. Antibody dependent lateral flow test has been developed in two basic formats that are sandwich and competitive [1, 16].

1.15. Sandwich Format

Sandwich format LFA is the most preferred format type LFA. It is used to detect large molecular analytes with more than one antigenic determinant. Three different antibodies are used in this format. The first antibody is specific to the antigen. It is known as reaction antibody or secondary antibody. This antibody is adsorbed onto the conjugate pad in conjugated form with a label. When the sample is added, the labeled antibody rehydrates and forms a complex with the labeled antibody. It flows towards the membrane by capillary force. The second antibody is specific to the second antigenic region of the antigen. It is known as the detection antibody or primary antibody and is fixed on the test line. The third antibody is a species-specific anti-immunoglobulin antibody. It is fixed on the control line. The conjugate binds to the first antibody present on the pad [1, 6].

When a sample containing analyte is applied to the test; forms a complex with the labeled antibody on the conjugate pad. This complex flows across the nitrocellulose membrane by capillary force. The analyte in complex with the labeled antibody binds from its other exposed epitope to the detection antibody in the test line. A sandwich-linked structure is formed on the test line. Some of the conjugates are not retained in the test line but continue to flow towards the control line. The labeled antibody binds to the anti-immunoglobulin found in the control line and forms a line. Absorbent pad absorbs excess liquid and prevents backflow. When a sample without analyte is applied to the test; cannot be complexed with the labeled antibody on the conjugate pad. The labeled antibody flows across the membrane by capillary force. Since there is no analyte that binds to the antibody in the test line, a sandwich structure is not formed. The labeled antibody binds to the anti-immunoglobulin found in the control line and forms a line. Thus, a single line is formed in the absence of the analyte and the result is negative [8, 11, 16].

1.16. Competitive Format

Competitive format LFA is used to detect small molecular analytes that can't bind to two antibodies at the same time and have a single antigenic determinant [16]. Two different antibodies are generally used in this format [1]. The control line contains a species-specific anti-immunoglobulin [45]. This anti-immunoglobulin binds to the labeled antibody on the conjugate pad regardless of the presence of the antigen and the control line is formed [11].

The competitive format can occur in two different systems. In the first system, the labeled analyte-specific primary antibody is present on the conjugate pad. In the test line, the antigen conjugated with a protein carrier molecule such as BSA is fixed, while the control line contains the secondary antibody, anti-immunoglobulin. When the sample is applied, the conjugate in the conjugate pad is hydrated and begins to move through the NC membrane with the incoming liquid. The antigen in the

applied sample and the antigen immobilized on the test line of the strip compete for binding with the labeled conjugate. When the sample contains the target analyte, it binds to the labeled antibody, the labeled antibody does not bind to the test line and the line can't form. When the analyte is absent or low in the sample, some regions of the labeled antibody conjugate remain empty and the labeled antibody in the conjugate pad binds to the analyte in the test line [1, 6, 11].

In the second system, the analyte is labeled conjugated to the conjugate pad and the test line contains the primary antibody. When the antigen-containing sample is applied, the analyte and the labeled analyte compete for binding with the primary antibody in the test line. The unlabeled analyte binds to the test line, the line doesn't form because the labeled analyte can't bind, and the result is negative. When the sample doesn't contain the target analyte, the labeled analyte binds to the antibody in the test line. Thus, the line forms and the result are negative [1, 11].

1.17. Nucleic Acid Dependent LFA

Nucleic acid amplification products are used in these systems for detection. It is generally used in the detection of pathogens in food, feed and the environment. This system is created in two different formats: so-called antibody-dependent and antibody-independent format, depending on the use of antibodies. The antibody-dependent form is referred as nucleic acid lateral flow immunoassay (NALFIA) because it uses nucleic acid-antibody interaction and a specific labeled double-stranded amplicon. Two differently labeled primers are used to detect the amplified double-stranded nucleic acid. The labeled specific antibody is fixed on the NC membrane to interact with the target analyte and the NP. Avidin-labeled AuNPs can be used to enable visualization [1, 6, 10, 11].

The antibody-independent form relies on the joint use of two linkers, such as a biotinylated probe or amplicon and a streptavidin [6]. Oligonucleotide capture probes are fixed to the

nitrocellulose membrane directly (via passive adsorption), using the BSA oligonucleotide conjugate probe, or by biotin-avidin interaction [11]. There is a relationship between the response in the presence of the analyte and the amount of analyte [1]. Aptamers can also be used in this system. As with antibody based LFA, there are sandwich and competitive formats.

1.18. Application Areas

LFAs are used in pesticides, biotoxins, heavy metals, infectious diseases, biological warfare agents and drug detection. It is also applied in medical, environmental, food and water safety.

1.19. Clinical Applications

The importance of diagnosing at home before reaching the hospital was further understood with the COVID-19 outbreak. Viruses, bacteria, fungi and parasites that cause clinical epidemics threaten health. Detection of these at home prevents transmission to other people. Infectious microorganisms are found abundantly in body fluids which are used for clinical detection. Thanks to the immune system, it is possible to encounter antibodies in body fluids, so LFA systems based on both the antigen and the antibody developed during infection can be performed. LFAs have been used frequently for the detection of IgM and IgG in serum. The first and pioneering study for the detection of COVID-19 was reported in 2020. 40 nm AuNP conjugated to SARS-CoV-2 recombinant protein and rabbit IgG were used in the conjugate pad. This assay, which has given results within 15 minutes, has a sensitivity of 88.7% and a specificity of 90.6% [20].

LFAs were recommended for detection in infectious diseases such as cholera, cryptococcal meningitis, dengue virus, hepatitis B and C, HIV (Human Immunodeficiency Virus), influenza A and B, malaria, syphilis, tuberculosis, and visceral leishmaniasis by WHO [21]. A rapid, qualitative LFA has been developed by Artron Laboratories Inc for the detection of two immunoglobin

antibodies against Zika virus, namely IgG and IgM, in whole blood, plasma or serum. The test has given results within 10 minutes and the result is easy to detect visually. Two test lines indicate a positive result for a possible primary or secondary Zika infection [22].

Influenza virus is one of the most common disease-causing viruses in winter. Specific many LFA studies have been performed on the surface protein of this virus. They have been labeled with AuNP, silica nanoparticles, SERS-LFA and QD [20].

Another virus that threatens human life is the mosquito-borne Zika virus. It causes microcephaly in infants. Dengue fever, another mosquito-borne virus, causes fever, headache and severe muscle aches. If someone infected with Dengue is infected with the Zika virus, the severity of the disease increases considerably. In 2019, LFA was developed for dengue-1 RNA detection using the sandwich LFA format. Dextrin-capped AuNP was used as the label. After one step signal amplification, target Dengue-1 RNA was tested in LFA within 20 minutes [20, 23].

Human immunodeficiency virus type 1 (HIV-1), a retro virus, affects the human immune system. This sexually transmitted virus can transform into acquired immunodeficiency (AIDS). SERS-based LFAs have been studied for HIV-1 DNA detection. Oligonucleotide-linked AuNPs have used. After adsorbing a Raman reporter (MGITC molecules) to the surface of the AuNPs, the thiolated DNAs have conjugated onto them. It has been seen that the results read with the help of the device are 1000 times more sensitive [24]. In 2019, HIV1 DNA was detected using fluorescent LFA. The QD nanocomposite particles were determined to be 4 ± 1.5 nm in diameter by transmission electron microscopy (TEM). DNA and QD were joined by a hydrothermal process. In this LFA using streptavidin and biotin interaction, the LOD (Limit of detection) was determined as 1 pM and 10 nM [25].

In addition to microorganisms, LFAs are preferred for the detection of hormones, cancer-causing genes and proteins. Early diagnosis is facilitated by LFAs. Circulating tumor DNA (ctDNA) and microRNA (miRNA) found in serum are known to contain tumor-specific mutations. Therefore, studies are carried out on LFAs, which use body fluids for early diagnosis in cancer treatment. Tumor-specific mutations of tumor DNA (ctDNA) and microRNA (miRNA) are found in blood. LFA has been developed for the detection of Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations in colorectal cancer (CRC) cells and DNA samples extracted from blood. Gold nanoparticles were used as labels and blood as samples. KRAS can act as a therapeutic biomarker [26].

1.20. Food Safety

Unsafe foods can cause more than 200 diseases ranging from digestive system infection to cancer. Detection of possible toxic substances, veterinary drugs, heavy metals, pesticides, toxins, fertilizers, pathogens and allergenic components is important to ensure food safety [21].

Methods such as immunohistochemical staining, western blot and ELISA are carried out to determine the target protein in genetically modified (GM) detection. LFAs using antibodies specific to the GM protein, are preferred in agricultural products for quality control instead of these methods [27].

Additionally, LFAs using Up-converting phosphor (UCP) particles as reporter have been developed for 10 foodborne pathogens which are *Escherichia coli* O157:H7, *Salmonella* paratyphi A, *Salmonella* paratyphi B, *Salmonella* paratyphi C, *Salmonella* typhi, *Salmonella* enteritidis, *Salmonella* choleraesuis, *Vibrio cholera* O1, *Vibrio cholera* O139, and *Vibrio parahaemolyticus* [28].

1.21. Pesticides

Due to the low molecular weight of pesticide molecules, it is mostly used LFA systems with aptamers. Apart from aptamer,

molecular imprinted polymers (MIPs) are used. MIPs are synthetic polymers with recognition sites where analyte specificity is achieved through non-covalent binding patterns, electrostatic interactions or inclusion/exclusion dimension. MIPs are more economically viable given the high cost of production of antibodies. In a study with MIP, the LOD was determined as 20 μg/L in the sepsis of triazophos in water [29].

AuNP is mostly preferred label for pesticide detection. AuNP has been used for organophosphate, carbamates, and pesticides such as pyrethrin and pyrethroid. Separately, Raman has been used in surface-enhanced dispersion labels (SERS). Carbon nanoparticles, Uv-converting nanoparticles (UCNPs), QDs, Magnetic nanoparticles (MNPs) and enzymes have been used. QD has been shown to give better results than AuNPs. When using a QD-mAb conjugate, acetamipride's LOD was determined to be 1 ng/mL by the naked eye. When AuNP was used, the LOD was found to be 10 ng/mL. At another study, a test in which 1.0 ng/mL 3,5,6-trichloropyridinol (TCP) can be detected with QD was found to give results in a longer time when set up with AuNP. It was observed that the detection limit increased 40 times when Fe_3O_4 magnetic nanoparticles were used in the detection of pesticide paraoxon methyl [29].

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Bahadır EB, Sezgintürk MK. Lateral flow assays: principles, designs and labels. 87th ed. Trends in Analytical Chemistry. 2016. p. 286-306. DOI: 10.1016/j.trac.2016.06.006.
[2] Qriouet Z, Cherrah Y, Sefrioui H, Qmichou Z. Monoclonal Antibodies Application in Lateral Flow Immunochromatographic Assays for Drugs of Abuse Detection. Molecules. 2021; 26, 1058. DOI: 10.3390/molecules26041058
[3] Miocˇ evic´ O, Cole CR, Laughlin MJ, Buck RL, Slowey PD and Shirtcliff EA. Quantitative Lateral Flow Assays for Salivary

Biomarker Assessment: A Review. Frontiers in Public Health. 2017; 14; 5:133. DOI: 10.3389/fpubh.2017.00133
[4] Koczula KM, Gallotta A. Lateral flow assays. Essays in Biochemistry. 2016; 60 (1): 111–120. DOI: 10.1042/EBC20150012
[5] Jara MDL, Alvarez LAC, Guimarães MCC, Antunes PWP, Oliveira JP. Lateral flow assay applied to pesticides detection: recent trends and progress. Environmental Science and Pollution Research. 2022; 31: 46487-46508. DOI: 10.1007/s11356-022-20426-4
[6] Raeisossadatia MJ, Daneshb NM, Bornac F, Gholamzadd M, Ramezanib M, Abnouse K, Taghdisif SM. Lateral flow based immunobiosensors for detection of food contaminants. Biosensors and Bioelectronic. 2016; 86:235-246. DOI: 10.1016/j.bios.2016.06.061
[7] Xiao M, Xie K, Dong X, Wang L, Huang C, Xu F, Xiao W, Jin M, Huang B, Tang Y. Ultrasensitive detection of avian influenza A (H7N9) virus using surface-enhanced Raman scattering-based lateral flow immunoassay strips. Analytica Chimica Acta. 2019; 1053: 139-147. DOI: 10.1016/j.aca.2018.11.056
[8] Nguyen VT, Song S, Park S, Joo C. Recent advances in high sensitivity detection methods for paper-based lateral-flow assay. Biosensors and Bioelectronics. 2020; 152: 112015. DOI: 10.1016/j.bios.2020.112015
[9] Moyano A, Serrano-Pertierra E, Salvador M, Martínez-García JC, Rivas M, Blanco-López MC. Magnetic Lateral Flow Immunoassays. Diagnostics. 2020; 10: 288. DOI:10.3390/diagnostics10050288
[10] Posthuma-Trumpie GA, Korf, J, Van Amerongen A. Lateral flow (immuno)assay: Its strengths, weaknesses, opportunities and threats. A literature surveys. Analytical and Bioanalytical Chemistry. 2009; 393: 569–582. DOI: 10.1007/s00216-008-2287-2
[11] Sajid M, Kawde AN, Daud M. Designs, formats and applications of lateral flow assay: A literature review. Journal of Saudi Chemical Society. 2015; 19: 689–705. DOI: 10.1016/j.jscs.2014.09.001

[12] O'Farrell B. Lateral Flow Technology for Field-Based Applications Basics and Advanced Developments. Topics in Companion Animal Medicine, 2015; 30: 139–47. DOI: 10.1053/j.tcam.2015.12.003.
[13] Sharma S, Byrne H, O'Kennedy RJ. Antibodies and antibody-derived analytical biosensors, Essays in Biochemistry, 2016; 60: 9–18. DOI: 10.1042/EBC20150002
[14] Schüling T, Eilers A, Scheper T, Walter J. Aptamer-based lateral flow assays. AIMS Bioengineering. 2018. p. 78–102. DOI: 10.3934/bioeng.2018.2.78
[15] Parray HA, Shukla S, Samal S, Shrivastava T, Ahmed S, Sharma C, Kumar R. Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives. International Immunopharmacology. 2020; 85:106639. DOI: /10.1016/j.intimp.2020.106639
[16] Liu R, McConnell EM, Li J, Li Y. Advances in Functional Nucleic Acid Based Paper Sensors. Journal of Materials Chemistry B. 2020; 8(16): 3213–3230. DOI: 10.1039/C9TB02584G
[17] Singh J, Sharma S, Nara S. Evaluation of Gold Nanoparticle Based Lateral Flow Assays for Diagnosis of Enterobacteriaceae Members in Food and Water. Food Chemistry; 2015; 170:470-83. DOI: 10.1016/j.foodchem.2014.08.092
[18] Luo K, Kim HY, Oh MH, Kim YR. Paper-based lateral flow strip assay for the detection of foodborne pathogens: principles, applications, technological challenges and opportunities. Critical Reviews in Food Science and Nutrition. 2020; 60: 157-170. DOI: 10.1080/10408398.2018.1516623.
[19] Huang X, Aguilar ZP, Xu H, Lai W, Xiong Y, Membrane-based lateral flow immunochromatographic strip with nanoparticles as reporters for detection: A review. Biosensors and Bioelectronics. 2016; 75: 166-180. DOI: 10.1016/j.bios.2015.08.032.
[20] Ince B, Sezgintürk MK. Lateral flow assays for viruses' diagnosis: Up-to-date technology and future prospects. Trends in Analytical Chemistry. 2022; 157: 116725. DOI: 10.1016/j.trac.2022.116725

[21] Di Nardo F, Chiarello M, Cavalera S, Baggiani C, Anfossi L. Ten Years of Lateral Flow Immunoassay Technique Applications: Trends, Challenges and Future Perspectives. Sensors. 2021; 21, 5185. DOI: 10.3390/s21155185.
[22] Jiang N, Ahmed R, Damayantharan Münal B, Butt H, Yetisen AK. Lateral and Vertical Flow Assays for Point-of-Care Diagnostics. 2019; 8(14): e1900244. DOI: 10.1002/adhm.201900244.
[23] Yrad FM, Castañares JM, Alocilja EC. Visual detection of dengue-1 RNA using gold nanoparticle-based lateral flow biosensor. Diagnostics. 2019; 9:74. DOI: 10.3390/diagnostics9030074
[24] Fu X, Cheng Z, Yu J, Choo P, Chen L, Choo J. A SERS-based lateral flow assay biosensor for highly sensitive detection of HIV-1 DNA. Biosensors and Bioelectronics. 2016; 78:530–537. DOI: 10.1016/j.bios.2015.11.099
[25] Deng X, Wang C, Gao Y, Li J, Wen W, Zhang X, Wang S. Applying strand displacement amplification to quantum dots-based fluorescent lateral flow assay strips for HIV-DNA detection. Biosensors and Bioelectronics. 2018; 105:211–217. DOI: 10.1016/j.bios.2018.01.039
[26] Dyan B, Seele PP, Skepu A, Mdluli PS, Mosebi S, Sibuyi NRS. A Review of the Nucleic Acid-Based Lateral Flow Assay for Detection of Breast Cancer from Circulating Biomarkers at a Point-of-Care in Low Income Countries. Diagnostics. 2022; 12: 1973. DOI: 10.3390/ diagnostics12081973
[27] Grothaus GD, Bandla M, Currier T, Giroux R, Jenkins GR, Lipp M, Shan G, Stave JW, Pantella V. Immunoassay as an analytical tool in agricultural biotechnology. Journal of AOAC international. 2006; 89(4): 913-928.
[28] Zhao Y, Wang H, Zhang P, Sun C, Wang X, Wang X, Yang R, Wang C, Zhou L. Rapid multiplex detection of 10 foodborne pathogens with an up-converting phosphor technology-based 10-channel lateral flow assay. Scientific Reports. 2016; 6: 21342. DOI: 10.1038/srep21342
[29] Marcia Daniela Lazo Jara, Luis Alberto Contreras Alvarez, Marco C C Guimarães, Paulo Wagnner Pereira Antunes, Jairo Pinto de Oliveira. Lateral flow assay applied to pesticides detection: recent trends and progress. Environmental Science

and Pollution Research. 2022; 29(31): 46487–46508. DOI: 10.1007/s11356-022-20426-4

CHAPTER 11

Recent Antidiabetic Potentials of Selected *Cucurbitaceae* Family Plant Species

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Recent Antidiabetic Potentials of Selected *Cucurbitaceae* Family Plant Species

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Abstract

Diabetes is a metabolic and chronic disorder characterized by elevated glucose concentrations in the body. It contributes to various diseases, including heart attack, blindness, and stroke. Further, the *Cucurbitaceae* family plant species are well known for managing diabetes in traditional medicine. They have been used for several years for healing other disorders too. Several compounds such as arachidic acid, avenasterol, behenic acid, betulin, and bryonolic acid were isolated from the *Cucurbitaceae* family plant species. The diverse bioactivities of these compounds were studied in various assays and models. This chapter concentrates on the most recently published antidiabetic activity studies of selected plant species from the *Cucurbitaceae* family.

Keywords: Antidiabetic activity, *Cucurbitaceae*, *Citrullus colocynthis*, Diabetes, *Coccinia grandis*

1. Introduction

Diabetes affected about 537 million individuals aged 20 to 79 worldwide in 2021, making it one of the leading causes of death. By 2030, this figure is expected to climb to 643 million, and by

2045 it is expected to reach 783 million. Moreover, the West Pacific region has the greatest number of people with diabetes (206 million), followed by Southeast Asia (90 million), the Middle East and North Africa (73 million), Europe (61 million), North America and the Caribbean (51 million), South and Central America (32 million), and Africa (24 million). Type 2 diabetes impacts most people (90%), while type 1 diabetes impacts only 10%. More than three-quarters of individuals with diabetes reside in countries with low or middle incomes. Also, diabetes caused 6.7 million deaths in 2021, one every 5 seconds. Diabetes resulted in a minimum of USD 966 billion in healthcare spending, a 316% rise over the previous 15 years. Impaired Glucose Tolerance affects 541 million individuals, placing them at high risk of type 2 diabetes [1].

Diabetes is a metabolic and chronic disorder characterized by elevated glucose amounts in the body. Diabetes contributes to renal failure, stroke, blindness, heart attack, amputation, and other problems [1]. Additionally, type 1 diabetes, type 2 diabetes, and gestational diabetes are the three types of diabetes. Age, a family history of diabetes, and genetics are all associated risks for developing type 2 diabetes [2]. Obesity, being overweight, eating unhealthy foods, and physical inactivity are all risk factors. Further, essential activities such as healthy eating, avoiding excessive weight gain, being physically active, monitoring glucose concentrations, and observing medical guidance may lessen the chances of developing diabetes [3]. Insulin injections are used to manage type 1 diabetes. Leonard Thompson had the first effective insulin injection 101 years ago. Moreover, every year on November 14, the birthday of Sir Frederick Banting, who jointly discovered insulin with Charles Best in 1922, is recognized as World Diabetes Day [4]. Metformin and Sulfonylureas (Glimepiride, Glipizide, Tolbutamide, Gliclazide, and Glibenclamide) are taken to manage type 2 diabetes [5]. A metallic taste, stomach pain, constipation, severe dizziness, dark urine, mood changes, weight gain, easy bleeding, feet swelling, fever, diarrhea, hands swelling, sore throat, vomiting, stomach upset, itching, yellowing skin, swelling, unusual tiredness, loss of appetite,

nausea, rash, weight gain, yellowing eyes, seizures, weakness, and trouble breathing are all typical side effects of these drugs [6,7].

Cucurbitaceae Juss. family contains 101 accepted genera, and they are *Abobra* Naudin, *Acanthosicyos* Welw. ex Benth. & Hook.f., *Actinostemma* Griff., *Alsomitra* (Blume) Spach, *Ampelosicyos* Thouars, *Apodanthera* Arn., *Austrobryonia* H. Schaef. *Baijiania* A.M. Lu & J.Q. Li, *Bambekea* Cogn., *Bayabusua* W.J.de Wilde, *Benincasa* Savi, *Blastania* Kotschy & Peyr., *Bolbostemma* Franquet, *Borneosicyos* W.J.de Wilde, *Brandegea* Cogn., *Bryonia* L., *Calycophysum* H. Karst. & Triana, *Cayaponia* Silva Manso, *Cephalopentandra* Chiov., *Ceratosanthes* Burm. ex Adans., *Cionosicys* Griseb., *Citrullus* Schrad. ex Eckl. & Zeyh., *Coccinia* Wight & Arn., *Cogniauxia* Baill., *Corallocarpus* Welw. ex-Hook.f., *Cucumis* L., *Cucurbita* L., *Cucurbitella* Walp., *Cyclanthera* Schrad., *Cyclantheropsis* Harms, *Dactyliandra* (Hook.f.) Hook.f., *Dendrosicyos* Balf.f., *Diplocyclos* (Endl.) Post & Kuntze, *Doyerea* Grosourdy, *Ecballium* A. Rich., *Echinocystis* Torr. & A. Gray, *Echinopepon* Naudin, *Eureiandra* Hook.f., *Fevillea* L., *Gerrardanthus* Harv. ex Benth. & Hook.f., *Gomphogyne* Griff., *Gurania* (Schltdl.) Cogn., *Gynostemma* Blume, *Halosicyos* Mart.Crov., *Hanburia* Seem., *Hodgsonia* Hook.f. & Thomson, *Ibervillea* Greene, *Indofevillea* Chatterjee, *Indomelothria* W.J.de Wilde & Duyfjes, *Kedrostis* Medik., Khmeriosicyos W.J.de Wilde & Duyfjes, *Lagenaria* Ser., *Lemurosicyos* Keraudren, *Linnaeosicyos* H. Schaef. & Kocyan, *Luffa* Mill., *Marah* Kellogg, *Melothria* L., *Microsechium* Naudin, *Momordica* L., *Muellerargia* Cogn., *Neoalsomitra* Hutch., *Nothoalsomitra* I.Telford, *Oreosyce* Hook.f., *Papuasicyos* Duyfjes, *Parasicyos* Dieterle, *Penelopeia* Urb., *Peponium* Engl., *Peponopsis* Naudin, *Polyclathra* Bertol., *Psiguria* Arn., *Pteropepon* Cogn., *Raphidiocystis* Hook.f., *Ruthalicia* C.Jeffrey, *Schizocarpum* Schrad., *Schizopepon* Maxim., *Scopellaria* W.J.de Wilde & Duyfjes, *Sechiopsis* Naudin, *Selysia* Cogn., *Seyrigia* Keraudren, *Sicana* Naudin, *Sicydium* Schltdl., *Sicyocaulis* Wiggins, *Sicyos* L., *Sicyosperma* A.Gray, *Sinobaijiania* C.Jeffrey & W.J.de Wilde, *Siolmatra* Baill., *Siraitia* Merr., *Solena* Lour., *Tecunumania* Standl. &

Steyerm., *Telfairia* Hook., *Thladiantha* Bunge, *Trichosanthes* L., *Trochomeria* Hook.f., *Trochomeriopsis* Cogn., *Wilbrandia* Silva Manso, *Xerosicyos* Humbert, *Zanonia* L., and *Zehneria* Endl. [8].

Cucurbitaceae family plant species have antiangiogenic, antibacterial, antifungal, antidiabetic, antihyperlipidemic, anti-inflammatory, anticancer, hepatoprotective, cardioprotective, antihistaminic, antioxidant, antiwrinkle, antiaging, antihypertensive, immunomodulatory, antiviral, and gastroprotective activities [9-25]. Compounds including 3-octadecenoic acid, acutoside C, acutoside D, apigenin, arachidic acid, avenasterol, behenic acid, betulin, bryonolic acid, butyrospermol, caffeic acid, campesterol, catechin, cephalandrin A, cephalandrin B, charantoside, chlorogenic acid, chrysanthemaxanthin, colocynthoside A, colocynthoside B, cucumegastigmane I, cucumegastigmane II, cucumerin A, cucurbitacin G, cucurbitaxanthin, echinatin, echinatol A, echinatol B, ellagic acid, eugenol, euphol, ferulic acid, flavoxanthin, fucosterol, gallic acid, gibberellin, gypsogenin, hederagenin, hespiridin, isocucurbitacin B, isocucurbitacin D, isosaponarin, isovitexin, karounidiol, khekadaengoside E, lignoceric acid, linoleic acid, lucyin A, lucyoside, luffaculin, luffacylin, luffangulin, luffin P1, luffin S, lupeol, lutein, luteoxanthin, lycopene, maslinic acid, m-coumaric acid, methyl palmitate, momordicin I, momordicin IV, momordin, multiflorenol, naringenin, oleanolic acid, oleic acid, oleuropein, orientin, p- hydroxybenzoic acid, palmitic acid, p-coumaric acid, phenylacetic acid, phloretin, phloridzin, p-hydroxybenzoic acid, pleuchiol, polyprenol 1, protocatechuic acid, quercetin, saponarin, sitosterol, spinasterol, squalene, stearic acid, stigmasterol, suberic acid, syringic acid, taraxerol, taraxerone, trichosanthin, trilinolenin, ursolic acid, vanillic acid, vanillin, vicine, vitexin, zeaxanthine, α-amyrin, α-carotene, α-cryptoxanthin, α-linolenic acid, α-spiranosterol, α-tocopherol, β-amyrin, β-carotene, β-cryptoxanthin, β-sitosterol, and γ-amino butyric acid were discovered from this group of plants [15-38]. Only selected *Cucurbitaceae* family plant species with recently

reported antidiabetic activity studies are discussed in this chapter.

1.1. *Citrullus colocynthis* (L.) Schrad.

Citrullus colocynthis (L.) Schrad. is an annual or subshrub that grows in a subtropical environment. The synonyms of this plant species are *Citrullus colocynthoides* Pangalo, *Citrullus pseudocolocynthis* M. Roem., *Colocynthis officinalis* Schrad., *Colocynthis vulgaris* Schrad., *Cucumis bipinnatifidus* Wight ex Naudin, *Cucumis colocynthis* L., *Cucumis colocynthoides* Schult., and *Cucurbita colocyntha* Link. In addition, *C. colocynthis* is distributed in Yemen, Afghanistan, Algeria, Iraq, Italy, India, Iran, Ivory Coast, Djibouti, Saudi Arabia, Senegal, Somalia, Spain, Sri Lanka, Sudan, Syria, Mali, Mauritania, Morocco, Myanmar, Greece, Lebanon, Libya, Cape Verde, Chad, Cyprus, Niger, Oman, Kenya, Kuwait, Egypt, Eritrea, Ethiopia, Tunisia, Turkey, Pakistan, Palestine, Turkmenistan, Western Sahara, Bangladesh, Benin, Burkina Faso, Argentina, Australia, Bulgaria, Hungary, Italy, Paraguay, Romania, USA, and Uzbekistan.

Recently reported antidiabetic activities of this plant species are listed in Table 11.1 Among these studies, the following was the most effective. An *in vivo* study was conducted using *C. colocynthis* fruit ethanol extract at a dose of 100 mg/kg. The extract was orally administered to the Streptozotocin model for 21 days. The results showed that there was a significant reduction in the blood sugar level [40].

Table 11.1. Recently published relevant antidiabetic investigations of *C. colocynthis*

Level of evidence	Part used	Extract/ compound	Bioassay/ model	Dose/ concentration	Duration	Ref
Clinical	NS	Aqueous	Type 2 diabetic	2%	10 d	[39]
In vivo	Fruit	Ethanol	Streptozotocin	100 mg/kg	21 d	[40]
In vivo	Fruit	Not applicable	Streptozotocin	300 mg/kg	28 d	[41]
In vivo	Fruit, Seed	Ethanol	Streptozotocin	150 mg/kg	14 d	[42]
In vitro	Seed	Aqueous	3T3-L1 cell	20 μg/ml	Not applicable	[43]
In vitro	Fruit	Aqueous	3T3-L1 cell	100 μg/ml	Not applicable	[43]
In vitro	Seed	Ethanol	α-Glucosidase	Not stated	Not applicable	[42]

1.2. *Citrullus lanatus* (Thunb.) Matsum. & Naka

Citrullus lanatus (Thunb.) Matsum. & Nakai is a perennial that flourishes in the dry tropical environment. *C. lanatus* is used as fuel, toxin, and animal food, and for food and medicine. The synonyms of *C. lanatus* are *Anguria citrullus* Mill., *Citrullus anguria* (Duchesne) H. Hara, *Citrullus aquosus* Schur, *Citrullus battich* Forssk., *Citrullus caffrorum* Schrad., *Citrullus chodospermus* Falc. & Dunal, *Citrullus* H. Karst., *Citrullus citrullus* Small, *Citrullus edulis* Pangalo, *Citrullus edulis* Spach, *Citrullus pasteca* Sageret, *Citrullus vulgaris* Schrad., *Colocynthis amarissima* Schrad., *Colocynthis citrullus* (L.) Kuntze, *Cucumis amarissimus* Schrad., *Cucumis citrullus* (L.) Ser., *Cucumis dissectus* Decne., *Cucumis edulis* Steud., *Cucumis laciniosus* Eckl. ex Steud., *Cucumis purpureus* Noronha, *Cucumis vulgaris* (Schrad.) E.H.L. Krause, *Cucurbita anguria* Duchesne, *Cucurbita caffra* Eckl. & Zeyh., *Cucurbita citrullus* L., *Cucurbita gigantea* Salisb., *Cucurbita pinnatifida* Schrank, and *Momordica lanata* Thunb. Furthermore, this plant species is found in Afghanistan, Albania, Angola, Argentina, Australia, Austria, Bahamas, Bangladesh, Benin, Bolivia, Botswana, Brazil, Burkina Faso, Burundi, Cameroon, Central African

Republic, Chad, China, Colombia, Congo, Costa Rica, Cuba, Czech Republic, Denmark, Djibouti, Dominican Republic, Ecuador, Egypt, El Salvador, Eritrea, Ethiopia, Finland, Gabon, Gambia, Ghana, Greece, Guatemala, Guinea, Haiti, India, Indonesia, Iraq, Ivory Coast, Kazakhstan, Kenya, Laos, Lesotho, Libya, Malawi, Mali, Mauritania, Mauritius, Mexico, Mozambique, Namibia, Nauru, Nepal, New Caledonia, New Zealand, Nicaragua, Niger, Nigeria, North Korea, Norway, Oman, Pakistan, Portugal, Puerto Rico, Réunion, Romania, Rwanda, Samoa, Saudi Arabia, Senegal, Sierra Leone, Slovakia, Somalia, South Africa, South Korea, Sri Lanka, Sudan, Swaziland, Sweden, Tadzhikistan, Tanzania, Togo, Trinidad-Tobago, Turkey, Turkmenistan, Uganda, USA, Uzbekistan, Vanuatu, Venezuela, Vietnam, Zaïre, Zambia, and Zimbabwe.

Table 11.2. Lately publicized suitable antidiabetic explorations of *C. lanatus*

Level of evidence	Part used	Extract/ compound	Bioassay/ model	Dose/ concentration	Duration	Ref
In vivo	Seed	Methanol	Streptozotocin	200 mg/kg	28 d	[44]
In vivo	Fruit	Juice	Alloxan	500 mg/kg	14 d	[45]
In vitro	Fruit	Juice	α-Amylase	0.2 g/ml	Not applicable	[45]
In vitro	Fruit	Juice	α-Glucosidase	0.05 g/ml	Not applicable	[45]
In vivo	Leaf	Ethanol	Streptozotocin	400 mg/kg	42 d	[46]
Clinical	Fruit	Not applicable	Diabetic	4 g	30 min	[47]

Newly documented antidiabetic activities of this plant species are listed in Table 11.2. Amongst these investigations, the following was considerably adequate. The impact of consuming microencapsulated *C. lanatus* rind and pulp on plasma levels of the amino acids L-citrulline, L-arginine, and L-ornithine, as well as glucose and insulin in healthy persons, were assessed in clinical research. All participants in the study were permitted to consume a 4 g capsules of *C. lanatus* rind and pulp. According to the findings, plasma glucose levels surged 30 minutes after consumption of *C. lanatus* rind and pulp capsules.

Additionally, 30 minutes after consumption of both capsules raised plasma insulin levels [47].

1.3. *Coccinia grandis* (L.) Voigt

Coccinia grandis (L.) Voigt is a creeping tuberous geophyte that thrives in periodically dry tropical ecosystems. *C. grandis* is utilized for food, medicine, and animal feed. The synonyms of plant species are *Bryonia acerifolia* D. Dietr., *Bryonia alceifolia* Willd., *Bryonia barbata* Buch. -Ham. ex Cogn., *Bryonia grandis* L., *Bryonia moimoi* Ser., *Bryonia sinuosa* Wall., *Cephalandra grandis* (L.) Kurz, *Cephalandra indica* Naudin, *Cephalandra moghadd* (Forssk. ex J.F. Gmel.) Broun & R.L. Massey, *Cephalandra schimperi* (Naudin) Naudin, *Coccinia helenae* Buscal. & Muschl., *Coccinia indica* Wight & Arn., *Coccinia loureiroana* M. Roem., *Coccinia moghadd* (Forssk. ex J.F. Gmel.) Asch., *Coccinia moimoi* (Ser.) M. Roem., *Coccinia palmatisecta* Kotschy, *Coccinia schimperi* Naudin, *Coccinia wightiana* M. Roem., *Cucumis pavel* Kostel., *Cucurbita dioica* Roxb. ex-Wight & Arn., *Cucurbita schimperiana* Hochst. ex Cogn., *Luffa moghadd* (Forssk. ex J.F. Gmel.) Peterm., *Momordica bicolor* Blume, *Momordica covel* Dennst., *Momordica monadelpha* Roxb., *Physedra gracilis* A. Chev., and *Turia moghadd* Forssk. ex J.F. Gmel. Further, *C. grandis* is distributed in India, Bangladesh, Benin, Malaysia, Cambodia, Cameroon, Central African Republic, Chad, China, Djibouti, Eritrea, Ethiopia, Gambia, Ghana, Guinea-Bissau, Ivory Coast, Indonesia, Kenya, Laos, Mali, Mauritania, Myanmar, Nepal, Niger, Nigeria, Pakistan, Philippines, Saudi Arabia, Senegal, Sierra Leone, Somalia, Sri Lanka, Sudan, Tanzania, Thailand, Togo, Uganda, Vietnam, Yemen, Zaïre, Brazil, Colombia, Cuba, Dominican Republic, Egypt, Fiji, USA, French Guiana, Guyana, Mauritius, Mozambique, Nicaragua, Australia, Puerto Rico, Suriname, Taiwan, Tonga, and Trinidad-Tobago. Just publicized antidiabetic activity studies of *C. grandis* are listed in Table 11.3. Among these analyses, the following was the most useful. In the glucocorticoid-induced insulin resistance experiment, the present investigation explores the antidiabetic, insulin-sensitizing, and hypolipidemic efficacy

of *C. grandis* leaf ethanol extract. The ethanol extract of the *C. grandis* leaf (1 mg/kg) was administered orally to one set of rats in this experiment to induce insulin resistance. At the start and end of the trial, fasting serum glucose, insulin, and lipid levels were estimated. On the one hand, tested animals showed a substantial dose-dependent reduction in lipids, insulin, hepatic steatosis, blood glucose, and insulin resistance. On the other hand, there was an increase in insulin sensitivity [53].

Table 11.3. Recently published appropriate antidiabetic examinations of *C. grandis*

Level of evidence	**Part used**	**Extract/ compound**	**Bioassay/ model**	**Dose concentration**	**Duration**	**Ref**
In vitro	Fruit	Aqueous	α-Amylase	Not stated	Not applicable	[48]
In vitro	Fruit	Not stated	α-Glucosidase	2.43 mg/ml	Not applicable	[49]
In vivo	Leaf	Ethanol	High fat diet fed	100 mg/kg	56 d	[50]
Clinical	Leaf	Aqueous	Type 2 diabetic	500 mg	90 d	[51]
In vivo	Fruit	β-sitosterol	High fat diet and streptozotocin	5 mg/kg	30 d	[52]
In vivo	Leaf	Ethanol	Glucocorticoid	1 g/kg	12 d	[53]

1.4. *Cucumis melo* L.

Cucumis melo L. is a climbing perennial that thrives in a periodically dry tropical environment. It is used as medicine, nourishment, animal food, poison, and fuel. *C. melo* has the synonyms of *Bryonia collosa* Rottler, *Cucumis acidus* Jacq., *Cucumis agrestis* (Naudin) Greb., *Cucumis alba* Nakai, *Cucumis ambiguus* Fenzl ex Hook.f., *Cucumis arenarius* Schumach. & Thonn., *Cucumis aromaticus* Royle, *Cucumis bardanus* Fenzl ex Naudin, *Cucumis bisexualis* A.M. Lu & G.C. Wang, *Cucumis campechianus* Kunth, *Cucumis cantalupensis* Haberle ex M. Roem., *Cucumis cantalupo* Rchb. This plant species is available in Afghanistan, Albania, Algeria, Angola, Argentina, Australia, Austria, Bahamas, Bangladesh, Belize, Benin, Bolivia, Burkina Faso, Cambodia, Cameroon, Canada, Cape Verde, Central African Republic, Chad, China, Colombia, Costa Rica, Cuba, Czech Republic, Iraq, Ivory Coast, Jamaica, Japan, Kazakhstan, Kenya, Kirgizstan, Laos, Lebanon, Libya, Malawi, Malaysia, Maldives, Mali, Mauritania, Mexico, Morocco, Mozambique, Myanmar, Nauru, Nepal, New Caledonia, New Guinea, Nicaragua, Niger, Nigeria, North Korea, Norway, Oman, Pakistan, Palestine, Philippines, Portugal, Russia, Samoa, Saudi Arabia, Senegal, Seychelles, Slovakia, Somalia, South Africa, South Korea, Spain, Sri Lanka, Uganda, USA, Uzbekistan, Venezuela, Vietnam, Yemen, Zaïre, Zambia, and Zimbabwe.

Table 11.4. Newly issued relevant antidiabetic studies of *C. melo*

Level of evidence	Part used	Extract / compound	Bioassay / model	Dose / concentration	Duration	Ref
In vivo	Leaf, Seed	Ethanol	Alloxan	250 mg/kg	45 d	[54]
In vitro	Leaf	Ethanol	Hemoglobin glycosylation	66.45μg/ml	Not applicable	[55]
In vitro	Leaf	Ethanol	Glucose diffusion	50 mg/ml	Not applicable	[55]
In vitro	Leaf	Ethanol	α-Glucosidase	269.47 μg/ml	Not applicable	[55]
In vitro	Leaf	Ethanol	Glucose uptake in yeast cell	Not stated	Not applicable	[55]

In vitro	Leaf	Ethanol	Glucose adsorption capacity	Not stated	Not applicable	[55]
In vivo	Flower and seed	Not stated	Not stated	500 mg/kg	Not stated	[54]
In vivo	Leaf	Ethanol	Nicotinamide-streptozotocin	300 mg/kg	21 d	[56]

Newly informed antidiabetic activity examinations of this plant species are listed in Table 11.4. Among these examinations, the following was the most convincing. A study utilized ethanol extract of *C. melo* to observe the blood glucose level, insulin level, oxidative stress, and lipid profile in Alloxan-induced diabetic rats. In this study, the diabetic treatment group received an oral ethanol extract dosage of 250 mg/kg body weight for 45 days. The diabetic rats treated with the extract exhibited a dose-dependent reduction in blood glucose levels [54].

1.5. *Cucurbita pepo* L.

Cucurbita pepo L. is a perennial that thrives in the subtropical environment. It is used for animal food, nourishment, medicine, and fuel. The synonyms of *C. pepo* are *Citrullus variegatus* Schrad. ex M. Roem., *Cucumis pepo* (L.) Dumort., *Cucumis zapallo* Steud., *Cucurbita aurantia* Willd., *Cucurbita ceratoceras* Haberle ex Mart., *Cucurbita clodiensis* Naccari, *Cucurbita courgero* Ser., *Cucurbita elongata* Bean ex Schrad., *Cucurbita esculenta* Gray, *Cucurbita fastuosa* Salisb., *Cucurbita hybrida* Bertol. ex Naudin, *Cucurbita lignosa* Mill., *Cucurbita mammeata* Molina, *Cucurbita mammosa* J.F. Gmel., *Cucurbita marsupiiformis* Haberle ex M. Roem., *Cucurbita oblonga* Link, *Cucurbita polymorpha* Duchesne, *Cucurbita pomiformis* M. Roem., *Cucurbita pyridaris* Duchesne ex Poir., *Cucurbita pyxidaris* DC., *Cucurbita succado* Nägeli ex Naudin, *Cucurbita succedo* Arn., *Cucurbita tuberculosa* Schrad., *Cucurbita urnigera* Schrad., *Cucurbita variegata* Steud., *Cucurbita venosa* Descourt., *Pepo aurantius* (Willd.) Peterm., *Pepo citrullus* Sageret, *Pepo clypeiformis* Poit., *Pepo oblongus* Poit., *Pepo potiron* Sageret, and *Pepo vulgaris*

Moench. This plant species is available in Albania, Argentina, Bahamas, Bangladesh, Belgium, Benin, Bolivia, Brazil, Burkina Faso, Cameroon, Canada, Cape Verde, China, Costa Rica, Cuba, Czech Republic, Denmark, Dominican Republic, Finland, France, Gabon, Gambia, Greece, Guatemala, Haiti, India, Italy, Kazakhstan, Mexico, Nauru, New Caledonia, New Zealand, Nigeria, North Korea, Norway, Oman, Puerto Rico, Romania, Sierra Leone, Slovakia, South Korea, Sweden, Tadzhikistan, Trinidad-Tobago, Turkey, Turkmenistan, Tuvalu, USA, Uzbekistan, Venezuela, Vietnam, and Zaïre.

Recently published antidiabetic activities of this plant species are listed in Table 11.5. Among these investigations, the following was the most effective. Thanh et al. (2021) examined the antidiabetic effects of a polysaccharide extracted from the fruit of the *C. pepo* plant using hot water. Blood glucose levels in Alloxan-induced diabetic mice were reduced by 29.8% after seven days of therapy at a dose of 100 mg/kg [57].

2. Conclusion

Various parts of the selected *Cucurbitaceae* plant species showed antidiabetic activities. They provided plenty of potentials to explore as an important source for traditional medicinal goals. These studies suggest that more clinical trials should be conducted using various extracts of these selected plant species as they had *in vitro* and *in vivo* evidence.

Table 11.5. Recently published antidiabetic investigations of *C. pepo*

Level of evidence	Part used	Extract/ compound	Bioassay/ model	Dose / concentration	Duration	Ref
In vivo	Fruit	Polysaccharide	Alloxan	100 mg/kg	7 d	[57]
In vitro	Fruit	Polysaccharide	α-Amylase	103.06 mg/ml	Not applicable	[57]
In vitro	Fruit	Polysaccharide	α-Glucosidase	110.32 mg/ml	Not applicable	[57]
In vitro	Leaf	Ethyl acetate	α-Glucosidase	22.29 μg/ml	Not applicable	[58]

It would be beneficial to isolate the antidiabetic compounds from the *Cucurbitaceae* family plant species. In addition, *in vitro, in vivo,* and clinical studies should be carried out on these antidiabetic compounds.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] World Health Organization. 2023. Available from: https://www.who.int/ (Accessed on 09.05.2023)

[2] European Association for the Study of Diabetes. 2023. Available from: https://www.easd.org/ (Accessed on 03.05.2023)

[3] Mayo Foundation for Medical Education and Research. 2023. Available from: https://www.mayo.edu/ (Accessed on 22.04.2023)

[4] International Diabetes Federation. 2023. Available from: https://idf.org/ (Accessed on 26.03.2023)

[5] Cleveland Clinic. 2023. Diabetes Center. Available from: https://my.clevelandclinic.org/departments/endocrinology-metabolism/depts/diabetes#what-we-treat-tab (Accessed on 03.05.2023)

[6] American Diabetes Association. 2023. Available from: https://diabetes.org/ (Accessed on 30.03.2023)

[7] WebMD LLC. 2023. Avaialbel from: https://www.webmd.com/ (Accessed on 30.03.2023)

[8] Kew Royal Botanic Gardens. Plants of the world online, *Cucurbitaceae* Juss, 2023. Available from: https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:30000781-2 (Accessed on 12.02.2023)

[9] Natarajan D., Lavarasan RJ, Chandra Babu S, Refai MA, Ansari LH. Antimicrobial studies on methanol extract of *Benincasa hispida*. Ancient Sci. Life. 2003; 22: 98-100

[10] Chakraborty K, Datta (Guha) M, Guha A, Choudhary R, De B. Hypoglycemic activity of the fruits of *Benincasa hispida*

found in state of Tripura, India. Der Pharma Chem. 2018;10 (8): 102-104
[11] Hussain AI, Rathore HA, Sattar MZ, Chatha SA, Sarker SD, Gilani AH. *Citrullus colocynthis* (L.) Schrad. (Bitter apple fruit): a review of its phytochemistry, pharmacology, traditional uses and nutritional potential. J. Ethnopharmacol. 2014; 155: 54-66
[12] Marzouk B, Marzouk Z, Décor R, Edziri H, Haloui E. Antibacterial and anticandidal screening of Tunisian *Citrullus colocynthis* Schrad. From medenine. J. Ethnopharmacol. 2009; 125: 344-349
[13] Pashmforosh M, Vardanjani HR, Vardanjani HR, Pashmforosh M, Khodayar M. Topical Anti-Inflammatory and analgesic activities of *Citrullus colocynthis* extract cream in rats Medicina. 2018; 54: 51
[14] Rezai M, Davoodi A, Asori M, Azadbakht M. Cytotoxic activity of *Citrullus colocynthis* (L.) Schrad fruit extract on gastric adenocarcinoma and breast cancer cell lines. Int. J. Pharmaceut. Sci. Rev. Res. 2017; 45: 175-178
[15] Gupta A, Singh A, Ranu P. A review on watermelon (*Citrullus lanatus*) medicinal seeds. J. Pharmacogn. Phytochem. 2018; 7: 2222-2225
[16] Taur DJ, Patil RY. Mast cell stabilizing, antianaphylactic and antihistaminic activity of *Coccinia grandis* fruits in asthma Chin. J. Nat. Med. 2011; 9: 359-362
[17] Sakharkar P, Chauhan B. Antibacterial, antioxidant and cell proliferative properties of *Coccinia grandis* fruits. Avicenna J. Phytomed. 2017: 7: 295-307
[18] Dhiman K, Gupta A, Sharma DK, Gill NS, and Goyal A. A review on the medicinally important plants of the family Cucurbitaceae. Asia Pac. J. Clin. Nutr. 2012; 4: 16-26
[19] Nema NK, Maity N, Sarkar B, Mukherjee PK. *Cucumis sativus* fruit potential antioxidant, anti-hyaluronidase, and anti-elastase agent. Arch. Dermatol. Res. 2011; 303: 247-252
[20] Sharma A, Sharma AK, Chand T, Khardiya J Yadav KC. Antidiabetic and antihyperlipidemic activity of *Cucurbita maxima* Duchense (Pumpkin) seeds on streptozotocin induced diabetic rats. J. Pharmacogn. Phytochem. 2013; 1: 1108-1116

[21] Rajasree RS, Sibi PI, Femi F, Helen W. Phytochemicals of Cucurbitaceae family – a review. Int. J. Pharmcog. Phytochem. Res. 2016; 8: 113-123
[22] Ahmed B, Alam T, Khan SA. Hepatoprotective activity of *Luffa echinata* fruits. J. Ethnopharmacol. 2001; 76: 187-189
[23] Venkateshwarlu M, Nagaraju M, Odelu G, Srilatha T, Ugandhar T. Studies on phytochemical analysis and biological activities in *Momordica dioica* Roxb. through fruit. J. Pharm. Innov. 2017; 6: 437-440
[24] Arawwala M, Thabrew I, Arambewela L. Gastroprotective activity of *Trichosanthese cucumerina* in rats. J. Ethanopharmacol. 2010; 127: 750-754
[25] Bhadoriyal SS, Mandoriya N. Immunomodulatory effect of tricosanthes dioica roxb. Asian Pac. J. Trop. Biomed. 2012; 2: S985-S987
[26] Nagarajaiah SB, Prakash J. Chemical composition and bioactive potential of dehydrated peels of *Benincasa hispida, Luffa acutangula,* and *Sechium edule.* J. Herbs, Spices, Med. Plants. 2015; 21: 193-202
[27] Suryanti V, Marliyana SD, Wulandari T. Antioxidant activity, total phenolics and flavonoids contents of *Luffa acutangula* (L.) Roxb fruit. J. Chem. Pharmaceut. Res. 2015; 7: 220-226
[28] Singha S, Biswas S, Dasgupta B, Kar A, Mukherjee PK. Standardizat ion of some plants of the Cucurbitaceae family by a validated high-performance thin-layer chromatography method. J. Plan. Chromatgr. - Modern TLC. 2020.10.1007/s00764-020-00061-w
[29] Prajapati RP, Kalariya M, Parmar SK, and Sheth NR. Phytochemical and pharmacological review of *Lagenaria sicereria*. J. Ayurveda Integr. Med. 2010; 1: 266-272
[30] Chen CR, Chen HW, Chang CI. D:C-Friedooleanane-type triter pen- -oids from *Lagenaria siceraria* and their cytotoxic activity Chem. Pharm. Bull. (Tokyo). 2008; 56: 385-388
[31] Chanda J, Mukherjee PK, Biswas R, Singha S, Kar A, Haldar PK. *Lagenaria siceraria* and it's bioactive constituents in carbonic anhydrase inhibition: a bioactivity guided LC–MS/MS approach. Phytochem. Anal. 2020; 32: 298-307

[32] Perez-Gutierrez RM. Review of *Cucurbita pepo* (pumpkin) its phytochemistry and pharmacology. Med. Chem. 2016; 6: 12-21
[33] Muchirah PN, Waihenya R, Muya S, Abubakar L, OzwaraH, Makokha A. Characterization and antioxidant activity of *Cucurbita maxima* Duchesne pulp and seed extracts. J. Phytopharmacol. 2018; 7: 134-140
[34] Rezig L, Chouaibi M, Msaada K, Hamdi S. Chemical composition and profile characterisation of pumpkin (*Cucurbita maxima*) seed oil. Ind. Crop. Prod. 2012; 37: 82-87
[35] Mukherjee PK, Nema NK, Maity N, Sarkar BK. Phytochemical and therapeutic potential of cucumber. Fitoterapia. 2013; 84: 227-236
[36] Mallek-Ayadi S, Bahloul N, Kechaou N. Characterization, phenolic compounds and functional properties of *Cucumis melo* L. peels. Food Chem. 2017; 221: 1691-1697
[37] Han XN, Liu CY, Liu YL, Xu QM, Li XR, Yang SL. New triterpenoids and other constituents from the fruits of *Benincasa hispida* (Thunb.) Cogn. J. Agric. Food Chem. 2013; 61: 12692-12699
[38] Ghosh K, Baghel MS. A pharmacognostical & physicochemical study of Benincasa hispida with Ayurvedic review. Int. J. Res. Ayurveda Pharm. 2011; 2: 1664-1668
[39] Ahangarpour A, Belali R, Bineshfar F, Javadzadeh S, Yazdanpanah L. Evaluation of skin absorption of the *Citrullus colocynthis* in treatment of type II diabetic patients. Journal of Diabetes & Metabolic Disorders. 2020. https://doi.org/10.1007/s40200-020-00509-0
[40] Ahmadi A, Roghani M, Parsianfard M, Seyedmomeni F, Gheraati S, Sobhanian SA. Antihyperglycemic and Antihyperlipidemic Evaluation of *Zingiber officinale*, *Anethum graveolens* and *Citrullus colocynthis* extracts with Different Polarities in Streptozotocin-Induced Diabetic Rats. Pharmaceutical Chemistry. 2022; 55: 1062–1070
[41] Ostovar M, Akbari A, Anbardar MH, Iraji A, Salmanpour M, Ghoran SH, Heydari M, Shams M. Effects of *Citrullus colocynthis* L. in a rat model of diabetic neuropathy. J Integr Med. 2020; 18(1): 59-67.

[42] Ghauri AO, Ahmad S, Rehman T. In vitro and in vivo anti-diabetic activity of *Citrullus colocynthis* pulpy flesh with seeds hydro-ethanolic extract. J. Complement. Integr. Med. 2020; 23: 17(2).
[43] Drissi F, Lahfa F, Gonzalez T, Peiretti F, Tanti J, Haddad M, Fabre N, Govers RA. *Citrullus colocynthis* fruit extract acutely enhances insulin induced GLUT4 translocation and glucose uptake in adipocytes by increasing PKB phosphorylation. Journal of Ethnopharmacology. 2021; 270
[44] Deshmukh CD, Jain A. Antidiabetic and antihyperlipidemic effects of methanolic extract of *Citrullus lanatus* seeds in rats. Int. J. Pharm. Pharm. Sci. 2015; 7 (10): 232-236
[45] Ajiboye BO, Shonibare MT, Oyinloye BE. Antidiabetic activity of watermelon (*Citrullus lanatus*) juice in alloxan-induced diabetic rats. J. Diabetes Metab. Disord. 2020; 19 (1): 343-352.
[46] Jibril MM, Haji-Hamid A, Abas F, Karrupan J, Mohammed AS, Jaafar AH, Dek MSP, Ramli NS. Watermelon (*Citrullus lanatus*) leaf extract attenuates biochemical and histological parameters in high-fat diet/streptozotocin-induced diabetic rats. J. Food Biochem. 2022; 46 (2).
[47] Volina-Souza M, Vieira Oliveira G, Vargas R, Carolina Tavares A, Conte-Junior CA, Alvares TS. Effect of microencapsulated watermelon (*Citrullus lanatus*) intake on plasma amino acids and glycemic response in healthy adults. Food Bioscience. 2022. 46; https://doi.org/10.1016/j.fbio.2022.101553.
[48] Shree VS, Sathishkumar T, Kumaresan K, Rapheal VS, Muthukumaran P, Muthukumaran V. Therapeutic effects of purified polyphenols from *Coccinia grandis*: correlation between hypertension and diabetes mellitus. Advances in Traditional Medicine. 2021; 21: 579–590
[49] Chanda J, Mukherjee PK, Kar A, Maitra PK, Singha S, Halder PK, Gajbhiye R, Vishnuvardh R. LC–QTOF–MS-based metabolite profiling and evaluation of α-glucosidase inhibitory kinetics of *Coccinia grandis* fruit. Biomedical chromatography. 2020; 34 (12)

[50] Siddiqua S, Jyoti FH, Saffoon N, Miah P, Lasker S, Hossain H, Akter R, Ahmed MD, Alam MDA. Ethanolic extract of *Coccinia grandis* prevented glucose intolerance, hyperlipidemia and oxidative stress in high fat diet fed rats. Phytomedicine Plus. 2021; 1 (4)
[51] Wasana KGP, Attanayake AP, Weerarathna TP, Jayatilaka KAPW. Efficacy and safety of an herbal drug of *Coccinia grandis* (Linn.) Voigt in patients with type 2 diabetes mellitus: A double blind randomized placebo controlled clinical trial. Phytomedicine. 2021; 81.
[52] Ramalingam S, Packirisamy M, Karuppiah, M, Vasu G, Gopalakrishnan R, Gothandam K, Thiruppathi M. Effect of β-sitosterol on glucose homeostasis by sensitization of insulin resistance via enhanced protein expression of PPRγ and glucose transporter 4 in high fat diet and streptozotocin-induced diabetic rats. Cytotechnology. 2020; 72(3): 357–366.
[53] Koyagura N, Kumar VH, Shanmugam C. Anti-Diabetic and Hypolipidemic Effect of *Coccinia indica* in Glucocorticoid Induced Insulin Resistance. Biomedical & Pharmacology Journal, 14(1). 2021; 133-140.
[54] Yadav J P, Singh A K, Grishinad M, Pathakd P, Patel D K. *Cucumis melo* Var. agrestis Naudin as a potent antidiabetic: Investigation via experimental methods. Phytomedicine Plus. 2022. https://doi.org/10.1016/j.phyplu.2022.100340.
[55] Gopalasatheeskumar K, Kalaichelvan VK, Ariharasivakumar G, Sengottuvel T, Sanish DV. In vitro antidiabetic activity of hydroalcoholic leaf extract of *Cucumis melo* var agrestis (Cucurbitaceae). Research journal of pharmacy and technology. 2020a; 13 (12): 5851-5854
[56] Gopalasatheeskumar K, Ariharasivakumar G, Kalaichelvan VK, Sengottuvel T, Sanish Devan V, Srividhya V. Antihyperglycemic and antihyperlipidemic activities of wild musk melon (*Cucumis melo* var. agrestis) in streptozotocin-nicotinamide induced diabetic rats. Chinese Herbal Medicines. 2020b; 12 (4): 399-405
[57] Thanh TTT, Quach TTM, Yuguchi Y, Nguyen NT, Ngo QV, Bui N. V, Kawashima S, Ho CD. Molecular structure and anti-diabetic activity of a polysaccharide extracted from

pumpkin *Cucurbita pepo*. Journal of Molecular Structure. 2021; 1239.
[58] Chigurupati S, AlGobaisy YK, Alkhalifah B. Alhowail A. Bhatia S, Das S. Vijayabalan S. Antioxidant and antidiabetic potentials of Cucurbita pepo leaves extract from the gulf region. Rasayan J. Chem., 2021; 14 (4): 2357-2362

CHAPTER 12

Evaluation of Enzyme Activity with Nanoparticles Conjugation

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Evaluation of Enzyme Activity with Nanoparticles Conjugation

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Abstract

Nanoparticles enhance enzyme activity, improving catalytic efficiency and application potential. Biocatalysts like enzymes are essential to biological and industrial processes. Their poor stability, restricted substrate specificity, and constrained operating circumstances have led to initiatives to maximize their performance. Recently, conjugating enzymes with nanoparticles has shown promise in overcoming these restrictions and boosting enzyme activity. Nanoparticles may modify and improve enzymes due to their high surface area, variable size and shape, and good stability. This chapter discusses enzyme conjugation using metal, metal oxide, quantum dot, and carbon-based nanoparticles. The chapter begins with a review of nanoparticle conjugation with enzymes, emphasizing surface modification and coupling chemistry to guarantee stable and effective enzyme-nanoparticle interactions. Enhancing substrate binding, enzyme stability, and denaturing resistance boost enzyme activity. The chapter also examines how nanoparticle conjugation affects enzyme catalytic parameters as reaction kinetics, substrate specificity, and product selectivity. It explains how nanoparticle size, shape, surface chemistry, and enzyme spatial organization affect enzyme-nanoparticle interactions. The chapter also discusses nanoparticle-conjugated enzyme applications in biocatalysis, biosensors, bioremediation, and medication delivery. It presents contemporary enzyme-nanoparticle systems that improve process efficiency, sensitivity, and selectivity.

1. Introduction

It has been known for a long time that enzymes have the capacity to catalyze complex reactions; many of these reactions are now hard to recreate using synthetic chemical processes. Natural products have extensive industrial uses due to their use in bioenergy and healthcare [1]. Recent advances in metagenomics and the proliferation of genetic data over the last decade have greatly aided in the discovery of previously unknown enzymes with the potential to catalyze previously unimaginable events [2, 3]. Proteins responsible for carrying out the specified reactions are becoming easier to identify thanks to the development of genome editing techniques like CAS/CRISPR [4]. Since their discovery, enzymes have found several uses in business, from drug manufacturing to textile processing, and the list keeps growing [5, 6]. Recently, in the context of synthetic biology, the enormous potential of utilizing enzyme cascades for various complicated syntheses has been brought to the fore [7, 8]. There is a growing understanding that cell-free synthetic techniques based on multienzyme cascades also have much to offer for these reasons, despite the fact that much present research in this area is focused on establishing first-generation cell-based platforms [9, 10].

Immobilization of enzymes onto macro or micro surfaces is often necessary to allow their usage in biosensors, materials science, and other non-natural uses for restoration of the environment, biological reactors, and several other industrial biological technology areas [11, 12]. Multiple variables, such as the distortion of native protein conformation[13, 14], steric hindrance, leading to a slowed diffusion rate of the entering substrate at the bulk surface [15, 16], contribute to the reduced performance of enzymes that have been immobilized onto planar surfaces. Researchers have started using nanoparticles (NPs) as enzyme carriers to avoid the requirement to modify enzymes and to prevent the detrimental consequences of immobilization through enzyme on the surfaces.

Enzyme immobilization on large-scale flat surfaces and microparticles has both proven effective; nevertheless, NPs (materials with a single dimension 100 nm in size; henceforth referred to as "NPs") provide unique advantages [17, 18]. NPs have many unique and advantageous physicochemical capabilities, such as high surface-to-volume values that enhance catalytic activity, the chemical composition of the surface that is favorable to bioconjugation, and ranges of length that incorporate well into and accordingly influence biologic functions such as cell uptake/metabolism and the expression of genes. [19]. Biologically focused applications benefit from NPs due to their unique characteristics, such as biological/chemical sensing [20, 21], renewable energy generation [21], biodiesel synthesis [22], medicine delivery [23], and disease diagnostics[24]. One of the most interesting features of NPs is their potential to improve the performance and activity of immobilized enzymes [25]. This article discusses the cutting-edge nanoparticles being introduced into such systems and the processes that underpin the improved performance of enzyme-NP conjugates.

1.1. Strategy for Enzyme Loading

Since Nelson and Griffin [26] initially loaded enzymes onto the solid surface of charcoal, other enzyme loading techniques have been devised. There are several ways to immobilize enzymes, but they may be broken down into three primary categories: binding to a support, trapping, and cross-linking [27, 28]. Enzyme activity may be increased in extreme environments by physically attaching to a support. Here, enzyme stability is improved by adsorption, ionic, and covalent binding to the substrate, with the latter providing a more persistent attachment to the support [27, 29-32]. When an enzyme is entrapped in a matrix of inorganic or organic polymers, its activity often depends on its being released via a membrane or biological gates [33]. Covalent bonding may be used to overcome some of the method's drawbacks, such as enzyme leakage. Enzymes bond to one other in the cross-linking process,

which makes use of a multifunctional reagent. Enzyme activity might diminish or cease altogether as a result of conformational alterations [34]. This approach may make useful contributions to biological activity and does not need a support matrix.

1.2. The Effects of Nanomaterials on Enzymes

Enzymes are now able to interact with nanoparticles and nanomaterials much more often because of the fast advancement of nanotechnology. Biomedical, biochemistry technology, tracking the environment, nanoparticle design, and biosensor applications all have growing needs for combining nanoparticles and enzymes. Since 2014, enzymatic modification, degradation, immobilization, and biosensors utilizing nanoparticles have made substantial advances. Nanoparticles have been used in recent years to manipulate enzyme activity and alter the structures and activities of enzymes. At the same time, reports surfaced of enzymes that potentially change the characteristics of nanoparticles and other nanomaterials in order to facilitate the formation of assemblies and conjugates. Rational design and optimization of immobilization of enzymes on nanoparticles have significantly enhance their catalytic efficiency. Enzyme-based biosensors benefit from the incorporation of nanoparticles because the biological sensors take on the nanoparticles' or nanomaterials' unique inherent features, making them more stable and sensitive. In addition to these applications, research into the enzymatic degradation of nanoparticles has been prompted by the potential environmental risks of nanoparticles [35, 36]. Enzyme and mimics of nanoparticles have been produced to address the limitations of natural enzymes, such as their susceptibility to inactivation and the length of time required for extraction [37]. Despite the fact that several studies have concentrated in on the relationship and interactions between proteins and nanoparticles [38-40], enzymes have been largely overlooked. Enzymes, in contrast to other proteins, are catalysts that perform crucial functions in biological control and metabolism inside living organisms. Thus, in this Chapter, we shed light on the mechanisms by which enzymes and nanoparticles interact, as well as on the

enzymes that function as catalysts nanoparticles. With a focus on recent literature, this chapter addresses key questions concerning nanoparticle and nanomaterial interactions with enzymes. Over the last three years, more than twenty-five nanoparticles have come into contact with more than forty enzymes.

1.3. Changes in Enzyme Properties Caused by Nanoparticle Interactions

The usage of nanoparticles in several fields has been on the rise recently [41-45]. Enzymes play a substantial part in many of these applications, and their involvement may alter the physicochemical characteristics of the enzymes or nanoparticles in either a positive or negative way. Enzymes, for instance, may damage single-walled carbon nanotubes (SWCNTs) by nonspecifically adsorbing onto their surfaces [46]. Nanoparticles may acquire a biological identity when they attach to proteins or enzymes, creating what is called a "nanoparticle-protein corona" [40, 47-49]. Nanoparticles' structural and functional effects on enzymes are necessary to be studied. When enzymes bind to nanoparticles, they undergo conformational changes that may either reduce or increase their activity. It has been shown that certain nanoparticles, such as carbon nanotubes (CNTs), graphene, fullerene derivatives, and metal nanoparticles (Tables 12.1 and 12.2), may alter the structure or activity of enzymes in distinct ways. Enzyme composition and orientation, nanomaterial size and shape, chemical functional groups, and environmental factors all have a role in the wide range of observed effects [25, 50-52]. Enzymes vary in their amino acid makeup and 3D architectures, which causes them to interact with nanoparticles in unique ways. The orientation of enzymes with respect to nanoparticles is crucial because nanoparticles and other nearby molecules might obstruct or inhibit the catalytic active sites if the enzymes are oriented incorrectly [25]. The physicochemical characteristics of nanoparticles and the surrounding environmental conditions (such as pH and temperature) all impact the attachment positions and activities of nanoparticles with enzymes, enzyme stability, and substrate

availability. The influence of nanomaterials on the molecular structure and activity of a variety of the enzymes have been examined, including protein tyrosine phosphatase 1B, l, lignin peroxidase, horseradish peroxidase, NADPH oxidase, and b-galactosidase.

This chapter begins by providing a discussion of lysozyme, which has been used extensively as a model enzyme to investigate how nanoparticles and enzymes interact. According to reports, arginine prevents lysozymes from adsorbing onto SWCNTs by blocking the interactions between the amino acid residues of arginine and the carbon nanotubes [46]. Lysozyme adsorption onto graphitized, carboxylated, and hydroxylated multi-walled carbon nanotubes (MWCNTs) was studied by Du et al. [60]. The adsorption capability of hydroxylated MWCNTs was highest, followed by carboxylated and graphitized MWCNTs. Significant activity reduction was seen when Pan et al. [53] investigated the structural basis and adsorption of T4 lysozyme onto silica nanoparticles. Another enzyme often studied to see how nanoparticles affect enzymatic function is a-chymotrypsin.

Carboxylated SWCNTs can control a-chymotrypsin activity. Adsorption of a-chymotrypsin onto CNTs, as discovered by Zhao and Zhou [55], modifies the protein's secondary structure. The scientists went on to note that although the interaction between a-chymotrypsin and pure CNTs is noncompetitive, binding to carboxylated CNTs may limit enzyme activity via a competitive-like manner. Several enzymes, including lysozyme and chymotrypsin, have made contact with nanoparticles. Inhibition of protein tyrosine phosphatase 1B by fullerene derivatives has been observed in one research [64]. The C termini of various enzymes produced by microorganisms, such as laccase, haloalkane, lignin peroxidase, 1,2-dioxygenase, and manganese peroxidase, undergo significant conformational changes once SWCNTs are employed to undergo oxidation organic substances or lignin framework substances, as reported by Chen et al. [54]. Horseradish peroxidase (HRP) activity and stability are affected

differently by graphene, graphene oxide (GO), and reduced graphene oxide (RGO) [67]. While graphene and GO reduce enzymatic stability, RGO improves it. The secondary structure of HRP is altered by all of these graphene-based nanomaterials. TiO_2's impact on NADPH oxidase activity has also been studied. Without the traditional activator, acid, TiO_2 cannot stimulate this enzyme's activity. TiO_2 nanoparticles inhibited NADPH oxidase's ability to generate superoxide anion in the absence of arachidonic acid [71] but stimulated its activity in the presence of the fatty acid. Investigation of copper oxide nanoparticles' interaction with b-galactosidase revealed that the enzyme's structure and activity were disturbed by the particles [72]. Enzyme-nanoparticle interactions and the resulting modifications to enzyme properties have been studied. Researchers are interested in using nanoparticles as supports for enzyme immobilization because their features (such as shape, size, and surface chemistry) may increase enzymatic function if rational design is conducted.

Table 12.1. Enzyme-carbon nano material interactions

Nanomaterial	Enzyme	Cause of enzyme interactions	Refs
SWCNT	Lysozyme	Studying the adsorption of lysozyme onto SWCNTs	[46]
	Laccase	Examining the effects of SWCNT on oxidation reactions mediated by enzymes	[54]
	Haloalkane dehalogenase	Examining the effects of SWCNT on oxidation reactions mediated by enzymes	[54]
	Lignin peroxidase	Understanding how SWCNT influences oxidation reactions mediated by enzymes	[54]
	Naphthalene 1,2-dioxygenase	Investigating how SWCNT affects oxidation reactions mediated by enzymes	[54]
	Manganese peroxidase	Examining the effects of SWCNT on oxidation reactions mediated by enzymes	[54]
	a-Chymotrypsin	Studying the Inhibition of a-Chymotrypsin by SWCNTs	[55]
	Horseradish peroxidase	The SWCNTs biodegrade process	[56]

	Lactoperoxidase	The SWCNTs biodegrade process	[57]
	Myeloperoxidase	The SWCNTs biodegrade process	[58]
	Eosinophil peroxidase	The SWCNTs biodegrade process	[59]
	Lysozyme	Lysozyme adsorption studies on multiwall carbon nanotubes	[60]
	Human monoamine oxidase B	Developing an electrochemical biosensor that is responsive to monoamines	[61]
Nanomaterial	**Enzyme**	**Cause of enzyme interactions**	**Refs**
MWCNT	Horseradish peroxidase	MWCNTr biodegradation	[56]
	Laccase	Immobilization through enzymes	[62]
	Cellulase	Immobilization through enzymes	[63]
Fullerene	Protein tyrosine phosphatase 1B	Examining how protein tyrosine phosphatase 1B is inhibited by fullerene analogues.	[64]
Graphene	Diamine oxidase	A biosensor for histamine	[65]
	Plant esterase	Methyl parathion and malathion biosensor.	[66]
	Horseradish peroxidase	Examining how graphene-based nanomaterials affect horseradish peroxidase activity and stability.	[67]
	a-Galactosidase	Immobilization through enzymes	[68]
Graphene oxide	Horseradish peroxidase	Examining how graphene-based nanomaterials affect horseradish peroxidase activity and stability.	[67]
Reduced graphene oxide	Lipase	Immobilization through enzymes	[69]
	Glucose oxidase	Biosensing and glucose immobilization using enzymes	[70]
	Horseradish peroxidase	Investigating the impacts of graphene-based nanomaterials on the activity or stability of horseradish peroxidase	[67]

Table 12.2. Enzyme Analysis of Inorganic Nanoparticles

Nanoparticle/ nanomaterial	Enzyme	Reason for their interaction	Refs
Fe_3O_4	Aminoacylase	Immobilization through enzymes	[73]
	Glucose oxidase	Immobilization through enzymes	[74]
	Feruloyl esterases	Immobilization through enzymes	[75]
	Acetylxylan esterase	Immobilization through enzymes	[76]
	DNA ligase	Enzyme mimic biosensor for genomic DNA detection	[77]
	Peroxidase	Artificial enzyme	[78, 79]
Au	Bovine serum amine oxidase	The generation of a bioconjugation system	[80]
	Cytochrome bd oxidase	Identifying Cytochrome bd Oxidase Inhibitors	[12]
	Glucose oxidase	Biosensor for glucose	[81-83]
	Glucose dehydrogenase	Creating a bioanode with low redox potential, high current, and the absence of mediators	[84]
	Glutathione-S transferase	Achieving an Immunosensor for Glutathione S-Transferase	[85]
	Horseradish peroxidase	Creating an Immunosensor for Glutathione S-Transferase	[85]
Nanoparticle/ nanomaterial	**Enzyme**	**Reason for their interaction**	**Refs**
Au	DNA methyltransferase	Biosensor utilizing electrochemiluminescence to detect DNA methyltransferase activity	[86]
	Protein disulfide isomerase	Investigating the Protein Disulfide Isomerase from a Hydrodynamic Perspective	[87]
	DNA ligase	Genomic DNA Detection Employing a Biosensor	[77]
	Plant esterase	Methyl parathion and malathion biosensor.	[66]
	DNA methyltransferase	DNA Methyltransferase Detection	[88]
	Peroxidase	Virus detection for influenza	[89]
	Horseradish peroxidase	Measurement of cyanide using biosensors	[90]

Ag	Glucose oxidase	Biosensing and glucose immobilization employing enzymes	[70]
	Nitrate reductase	Analysis of Soil Nitrates	[91]
AgX (X is Cl, I or Br)	Peroxidase	Artificial enzyme	[92]
SiO_2	Laccase	Developing multilayered structures of electroactive nanobiomolecules	[93]
	Lysozyme	Examining the adsorption of lysozyme onto silica nanoparticles	[53]
	Pullulanase	Immobilization through enzymes	[94]
	Inulinase	Immobilization through enzymes	[95]
Nanoparticle/ nanomaterial	**Enzyme**	**Reason for their interaction**	**Refs**
CdS	Sulfite oxidase	Polyethylenimine-encapsulated CdS nanoparticles on an ITO electrode for human sulfite oxidase assembly.	[96]
	DNA methyltransferase	A biosensor based on electrochemiluminescence for the detection of DNA methyltransferase activity	[86]
Pt	Peroxidase	RNA and DNA detection	[97]
	Diamine oxidase	Histamine detection biosensors	[65]
Cobalt oxide	Microbial esterases	Enzymatic immobilization	[98]

1.4. Nanoparticle-Based Enzyme Immobilization for Enhanced Activity

Optimal conditions are typically needed for processes mediated by enzymes. In the real world, free enzymes are quickly rendered ineffective [56]. Furthermore, they are hard to reuse [57]. These issues should be resolved by immobilizing enzymes on nanoparticles. However, enzyme immobilization may distort enzyme structures and reduce activity, preventing enzymatic activity improvement. Therefore, research into the best immobilization conditions is required. Recently, magnetic nanoparticles have gained popularity as enzymatic immobilization supports due to their convenience in recycling

the enzymes through magnetic collection. Aminoacylase now has a home on magnetic Fe_3O_4 nanoparticles. Then, it was shown that aminoacylase could be immobilized on Fe_3O_4 nanoparticles with the help of 3-(aminopropyl) triethoxysilane [58]. In their research, Yang et al. [57] demonstrated a nanohybrid of glucose oxidase (GOX) on Fe_3O_4@C-silica. GOX encapsulated on the nanomaterials enhanced the temperature range of stable enzyme activity and retained the activity after reuse, demonstrating the excellent thermal and operational stability of the enzyme nanoparticles. To increase the thermal and operational stability of feruloyl esterases for use in ferulic acid synthesis, He et al. [56] immobilized them on magnetic Fe_3O_4 nanoparticles. Immobilized recombinant acetyl xylan esterase (rAXE) on Fe_3O_4 magnetic nanoparticles coated with chitosan was more stable than free rAXE throughout a larger pH and temperature range [59].

Due to their distinct physicochemical features (Table 12.2), gold nanoparticles have gained attention for enzyme immobilization. Scholars Venditti et al. described the biological conjugation of AuNPs, poly(3-dimethylammonium-1-propyne hydrochloride), and bovine serum amine oxidase [61]. This bioconjugation increased the enzyme's activity by as much as 40% compared to its natural state. In order to investigate the catalytic activity and inhibition of Escherichia coli cytochromes bd oxidase, Fournier et al. [62] immobilized the enzyme on gold nanoparticle-modified electrodes.

Recent research has also focused on the topic of immobilizing enzymes onto nanoparticles of materials as nanosilica, nano-ZnO, cobalt oxide, and CdS. A glutaraldehyde spacer arm immobilized a novel pullulanase from Fontibacillus sp. strain DSHK 107 on nanosilica [63]. Immobilized pullulanase was more thermally stable than free enzyme.

The optimal pH for this immobilized pullulanase also changed, going from 6.0 to 5.0. Using laccase as a biocatalyst, cytochrome c as an electron shuttle, and silica nanoparticles as an artificial matrix, researchers have built electroactive

nanobiomolecular multilayered structures [65]. The thermostability and reusability of inulinase from Aspergillus niger were improved by octadecyl substituted nanoporous silica compared to free enzyme [66]. Similarly, diastase a-amylase immobilized on nano-ZnO was more stable at high temperatures than the loose enzyme [68]. The bacterial esterases were immobilized using cobalt oxide nanoparticles coated with a Bacillus subtilis template. After 15 reuses, the enzyme-based nanostructure had 85% activity [69]. Polyethylenimine-entrapped CdS nanoparticles on ITO electrodes improved human sulfite oxidase binding and electron transfer [70]. Laccase and cellulase may be immobilized on MWCNTs with the use of these nanotubes (Table 12.1). Enzyme concentrations, pH, temperature, and contact length improve immobilized laccase [75] and cellulase [74]. When immobilized on CNTs, Ganoderma lucidum and Pleurotus ostreatus enzymes increased lignin peroxidase activity by 27- and 18-fold, respectively [99]. Functionalized graphene and GO were used with CNTs to immobilize cicer a galactosidase [76] and Rhizopus oryzae lipase [80]. Graphene and GO increased enzyme thermal stability.

Significant improvements in enzymatic activity have been seen for enzymes immobilized on a variety of nanoparticles; however, these improvements are highly dependent on the specific enzyme types, support materials, and immobilization conditions used. The finding the ideal pH, temperature, enzyme concentration, immobilization period, and nanoparticle concentration for immobilized enzymes requires extensive experimentation. Enzymes that were immobilized on nanoparticles and then optimized demonstrated four benefits over their native counterparts: (i) stronger thermostability, (ii) more thermostability at elevated temperatures, (iii) greater thermostability at elevated pH, and (iv) enhanced reusability. Nanoparticles' enhanced enzyme loading, diffusion of immobilized enzymes, and catalytic activity are all influenced by their high surface-to-volume ratio and chemical, electrical, and optical characteristics [12]. Nanoparticles and nanomaterials are not only constructed into biosensors for the detection of

diverse chemicals but are also used for enzymatic immobilization.

1.5. Nanoparticle-Enzyme-Based Biosensors

Enzyme-based electrochemical biosensors are a kind of analytical equipment for detecting a wide range of target chemicals. Due to the inherent properties of nanoparticles (such as large surface area, good electrical conductivity, unique optical properties, and nanoscale structures), enzyme-based biosensors exhibit new properties after being modified by nanoparticles, including enhanced selectivity, analytical signaling, and sensitivity [93, 94]. In contrast to the sluggish testing speed and logistical challenges of standard detection techniques like chromatography in the field, enzyme-based biosensors with nanoparticles provide quick, sensitive, in-time detection of substances. Biosensors for target molecule detection employ nanoparticles like gold, silver, carbon, Fe_3O_4, magnetic, platinum, and ceria nanospheres. In the meanwhile, several different enzymes, such as glutathione peroxidase, heme oxygenase, ribonuclease P, protein disulfide isomerase, DNA ligase, organophorous hydrolases, diamine oxidase, human monoamine oxidase B, and nitrate reductase, have all played roles in the development of biosensors. Glucose, cyanide, histamine, herbicides, and enzymes are only few of the known target compounds for biosensor detection.

Many studies have shown the success of employing gold nanoparticles as a glucose biosensor in a variety of settings, including the food sector, biodetection, and biomedicine.

A glucose biosensor based on GOX and ZnO nanoarrays benefits from the addition of gold nanoparticles [51]. Surface factors affect GOX conformation and bioactivity on gold nanoparticle surfaces [100]. GOX immobilized on an RGO/silver nanocomposite modified electrode showed good glucose-sensitive electrocatalytic activity [96]. Ratautas et al. [101] studied glucose dehydrogenase from Ewingella americana catalyzing glucose oxidation on gold nanoparticles

functionalized with 4-mercaptobenzoic acid and 4-aminothiophenol. Gold nanoparticles on polyaniline nanowires and GOX created a high-performance 3D glucose sensing bioplatform. The produced entity is stable, selective, and detectable at 0.05 μM [98]. AuNPs improve glucose biosensor performance through surface functionalization and interaction with other materials.

Biosensors based on gold nanoparticles have attracted significant interest for the detection of cyanide, enzymes, and genomic DNA (Table 12.2), in addition to glucose. For the detection of cyanide, researchers have created a biosensor comprised of HRP and a gold sononanoparticle [77]. Lu et al. [78] detailed the development of a double-layer gold nanoparticle-based GST immunosensor that could detect concentrations as low as 0.03 pg/mL of GST. In order to detect DNA methyltransferase activity, Zhou et al. [79] developed an electrochemiluminescence biosensor using CdS quantum dots and gold nanoparticles. The electrochemiluminescence of CdS quantum dots is significantly improved by the addition of gold nanoparticles. Another team used CuS nanoparticles and functionalized gold nanoparticles to detect DNA methyltransferase without costly equipment [81]. Zheng et al. [82] used citrate-capped AuNP probes and dynamic light scattering to study protein disulfide isomerase hydrodynamics. Researchers calculated the enzyme's hydrodynamic size by measuring gold nanoparticles' average diameter before and after protein corona formation. AuNPs, Fe_3O_4 magnetic nanoparticles, DNA ligase, and a modified gold electrode were used to detect genomic DNA [83].

Paraoxon, coumaphos, and parathion are just a few examples of organophosphorus insecticides that may be broken down by organophosphorus hydrolases. The widespread use of organophosphate insecticides has resulted in a toxic buildup of the chemical in the environment. Therefore, there is an immediate need for methods to measure environmental concentrations of organophosphates. Enzyme and nanoparticle-based biosensors have shown to be a reliable way for monitoring

these substances. Khaksarinejad et al. [84] developed a 5×10^{-6} μM paraoxon biosensor using organophosphorus hydrolase and silica-coated magnetic nanoparticles. Plant esterase, gold nanoparticles, chitosan, and graphene nanosheets formed a biosensor for methyl parathion and malathion [85]. Chitosan, gold nanoparticles, and graphene nanosheets improve electron transport and electric conductivity. Histamine (2-(4-imidazolyl) ethylamine), total monoamines, putrescine (1,4-diaminobutane), and soil nitrate (NO_3) may all be detected with high sensitivity thanks to the incorporation of nanoparticles and nanomaterials into biosensors.

Two amines that serve as quality markers include histamine and putrescine. Monoamines, which include serotonin and dopamine, are involved in a wide range of physiological processes and have been related to a number of illnesses. Nitrate is a widespread environmental contaminant. The detection of these molecules is crucial since it allows for the evaluation of food quality, the aid in the diagnosis of diseases, and the accomplishment of environmental monitoring. Histamine biosensors with low detection limits and high sensitivities were developed using a modified carbon screen-printed electrode, diamine oxidase, graphene, platinum particles, and chitosan [86].

Aigner et al. [87] built a monoamine-sensitive electrochemical biosensor using MWCNTs and human monoamine oxidase B. Diamine oxidase on ceria nanospheres measured tiger prawn putrescine levels [88]. Epoxy bonded silver nanoparticles were immobilized nitrate reductase, creating epoxy/AgNPs/NR conjugates for soil nitrate concentrations [89]. Nanoparticles pose threats to the environment despite their usefulness in improving biosensor performance when measuring target molecules.

2. Conclusion

In this chapter, we explored the exciting field of enzyme activity enhancement through the utilization of nanoparticle conjugation. Enzymes are remarkable catalysts that play a crucial role in various biological and industrial processes. However, their inherent limitations, such as low stability, substrate specificity, and limited operational conditions, have prompted scientists to seek innovative strategies to enhance their performance. Nanoparticles have emerged as promising tools for enzyme activity improvement due to their unique properties, including high surface area, tunable surface chemistry, and excellent stability. The conjugation of enzymes with nanoparticles offers a multitude of advantages, such as enhanced stability, improved catalytic efficiency, increased substrate affinity, and expanded operational conditions. These benefits arise from the synergistic effects between the nanoparticles and enzymes, which enable a new level of control over enzyme activity. One of the key findings discussed in this chapter was the impact of nanoparticle size and composition on enzyme activity. It was observed that nanoparticles with appropriate sizes and surface functionalities can create a favorable microenvironment for enzymes, leading to increased catalytic rates and enhanced substrate binding. Moreover, the introduction of specific functional groups on nanoparticle surfaces allowed for tailored interactions with enzymes, further optimizing their activity.

The chapter also delved into various techniques employed for nanoparticle conjugation with enzymes. Covalent binding, physical adsorption, and encapsulation within nanoparticle structures were explored as effective methods to establish stable and functional enzyme-nanoparticle complexes. Each technique demonstrated its own advantages and limitations, highlighting the importance of selecting an appropriate conjugation strategy based on the specific enzyme and application. Furthermore, the chapter highlighted some notable applications of enzyme-nanoparticle conjugates in diverse fields, including biomedicine, biocatalysis, and environmental remediation. Nanoparticle

conjugation has proven to be particularly valuable in the development of biosensors, drug delivery systems, and enzymatic reactors. These applications capitalize on the enhanced enzyme activity conferred by nanoparticles, allowing for improved detection limits, controlled drug release, and efficient transformation of target compounds. While the research and development in this field are still ongoing, the chapter concludes with a note on the future prospects and challenges in the realm of enzyme-nanoparticle conjugation. The optimization of nanoparticle properties, such as size, shape, and surface chemistry, remains an active area of investigation. Additionally, the development of novel techniques for precise control over enzyme orientation and spatial arrangement on nanoparticle surfaces holds great potential for further improving enzyme activity. In conclusion, the conjugation of enzymes with nanoparticles represents a powerful approach to enhance enzyme activity and expand their potential applications. The synergistic effects achieved through this combination have enabled the development of advanced enzyme systems with improved catalytic efficiency, stability, and versatility. As scientists continue to explore and innovate in this field, the integration of enzyme-nanoparticle conjugates is poised to revolutionize diverse industries and contribute to the advancement of biotechnology and nanomedicine.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Adrio, J.L. and A.L. Demain, Microbial enzymes: tools for biotechnological processes. Biomolecules, 2014. 4(1): p. 117-139.
[2] Alma'abadi, A.D., T. Gojobori, and K. Mineta, Marine metagenome as a resource for novel enzymes. Genomics, proteomics & bioinformatics, 2015. 13(5): p. 290-295.
[3] Rashid, M. and U. Stingl, Contemporary molecular tools in microbial ecology and their application to advancing

biotechnology. Biotechnology advances, 2015. 33(8): p. 1755-1773.
[4] Wright, A.V., J.K. Nuñez, and J.A. Doudna, Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering. Cell, 2016. 164(1-2): p. 29-44.
[5] Kirk, O., T.V. Borchert, and C.C. Fuglsang, Industrial enzyme applications. Current opinion in biotechnology, 2002. 13(4): p. 345-351.
[6] Volpato, G., R. C Rodrigues, and R. Fernandez-Lafuente, Use of enzymes in the production of semi-synthetic penicillins and cephalosporins: drawbacks and perspectives. Current medicinal chemistry, 2010. 17(32): p. 3855-3873.
[7] Nielsen, J. and J.D. Keasling, Engineering cellular metabolism. Cell, 2016. 164(6): p. 1185-1197.
[8] Woolston, B., Edgar S. Stephanopoulos G. Metabolic engineering: past and future. Annu Rev Chem Biomol Eng, 2013. 4: p. 259-288.
[9] Friedman, D.C., Industrialization of Biology. A Roadmap to Accelerate the Advanced Manufacturing of Chemicals. 2015.
[10] Vranish, J.N., et al., Enhancing coupled enzymatic activity by conjugating one enzyme to a nanoparticle. Nanoscale, 2017. 9(16): p. 5172-5187.
[11] Palmer, T. and P.L. Bonner, Enzymes: biochemistry, biotechnology, clinical chemistry. 2007: Elsevier.
[12] Ansari, S.A. and Q. Husain, Potential applications of enzymes immobilized on/in nano materials: A review. Biotechnology advances, 2012. 30(3): p. 512-523.
[13] Bryjak, J. and B. Kolarz, Immobilisation of trypsin on acrylic copolymers. Process biochemistry, 1998. 33(4): p. 409-417.
[14] Janssen, M.H., et al., Evaluation of the performance of immobilized penicillin G acylase using active-site titration. Biotechnology and bioengineering, 2002. 78(4): p. 425-432.
[15] Fernandez-Lafuente, R., Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. Enzyme and microbial technology, 2009. 45(6-7): p. 405-418.

[16] Talbert, J.N. and J.M. Goddard, Enzymes on material surfaces. Colloids and Surfaces B: Biointerfaces, 2012. 93: p. 8-19.
[17] Höldrich, M., A. Sievers-Engler, and M. Lämmerhofer, Gold nanoparticle-conjugated pepsin for efficient solution-like heterogeneous biocatalysis in analytical sample preparation protocols. Analytical and bioanalytical chemistry, 2016. 408: p. 5415-5427.
[18] Ellis, G.A., S.A. Díaz, and I.L. Medintz, Enhancing enzymatic performance with nanoparticle immobilization: improved analytical and control capability for synthetic biochemistry. Current Opinion in Biotechnology, 2021. 71: p. 77-90.
[19] Albanese, A., P.S. Tang, and W.C. Chan, The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annual review of biomedical engineering, 2012. 14: p. 1-16.
[20] Hwang, E.T. and M.B. Gu, Enzyme stabilization by nano/microsized hybrid materials. Engineering in Life Sciences, 2013. 13(1): p. 49-61.
[21] Sotiropoulou, S. and N.A. Chaniotakis, Carbon nanotube array-based biosensor. Analytical and Bioanalytical Chemistry, 2003. 375: p. 103-105.
[22] Dizge, N. and B. Keskinler, Enzymatic production of biodiesel from canola oil using immobilized lipase. Biomass and bioenergy, 2008. 32(12): p. 1274-1278.
[23] Tao, Y., M. Ning, and H. Dou, WITHDRAWN: A novel therapeutic system for malignant glioma: nanoformulation, pharmacokinetic, and anticancer properties of cell-nano-drug delivery. 2012, Elsevier.
[24] Liu, J., et al., Flexible gold electrode array for multiplexed immunoelectrochemical measurement of three protein biomarkers for prostate cancer. ACS applied materials & interfaces, 2014. 6(22): p. 20137-20143.
[25] Johnson, B.J., et al., Understanding enzymatic acceleration at nanoparticle interfaces: Approaches and challenges. Nano Today, 2014. 9(1): p. 102-131.

[26] Nelson, J. and E.G. Griffin, Adsorption of invertase. Journal of the American Chemical Society, 1916. 38(5): p. 1109-1115.
[27] Cantone, S., et al., Efficient immobilisation of industrial biocatalysts: criteria and constraints for the selection of organic polymeric carriers and immobilisation methods. Chemical Society Reviews, 2013. 42(15): p. 6262-6276.
[28] Sheldon, R.A. and S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how. Chemical society reviews, 2013. 42(15): p. 6223-6235.
[29] Sharifi, M., et al., Strategies of enzyme immobilization on nanomatrix supports and their intracellular delivery. Journal of biomolecular structure and Dynamics, 2020. 38(9): p. 2746-2762.
[30] Poorakbar, E., et al., Synthesis of magnetic gold mesoporous silica nanoparticles core shell for cellulase enzyme immobilization: improvement of enzymatic activity and thermal stability. Process biochemistry, 2018. 71: p. 92-100.
[31] Besharati Vineh, M., et al., Physical adsorption of horseradish peroxidase on reduced graphene oxide nanosheets functionalized by amine: a good system for biodegradation of high phenol concentration in wastewater. International Journal of Environmental Research, 2018. 12: p. 45-57.
[32] Vineh, M.B., et al., Stability and activity improvement of horseradish peroxidase by covalent immobilization on functionalized reduced graphene oxide and biodegradation of high phenol concentration. International journal of biological macromolecules, 2018. 106: p. 1314-1322.
[33] Ursoiu, A., et al., Sol-gel entrapped Candida antarctica lipase B—A biocatalyst with excellent stability for kinetic resolution of secondary alcohols. Molecules, 2012. 17(11): p. 13045-13061.
[34] Talekar, S., et al., Immobilized enzyme mediated synthesis of silver nanoparticles using cross-linked enzyme aggregates (CLEAs) of NADH-dependent nitrate reductase. Nanostructures & Nano-Objects, 2016. 6: p. 23-33.
[35] Chen, M., X. Qin, and G. Zeng, Biodegradation of carbon nanotubes, graphene, and their derivatives. Trends in biotechnology, 2017. 35(9): p. 836-846.

[36] Chen, M., et al., Understanding enzymatic degradation of single-walled carbon nanotubes triggered by functionalization using molecular dynamics simulation. Environmental Science: Nano, 2017. 4(3): p. 720-727.
[37] Tao, Y., et al., Polypyrrole nanoparticles as promising enzyme mimics for sensitive hydrogen peroxide detection. Chemical Communications, 2014. 50(23): p. 3030-3032.
[38] Shemetov, A.A., I. Nabiev, and A. Sukhanova, Molecular interaction of proteins and peptides with nanoparticles. ACS nano, 2012. 6(6): p. 4585-4602.
[39] Lynch, I. and K.A. Dawson, Protein-nanoparticle interactions. Nano today, 2008. 3(1-2): p. 40-47.
[40] Monopoli, M.P., et al., Biomolecular coronas provide the biological identity of nanosized materials. Nature nanotechnology, 2012. 7(12): p. 779-786.
[41] Xu, P., et al., Use of iron oxide nanomaterials in wastewater treatment: a review. Science of the total environment, 2012. 424: p. 1-10.
[42] Gong, J.-L., et al., Removal of cationic dyes from aqueous solution using magnetic multi-wall carbon nanotube nanocomposite as adsorbent. Journal of hazardous materials, 2009. 164(2-3): p. 1517-1522.
[43] Lai, C., et al., Synthesis of surface molecular imprinted TiO2/graphene photocatalyst and its highly efficient photocatalytic degradation of target pollutant under visible light irradiation. Applied Surface Science, 2016. 390: p. 368-376.
[44] De Crozals, G., et al., Nanoparticles with multiple properties for biomedical applications: A strategic guide. Nano Today, 2016. 11(4): p. 435-463.
[45] Thorkelsson, K., P. Bai, and T. Xu, Self-assembly and applications of anisotropic nanomaterials: A review. Nano Today, 2015. 10(1): p. 48-66.
[46] Iwashita, K., et al., Arginine suppresses the adsorption of lysozyme onto single-wall carbon nanotubes. Chemistry Letters, 2016. 45(8): p. 952-954.
[47] Cedervall, T., et al., Understanding the nanoparticle–protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proceedings of the National Academy of Sciences, 2007. 104(7): p. 2050-2055.

[48] Kelly, P.M., et al., Mapping protein binding sites on the biomolecular corona of nanoparticles. Nature nanotechnology, 2015. 10(5): p. 472-479.
[49] Lo Giudice, M.C., et al., In situ characterization of nanoparticle biomolecular interactions in complex biological media by flow cytometry. Nature communications, 2016. 7(1): p. 13475.
[50] Castagnola, V., et al., Towards a classification strategy for complex nanostructures. Nanoscale Horizons, 2017. 2(4): p. 187-198.
[51] Zhao, X., et al., Influence of the surface functional group density on the carbon-nanotube-induced α-chymotrypsin structure and activity alterations. ACS applied materials & interfaces, 2015. 7(33): p. 18880-18890.
[52] Chen, M., et al., Interaction of carbon nanotubes with microbial enzymes: conformational transitions and potential toxicity. Environmental Science: Nano, 2017. 4(10): p. 1954-1960.
[53] Pan, Y., et al., Probing the structural basis and adsorption mechanism of an enzyme on nano-sized protein carriers. Nanoscale, 2017. 9(10): p. 3512-3523.
[54] Chen, M., X. Qin, and G. Zeng, Single-walled carbon nanotube release affects the microbial enzyme-catalyzed oxidation processes of organic pollutants and lignin model compounds in nature. Chemosphere, 2016. 163: p. 217-226.
[55] Zhao, D. and J. Zhou, Electrostatics-mediated α-chymotrypsin inhibition by functionalized single-walled carbon nanotubes. Physical Chemistry Chemical Physics, 2017. 19(2): p. 986-995.
[56] He, F., S. Zhang, and X. Liu, Immobilization of feruloyl esterases on magnetic nanoparticles and its potential in production of ferulic acid. Journal of bioscience and bioengineering, 2015. 120(3): p. 330-334.
[57] Yang, Y., et al., Robust glucose oxidase with a Fe 3 O 4@ C-silica nanohybrid structure. Journal of Materials Chemistry B, 2016. 4(27): p. 4726-4731.
[58] Feng, J., et al., Enhancement of the catalytic activity and stability of immobilized aminoacylase using modified magnetic

Fe3O4 nanoparticles. Chemical Engineering Journal, 2016. 286: p. 216-222.
[59] Saravanakumar, T., et al., Optimized immobilization of peracetic acid producing recombinant acetyl xylan esterase on chitosan coated-Fe3O4 magnetic nanoparticles. Process Biochemistry, 2014. 49(11): p. 1920-1928.
[60] Du, P., et al., Adsorption of bovine serum albumin and lysozyme on functionalized carbon nanotubes. The Journal of Physical Chemistry C, 2014. 118(38): p. 22249-22257.
[61] Venditti, I., et al., Bioconjugation of gold-polymer core–shell nanoparticles with bovine serum amine oxidase for biomedical applications. Colloids and Surfaces B: Biointerfaces, 2015. 134: p. 314-321.
[62] Fournier, E., et al., Creation of a gold nanoparticle based electrochemical assay for the detection of inhibitors of bacterial cytochrome bd oxidases. Bioelectrochemistry, 2016. 111: p. 109-114.
[63] Alagöz, D., et al., Covalent immobilization and characterization of a novel pullulanase from Fontibacillus sp. strain DSHK 107 onto Florisil® and nano-silica for pullulan hydrolysis. Applied biochemistry and biotechnology, 2016. 179: p. 1262-1274.
[64] Qian, M., et al., Structural basis of fullerene derivatives as novel potent inhibitors of protein tyrosine phosphatase 1B: insight into the inhibitory mechanism through molecular modeling studies. Journal of chemical information and modeling, 2016. 56(10): p. 2024-2034.
[65] Feifel, S.C., A. Kapp, and F. Lisdat, Electroactive nanobiomolecular architectures of laccase and cytochrome c on electrodes: applying silica nanoparticles as artificial matrix. Langmuir, 2014. 30(19): p. 5363-5367.
[66] Karimi, M., et al., Immobilization of inulinase from Aspergillus niger on octadecyl substituted nanoporous silica: Inulin hydrolysis in a continuous mode operation. Biocatalysis and agricultural biotechnology, 2016. 7: p. 174-180.
[67] Zhang, C., et al., Reduced graphene oxide enhances horseradish peroxidase stability by serving as radical scavenger and redox mediator. Carbon, 2015. 94: p. 531-538.

[68] Antony, N., S. Balachandran, and P. Mohanan, Immobilization of diastase α-amylase on nano zinc oxide. Food chemistry, 2016. 211: p. 624-630.
[69] Jang, E., et al., Adsorption of microbial esterases on Bacillus subtilis-templated cobalt oxide nanoparticles. International journal of biological macromolecules, 2014. 65: p. 188-192.
[70] Zeng, T., et al., Effective electrochemistry of human sulfite oxidase immobilized on quantum-dots-modified indium tin oxide electrode. ACS Applied Materials & Interfaces, 2015. 7(38): p. 21487-21494.
[71] Masoud, R., et al., Titanium dioxide nanoparticles increase superoxide anion production by acting on NADPH oxidase. PloS one, 2015. 10(12): p. e0144829.
[72] Rabbani, G., et al., Effect of copper oxide nanoparticles on the conformation and activity of β-galactosidase. Colloids and Surfaces B: Biointerfaces, 2014. 123: p. 96-105.
[73] Vickers, N.J., Animal communication: when i'm calling you, will you answer too? Current biology, 2017. 27(14): p. R713-R715.
[74] Mubarak, N., et al., Immobilization of cellulase enzyme on functionalized multiwall carbon nanotubes. Journal of Molecular Catalysis B: Enzymatic, 2014. 107: p. 124-131.
[75] Tavares, A.P., et al., Laccase immobilization over multi-walled carbon nanotubes: kinetic, thermodynamic and stability studies. Journal of colloid and interface science, 2015. 454: p. 52-60.
[76] Singh, N., et al., Cicer α-galactosidase immobilization onto functionalized graphene nanosheets using response surface method and its applications. Food Chemistry, 2014. 142: p. 430-438.
[77] Attar, A., et al., Amperometric inhibition biosensors based on horseradish peroxidase and gold sononanoparticles immobilized onto different electrodes for cyanide measurements. Bioelectrochemistry, 2015. 101: p. 84-91.
[78] Lu, D., F. Lu, and G. Pang, A novel glutathione-S transferase immunosensor based on horseradish peroxidase and double-layer gold nanoparticles. Biomedical microdevices, 2016. 18: p. 1-9.

[79] Zhou, H., et al., Efficient enhancement of electrochemiluminescence from cadmium sulfide quantum dots by glucose oxidase mimicking gold nanoparticles for highly sensitive assay of methyltransferase activity. Analytical chemistry, 2016. 88(5): p. 2976-2983.
[80] Hermanová, S., et al., Graphene oxide immobilized enzymes show high thermal and solvent stability. Nanoscale, 2015. 7(13): p. 5852-5858.
[81] Zhang, X., et al., Visual detection of single-nucleotide polymorphisms and DNA methyltransferase based on cation-exchange of CuS nanoparticles and click chemistry of functionalized gold nanoparticles. Chemical Communications, 2016. 52(90): p. 13261-13264.
[82] Zheng, T., et al., A simple and fast method to study the hydrodynamic size difference of protein disulfide isomerase in oxidized and reduced form using gold nanoparticles and dynamic light scattering. Analyst, 2016. 141(3): p. 934-938.
[83] Hushiarian, R., et al., Facilitating the indirect detection of genomic DNA in an electrochemical DNA biosensor using magnetic nanoparticles and DNA ligase. Analytical Chemistry Research, 2015. 6: p. 17-25.
[84] Khaksarinejad, R., et al., An organophosphorus hydrolase-based biosensor for direct detection of paraoxon using silica-coated magnetic nanoparticles. Applied biochemistry and biotechnology, 2015. 176: p. 359-371.
[85] Bao, J., et al., Plant esterase–chitosan/gold nanoparticles–graphene nanosheet composite-based biosensor for the ultrasensitive detection of organophosphate pesticides. Journal of agricultural and food chemistry, 2015. 63(47): p. 10319-10326.
[86] Apetrei, I.M. and C. Apetrei, Amperometric biosensor based on diamine oxidase/platinum nanoparticles/graphene/chitosan modified screen-printed carbon electrode for histamine detection. Sensors, 2016. 16(4): p. 422.
[87] Aigner, M., et al., Determination of total monoamines in rat brain via nanotubes based human monoamine oxidase B biosensor. Electroanalysis, 2017. 29(2): p. 367-373.
[88] Gumpu, M.B., et al., Determination of putrescine in tiger prawn using an amperometric biosensor based on

immobilization of diamine oxidase onto ceria nanospheres. Food and bioprocess technology, 2016. 9: p. 717-724.
[89] Sachdeva, V. and V. Hooda, Immobilization of nitrate reductase onto epoxy affixed silver nanoparticles for determination of soil nitrates. International journal of biological macromolecules, 2015. 79: p. 240-247.
[90] Chen, M., et al., Interactions of carbon nanotubes and/or graphene with manganese peroxidase during biodegradation of endocrine disruptors and triclosan. Chemosphere, 2017. 184: p. 127-136.
[91] Flores-Cervantes, D.X., et al., Slow biotransformation of carbon nanotubes by horseradish peroxidase. Environmental science & technology, 2014. 48(9): p. 4826-4834.
[92] Bhattacharya, K., et al., Lactoperoxidase-mediated degradation of single-walled carbon nanotubes in the presence of pulmonary surfactant. Carbon, 2015. 91: p. 506-517.
[93] Rocha-Santos, T.A., Sensors and biosensors based on magnetic nanoparticles. TrAC Trends in Analytical Chemistry, 2014. 62: p. 28-36.
[94] Sapountzi, E., et al., gold nanoparticles assembly on electrospun poly (vinyl alcohol)/poly (ethyleneimine)/glucose oxidase nanofibers for ultrasensitive electrochemical glucose biosensing. Sensors and Actuators B: Chemical, 2017. 238: p. 392-401.
[95] Liu, G., W. Guo, and Z. Yin, Covalent fabrication of methyl parathion hydrolase on gold nanoparticles modified carbon substrates for designing a methyl parathion biosensor. Biosensors and Bioelectronics, 2014. 53: p. 440-446.
[96] Palanisamy, S., C. Karuppiah, and S.-M. Chen, Direct electrochemistry and electrocatalysis of glucose oxidase immobilized on reduced graphene oxide and silver nanoparticles nanocomposite modified electrode. Colloids and Surfaces B: Biointerfaces, 2014. 114: p. 164-169.
[97] Kagan, V.E., et al., Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. Nature nanotechnology, 2010. 5(5): p. 354-359.
[98] Lou, F., et al., A 3D bio-platform constructed by glucose oxidase adsorbed on Au nanoparticles assembled polyaniline nanowires to sensitively detect glucose by

electrochemiluminescence. Journal of Electroanalytical Chemistry, 2017. 787: p. 125-131.
[99] Oliveira, S.F., et al., Enzymatic extract containing lignin peroxidase immobilized on carbon nanotubes: Potential biocatalyst in dye decolourization. Saudi journal of biological sciences, 2018. 25(4): p. 651-659.
[100] Song, Y., et al., Conformation, bioactivity and electrochemical performance of glucose oxidase immobilized on surface of gold nanoparticles. Electrochimica Acta, 2015. 158: p. 56-63.
[101] Ratautas, D., et al., High current, low redox potential mediatorless bioanode based on gold nanoparticles and glucose dehydrogenase from Ewingella americana. Electrochimica Acta, 2016. 199: p. 254-260.

CHAPTER 13

MicroRNAs Modulating Autophagy Pathway

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MicroRNAs Modulating Autophagy Pathway

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Abstract

MicroRNAs (miRNAs) are small non-coding, endogenous RNAs and take part in post-transcriptional regulation. miRNA interacts with complementary mRNA and leads to silencing or degradation of mRNA target. They also regulate various molecular biological processes including cell proliferation, migration, apoptosis, autophagy. Autophagy is an intracellular degradation process that removes damaged organelles, unused proteins, and pathogen microorganisms to maintain cell homeostasis. An increasing number of studies have shown this pathway is highly regulated by miRNAs. Dysregulation of autophagy-related miRNA causes many diseases such as cancer, neurodegenerative, cardiovascular disorders, and infectious diseases. Links between autophagy and miRNAs are complex and still unclear. Therefore, A comprehensive analysis of miRNA regulating autophagy is still required. In this review, we will summarize how miRNAs regulate the autophagy-related genes, a better understanding of the relationship between miRNA and autophagy might be useful for identifying new therapeutic targets.

Keywords: miRNA, autophagy, autophagy related genes, autophagy regulation, non-coding RNA

1. Introduction

Micro(mi)RNAs are endogenous, single-stranded, small non-coding RNAs (19-24 bp) and regulate post-transcriptional

mRNA expression which results in silencing or degradation of mRNA [1]. miRNAs can be located in exon, intron, and intergenic regions. They involve in critical cellular events including cell proliferation, differentiation, metabolism, motility, and apoptosis. miRNAs are transcribed by RNA polymerase II enzyme as a long primary transcript (pri-miRNAs) in the nucleus. The pre-miRNAs are exported to the cytoplasm via Exportin-5 in Ran-GTP- dependent manner after DROSHA-mediated cleavage of pri-miRNAs convert to pre-miRNAs. The mature miRNAs and AGO2 are integrated into the RNA-induced silencing complex (RISC) to form a miRISC complex, following the pre-miRNAs are cleaved by RNAase III enzyme DICER. The miRISC complex binding to the target mRNA throughout complementary sequence to the miRNA 5` UTR and mRNA 3` UTR occur the degradation of mRNA and inhibit protein translation [1]. miRNAs may serve as oncogenes (OncomiRs) which drive cell proliferation, migration or as tumor suppressor miRNAs (miRsupps) which suppress tumor progression. Dysregulation of miRNAs are commonly associated with many human diseases. Furthermore, miRNAs are a major regulator of several biological processes including autophagy.

Autophagy is a cellular process that degrades long-lived proteins, damaged organelles, microorganisms, and viruses by lysosomes [2]. Autophagy is induced by cellular stress such as starvation, hypoxia, DNA damage. It also plays a role in embryonic development, aging, immune system [3]. Deficient or excessive autophagic activity is observed in a variety of diseases such as cancer, neurodegenerative while its activity maintains at basal levels in healthy cells [4]. The process of autophagy consists of four-phase; I-Induction and nucleation II- Elongation III-Maturation IV- Fusion and degradation. Numerous autophagy-related genes (ATG) drive this process.

mTOR (Mammalian Target of Rapamycin), serine-threonine protein kinase, involves in cell survival, proliferation, motility, and is an important regulator of the autophagy pathway. The autophagy initiation phase is triggered by inactivated mTOR

under nutrients deprived conditions. ULK1/2 dissociate from mTOR and bind ATG13, FIP200, ATG101 and activated. Nucleation phase, the class III phosphatidylinositol-3-kinase (PI3 kinase) complex including Beclin-1 (ATG6), Vps34 (PI3KC3), p150 (Vps15, PI3KR4), UVRAG, ATG Atg14L, Ambra1, Bif-1 compose to autophagosome membrane [5].

Vesicle elongation includes two ubiquitin-like conjugation systems ATG12- ATG 5- ATG16 and ATG8/LC3-PE (phosphatidylethanolamine). Firstly, ATG 12 is activated by E1 like ATG 7 and ATG 5 is conjugated to ATG12 by E2 like ATG 10 and ATG 3, respectively. Then ATG 12-5 conjugate form bind ATG16L1. These conjugation systems cause activation of ATG 8/LC3-PE conjugation system [6]. ATG7 and ATG3 ensure conjugation of ATG8/LC3-PE as well. C terminal of LC3 is cleaved by ATG4 cysteine protease and converted to LC3-I. LC3-II is formed when LC3-I binds PE and is used commonly as an autophagy marker [7]. ATG 8/LC3-PE is an important step for membrane elongation and closure of vesicle. The outer membrane of autophagosome fuses with the lysosome to form an autolysosome and it is degraded by lysosomal cysteine proteases such as cathepsin B, D, L [7].

The autophagy pathway is a tightly regulated by variety of mechanisms. Recently, many studies have revealed that miRNAs are involved in regulating each step of the autophagic pathway. miR-378 is a positive regulator of mTOR-ULK1 and triggers autophagy [8]. miR-17-5p is an oncomiR overexpressed in many different types of cancer including lung, breast, stomach, prostate, colon. It was shown miR-17-5p stimulates ULK1 by inhibiting mTOR and promotes autophagy [9], as well as miR-20a, miR-106b, and miR-290-295 cluster were reported to be responsible for ULK1 regulation [10, 11]. In addition, miR-7, miR-99a, miR-338, miR-375 and miR-382 regulate autophagy through mTOR [12, 16]. miR-224-3p directly suppresses FIP200 in cervical cancer [17]. On the other hand, Guo et al. observed that miR-224-3p binds both ATG5 and FIP200 as a negative regulator, resulting in suppress autophagy in glioblastoma cell [18]. miR-125b-5p activates autophagy

through increasing UVRAG expression in the systemic lupus erythematosus diseases [19].

miR-30a binds 3' UTR of Beclin-1, one of the major genes of autophagy, and inhibits autophagy through Beclin-1 mRNA degradation [20]. Chen et al. showed that miR-30a mediated Beclin-1 diminishes imatinib resistance in gastrointestinal stromal tumors in vitro and in vivo [21]. miR376, miR-409-3p, miR-216a and miR-199a-5p were shown to target Beclin-1 in different cancer [22, 25]. Huang et al. found that overexpressed miR-181a, miR-519a, miR-374a, and miR-630 using mimics decrease UVRAG, Beclin-1, ATG10, ATG12, ATG16L1 and ATG5 [26]. miR-214-3p regulates autophagy via directly targeting ATG5 in atherosclerosis [27]. Moreover, miR130a suppresses HCV infection by downregulating ATG5 [28].

Vps34 is required for autophagophore and intracellular membrane trafficking and also regulates cell-cycle progression, development. miR-338-5p expression was found to increase in colorectal cancer and silencing miR-338-5p hinders Vps34 [29]. Additionally, miR-148b, miR-30b, miR-663 directly regulate Vps34 [30, 32]. miR-376b, miR-101 target ATG4C, ATG4D, respectively [22, 33]. miR-375 binds directly to ATG7, causing a decline in its expression and autophagic activity [15]. Likewise, miR-17 inhibits ATG7 binding 3' UTR, and downregulation of miR-17 leads to increase sensitization of chemotherapy in glioblastoma cell line [34]. Independent studies demonstrated that miR-20a, miR-137, miR-210, miR-202-5p, miR-1343-3p also play a role in the regulation of ATG7 [35, 39]. miR-148b-3p, miR-424-5p, miR-29c-3p were reported to be associated with ATG14 [40, 42]. miR-23b regulates ATG12 which is responsible for vesicle elongation, and downexpression of miR-23b leads to a decrease in both autophagy and radio-sensitize in pancreatic cancer cells [43]. miR-93 downregulating ATG12 decreases autophagy in colorectal cancer [44]. Serum expression levels of miR-152, miR-24 were found to be lower in uterine sarcoma patients, and upregulated these miRNAs modulate autophagy by deacetylating LC3 [45]. Moreover, miR-342-3p, miR-204, miR34a are involved in autophagy regulation

through LC3, further miR-204 inhibit both autophagy and renal clear cell proliferation [46, 48].

2. Conclusion

Tremendous progress has been made about miRNAs since their discovery in 1993 and to date, 2600 human mature miRNAs have been identified. Numerous studies have shown that miRNAs are major gene regulators and play a role in both transcription and translation. miRNAs directly or indirectly might alter the expression of autophagy-related proteins. Autophagy is an important cellular degradation process, but it has a dual role in cell survival or death depending on stress type and cell context. The different miRNAs may target the same autophagy gene, or the same miRNA may target several autophagy genes. There is still a need for comprehensive analysis of miRNA regulating autophagy. A growing number of works have shown alteration in the expression of these miRNA leads to various human diseases. Therefore, it is thought miRNA could be a marker and therapeutic for these diseases. Links between autophagy and miRNAs are complex and still unclear, so a better understanding of autophagy-mediated miRNA could be useful in the development of new therapeutic strategies.

Conflict of Interest

The authors declare no conflict of interest.

Financial Disclosure

There are no financial supports.

References

[1] Ling, H., M. Fabbri, and G.A. Calin, MicroRNAs and other non-coding RNAs as targets for anticancer drug development. Nat Rev Drug Discov, 2013. 12(11): p. 847-65.

[2] Eisenberg-Lerner, A. and A. Kimchi, The paradox of autophagy and its implication in cancer etiology and therapy. Apoptosis, 2009. 14(4): p. 376-91.
[3] Klionsky, D.J. and S.D. Emr, Autophagy as a regulated pathway of cellular degradation. Science, 2000. 290(5497): p. 1717-21.
[4] Dalby, K.N., et al., Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy, 2010. 6(3): p. 322-9.
[5] Parzych, K.R. and D.J. Klionsky, An overview of autophagy: morphology, mechanism, and regulation. Antioxid Redox Signal, 2014. 20(3): p. 460-73.
[6] Hanada, T., et al., The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J Biol Chem, 2007. 282(52): p. 37298-302.
[7] Tanida, I., T. Ueno, and E. Kominami, LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol, 2004. 36(12): p. 2503-18.
[8] Li, Y., et al., microRNA-378 promotes autophagy and inhibits apoptosis in skeletal muscle. Proc Natl Acad Sci U S A, 2018. 115(46): p. E10849-E10858.
[9] Bobbili, M.R., et al., OncomiR-17-5p: alarm signal in cancer? Oncotarget, 2017. 8(41): p. 71206-71222.
[10] Wu, H., et al., MiR-20a and miR-106b negatively regulate autophagy induced by leucine deprivation via suppression of ULK1 expression in C2C12 myoblasts. Cell Signal, 2012. 24(11): p. 2179-86.
[11] Chen, Y., R. Liersch, and M. Detmar, the miR-290-295 cluster suppresses autophagic cell death of melanoma cells. Sci Rep, 2012. 2: p. 808.
[12] Gu, D.N., et al., microRNA-7 impairs autophagy-derived pools of glucose to suppress pancreatic cancer progression. Cancer Lett, 2017. 400: p. 69-78.
[13] Yang, Z., et al., miR-99a directly targets the mTOR signalling pathway in breast cancer side population cells. Cell Prolif, 2014. 47(6): p. 587-95.
[14] Lu, R., et al., miR-338 modulates proliferation and autophagy by PI3K/AKT/mTOR signaling pathway in cervical cancer. Biomed Pharmacother, 2018. 105: p. 633-644.

[15] Chang, Y., et al., miR-375 inhibits autophagy and reduces viability of hepatocellular carcinoma cells under hypoxic conditions. Gastroenterology, 2012. 143(1): p. 177-87 e8.
[16] Feng, J., et al., miR-382 functions as a tumor suppressor against esophageal squamous cell carcinoma. World J Gastroenterol, 2017. 23(23): p. 4243-4251.
[17] Fang, W., et al., miR-224-3p inhibits autophagy in cervical cancer cells by targeting FIP200. Sci Rep, 2016. 6: p. 33229.
[18] Guo, X., et al., MiR224-3p inhibits hypoxia-induced autophagy by targeting autophagy-related genes in human glioblastoma cells. Oncotarget, 2015. 6(39): p. 41620-37.
[19] Cao, W., et al., miR-125b is downregulated in systemic lupus erythematosus patients and inhibits autophagy by targeting UVRAG. Biomed Pharmacother, 2018. 99: p. 791-797.
[20] Wang, P., et al., Down-regulation of miRNA-30a alleviates cerebral ischemic injury through enhancing beclin 1-mediated autophagy. Neurochem Res, 2014. 39(7): p. 1279-91.
[21] Chen, W., et al., MicroRNA-30a targets BECLIN-1 to inactivate autophagy and sensitizes gastrointestinal stromal tumor cells to imatinib. Cell Death Dis, 2020. 11(3): p. 198.
[22] Korkmaz, G., et al., miR-376b controls starvation and mTOR inhibition-related autophagy by targeting ATG4C and BECN1. Autophagy, 2012. 8(2): p. 165-76.
[23] Yi, H., et al., Differential roles of miR-199a-5p in radiation-induced autophagy in breast cancer cells. FEBS Lett, 2013. 587(5): p. 436-43.
[24] Tan, S., et al., miR-409-3p sensitizes colon cancer cells to oxaliplatin by inhibiting Beclin-1-mediated autophagy. Int J Mol Med, 2016. 37(4): p. 1030-8.
[25] Zhang, X., et al., MicroRNA-216a enhances the radiosensitivity of pancreatic cancer cells by inhibiting beclin-1-mediated autophagy. Oncol Rep, 2015. 34(3): p. 1557-64.
[26] Huang, Y., R. Guerrero-Preston, and E.A. Ratovitski, Phospho-DeltaNp63alpha-dependent regulation of autophagic signaling through transcription and micro-RNA modulation. Cell Cycle, 2012. 11(6): p. 1247-59.

[27] Wang, J., et al., MicroRNA-214-3p: A link between autophagy and endothelial cell dysfunction in atherosclerosis. Acta Physiol (Oxf), 2018. 222(3).
[28] Duan, X., et al., Microrna-130a Downregulates HCV Replication through an atg5-Dependent Autophagy Pathway. Cells, 2019. 8(4).
[29] Chu, C.A., et al., MiR-338-5p promotes metastasis of colorectal cancer by inhibition of phosphatidylinositol 3-kinase, catalytic subunit type 3-mediated autophagy pathway. EBioMedicine, 2019. 43: p. 270-281.
[30] Cimino, D., et al., miR148b is a major coordinator of breast cancer progression in a relapse-associated microRNA signature by targeting ITGA5, ROCK1, PIK3CA, NRAS, and CSF1. FASEB J, 2013. 27(3): p. 1223-35.
[31] Liao, W.T., et al., MicroRNA-30b functions as a tumour suppressor in human colorectal cancer by targeting KRAS, PIK3CD and BCL2. J Pathol, 2014. 232(4): p. 415-27.
[32] Shi, Y., et al., Primate-specific miR-663 functions as a tumor suppressor by targeting PIK3CD and predicts the prognosis of human glioblastoma. Clin Cancer Res, 2014. 20(7): p. 1803-13.
[33] Frankel, L.B., et al., microRNA-101 is a potent inhibitor of autophagy. Embo j, 2011. 30(22): p. 4628-41.
[34] Comincini, S., et al., microRNA-17 regulates the expression of ATG7 and modulates the autophagy process, improving the sensitivity to temozolomide and low-dose ionizing radiation treatments in human glioblastoma cells. Cancer Biol Ther, 2013. 14(7): p. 574-86.
[35] Zhao, S., et al., MiR-20a promotes cervical cancer proliferation and metastasis in vitro and in vivo. PLoS One, 2015. 10(3): p. e0120905.
[36] Zeng, Y., et al., MIR137 Regulates Starvation-Induced Autophagy by Targeting ATG7. J Mol Neurosci, 2015. 56(4): p. 815-821.
[37] Ju, S., et al., The effect and mechanism of miR-210 in down-regulating the autophagy of lung cancer cells. Pathol Res Pract, 2019. 215(3): p. 453-458.

[38] Chen, J., et al., Effect of miR-202-5p-mediated ATG7 on autophagy and apoptosis of degenerative nucleus pulposus cells. Eur Rev Med Pharmacol Sci, 2020. 24(2): p. 517-525.
[39] Qin, Y., et al., ATF2-Induced lncRNA GAS8-AS1 Promotes Autophagy of Thyroid Cancer Cells by Targeting the miR-187-3p/ATG5 and miR-1343-3p/ATG7 Axes. Mol Ther Nucleic Acids, 2020. 22: p. 584-600.
[40] Jiang, X., et al., [miR-148b-3p inhibits the proliferation and autophagy of acute myeloid leukemia cells by targeting ATG14]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi, 2021. 37(10): p. 881-890.
[41] Zhao, Z., et al., [MicroRNA-424 inhibits autophagy and proliferation of hepatocellular carcinoma cells by targeting ATG14]. Nan Fang Yi Ke Da Xue Xue Bao, 2021. 41(7): p. 1012-1021.
[42] Hu, Z., et al., miR-29c-3p inhibits autophagy and cisplatin resistance in ovarian cancer by regulating FOXP1/ATG14 pathway. Cell Cycle, 2020. 19(2): p. 193-206.
[43] Wang, P., et al., MicroRNA 23b regulates autophagy associated with radioresistance of pancreatic cancer cells. Gastroenterology, 2013. 145(5): p. 1133-1143 e12.
[44] Liu, Y.Q., et al., Long non-coding RNA HOTAIR knockdown enhances radiosensitivity through regulating microRNA-93/ATG12 axis in colorectal cancer. Cell Death & Disease, 2020. 11(3).
[45] Tong, X., et al., Elevated levels of serum MiR-152 and miR-24 in uterine sarcoma: potential for inducing autophagy via SIRT1 and deacetylated LC3. Br J Biomed Sci, 2018. 75(1): p. 7-12.
[46] Zhang, M.Y., et al., Epigenetic silencing of miR-342-3p in B cell lymphoma and its impact on autophagy. Clin Epigenetics, 2020. 12(1): p. 150.
[47] Xiao, J., et al., MiR-204 regulates cardiomyocyte autophagy induced by ischemia-reperfusion through LC3-II. J Biomed Sci, 2011. 18: p. 35.
[48] Rupaimoole, R., et al., A High-Throughput Small Molecule Screen Identifies Ouabain as Synergistic with miR-34a in Killing Lung Cancer Cells. iScience, 2020. 23(2): p. 100878.

CHAPTER 14

Overview of Animal and Plant Genomic Research

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Overview of Animal and Plant Genomic Research

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Abstract

Agriculture is of paramount importance in addressing the challenges posed by a changing environment, growing populations and increasing nutritional demands. Genetic developments such as genomic selection (GS) and gene editing offer promising solutions in agriculture. GS uses genomic information from markers distributed throughout the genome to predict breeding values and select for desired agronomic traits. Recent advances in genomic technologies have greatly expanded agricultural genetic resources, enabling breeders to link genotypes and phenotypes.

The availability of sequence data, well-characterized marker sets, and curated genomic databases is crucial for understanding genetic variation and its impact on agricultural improvement. Genomic technologies have revolutionized crop and livestock breeding and management through agrigenomics. Genomic applications in agriculture focus on improving various agronomic traits including sustainability, disease resistance, nutrition, reproduction, yield, growth and abiotic stress adaptation in different plant and animal species. Based on the cumulative effect of a large number of genetic markers, GS enables breeders to improve breeding programs and select for desirable traits without prior knowledge of specific genes. Furthermore, gene editing technologies such as CRISPR/Cas and TALEN allow targeted modifications of genome sequences, generating new variations with potential benefits for crop and livestock improvement.

Next-generation sequencing technologies, together with metagenomic engineering, contribute to ecodiversity studies, environmental DNA analysis, fisheries and aquaculture management, disease profiling, pathogen surveillance and microbiome analysis. Genomics offers valuable insights into microbial ecosystems associated with plants and animals, providing opportunities for improved productivity, stress tolerance and disease resistance in agriculture.

Keywords: agriculture, improvement, next generation sequence, bioinformatics, sequence.

1. Introduction

Agriculture, which encompasses the examination and application of techniques for cultivating plants and animals to obtain sustenance, textile resources, and energy sources, has been instrumental in supporting and enhancing the human way of life throughout history. The optimization of agricultural practices assumes paramount importance considering the dynamic environmental conditions, burgeoning global population, and escalating nutritional requirements [1]. Incorporating genetic advancements within the field of agriculture holds the potential to facilitate enhanced productivity and offer sustainable resolutions to these challenges in the long run.

Significant advancements in genomic selection (GS) and, in recent years, gene editing techniques have considerably expedited the progress in developing crops and livestock possessing desirable agronomic characteristics [2]. The fundamental principle underlying GS revolves around leveraging information derived from a vast array of genetic markers scattered across the genome, enabling the capture of population variations and accurate prediction of breeding values [3]. The concept was first proposed in 2001, and it is based on building an agricultural equation with specified qualities using an experimental population. Prominent objectives driving agricultural progress encompass heightened production levels,

enhanced stress tolerance, improved disease resistance, and long-term sustainability [4]. Historically, breeders used a restricted range of molecular markers; however, the development of genome-wide technologies has resulted in a tremendous increase in the breadth and depth of accessible genetic resources within the agricultural sector.

The field of agriculture greatly benefits from the remarkable advancements in genetics, bioinformatics, and biotechnology, equipping breeders with powerful tools for agricultural development. The connection between genotypes and phenotypes can be established by leveraging sequence data and well-characterized marker sets, while the curation of comprehensive genomic databases assumes pivotal importance in comprehending genetic variations within human populations and ecosystems [5]. These invaluable datasets facilitate the identification of novel species, facilitate meta-analyses, unravel intricate traits, and ultimately pave the way for the implementation of GS and gene editing techniques in agricultural enhancement [6]. The application of genomic technologies has ushered in a transformative era in crop and livestock breeding and management, leading to the emergence of the discipline of agrigenomics [7].

1.1. What is Genome?

A genome is found in all living things, from the earliest forms of life to the most sophisticated species. Deoxyribonucleic acid (DNA) is a molecule that contains genetic information and is commonly referred to as an organism's blueprint. DNA is responsible for encoding and controlling genes, which are ultimately translated to proteins (RNAs). The biological information recorded inside the DNA genome eventually organizes the growth of an organism. In all species, basic molecular substance compound composition of DNA is the same. It is made up of two polymeric strands that are wrapped around one other to produce the well-known double helix structure. It is composed of two polymeric strands wrapped around one another to form the well-known double-stranded

structure. Every single strand of DNA is composed of up of a succession of nucleotide components. Each nucleotide block of DNA is made up of one phosphate group, a type of monosaccharide, and a variety of nitrogen-containing base. Each nucleotide subunit is made up of a phosphate group, a monosaccharide, and a variable nitrogen-containing base. The four possible base subunits of DNA are adenine (A), guanine (G), cytosine (C), and thymine (T). The structure of the double helix is maintained by highly accurate hydrogen bonding between complementary subunits: A is paired with T, and G is paired with C. For this binding to occur, the two strands must be in a reverse and complementary orientation to one other. Genes are regarded to be the basic unit of heredity in the genome, defining the traits that define an organism. Genes are sequences of DNA that encode proteins or regulatory RNAs. [8, 9].

1.2. Genomic Changes: Variants of Gene

Typically, plant and animal genomes consist of duplicated sets of chromosomes, which are large, distinct DNA molecules wound around structural proteins and inherited from both parents. As a result, diploid organisms, possessing a pair of duplicated chromosomes, harbor two alleles for each gene. One allele represents the chromosome inherited from the maternal parent, while the other represents the chromosome inherited from the paternal parent. These alleles serve as distinct variants of the same gene, exhibiting slight differences in their nucleotide sequences. The genetic makeup, or genotype, of an individual organism is determined by the combination of alleles inherited by that particular individual [10, 11].

The wide range of observable traits within and among species can be attributed to the combination of genetic variability and environmental influences. While the majority of the genome remains conserved across individuals of the same species, there exist specific crucial regions that display inter-individual variability. These regions are referred to as polymorphisms, representing sites of DNA variation within the genomes of a given species. While some polymorphisms may have minor

phenotypic influence, many others contribute to the unique characteristics that distinguish an individual from another. Polymorphisms are defined by their sequence and length and include nucleotide changes, deletions, insertions or repeats. They might be as little as single nucleotide polymorphisms (SNPs) or as large as structural differences spanning tens to thousands of nucleotides [12]. Similarly, the functional effects of a genetic variant on a particular trait may or may not be influential in terms of expression. Due to the complex and multivariate nature of most agronomic traits, their penetrance typically falls within an intermediate to poor range [13]. The recent advancements in next-generation sequencing (NGS) technologies have revolutionized the field by enabling the isolation and sequencing of DNA or RNA from diverse sources, amplification and sequencing of genomic regions, as well as the sequencing and assembly of complete genomes. These developments have significantly enhanced our capacity to identify and characterize polymorphisms that contribute to the phenotypic diversity observed in crops and cattle, resulting in more comprehensive and robust analyses [14].

1.3. Genomic Applications in Agriculture

Animals and plants undergo evolutionary processes within intricate ecosystems, acquiring the ability to adapt to various factors such as predators, soil conditions, and climate dynamics [15]. The majority of desirable agricultural traits, known as phenotypes, fall under the category of complex or quantitative characteristics. These complex traits do not exhibit continuous variation solely due to the influence of a single gene; instead, they are influenced by the combined effects of multiple genes [16]. Extensive research endeavors have been dedicated to unraveling the genetic basis of complex phenotypic variations by identifying specific segments of the genome that contribute to these traits. These identified regions, termed quantitative trait loci (QTL), play a pivotal role in the discovery and dissemination of genetic variations that confer favorable agronomic features [17].

In the past, breeding selection practices primarily relied on estimated breeding values (EBVs) rather than pinpointing the specific genes associated with particular traits. EBVs were computed based on pedigree information, phenotypic data, and an understanding of the heritability of each trait. However, the effectiveness of this approach diminishes when applied to characteristics that are challenging to measure, exhibit low heritability, or necessitate extended periods and/or multiple generations for measurement. Consequently, a significant focus of agrigenomics research revolves around the identification of genes underlying key attributes in both plants and animals [18, 19].

The application of GS in agricultural breeding can be traced back to the utilization of marker-assisted selection (MAS), a technique where breeders employ markers (or QTLs) associated with a specific trait of interest to indirectly select for the inheritance of that trait. However, the impact of QTL research on promoting MAS in agricultural and livestock development has been somewhat limited thus far [17]. Early studies faced challenges in terms of genetic precision, resulting in sparse marker coverage that lacked universal relevance across different populations and contexts. Nonetheless, significant advancements in genomic technology, including the development of genome microarrays and the introduction of NGS platforms, have greatly enhanced researchers' ability to identify genetic markers that account for phenotypic variation in complex traits [20]. The recent genome-wide investigations encompassing diverse populations and ecosystems have wider implications for the broader implementation of GS in crop and livestock breeding programs [21].

GS is grounded on the principle that information obtained from a vast array of markers can be utilized to estimate breeding values, even without precise knowledge of the location or existence of specific genes. In this regard, GS shares similarities with traditional marker-assisted selection (MAS), where genetic information is employed. However, instead of focusing on a limited subset of designated markers (such as QTLs) associated

with a specific trait as in MAS, GS allows for the selection of markers from the entire genome, utilizing a training population exhibiting favorable phenotypes. Genomic estimated breeding values (gEBVs) are derived in GS by considering the collective influence of numerous genetic markers and subsequently utilized to evaluate potential breeding partners [22, 23].

The successful implementation of GS relies on the availability of representative cohorts of individuals within the same species, alongside genomic prediction algorithms that integrate genetic information with phenotypic and/or pedigree data. Compared to MAS, GS has gained significant traction due to the accessibility of cost-effective genotyping resources, such as NGS and genome arrays [26]. As an illustrative example, the dairy cow industry in the United States has extensively adopted GS methodologies to enhance livestock breeding practices. This has resulted in the genotyping of over 3 million animals since 2008, leading to reduced generation intervals for bulls and substantial improvements in animal health, fertility, and lifespan [27]. Additionally, the combination of GS with high-throughput phenotyping (HTP) is garnering interest for accelerating crop breeding programs aimed at enhancing grain yield. Undoubtedly, GS has demonstrated its effectiveness as a valuable tool in enhancing the efficiency of both crop and livestock breeding endeavors.

Table 14.1. Recently reported examples of phenotypic features of agricultural significance that are genetically influenced by QTLs [24, 25].

Agronomic Characteristic	Species
Sustainability	Rice, Barley, Chickpea, Cattle, Cod, Tuna
Disease resistance	Wheat, Rice, Barley, Potato, Sugar beet, Sugarcane, Peanut, Hot pepper, Cattle, Haddock, Catfish
Nutrition/End-use quality	Wheat, Maize, Apple, Potato, Tomato, Olive, Peanut, Carrot, Sheep, Pig, Cattle
Reproduction	Wheat, Millet, Yam, Cattle, Salmon, Yellow Drum, Catfish
Yield and growth	Wheat, Rice, Maize, Millet, Potato, Canola, Spinach, Carrot, Sheep, Cattle, Salmon, Yellow Drum
Abiotic stress adaptation	Wheat, Soybean, Barley, Chickpea, Common bean, Wheat, Millet, Grape, Cattle, Sheep, Goat, Yak, Cod, Catfish

1.4. Reference of Genome

To date, a considerable number of species have been subjected to high-coverage sequencing, and the resulting data are accessible in public databases. However, in cases where a reference genome is absent or of suboptimal quality, the undertaking of de novo sequencing becomes necessary. Since 1990, substantial investments in nucleotide sequencing technology, coupled with the advent of next-generation sequencing (NGS), have precipitated a staggering 100-million-fold reduction in the cost per genome. At present, the cost of sequencing one megabase (Mb) of raw DNA sequence is less than $0.1, contributing to the widespread utilization of de novo sequencing initiatives [2]. The abundance and proportion of repeated sequences within plant and animal genomes exhibit considerable variation. While the genomes of farm animals, with a few exceptions in fish species, tend to be diploid, plant genomes are often considerably more intricate, featuring

multiple sets of homologous chromosomes—a phenomenon known as polyploidy. To tackle the complexity of polyploid genomes, researchers have adopted the strategy of developing a pan-genome, which involves sequencing individuals from various closely related species, thus providing a more comprehensive understanding of these intricate genetic structures [3].

Researchers may opt to dissect the genome of a polyploid crop into its constituent subgenomes. Plant genomes, with their multiple sets of homologous chromosomes, also exhibit a higher proportion of repetitive sequences, rendering their accurate reconstruction a more intricate task. Likewise, uncovering the ancestral origins of structural variations within plant and animal genomes requires the application of specialized methodologies capable of resolving haplotypes the specific combinations of alleles likely to be inherited together [28-30].

Significant progress has been made in recent times in the field of genomic DNA library preparation, chromosomal architectural assays, and genome assembly algorithms. These advancements have facilitated the generation of reference genomes that are not only more accurate but also exhibit improved contiguity, all at a reduced cost. The availability of highly contiguous and high-quality reference genomes plays a pivotal role in gene annotation, facilitates the comprehension of chromosomal evolution and genetic diversity, and holds indispensable significance for the practical implementation of gene editing technologies [31, 32].

Following genome assembly, the identification of an effective approach for capturing the genetic diversity within a species becomes paramount. This often involves gathering data from informative cohorts of individuals representing the species or population of interest. By sequencing and comparing numerous individuals from a community, a wealth of genetic markers can be discovered. With the availability of a reference sequence, additional genomes can be assembled through alignment to the reference. Table 14.2 showcases online

databases that house some of the consensus-accepted references derived from research studies. Despite the significant decrease in the cost of whole-genome sequencing over the past decades, the comprehensive analysis of the complete genomes of large populations within a study or breeding program remains prohibitively expensive [33, 35].

Table 14.2. The most widely used and recognized online databases containing reference sequences used in genomic studies.

Available Resources	Connection Link
NCBI Reference Genomes	https://www.ncbi.nlm.nih.gov/genome
The Genome 10K project	https://genome10k.soe.ucsc.edu
Vertebrate Genomes Project	https://genome10k.soe.ucsc.edu
The Genome OnLine Database (GOLD)	https://gold.jgi.doe.gov/index
International Wheat Genome Sequencing Consortium (IWGSC)	www.wheatgenome.org

Genotyping arrays, also referred to as microarrays, employ oligonucleotide probes to simultaneously detect a multitude of genetic markers, numbering in the hundreds of thousands. These arrays are designed to capture representative polymorphisms, such as single nucleotide polymorphisms (SNPs), that serve to characterize genetic diversity within a population. The development of a genotyping microarray platform entails the initial sequencing of a diverse sample of individuals to identify haplotypes [36]. Leveraging DNA arrays and whole-genome SNP imputation has revolutionized the rapid genotyping of large cohorts of individuals, owing to its affordability, straightforward analysis, and robust parallel-processing and automation capabilities. Imputation leverages a reference library consisting of fully sequenced genomes to predict genotypes for genetic markers that may not have been directly measured within a larger sample of individuals [37]. The process involves initially reconstructing haplotypes for the target samples through the utilization of haplotypes from the reference set (haplotype phasing), followed by genotype estimation. Arrays and imputation methods have found wide-ranging applications in

human, animal, and plant research, with several platforms specifically designed for agricultural purposes [38].

Genotyping by sequencing (GBS) encompasses a range of genotyping techniques that leverage sequencing technology. GBS approaches involve either whole genome resequencing or reduced-representation sequencing [39]. In the first GBS technique, whole-genome libraries are combined with low-coverage "skim" sequencing. A second GBS technique reduces genomic complexity by generating reduced representation libraries (RRLs). These libraries are typically constructed by fragmenting the genome using restriction enzymes (REs). Alternatively, barcoded adapters can be employed to target specific genomic regions, enabling multiplexing, which facilitates the study of multiple individuals simultaneously. The resulting RRLs are subjected to high-coverage sequencing. One advantage of this GBS method is that it does not rely on prior knowledge of the sequence under investigation, making it particularly valuable in situations where information about marker spacing along chromosomes is limited. GBS finds extensive use in animal studies, particularly when reference genomes or genotyping arrays are not available. By harnessing the advantages of NGS, GBS allows for the efficient and cost-effective scanning of genomes in species with or without a reference genome assembly [40, 42].

The successful utilization of GS in crop and livestock breeding necessitates a thorough understanding of the genetic foundations of fitness and adaptability. Consequently, its implementation relies on the availability of well-established breeding programs that collect and document genomic, phenotypic, and pedigree information. This valuable data serves as the basis for breeders to make informed decisions and selectively breed individuals with the most desirable traits. The advantages of GS are manifold, including the ability to implement it at an early stage of an organism's life, thereby reducing generation intervals. Additionally, GS is not limited to a specific sex and proves particularly effective in improving traits that are otherwise challenging to enhance [43, 45].

1.5. Gene Editing

The process of crop and livestock development often faces challenges in keeping up with the increasing demands for sustainable and nutritious food, resulting in lengthy and laborious procedures [46, 47]. However, advancements in genome editing technologies such as CRISPR/Cas (clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein [CRISPR/Cas]) and TALENs (transcription activator-like effector nucleases [TALENs]) now offer targeted modification of genome sequences, enabling the generation of novel and beneficial genetic variations [48, 49]. In addition to gene editing, the utilization of microbiome therapies presents a promising strategy for enhancing crop and animal productivity, health, and sustainability [50]. The integration of NGS technologies into gene editing pipelines facilitates the creation of reference genomes, identification of targets for genome editing through functional genomics, and assessment of the specificity of genome-edited lines. Genomic and metagenomic engineering are emerging as viable breeding options for agricultural and animal species, opening up new possibilities for future advancements.

1.6. Guidance To Better Production: Genomic Monitoring and Management

The advent of NGS has revolutionized crop and animal breeding practices by providing unprecedented access to genomic information, even for non-model organisms. Beyond its application in enhancing breeding strategies, genomics plays a crucial role in understanding the biological status of vital resources such as fisheries, crop and livestock health, as well as food safety and authenticity [44]. NGS is employed to explore ecological diversity by identifying species present in distinct environments. Organisms release DNA into their surroundings, which can be easily collected and referred to as environmental DNA (eDNA). Through the use of "DNA barcoding," a technique that utilizes unique genetic markers, the species

composition within a specific habitat or sample can be identified through eDNA analysis [51]. Consequently, eDNA is a valuable tool for identifying the array of organisms in various environments, ranging from seawater and soil to food samples [52]. These emerging genomics applications, including eDNA analysis, are shaping the best practices for monitoring and managing agricultural resources [53].

Seafood plays a vital role as a source of animal protein for human consumption, and its significance in addressing future nutritional demands continues to grow. With natural fisheries facing decline and the demand for seafood on the rise, aquaculture has emerged as the fastest-growing sector in agricultural food production [4]. Despite its potential, the use of genomics in fisheries and aquaculture management has been slower compared to its application in crops and livestock. Genomics has the potential to provide valuable insights into adaptive variation and enhance breeding and management practices, thereby improving productivity and sustainability in fisheries and aquaculture [54]. Genomic data has been utilized to study the geographical and structural dynamics of aquatic populations, identify aquatic species, understand the genetic basis of desirable aquaculture traits, and combat illegal fishing and food fraud. Infectious diseases pose a significant threat to agricultural and livestock productivity, as well as human health and food security [55]. Whole genome sequencing, gene expression profiling, and metagenomics have yielded valuable insights into disease epidemiology and pathophysiology, with their application in disease profiling and pathogen surveillance within the agricultural sector expanding [56]. NGS technology enables the monitoring of disease progression, detection and characterization of pathogenic organisms, and a deeper understanding of host-pathogen interactions.

NGS and other genomic technologies have revolutionized the study of microbial ecosystems that surround us. Shotgun metagenomic sequencing has become a common approach to classify and characterize microbial communities directly from their natural habitats. The advancement of technology, coupled

with the growing interest in plant- and animal-associated microbes, has led to initiatives such as the Earth Microbiome Project, aimed at comprehensively studying microbial diversity [57]. Microbiomes associated with plants and animals have coevolved with their hosts, forming intricate symbiotic relationships. While research into the varied taxa and functions of microbiomes is continuing, adopting microbiome alteration and management methods in agriculture will necessitate enormous scale and integrated metagenomics frameworks [58, 59]. Table 14.1 provides a summary of the traits associated with organisms currently utilized in these strategies.

Metagenomic sequencing of plant-associated microbiomes has revealed diverse community structures comprising various species with distinct functional roles [60]. The microbiome of plants exerts influence on host nutrition, tissue growth, and susceptibility to diseases. It has been observed that microbial communities colonize plants from the soil, forming the rhizosphere and endosphere compartments [61]. Studies have extensively investigated nitrogen-fixing bacteria associated with root nodules of legumes. Plant hosts serve as a significant carbon source for associated microorganisms, and these communities, in turn, contribute to host productivity. Despite rising understanding of the importance of microbiomes in plant productivity, environmental stress tolerance, and resistance to diseases, the mechanisms driving crop-microbial interactions remain largely unknown [62, 63]. Understanding the intricate connections among the microbiome, host genetics, and environmental factors, particularly, will enable the development of agricultural management systems that meet productivity and sustainability objectives.

2. Conclusion

NGS technology has revolutionized the collection of genomic data, enabling comprehensive studies on numerous species that were previously challenging to investigate. The declining costs and advancements in methodologies have facilitated the widespread adoption of NGS, allowing for more accurate and

extensive examination of plants, animals, and their associated microbiomes. This unparalleled availability to quality genome data has resulted in the development of low-cost sequencing technologies and the discovery of the genetic bases of important agronomic features. Furthermore, reference sequences of genomes have enabled the use of gene editing technologies, allowing for the quick integration of desirable traits into plants and animals. Exploiting this plethora of genetic data is propelling breakthroughs in breeding tactics that incorporate the holobiont idea, which takes into account the combined effect of several species within an ecosystem. The application of genomic and metagenomic engineering to improve agricultural and animal production, in response to rising demands for sustainable agriculture, is the future of agrigenomics.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] M. C. Boliko: "FAO and the Situation of Food Security and Nutrition in the World." J Nutr Sci Vitaminol (Tokyo). 2019; S4-S8-Supplement. 10.3177/jnsv.65. S4
[2] M. W. Bevan, C. Uauy, B. B. Wulff, J. Zhou, K. Krasileva, and M. D. Clark: "Genomic innovation for crop improvement." Nature. 2017; 346-354-7645. 10.1038/nature22011
[3] M. Georges, C. Charlier, and B. Hayes: "Harnessing genomic information for livestock improvement." Nat Rev Genet. 2019; 135-156-3. 10.1038/s41576-018-0082-2
[4] L. Bernatchez *et al.*: "Harnessing the Power of Genomics to Secure the Future of Seafood." Trends Ecol Evol. 2017; 665-680-9. 10.1016/j.tree.2017.06.010
[5] P. Lidder and A. Sonnino: "Biotechnologies for the management of genetic resources for food and agriculture." Adv Genet. 2012; 1-167-10.1016/B978-0-12-394394-1.00001-8
[6] H. Hu, A. Scheben, and D. Edwards: "Advances in integrating genomics and bioinformatics in the plant breeding pipeline." Agriculture. 2018; 75-6.

[7] M. E. Goddard and B. J. Hayes: "Genomic selection." J Anim Breed Genet. 2007; 323-30-6. 10.1111/j.1439-0388.2007. 00702.x
[8] A. Stencel and B. Crespi: "What is a genome?" Mol Ecol. 2013; 3437-43-13. 10.1111/mec.12355
[9] C. P. Ponting and R. C. Hardison: "What fraction of the human genome is functional?" Genome Res. 2011; 1769-76-11. 10.1101/gr.116814.110
[10] S. K. Burley: "An overview of structural genomics." Nat Struct Biol. 2000; 932-4-10.1038/80697.
[11] J. S. Heslop-Harrison and T. Schwarzacher: "Organisation of the plant genome in chromosomes." Plant J. 2011; 18-33-1. 10.1111/j.1365-313X.2011. 04544.x
[12] E. Khlestkina and E. Salina: "SNP markers: methods of analysis, ways of development, and comparison on an example of common wheat." Russian Journal of Genetics. 2006; 585-594-.
[13] J.-P. Brog, C.-L. Chanez, A. Crochet, and K. M. Fromm: "Polymorphism, what it is and how to identify it: a systematic review." Rsc Advances. 2013; 16905-16931-38.
[14] S. Behjati and P. S. Tarpey: "What is next generation sequencing?" Arch Dis Child Educ Pract Ed. 2013; 236-8-6. 10.1136/archdischild-2013-304340
[15] J. T. Anderson, M. R. Wagner, C. A. Rushworth, K. V. Prasad, and T. Mitchell-Olds: "The evolution of quantitative traits in complex environments." Heredity (Edinb). 2014; 4-12-1. 10.1038/hdy.2013.33
[16] E. A. Boyle, Y. I. Li, and J. K. Pritchard: "An Expanded View of Complex Traits: From Polygenic to Omnigenic." Cell. 2017; 1177-1186-7. 10.1016/j.cell.2017.05.038
[17] Z. L. Hu, C. A. Park, and J. M. Reecy: "Building a livestock genetic and genomic information knowledgebase through integrative developments of Animal QTLdb and CorrDB." Nucleic Acids Res. 2019; D701-D710-D1. 10.1093/nar/gky1084
[18] B. Raymond, A. C. Bouwman, C. Schrooten, J. Houwing-Duistermaat, and R. F. Veerkamp: "Utility of whole-genome sequence data for a cross-breed genomic prediction." Genet Sel Evol. 2018; 27-1. 10.1186/s12711-018-0396-8

[19] C. Ning *et al.*: "Performance Gains in Genome-Wide Association Studies for Longitudinal Traits via Modeling Time-varied effects." Sci Rep. 2017; 590-1. 10.1038/s41598-017-00638-2
[20] F. W. Nicholas: "Online Mendelian Inheritance in Animals (OMIA): a comparative knowledgebase of genetic disorders and other familial traits in non-laboratory animals." Nucleic Acids Res. 2003; 275-7-1. 10.1093/nar/gkg074
[21] M. K. Tello-Ruiz *et al.*: "Gramene 2018: unifying comparative genomics and pathway resources for plant research." Nucleic Acids Res. 2018; D1181-D1189-D1. 10.1093/nar/gkx1111
[22] A. Garcia-Ruiz, J. B. Cole, P. M. VanRaden, G. R. Wiggans, F. J. Ruiz-Lopez, and C. P. Van Tassell: "Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection." Proc Natl Acad Sci U S A. 2016; E3995-4004-28. 10.1073/pnas.1519061113
[23] G. R. Wiggans, J. B. Cole, S. M. Hubbard, and T. S. Sonstegard: "Genomic Selection in Dairy Cattle: The USDA Experience." Annu Rev Anim Biosci. 2017; 309-327-10.1146/annurev-animal-021815-111422
[24] S. G. Milner *et al.*: "Genebank genomics highlights the diversity of a global barley collection." Nat Genet. 2019; 319-326-2. 10.1038/s41588-018-0266-x
[25] C. International Wheat Genome Sequencing: "Shifting the limits in wheat research and breeding using a fully annotated reference genome." Science. 2018; 6403. 10.1126/science. aar7191
[26] J. Crossa *et al.*: "Genomic Selection in Plant Breeding: Methods, Models, and Perspectives." Trends Plant Sci. 2017; 961-975-11. 10.1016/j.tplants.2017.08.011
[27] J. L. Araus, S. C. Kefauver, M. Zaman-Allah, M. S. Olsen, and J. E. Cairns: "Translating High-Throughput Phenotyping into Genetic Gain." Trends Plant Sci. 2018; 451-466-5. 10.1016/j.tplants.2018.02.001
[28] J. Xu, K. Hua, and Z. Lang: "Genome editing for horticultural crop improvement." Horticulture research. 2019.

[29] S. Renny-Byfield and J. F. Wendel: "Doubling down on genomes: polyploidy and crop plants." Am J Bot. 2014; 1711-25-10. 10.3732/ajb.1400119
[30] M. Brozynska, A. Furtado, and R. J. Henry: "Genomics of crop wild relatives: expanding the gene pool for crop improvement." Plant Biotechnol J. 2016; 1070-85-4. 10.1111/pbi.12454
[31] K. P. Eagen: "Principles of Chromosome Architecture Revealed by Hi-C." Trends Biochem Sci. 2018; 469-478-6. 10.1016/j.tibs.2018.03.006
[32] A. M. Phillippy: "New advances in sequence assembly." Genome Res. 2017; xi-xiii-5. 10.1101/gr.223057.117
[33] I. W. G. S. Consortium *et al.*: "Shifting the limits in wheat research and breeding using a fully annotated reference genome." Science. 2018; eaar7191-6403.
[34] S. Wu *et al.*: "Genome sequences of two diploid wild relatives of cultivated sweetpotato reveal targets for genetic improvement." Nat Commun. 2018; 4580-1. 10.1038/s41467-018-06983-8
[35] D. M. Bickhart *et al.*: "Single-molecule sequencing and chromatin conformation capture enable de novo reference assembly of the domestic goat genome." Nat Genet. 2017; 643-650-4. 10.1038/ng.3802
[36] D. P. Berry, J. F. Garcia, and D. J. Garrick: "Development and implementation of genomic predictions in beef cattle." Animal Frontiers. 2016; 32-38-.
[37] A. Rasheed *et al.*: "Crop Breeding Chips and Genotyping Platforms: Progress, Challenges, and Perspectives." Mol Plant. 2017; 1047-1064-8. 10.1016/j.molp.2017.06.008
[38] S. R. Loughnan, C. Smith-Keune, D. R. Jerry, L. B. Beheregaray, and N. A. Robinson: "Genetic diversity and relatedness estimates for captive barramundi (Lates calcarifer, B loch) broodstock informs efforts to form a base population for selective breeding." Aquaculture Research. 2016; 3570-3584-11.
[39] M. M. Malmberg *et al.*: "Evaluation and Recommendations for Routine Genotyping Using Skim Whole Genome Re-sequencing in Canola." Front Plant Sci. 2018; 1809-10.3389/fpls.2018.01809

[40] A. T. Melo, R. Bartaula, and I. Hale: "GBS-SNP-CROP: a reference-optional pipeline for SNP discovery and plant germplasm characterization using variable length, paired-end genotyping-by-sequencing data." BMC Bioinformatics. 2016; 29-10.1186/s12859-016-0879-y
[41] X. Huang *et al.*: "High-throughput genotyping by whole-genome resequencing." Genome Res. 2009; 1068-76-6. 10.1101/gr.089516.108
[42] D. Torkamaneh, J. Laroche, M. Bastien, A. Abed, and F. Belzile: "Fast-GBS: a new pipeline for the efficient and highly accurate calling of SNPs from genotyping-by-sequencing data." BMC Bioinformatics. 2017; 5-1. 10.1186/s12859-016-1431-9
[43] N. Heslot, J. Rutkoski, J. Poland, J. L. Jannink, and M. E. Sorrells: "Impact of marker ascertainment bias on genomic selection accuracy and estimates of genetic diversity." PLoS One. 2013; e74612-9. 10.1371/journal.pone.0074612
[44] J. A. Bhat *et al.*: "Genomic Selection in the Era of Next Generation Sequencing for Complex Traits in Plant Breeding." Front Genet. 2016; 221-10.3389/fgene.2016.00221
[45] V. G. Lebedev, T. N. Lebedeva, A. I. Chernodubov, and K. A. Shestibratov: "Genomic selection for forest tree improvement: Methods, achievements and perspectives." Forests. 2020; 1190-11.
[46] A. Ricroch, P. Clairand, and W. Harwood: "Use of CRISPR systems in plant genome editing toward new opportunities in agriculture." Emerg Top Life Sci. 2017; 169-182-2. 10.1042/ETLS20170085
[47] D. F. Voytas and C. Gao: "Precision genome engineering and agriculture: opportunities and regulatory challenges." PLoS Biol. 2014; e1001877-6. 10.1371/journal.pbio.1001877
[48] A. L. Van Eenennaam: "Genetic modification of food animals." Curr Opin Biotechnol. 2017; 27-34-10.1016/j.copbio.2016.10.007
[49] A. Scheben, F. Wolter, J. Batley, H. Puchta, and D. Edwards: "Towards CRISPR/Cas crops - bringing together genomics and genome editing." New Phytol. 2017; 682-698-3. 10.1111/nph.14702
[50] Z. Kong, M. Hart, and H. Liu: "Paving the Way from the Lab to the Field: Using Synthetic Microbial Consortia to

Produce High-Quality Crops." Front Plant Sci. 2018; 1467-10.3389/fpls.2018.01467
[51] M. Balint *et al.*: "Environmental DNA Time Series in Ecology." Trends Ecol Evol. 2018; 945-957-12. 10.1016/j.tree.2018.09.003
[52] K. C. Beng and R. T. Corlett: "Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects." Biodiversity and Conservation. 2020; 2089-2121-.
[53] M. Stat *et al.*: "Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment." Sci Rep. 2017; 12240-1. 10.1038/s41598-017-12501-5
[54] J. Casey, E. Jardim, and J. T. Martinsohn: "The role of genetics in fisheries management under the E.U. common fisheries policy." J Fish Biol. 2016; 2755-2767-6. 10.1111/jfb.13151
[55] R. Zanella: "Genomic Tools and Animal Health." Vet Sci. 2016; 3. 10.3390/vetsci3030021
[56] J. C. S. Harding *et al.*: "Novel insights into host responses and reproductive pathophysiology of porcine reproductive and respiratory syndrome caused by PRRSV-2." Vet Microbiol. 2017; 114-123-10.1016/j.vetmic.2017.02.019
[57] L. R. Thompson *et al.*: "A communal catalogue reveals Earth's multiscale microbial diversity." Nature. 2017; 457-463-7681. 10.1038/nature24621
[58] W. Ikeda-Ohtsubo, S. Brugman, C. H. Warden, J. M. J. Rebel, G. Folkerts, and C. M. J. Pieterse: "How Can We Define "Optimal Microbiota?": A Comparative Review of Structure and Functions of Microbiota of Animals, Fish, and Plants in Agriculture." Front Nutr. 2018; 90-10.3389/fnut.2018.00090
[59] M. K. Waldor *et al.*: "Where next for microbiome research?" PLoS Biol. 2015; e1002050-1. 10.1371/journal.pbio.1002050
[60] M. G. van der Heijden and M. Hartmann: "Networking in the Plant Microbiome." PLoS Biol. 2016; e1002378-2. 10.1371/journal.pbio.1002378

[61] C. M. J. Pieterse, R. de Jonge, and R. L. Berendsen: "The Soil-Borne Supremacy." Trends Plant Sci. 2016; 171-173-3. 10.1016/j.tplants.2016.01.018
[62] S. Kroll, M. T. Agler, and E. Kemen: "Genomic dissection of host-microbe and microbe-microbe interactions for advanced plant breeding." Curr Opin Plant Biol. 2017; 71-78-10.1016/j.pbi.2017.01.004
[63] P. Bakker, C. M. J. Pieterse, R. de Jonge, and R. L. Berendsen: "The Soil-Borne Legacy." Cell. 2018; 1178-1180-6. 10.1016/j.cell.2018.02.024

CHAPTER 15

Oxidative Stress Induced Toxicity

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Oxidative Stress Induced Toxicity

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Abstract

Oxygen is a key element of life. It is well known for its reaction with carbon-based substances such as wood and petrol. This reaction releases energy in the form of heat but requires high temperatures to occur. However, with the presence of enzymes, this reaction can also take place at 37°C. Cellular respiration enzymes have the unique ability to bind both substrate and oxygen molecules, bringing them close enough to facilitate a chemical reaction. This reaction results in the release of energy that can be stored as ATP and used later by other parts of the cell. While oxygen is essential for life, exposure to highly concentrated levels can result in cellular and tissue damage. Advances in technology have made it easier to measure oxidative damage within the body under normal cellular conditions. The key factor in preventing oxidative stress-induced toxicity is maintaining balance of oxidants and antioxidants. When this balance is impaired, oxidants or their metabolites can cause cellular toxicity. In the following chapter, this phenomenon will be explained in greater detail.

Keywords: Free radicals, oxidative damage, oxidative stress, reactive oxygen species, antioxidants.

1. Introduction

Free radicals are molecules that show high reactivity because of their unpaired electrons at the outer orbitals. This unpaired electron makes them unstable and willing to bond with another electron, which can lead to reactions with other molecules [1]. Free radicals are naturally produced as a result of normal

metabolic processes such as energy production in cells by mitochondria. However, they can also be produced by external factors such as exposure to environmental toxins and radiation.

When free radicals react with other molecules, it can result with oxidative damage to cells and tissues in body. For example, free radicals may damage cell membranes, proteins, and DNA therefore leading to mutations and cell death [1]. This damage can contribute to development of various diseases.

Despite their harmful effects, free radicals also play key roles in body. For example, free radicals can act as signaling molecules in certain physiological processes, such as immunity response to infections [2]. In addition, free radicals can be intentionally produced by cells to help kill bacteria and other pathogens.

1.1. Reactive Oxygen Species (ROS)

ROS are specific type of free radicals that contains oxygen. All of the ROS are part of free radicals, but not all the free radicals are ROS. Hydroxyl radical (•OH), superoxide (O_2^-), and singlet oxygen ($^1O_2$) are the important examples to ROS. Like other free radicals, ROS are highly reactive therefore may cause oxidative damage to the cells. Main difference between ROS and other free radicals is the ability of interacting with a variety of biological compounds due to the presence of oxygen. ROS can also react with other molecules in the body to produce other free radicals, for example, hydrogen peroxide can give a reaction with transition metals for producing hydroxyl radicals. Hydrogen peroxide itself show no oxidant effect, therefore product from its reaction with transition metals (Fenton Reaction) is highly oxidative, hydroxyl radical is one of the major oxidants [3].

There are several sources of ROS. Exposure to environmental pollutants such as air pollutants, pesticides, and heavy metals can increase the production of ROS. Lifestyle differences as smoking, alcohol consumption, and lacking physical activity

type of personal behaviors also stimulates the ROS production. Additionally, diets with saturated and trans fats, and low in antioxidants, can contribute to tissues vulnerable for ROS activity. Certain health problems, such as diabetes, cardiovascular diseases, and neurological diseases, can increase oxidative stress levels [4, 5]. As we age, the body's antioxidant defense mechanisms become less efficient, leading to an accumulation of ROS, and eventually increased tissue damage. This also works the same way with diseases; in diabetes, hyperglycemia promotes the production of free radicals, which can cause cellular injury [5].

ROS are highly reactive compounds, produced as byproduct of normal cellular metabolism activity. ROS are produced in various cellular compartments, including the mitochondria, endoplasmic reticulum, and peroxisomes. Although low levels of ROS can be necessary for cellular functions, excessive levels of ROS may lead oxidative damage to cellular components. Therefore, they can cause oxidative modification of biomolecules, leading to cellular damage and impaired cellular function.

In addition to ROS role in oxidative stress-induced toxicity, they involve in cell signalization and the regulation of cell activities, such as expression of genes, growth of cells, and induction of apoptosis. However, when ROS levels become excessive, they can disrupt normal cell signalization pathways and contribute to development of malfunctions [2].

1.2. Oxidative Stress

Oxidative stress is referring to the balance of ROS production and the ability of body to detoxify them. When ROS levels become excessive, they can cause damage to cellular components therefore it can cause to wide range of consequences, including impaired cellular function, oxidative modification of molecules, and ultimately cell death.

Oxidative stress is implicated to pathogenesis of diseases, including cancer, cardiovascular disease, neurological disorders, and ageing [4]. In cancer, oxidative stress may cause damage to DNA, which can result in mutations and contribute for cancer progress. In cardiovascular disease, oxidative stress can lead to foam cells formation. These foam cells accumulate in arterial wall, leading to the formation of plaques that can narrow or block the artery, thereby reducing blood flowing to vital organs, the heart and brain. Ultimately, this can lead to serious cardiovascular events such as atherosclerosis [6].

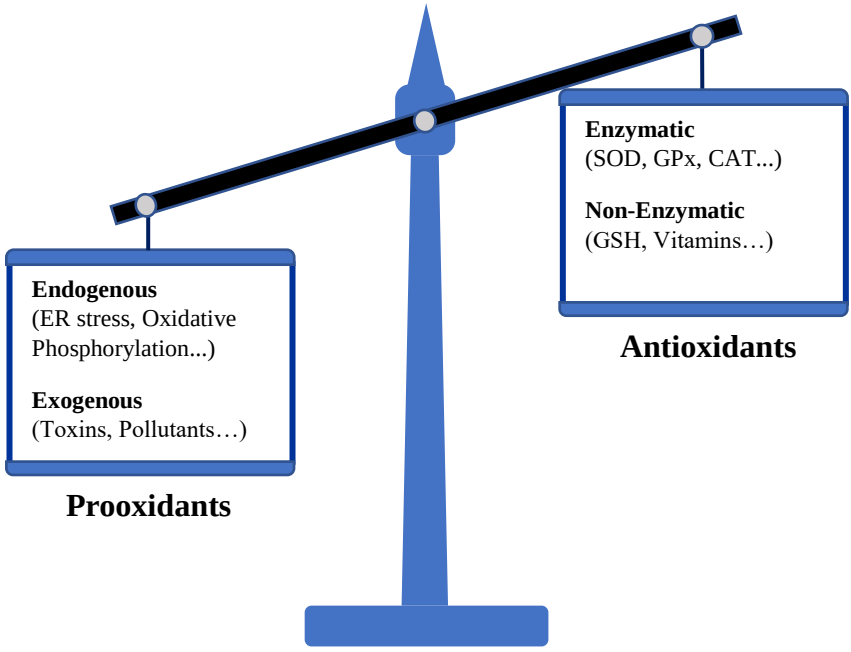

Figure 15.1. Oxidative Stress Balance

In neurodegenerative diseases, the accumulation of misfolded proteins and dysfunctional organelles in neurons can trigger an overproduction of ROS and reactive nitrogen species. In Alzheimer's disease, oxidative stress plays a central role in accumulation of amyloid-beta (Aβ) peptides, which form the characteristic plaques in the brain. Oxidative stress is also implicated with the accumulation of alpha-synuclein aggregates

in Parkinson's disease. Furthermore, oxidative stress may cause mitochondrial disorder, impaired protein homeostasis, and inflammation, therefore contributing for pathogenesis to neurodegenerative diseases [7].

Oxidative stress also plays a role to the decline in physical and cognitive function that occurs with aging [8]. This decline is characterized by a reduction in antioxidant defense mechanisms and increased levels of oxidative stress. As a result, it leads to cellular damage and may cause of age-related diseases [9]. Therefore, oxidative stress is a topic of significant scientific interest and has been the subject of numerous studies aimed at understanding its mechanisms and developing therapeutic strategies to counteract its effects [10].

1.3. Oxidative Stress Pathways

Oxidative stress pathways are important to study oxidative stress-induced toxicity, as they help us to determine the levels of ROS and the extent of oxidative damage to cellular components. The pathways involved in oxidative stress-induced toxicity include the activation of prooxidant enzymes, such as NADPH oxidases, and the mitochondrial electron transport chain, as well as the depletion of cellular antioxidant defenses, including antioxidant enzymes and antioxidants.

One of the main pathways involved in is the activation of NADPH oxidases, which are a family of enzymes that involve in production of ROS. NADPH oxidases produce superoxide anions, which can then be converted into other ROS, such as H_2O_2 and hydroxyl radicals [11]. The activation of NADPH oxidases can be triggered by various stimuli, including oxidative stress, inflammation, and cellular stress.

Another pathway involved in oxidative stress-induced toxicity is the mitochondrial electron transport chain, which is responsible for producing ATP, the primary energy source for cells. However, the mitochondrial electron transport chain also generates ROS as a byproduct of normal cellular metabolism

[11]. The levels of ROS produced by the mitochondrial electron transport chain can become extravagant in response to various stimuli, therefore leads for an accumulation.

In addition to the activation of prooxidant enzymes, oxidative stress-induced toxicity can also result from the depletion of cellular antioxidant defenses.

1.4. Cellular Damage

ROS can cause oxidative modification of biomolecules, leading to cellular damage and impaired cellular function. The cellular components that are most commonly affected by oxidative damage include enzymes, DNA, proteins, organelles, carbohydrates and lipids.

Organelles are involved in energy production, and they can be susceptible to oxidative damage because of the production of ROS during oxidative phosphorylation. Oxidative damage to mitochondria can lead to dysfunction, impaired energy production process, and high susceptibility to apoptosis.

Oxidative damage to carbohydrates can trigger the formation of advanced glycation end products (AGEs). AGEs can cause to the accumulation of abnormal protein aggregates. AGEs can also crosslink proteins, leading to tissue stiffening and impaired function. In addition to their direct effects on protein function, AGEs can also interact the receptors on surface of the cells, such as the receptor for advanced glycation end products (RAGE). RAGE activation can lead to the increase proinflammatory cytokines and activation of various signaling pathways, which can contribute to tissue damage and inflammation [12].

If oxidative damage occurs to enzymes, it can disrupt the normal functioning of the cell and the balance of the internal environment, potentially leads to a loss of activity, and altered function. The loss of enzyme activity can impair the ability of the cell to perform essential metabolic processes, which can

cause to the accumulation of toxic compounds and other harmful effects.

DNA can be susceptible to oxidative damage because of its high susceptibility to oxidative stress and its inability to repair damage as efficiently as other cellular components. Therefore, DNA damage can result in mutations and genomic instability. ROS can lead oxidative modification of DNA, causing DNA adducts and strand breaks. DNA damage can also trigger DNA repair processes, which can cause activation of cell cycle checkpoints and apoptosis.

Lipids are vulnerable to oxidative damage caused by ROS. ROS can cause oxidative modification of lipids. Lipid peroxidation products can occur such as acrolein, malondialdehyde and 4-hydroxy-2-nonenal. ROS can react with unsaturated fatty acids (UFA) within cell membranes, causing lipid peroxidation and subsequent membrane damage, leading to the formation of membrane vesicles and increased permeability. Lipid peroxidation can also trigger cellular signalization pathways which can cause to a cellular damage and apoptosis [11].

Proteins are more resistant than lipids to oxidative damage caused by ROS. ROS can cause oxidative modification to the amino acids in proteins, leading to the formation of protein carbonyls. In addition, unsaturated bonds and sulfur-containing molecules show higher reactivity against free radicals. Oxidatively modified proteins may become altered in their function. Proteins involved in cell signalization and regulation are vulnerable to oxidative damage, which can result in disrupted cell signalization pathways and contribute to development of various diseases.

1.5. Antioxidant Defense Mechanisms

The body has a variety of antioxidant defense mechanisms in place to defend the organism against oxidative stress. Enzymes,

antioxidants, minerals, flavonoids, carotenoids, and vitamins play key roles in reducing cellular damage.

Antioxidants are essential compounds can neutralize ROS and prevent oxidative damage to cellular components. There are several types of antioxidants, could be obtain from a diet or by food supplements or as medicines. Besides obtaining antioxidants from a source they can be synthesized by the body as well.

Enzymes too involve in antioxidant system. Enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and thioredoxin are produced by the body and act as primary enzymatic antioxidants. They involve in process of eliminating ROS and protecting the cells from oxidative damage.

SOD interacts with superoxide radicals converting them into H_2O_2 molecules, then converted into water and oxygen by CAT. Primary enzymatic antioxidants transform free radicals to less harmful or benign products. Secondary enzymatic antioxidants are glutathione reductase (GR) and glucose 6 phosphate dehydrogenases. They aim to neutralize free radicals therefore ROS [8, 13].

Vitamin C and vitamin E are also vital for antioxidant defense. Vitamin C is one of the potent antioxidants that helps neutralize ROS and regenerate other antioxidants. Vitamin E is an antioxidant that soluble in fat and it can protect lipid membranes from oxidative damage. These two vitamins are added to daily products as preservatives to prevent oxidation [13].

Carotenoids are a group of pigments, and they give vegetables and fruits bright colors. Lycopene, beta-carotene, and lutein are examples of carotenoids that have antioxidant properties [13]. Beta carotene is precursor of Vitamin A.

Flavonoids are a type of antioxidant that is present in many fruits and vegetables, such as berries, citrus fruits, and apples. Flavonoids have been linked with to have anti-cancer and anti-inflammatory properties [13]. Therefore, flavonoids are being studied for their potential medical uses and to produce as medicines [13].

In addition to these individual antioxidants, the body also has a variety of antioxidant mechanisms that work together to protect from oxidative stress. These mechanisms include the antioxidant network, which is a complex network of antioxidants that work together to neutralize ROS and protect cellular components. In the table below antioxidant classification is shown.

Table 15.1. Antioxidant Classification

Enzymatic Antioxidants			
Primary		**Secondary**	
Glutathione Peroxides (GPx)		Glucose 6-Phosphate Dehydrogenase	
Superoxide Dismutase (SOD)		Glutathione Reductase (GR)	
Catalase (CAT)	Thioredoxin (Trx)		
Non-Enzymatic Antioxidants			
Metabolic Antioxidants		**Nutritional Antioxidants**	
Glutathione	Selenium	Vitamin E	
Albumin	Bilirubin	Vitamin C	Vitamin A
L-arginine	Thiols	Rutin	Tetraterpenoids
Melatonin	Coenzyme Q10		

1.6. Oxidative Stress Markers

The measurement of oxidative stress biomarkers provides valuable information about the biological state of an individual and oxidative stress-induced cellular damage levels. It is crucial to comprehend the significance of oxidative stress biomarkers to evaluate the influence of oxidative stress on health and to establish efficacious approaches that mitigate oxidative stress and encourage wellbeing.

Lipid peroxidation products: MDA and 4-hydroxy-2-nonenal (HNE) are commonly used markers of lipid peroxidation. In addition to MDA and HNE, other markers of lipid peroxidation include isoprostanes, which are prostaglandin-like compounds that are produced by the peroxidation of arachidonic acid. MDA is a common measured marker of oxidative stress, and thiobarbituric acid reactive substances (TBARS) assay is used to show levels of MDA [14]. TBARS are formed when MDA reacts with thiobarbituric acid therefore we measure the absorbance in 532 nm with a UV spectrophotometer. By measuring the levels of these lipid peroxidation markers, researchers can gain insight into the extent of oxidative stress and cellular damage in various diseases and conditions, which can aid in the development of targeted therapies and preventative measures.

DNA oxidation products: 8-OHdG is a commonly used marker of DNA oxidation. Besides 8-OHdG, another commonly used marker of DNA oxidation is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). These biomarkers can be measured using various methods such as high-performance liquid chromatography (HPLC) with electrochemical or UV detection, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) or enzyme-linked immunosorbent assay (ELISA) [15].

Protein oxidation products: Protein carbonyls and 3-nitrotyrosine are commonly used markers of protein oxidation, they can be measured in serum or plasma samples using spectrophotometric or immunological techniques. In addition to protein carbonyls and 3-nitrotyrosine, advanced oxidation protein products (AOPPs) and oxidized low-density lipoproteins (oxLDL) are other markers of protein oxidation [16]. These biomarkers can be measured using methods such as ELISA, Western blot, or LC-MS/MS.

Reactive oxygen species (ROS) levels: H_2O_2, O_2^-, and •OH are commonly used markers of ROS levels. 2',7'-dichlorofluorescein (DCF) and dihydroethidium (DHE), which

are fluorescent dyes that can be used to visualize ROS production in cells and tissues. Alternatively, ROS levels can be measured using methods such as electron spin resonance (ESR) or chemiluminescence assays [17].

Antioxidant levels: Vitamin C, vitamin E, and GSH are commonly used markers of antioxidant levels they can be measured in serum or plasma samples using spectrophotometric or chromatographic techniques [13]. Besides individually measurement, total antioxidant capacity (TAC), which measures the ability of a biological sample to neutralize free radicals, and ferric reducing antioxidant power (FRAP), which measures the reducing capacity of antioxidants in a sample. These biomarkers can be measured using methods such as spectrophotometry, HPLC, or fluorometry [8].

Redox state markers: These markers reflect the balance or the imbalance between prooxidants and antioxidants in the body. Examples of redox state markers include ratio of the reduced to oxidized glutathione (GSH/GSSG) and redox potential of the plasma.

Inflammatory markers: These markers are a group of biomolecules, produced in response to the inflammation and cellular damage. These markers provide valuable information about the immune system's response to diseases and injuries, including infections, autoimmune diseases, and cancer. Inflammatory markers can be classified into acute-phase proteins, cytokines, chemokines, and adhesion molecules. Examples of acute-phase proteins include C-reactive protein (CRP) and fibrinogen. Elevated levels of these proteins indicate the presence of acute inflammation. Cytokines, such as interleukin-6 (IL-6), are signaling molecules that play a crucial role in the immune response to inflammation [11, 12]. Chemokines, such as monocyte chemoattractant protein-1 (MCP-1), are involved in the recruitment of immune cells to the site of inflammation. Adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), are involved in the adhesion of immune cells to the endothelium during the immune response.

By measuring the levels of these and other inflammatory markers, it can reflect the extent of systemic inflammation and oxidative stress-induced cellular damage [12].

Enzyme markers: SOD, CAT, GPx, GR, glutathione S-transferase (GST), and paraoxonase-1 (PON-1) are commonly used markers of antioxidant enzyme activity. These biomarkers can be measured using methods such as spectrophotometry, HPLC, or Western blot.

It is important to note that the choice of oxidative stress marker will depend on the research question, the biological system being studied, and the available methods for measuring oxidative stress markers. Additionally, the measurement of oxidative stress markers should always be considered in the context of other relevant biological parameters, such as inflammation and antioxidant defense mechanisms.

2. Conclusion

Despite the significant progress made in the field of oxidative stress research, several challenges still remain. One of the biggest challenges is the difficulty in accurately measuring oxidative stress markers, as the methods currently available are often invasive or limited in their sensitivity. Additionally, the complex and multifactorial nature of oxidative stress makes it difficult to identify a specific cause-and-effect relationship between oxidative stress and various diseases. Furthermore, there is a lack of consensus among researchers regarding the optimal dose and duration of antioxidant supplementation, as well as the safety of long-term antioxidant use.

Finally, the effects of oxidative stress can vary greatly between individuals, depending on factors such as age, gender, lifestyle, and genetics, making it difficult to generalize findings from studies and develop effective treatments.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL, et al. Oxygen radicals and human disease. Annals of internal medicine. 1987;107(4):526-45.
[2] Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radical Biology and Medicine. 2000;28(10):1456-62.
[3] Winterbourn CC. Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicology letters. 1995; 82:969-74.
[4] Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D, et al. Oxidative stress, aging, and diseases. Clinical interventions in aging. 2018:757-72.
[5] Tiwari BK, Pandey KB, Abidi A, Rizvi SI. Markers of oxidative stress during diabetes mellitus. Journal of biomarkers. 2013;2013.
[6] Ooi BK, Goh BH, Yap WHJIJoMS. Oxidative stress in cardiovascular diseases: involvement of Nrf2 antioxidant redox signaling in macrophage foam cells formation. 2017;18(11):2336.
[7] Casetta I, Govoni V, Granieri EJCpd. Oxidative stress, antioxidants and neurodegenerative diseases. 2005;11(16):2033-52.
[8] Kasapoglu M, Özben T. Alterations of antioxidant enzymes and oxidative stress markers in aging. Experimental gerontology. 2001;36(2):209-20.
[9] Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D, et al. Oxidative stress, aging, and diseases. 2018:757-72.
[10] Elfawy HA, Das BJLs. Crosstalk between mitochondrial dysfunction, oxidative stress, and age-related neurodegenerative disease: Etiologies and therapeutic strategies. 2019; 218:165-84.
[11] Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Current biology. 2014;24(10): R453-R62.

[12] Luc K, Schramm-Luc A, Guzik T, Mikolajczyk T. Oxidative stress and inflammatory markers in prediabetes and diabetes. J Physiol Pharmacol. 2019;70(6):809-24.
[13] Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress: A review. European journal of medicinal chemistry. 2015; 97:55-74.
[14] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry. 1979;95(2):351-8.
[15] Wu LL, Chiou C-C, Chang P-Y, Wu JT. Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clinica chimica acta. 2004;339(1-2):1-9.
[16] Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney international. 1996;49(5):1304-13.
[17] He W, Liu Y, Wamer WG, Yin J-J. Electron spin resonance spectroscopy for the study of nanomaterial-mediated generation of reactive oxygen species. Journal of food and drug analysis. 2014;22(1):49-63.

CHAPTER 16

Therapeutic Potential of Venom Peptides

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Therapeutic Potential of Venom Peptides

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Abstract

The biochemical composition of animal venoms (scorpions, snakes, spiders, cone snails, etc.) is diverse and contains short and large proteins with neurotoxic and enzymatic activities. Some short peptides act specifically to discriminate among Ca^{2+}, Na^{+}, and K^{+} ion channel subtypes, or act as inhibitors or activators of receptors that in turn could affect human physiology. Their selective affinities for ion channel subfamilies are also functional for mapping excitable cells. Furthermore, several of these short peptides have been proved to hyperpolarize peripheral neurons, which are associated with supplying sensation to the skin and skeletal muscles. For instance, some spider N-type calcium ion channel blockers may be important for the treatment of chronic pain. A special group of spider peptides are amphipathic and positively charged peptides. Their secondary structure is alpha-helical, and they insert into the lipid cell membrane of eukaryotic or prokaryotic cells leading to the formation of pores and subsequently depolarizing the cell membrane. Venom peptides from animal venoms represent an interesting source of molecules for the design of novel pharmaceutical drugs. This chapter presents the therapeutic properties of venom toxins and discusses the latest biochemical and molecular advances in the development of new therapies.

Keywords: Animal toxins, Drug discovery, Pharmacological tools, Therapeutics, Venom peptides

1. Introduction

The diversity of fauna is one of the most remarkable aspects of the Earth. It has been estimated that about ~ 15% of all animals on the planet are venomous species, which includes more than 220,000 species [1]. Among these species, some of them are well known for their sting or bites such as snakes, scorpions, spiders, cone snails, bees and sea anemones [2]. Venoms are deadly cocktails that include complex mixtures of bioactive components and proteins, peptides and small molecules which are of medical importance. For that reason, venomous animals can cause human envenomation that may result in mild symptoms to severe symptoms, even death [3]. For instance, the World Health Organisation (WHO) reported that although the precise number of snake bites is unclear, it is estimated that 5.4 million individuals are bitten a year, with up to 2.7 million of those bites resulting in envenoming. Each year, snake bites result in between 81,000 and 138,000 fatalities as well as three times as many amputations and other types of lifelong impairments [4]. Likewise, 1.2 million scorpion envenomation occurs, resulting in about 3,000 deaths annually [5]. To date, antivenoms are the only specific therapy shown to be effective against the threat of envenomation after being bitten and stung by venomous animals. The most commonly known method of producing an antivenom involves injecting small and then gradually increasing amounts of the venom into mammals (such as horses, lambs or rabbits) to obtain hyperimmunized animals. Antivenoms are polyclonal antibodies which purify from the plasma of hyperimmunized animals [6]. Antivenom production consists of four basic steps: the preparation of reference crude venom pool, obtaining of hyperimmune plasma, purification of polyclonal antibodies and formulation [7].

While venoms have been widely used since the late 1800s due to their immunogenic properties to produce antivenom, in recent years compounds from venoms were investigated as drug

discovery sources and were explored to be valuable therapeutic drug candidates. This is not a surprise, it is well-known that different animal venoms are used to treat many diseases in traditional medicine practices, especially in Asia and Africa [8]. The cobra was revered in ancient Egypt, and its image was used to design the crowns of Roman emperors. On the other hand, while the God of medicine was depicted with a stick entwined with a snake in the ancient Greek world, today the snake is a well-known symbol of medicine and pharmacy [9].

At the present time, the development of venom-based bioactive pharmaceuticals requires a new strategy to replace the traditional economic model of large pharmaceutical corporations. Many reasons are spurring efforts to incorporate new techniques into drug discovery, including the costs of patent protection, the expansion of the generic drug market, increased R&D spending and increased regulatory pressure [10]. Affecting human health such as cancer, increasing infections due to antibiotic resistance, and emerging diseases such as Covid-19 reveal the need for venom and venom-derived new peptide therapeutics. The WHO reports that cancer is one of the top causes of mortality globally, accounting for nearly 10 million fatalities or one in every six deaths in 2020. Furthermore, it has been observed that the most prevalent malignancies include breast, lung, colon, and rectum cancers, as well as prostate cancers [11]. It has been reported by the WHO that antibiotic resistance is currently one of the most serious risks to world health, food security, and development. As drugs become less effective, more diseases such as pneumonia, tuberculosis, gonorrhoea and salmonellosis are becoming increasingly difficult to treat. Antibiotic resistance results in longer hospital stay, higher medical expenses, and higher death rates [12]. It is essential for researchers to search for new drugs for such important diseases for which existing medicine may be insufficient. Since it is a fact known for thousands of years that humanity uses natural components as medicine, animal venoms are important natural resources for natural medicines [13]. With advances in modern venomic studies, proteome analyses are performed using state-of-the-art mass spectrometry devices even

for small venomous animals which produce lower quantities of venom. In addition, transcriptome techniques used in venom research provided more information on the composition of animal venoms and gave a chance to get an overview of the composition of animal venoms and compare the relative abundance of toxin transcripts [2]. Each component found in animal venom has a selective target against its prey, or for its defense. This shows that the bioactive components are formed with unique specificity according to the particular needs of the animal. Considering the scarcity of venom peptides identified so far and the venomous animals still undiscovered on Earth, we can calculate that there are components waiting to be discovered in huge amounts. As proteomic and transcriptomic approaches continue to identify new sequences, the therapeutic applications of these components will increase. Figure 16.1 shows an evolutionary tree of venomous animals.

It is well documented that throughout human history, venomous animals have had an impact on people's health, culture, and economic well-being [1,2]. However, they were aware of the deadly creatures' two distinctly contrasting aspects, A curse or a cure? Today, effective and Good Manufacturing Practices (GMP) approved safe antivenoms are produced and new antivenom products specific to the species of regions needed are developed. At the same time, new research on the beneficial effects of animal toxins provides hope and proof for the treatment of several relentless diseases. The therapeutic benefits of venom toxins are discussed in this chapter along with the most recent biochemical, molecular, and biotechnological developments in the creation of novel therapeutic perspectives for venom toxins in drug discovery.

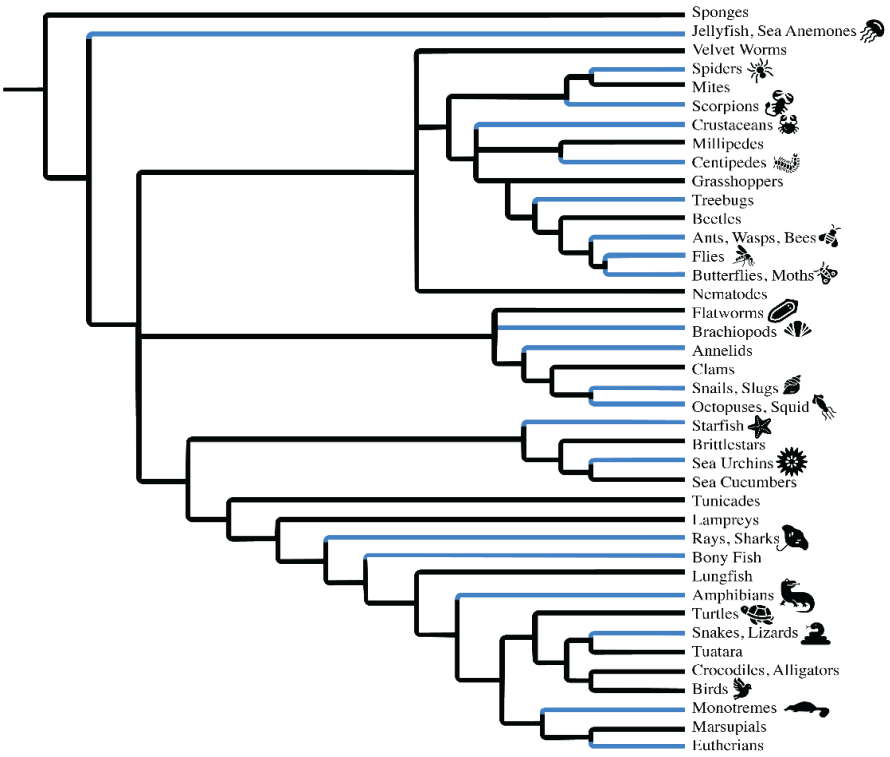

Figure 16.1. Evolutionary tree of animals modified from [1] Blue lines indicate venomous animals.

1.1. Biodiversity of Venoms

Animal venoms are complex mixtures of bioactive molecules that are used mainly for hunting their prey, or defense. These bioactive molecules can include proteins, enzymes, peptides, and other small organic molecules, and they can have a variety of effects on the target organism, such as neurotoxicity, cardiotoxicity, and cytotoxicity [14]. The biodiversity of animal venoms is vast. For instance, Arachnida spiders and scorpions represent more than 55,000 species. If we include snakes, cone snails, and some venomous arthropods such ants, bees, centipedes, wasps, and reduviids the number of species increases significantly [15,16]. Many of these species produce unique

venom components with particular biological activities. For example, some snake venom contains potent neurotoxins that paralyze their prey, while others have hemotoxic components that cause tissue damage and internal bleeding [17]. Scorpion, spider, and elapid venoms contain neurotoxins, cytotoxins, and enzymes that affect the nervous system and muscles of their victim [18]. Cone snail venoms contain a great diversity of peptides short disulfide bridged toxins that target specific receptors and ion channels, also in the nervous system and muscles, which lead to paralysis and death [19]. Reduviid venoms contain different types of proteases and phospholipases that liquefy the hemolymph proteins of their insect prey [16]. The study of venom biodiversity has led to the discovery of numerous bioactive molecules with potential therapeutic applications, such as painkillers, anticoagulants, antimicrobials, and cancer treatments. However, the diversity of venoms also poses a significant challenge for researchers trying to understand their mechanisms of action and develop new treatments based on them [20].

1.2. Composition of Animal Venoms

The composition of animal venoms varies widely between different species, is dependent on their ecological niches, and includes a variety of biomolecules such as small organic molecules, peptides, proteins, and enzymes [21]. Organic molecules found in animal venoms include lipids, alkaloids, some other heterocyclic compounds, and nucleotides. Alkaloids are nitrogen-containing organic molecules that can act as stimulants, hallucinogens, or toxins, depending on their structure and dose. Nucleotides, which are the building blocks of DNA and RNA, can act as signaling molecules and modulate the activity of cells in the target organism. Peptides are large and interesting components of many animal venoms, and they can have a wide range of bioactivities [22]. For example, some venom peptides can act as neurotoxins, others as antimicrobial agents, and still others as analgesics or anti-inflammatory agents [23]. Proteins and enzymes are the most abundant components of snake venoms, and they can have a variety of effects on the

target organism. For example, some venom proteins can disrupt the function of nerve cells, leading to paralysis or death, while others can target the cardiovascular system, causing heart failure or hypotension [24]. Phospholipases and hyaluronidases can disrupt cell membranes and facilitate the uptake of other venom components, respectively [25]. Overall, the composition of animal venoms is incredibly diverse, reflecting the evolutionary history and ecological niche of each venomous species.

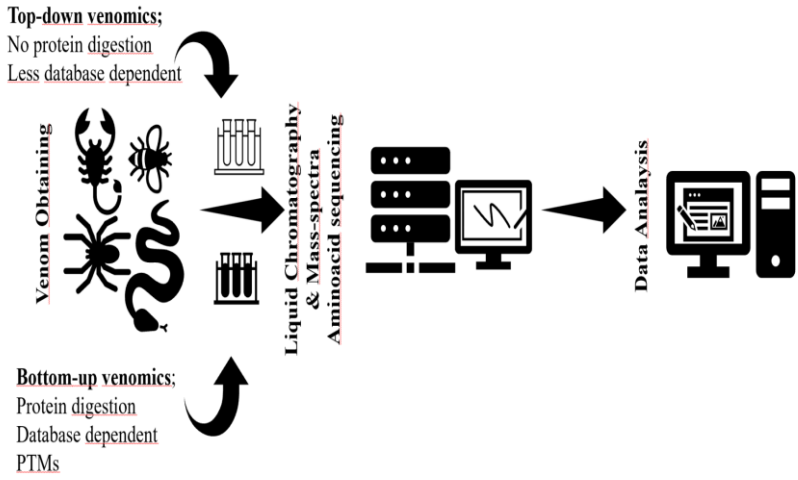

Figure 16.2. Scheme of venomic workflow.

1.3. Biological Screening of Animal Venoms

Since the biological screening of animal venoms is the process of testing the bioactive molecules found in venom for their potential pharmacological and therapeutic properties, this involves isolating and purifying individual venom components (See Fig. 2) and testing them for specific activities, such as analgesic, anti-inflammatory, or antitumor effects [2]. Pharmacological screening typically involves a series of *in vitro* and *in vivo* assays to evaluate the activity and toxicity of venom components [26].

In vitro, assays involve testing venom components in cell cultures to assess their effects on cellular function, while *in vivo* assays involve testing the effects of venom components on whole animals, typically rodents or other non-human animals. One important application of pharmacological screening of animal venoms is the discovery of new drug leads. Many bioactive molecules found in venoms have been shown to have potential therapeutic applications, such as painkillers, antimicrobials, anticoagulants, and cancer treatments [27]. For example, the peptide tetrodotoxin found in pufferfish venom is a potent painkiller that is currently being developed as a novel pain medication [28]. Another application of pharmacological screening of animal venoms is the development of new tools for studying biological systems. For example, many venom peptides have highly specific interactions with receptors and ion channels in the nervous system, making them valuable tools for studying the function of these systems [29]. The biological screening of animal venoms has led to the discovery of many important bioactive molecules and has the potential to lead to the development of new drugs and tools for studying physiological systems [21]. Even so, it also poses significant ethical and safety concerns, as many venom components can be highly toxic and require careful handling and disposal.

1.4. Approved Therapeutics from Venoms and Venom-Derived

There are several approved therapeutics that have been derived from animal venoms, demonstrating the potential of these complex mixtures of bioactive molecules to be used in medicine [30]. Some examples of approved therapeutics from venoms include Captopril, which is a drug used to treat hypertension and heart failure. It was derived from the venom of the Brazilian pit viper (*Bothrops jararaca*) and works by inhibiting the activity of an enzyme that regulates blood pressure. Another one is Eptifibatide, which is a drug used to prevent blood clots in patients with acute coronary syndrome [31]. It was derived from the venom of the southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*) and works by blocking

the activity of a protein involved in blood clotting [32]. A painkiller, Ziconotide is a drug used to treat severe chronic pain. It was derived from the venom of the cone snail (*Conus magus*) and works by blocking the activity of a specific type of calcium channel in nerve cells [33]. One more is Exenatide, which is a drug used to treat type 2 diabetes. It was derived from a protein found in the saliva of the Gila monster (*Heloderma suspectum*) and works by mimicking the activity of a hormone that regulates blood sugar levels [34]. Finally, Byetta, which is another drug used to treat type 2 diabetes, which is a synthetic version of exenatide from *Heloderma suspectum* [35]. These are some examples of the many approved therapeutics that have been derived from animal venoms. The discovery of these drugs highlights the potential of venom molecules as a rich source of novel pharmacological agents for treating a wide range of diseases.

1.5. Pharmacology of Animal Venoms

Currently, a number of venom-origin peptides are being studied and reported by researchers as promising molecules, including in the analgesia of chronic and neuropathic pain, in the treatments of chronic spinal cord injuries, multiple sclerosis, cardiovascular diseases, sickle cell anemia, arrhythmia, hypertension, atrial fibrillation, rheumatoid arthritis, urinary incontinence, psoriasis, stroke, epilepsy, Type 1 and Type 2 diabetes, Alzheimer's disease, Parkinson’s disease, tuberculosis and even human immunodeficiency virus (HIV). They are also well-recognized for their anti-tumor, anti-malarial, immunosuppressant, and antimicrobial properties [36-39]. Some selected venom-derived drugs for human use in clinical and pharmacological active peptides isolated from several animal venoms were listed in Table 16.1.

Table 16.1. Approved or in clinical trial venom-derived drugs for human use.

Source	Drug	Mechanism of Action	Use	Approved by FDA	Refs
Snake *(Bothrops jararaca)*	Captopril (Capoten ®)	Inhibitor of angiotensin converting enzyme (ACE)	Hypertension , cardiac failure	1981	[54]
Snake *(Sistrurus miliarius)*	Eptifibatide (Integrilin®)	Glycoprotein IIb/IIIa inhibitors	Acute coronary syndrome	1988	[22]
Snake *(Naja atra)*	Cobratide (Ketongning, cobratoxin)	Blockage of nicotinic receptors neuropathic headache	Chronic arthralgia, sciatica	1998	[55]
Snake *(Echis carinatus)*	Tirofiban (Aggrastat®)	Glycoprotein IIb/IIIa inhibitors	Hearth attack	1999	[36]
Snake *(Bothrops jararaca)*	Enalapril (Vasotec®)	Inhibitor of angiotensin converting enzyme (ACE)	Hypertension , cardiac failure	2001	[54]
Snake *(Bothrops atrox and Bothrops moojeni)*	Batroxobin (Defibrase®/ Plateltex-Act®/ Reptilase®)	Converts fibrinogen into fibrin through the release of fibrinopeptide A from fibrinogen	Stroke, pulmonary embolism, deep vein thrombosis, myocardial infarction, and perioperative bleeding.	Outside USA only	[36, 51]
Snake *(Calloselasma Rhodostoma)*	Ancrod (Viprinex®)	Anti-coagulant	Stroke, cerebral ischemia, brain infarction	Phase III	[37]
Scorpion *(Leiurus quinquestriatus Quinquestriatus)*	Chlorotoxin (TM-601, tozuleristide)	Chloride channel inhibitör, Binds MMP2, Annexin A2 and CLC-3 chloride channels on surface of glioma Cells and other tumors of neuroectodermal origin, impairing invasion ability	Glioma, Intraoperative imaging of solid cancer cells using as tumor paint	Clinical trials	[37, 42]

Scorpion *(Mesobuthus martensii)*	SVAP	Inhibit platelet aggregation in vitro, prolonged the occlusion time of carotid artery thrombosis in rats.	Antithrombotic agent, cardio and cerebral vascular disease	Preclinical phase	[51]
Scorpion *(Centruroides margaritatus)*	Margatoxin (MgTX)	Voltage-dependent potassium channels blocker; Kv1.1 Kv1.2, Kv1.3.	Immunosuppressant agent, treatment of cancer	Ongoing research	[56]
Scorpion *(Pandinus imperator)*	Scorpine	Inhibition of viral replication, bactericidal mechanism	Antiviral, antimalarial, and antibacterial agent	Ongoing research	[58]
Scorpion *(Scorpio maurus palmatus)*	Smp24	multiple modes of action, depending on the lipid content of the membrane	anticancer and antimicrobial agent	Ongoing research	[57]
Scorpion *(Hemiscorpius lepturus)*	Hemilipin	Reducing the expression of VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, HGF and CD105	Anti-angiogenesis	Ongoing research	[59]
Spider *(Hadronyche infensa)*	IB001	Blocks the signals that causes heart cells to die	Prevent damage caused by heart attacks and stroke	Phase I (Australia)	[60]
Spider *(Grammostola spatulate)*	GsMTx4	Mechanosensitive channel inhibitor	Analgesic, antibacterial, pain reaction blocker,	Ongoing research	[56, 52]
Spider *(Argiope aurantia)*	Delucemine ((NPS1506)	NMDA blocker	Protection of brain cells from ischemia, Neuroprotective drug for head trauma, hemorrhagic stroke, and ischemic stroke	Phase I (USA)	[61]
Honeybee *(Apis mellifera)*	Whole venom (Apitox®)	Anti-inflammatory action; alteration of the immune response via antigen competition	Pain associated with osteoarthritis. and multiple sclerosis	Clinical trails	[62, 42]

Wasp *(Vespula lewisii)*	Mastoparan	Breaking down the bacterial outer membrane	Antimicrobial, immunomodulator	Preclinical	[42]
Cone snails *(Conus magus)*	Ziconotide (Prialt®)	Blocks CaV2.2 voltage-gated calcium channe	Severe chronic pain	2004	[54]
Pufferfish *(Takifugu spp.)*	Tetrodotoxin (Tectin®)	Sodium channel blocker	Neuropathic pain caused by chemotherapy	Phase III	[51]
Lizard, Gila Monster *(Heloderma suspectum)*	Exenatide (Byetta®)	Glucagon-like peptide-1 receptor Agonist	Type 2 diabetes mellitus	2005	[54]
Lizard, Gila Monster *(Heloderma suspectum)*	Lixisenatide (Lyxumia®, Adlyxin®	Glucagon-like peptide-1 receptor Agonist	Type 2 diabetes mellitus	2016	[51, 54]
Leeches *(Hirudo medicinalis)*	Bivalirudin (Angiomax®)	Inhibits platelet aggregation and the coagulation cascade	Stroke, deep vein thrombosis and pulmonary embolism	2000	[54]
Leeches *(Hirudo medicinalis)*	Desirudin (Iprivask®)	Selective and near irreversible inhibitor of thrombin	Prevention of venous thrombotic events	2003	[54]
Sea anemone *(Stichodactyla helianthus)*	ShK	Voltage-dependent potassium channels blocker; Kv1.1, Kv1.3, Kv1.4, Kv1.6	Autoimmune disease targeting	Ongoing research	[31]

1.6. Snake Venoms

Snake venom contains bioactive peptides, polypeptides, enzymes, minerals, carbohydrates, and low-molecular-mass proteins, all having synergistic effects. Snake toxins can basically be divided into two main classes: enzymatic and non-enzymatic proteins. They may also be divided into three classes depending on their pharmacological properties, namely cardiotoxins, hemotoxins, and neurotoxins. To date, several components have been studied and identified concerning their

structure, and biological activities from snake venoms such as i. Phospholipase A2 (PLA2) enzyme complexes *(i.e., Covalent complexes, and non-covalent complexes such as homodimeric, heterodimeric, heterotrimeric and heteropentameric complexes)*. ii. Protease complexes *(metalloprotease complexes, dimeric disintegrins, serin protease complexes).* iii. L-amino acid oxidases, iv. Snake C-type lectins, v. Three-finger toxin complexes (*Covalent complexes, and non-covalent complexes, synergistic 3FTxs)* [40,41].

The structure of a bradykinin-potentiating peptide obtained from the venom of the Brazilian pit viper, *Bothrops jararaca*, was used to produce Captopril, the first ACE inhibitor licensed for human use. The discovery of Captopril demonstrated the ability of snake venom components to operate as lead molecules in modern drug development, as well as the various biochemical and pharmacological properties that have in snake venom components [42].

1.7. Scorpion Venoms

Scorpion venoms consist of several complex bioactive molecules such as polypeptides, enzymes, mucoproteins, free amino acids, nucleotides, lipids, amines, heterocyclic components, low molecular weight organic molecules, inorganic salts, enzyme inhibitors, and other unknown substances [2, 8]. The peptide components of scorpion venom are recognized to serve the most essential function in envenomation and biological activity, even though scorpion venoms are composed of extremely complicated combinations. These days, there are multiple alternative classifications for scorpion peptide venoms. The two groups of peptides that make up the present categorization are those with disulfide bridges (DBPs) and those without disulfide bridges (NDBPs). The pharmacological actions identified for ion channels of peptides containing disulfide bridges are divided into four classes: Na^+ channel toxins, K^+ channel toxins, Ca^{2+} channel toxins, and Cl^- channel toxins. Scorpion venom peptides that lack disulfide bridges are an important class of newly identified bioactive peptides. In

addition to these classifications, they may be classified into two types based on their molecular size: long-chain toxins and short-chain toxins.

They can also be classed as peptides unique to mammals, insects, and crustaceans based on their impact on different animal groups. Furthermore, they can be classed as neurotoxins or cytotoxins based on their method of action [43, 44]. For instance, Chlorotoxin is a stable peptide consisting of 36 amino acids in length and packaged with 4 disulfide bridges and it was isolated from the *Leiurus quinquestriatus* scorpion. The studies, which started after the discovery of the binding properties of Chlorotoxin to brain tumours in 1998, continue in three different categories: imaging (tumor staining), nanotechnology and radiotherapy. Moreover, it has been reported that in addition to the Chlorotoxin derivative TM-601, which is produced recombinantly, exists the radio-conjugate derivative 131I-TM601, which also has antiangiogenic and antineoplastic effects [45].

1.8. Spider Venoms

Spider venom is also a toxic blend of molecules where its major components are small molecular mass structures (from 350 to 1,000 Da) named acylpolyamines, which consist of an aromatic acyl group and a polyamine backbone (some with amino acids in the backbone) [23]. The acylpolyamines block ionotropic glutamate receptors to induce a fast paralysis of their insect prey [46]. Acylpolyamines produce a reversible paralysis in insects, and they act synergistically with ICK peptides to produce an irreversible paralysis. The venom of wolf spiders is also characterized for containing antimicrobial peptides [47]. The cysteine-rich and neurotoxic peptides are quite interesting molecules because of their various structural motifs, and their potential targets such as ion channels and membrane receptors [48].

1.9. Honeybee Venoms

Bee venoms consist of a complex mixture of bioactive peptides *(i.e., melittin, apamine, MCD, secapine, adolapin, cardiopep)*, enzymes (*i.e., phospholipase A2, phospholipase B, hyaluronidase, acid phosphomonoesterase*) bioactive amines *(i.e., histamines, catecholamines, and polyamines),* carbohydrates and pheromones. Melittin is an attractive candidate molecule for cancer therapy which constitutes approximately 40-50% of the total dry bee venom. Melittin is the most studied bee venom component together with phospholipase A2. Both crude honeybee venom and melittin, like other chemotherapeutic drugs, have demonstrated great effectiveness in producing apoptosis, necrosis, mitochondrial disruption, angiogenesis suppression, cell cycle arrest, and inhibiting the spread and invasion of cancer cells [41, 49].

1.10. Cone snail Venoms

Conotoxins are isolated from species of cone snails (Conus sp.), and they consist of a large family of short cysteine-rich peptides formed from 10 to 30 amino acid residues long. They are mainly classified into subfamilies based on their cysteine framework and mode of action. Each conus species has between 100 and 200 small, but highly organized venom peptides in their venom ducts. Depending on their prey, Conus species are divided into three groups: vermivorous (eating worms), molluscivorous (eating mollusk), and piscivorous (eating fish) [50,51]. A major milestone for Conus peptides was the approval by the US Food and Drug Administration in 2004 of the antinociceptive peptide named Prialt (Ziconotide), a peptide used for intractable pain [52]. Ziconotide is a 25 amino acid polybasic peptide analogue of omega-conotoxin, a peptide obtained from the sea snail *Conus magus* that also has analgesic effects. Ziconotide is a synthetic, non-opioid compound [53].

1.11. Other Animal Toxins

Not only the poisonous animals mentioned above but also the venom or saliva of many other venomous animals contain biologically active compounds such as Gila monsters, Wasps, Caterpillars, Centipedes, Pufferfishes, Leeches, Sea anemones, Jellyfish, Shrews, Bufo toads, Bats, Tics, Frogs, Ants, Sea squirts, Toads and Lizards. To date, several important and promising venom components have been isolated and identified from those noxious animals.

2. Conclusions and Future Directions

The study of animal venoms has been an important area of research for many decades, and it continues to generate significant scientific and medical advances. The diversity and complexity of venomous animals provide a vast and largely available source of bioactive molecules with a wide range of pharmacological properties. The discovery of approved therapeutics derived from animal venoms underscores the potential of venom molecules to be used in medicine. However, much remains to be explored and discovered about the biological activities of venom components and their mechanisms of action. Future directions of research on animal venoms include the identification and characterization of novel venom molecules and advances in analytical and bioinformatics tools, which support the rapid and efficient identification of novel drug leads. Also, the development of clinical treatments could launch the identification and characterization of novel molecules with potential therapeutic applications, such as new types of painkillers, anticoagulants, antibiotics, and anticancer agents. The study of venomous animals could provide insights into the evolution of venomous systems and the ecological interactions between venomous animals and their prey, predators, and competitors. Overall, the study of animal venoms is a rapidly evolving field that holds great promise for advancing our understanding of the natural world and developing novel therapies for a wide range of medical conditions.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Holford M, Daly M, King FG, Raymond SN. Venoms to the rescue. Science. 2018; 361 (6405) :842-844. DOI: 10.1126/science. aau7761

[2] Bjoern MVR, Anderluh G, Antunes A, Ayvazyan N, Beis D, Caliskan F, Crnković A, Damm M, Dutertre S, Ellgaard L, Gajski G, German H, Halassy B, Hempel BF, Hucho T, Igci N, Ikonomopoulou MP, Karbat I, Klapa MI, Koludarov I, Kool J, Lüddecke T, Mansour RB, Modica MV, Moran Y, Nalbantsoy A, Pachón-Ibáñez ME, Panagiotopoulos A, Reuveny E, Céspedes JS, Sombke A, Surm JM, Undheim EAB, Verdes A, Zancolli G. Modern venomics-Current insights, novel methods, and future perspectives in biological and applied animal venom research. GigaScience.2022;18;11: giac048. DOI:10.1093/gigascience/giac048

[3] Calvete JJ, Sanz L, Angulo Y, Lomonte B, Gutiérrez JM. Venoms, venomics, antivenomics. FEBS letters. 2009; 583(11), 1736–1743. DOI: 10.1016/j.febslet.2009.03.029

[4] World Health Organization (WHO) [Internet]. 2021. Available from: https://www.who.int/news-room/fact-sheets/detail/snakebite-envenoming [Accessed: 2023-04-01]

[5] Chippaux JP, Goyffon M. Epidemiology of scorpionism: a global appraisal. Acta tropica. 2008; 107(2), 71–79. DOI: 10.1016/j.actatropica.2008.05.021

[6] Bernard-Valle M, Neri-Castro EE, Fry BG, Boyer L, Cochran C, Alam M, Jackson TNW, Paniagua D, Olvera-Rodriguez F, Koludarov I, Sunagar K, Alagon A. Antivenom research and development. In: Fry BG, editor. Venomous Reptiles and Their Toxins. Evolution, Pathophysiology and Biodiscovery. Oxford University Press. New York; 2015. p. 62-72.

[7] León G, Vargas M, Segura Á, Herrera M, Villalta M, Sánchez A, Solano G, Gómez A, Sánchez M, Estrada R, Gutiérrez JM. Current technology for the industrial manufacture

of snake antivenoms. Toxicon. 2018; 151: 63-73. DOI: 10.1016/j.toxicon.2018.06.084.
[8] Ortiz E, Gurrola GB, Schwartz EF, Possani LD. Scorpion venom components as potential candidates for drug development. Toxicon: official journal of the International Society on Toxinology. 2015; 93, 125–135. DOI: 10.1016/j.toxicon.2014.11.233.
[9] Koh DCI, Armugam A, Jeyaseelan K. Snake venom components and their applications in biomedicine. Cell. Mol. Life Sci. 2006; 63, 3030–3041 DOI:10.1007/s00018-006-6315-0

[10] Escoubas P, King FG. Venomics as a drug discovery platform. Expert Review of Proteomics. 2009; 6:3, 221-224, DOI: 10.1586/epr.09.45
[11] World Health Organization (WHO) [Internet]. 2022. Available from: https://www.who.int/news-room/fact-sheets/detail/cancer [Accessed: 2023-04-01]
[12] World Health Organization (WHO) [Internet]. 2020. Available from: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance [Accessed: 2023-04-11]
[13] Robinson SD, Vetter I. Pharmacology and therapeutic potential of venom peptides. Biochem Pharmacol. 2020; 181: 114207, DOI: 10.1016/j.bcp.2020.114207
[14] Ageitos L, Torres MLA, De La Fuente-Nunez C. Biologically Active Peptides from Venoms: Applications in Antibiotic Resistance, Cancer, and Beyond. International Journal of Molecular Sciences. 2022; 23(23), 15437. DOI:10.3390/ijms232315437
[15] Agnarsson I. Grand challenges in research on arachnid diversity, conservation, and biogeography. Frontiers in Arachnid Science. 2023; 2. DOI:10.3389/frchs.2023.1101141
[16] Hernández-Vargas ML, Gil J, Lozano L, Pedraza-Escalona M, Ortiz E, Encarnación-Guevara S, Alagón A, Corzo G. Proteomic and transcriptomic analysis of saliva components from the hematophagous reduviid Triatoma pallidipennis. Journal of Proteomics. 2017; 162, 30–39. DOI: 10.1016/j.jprot.2017.04.022
[17] Vyas VK, Brahmbhatt KG, Bhatt HG, Parmar U. Therapeutic potential of snake venom in cancer therapy: current

perspectives. Asian Pacific Journal of Tropical Biomedicine. 2013; 3(2), 156–162. DOI:10.1016/s2221-1691(13)60042-8
[18] Herzig V, Cristofori-Armstrong B, Israel MR, Nixon SA, Vetter I, King GF. Animal toxins-Nature's evolutionary-refined toolkit for basic research and drug discovery. Biochemical Pharmacology. 2020; 181, 114096. DOI: 10.1016/j.bcp.2020.114096
[19] Duque HM, Dias SC, Franco OL. Structural and Functional Analyses of Cone Snail Toxins. Marine Drugs. 2019; 17(6), 370. DOI:10.3390/md17060370
[20] Schendel V, Isbister GK, Jenner RA, Undheim EAB. The Diversity of Venom: The Importance of Behavior and Venom System Morphology in Understanding Its Ecology and Evolution. Toxins. 2019; 11(11), 666. DOI:10.3390/toxins11110666
[21] El-Aziz TMA, Soares AJG, Stockand JD. Advances in venomics: Modern separation techniques and mass spectrometry. Journal of Chromatography B. 2020; 1160, 122352. DOI: 10.1016/j.jchromb.2020.122352
[22] Robinson SD, Undheim EAB, Ueberheide B, King GF. Venom peptides as therapeutics: advances, challenges and the future of venom-peptide discovery. Expert Review of Proteomics. 2017; 14(10), 931–939. DOI:10.1080/14789450.2017.1377613
[23] Langenegger N, Nentwig W, Kuhn-Nentwig L. Spider Venom: Components, Modes of Action, and Novel Strategies in Transcriptomic and Proteomic Analyses. Toxins. 2019; 11(10), 611. DOI:10.3390/toxins11100611
[24] Chan YS, Cheung RCF, Xia L, Wong J H, Ng TB, Chan WY. Snake venom toxins: toxicity and medicinal applications. Applied Microbiology and Biotechnology. 2016; 100(14), 6165–6181. DOI:10.1007/s00253-016-7610-9
[25] Fox JW. A brief review of the scientific history of several lesser-known snake venom proteins: l-amino acid oxidases, hyaluronidases and phosphodiesterases. Toxicon. 2013; 62, 75–82. DOI: 10.1016/j.toxicon.2012.09.009
[26] Alvarado D, Cardoso-Arenas S, Corrales-García LL, Clement H, Arenas IDR, Montero-Dominguez PA, Olamendi-Portugal T, Zamudio FZ, Csoti A, Borrego J, Panyi G, Papp F,

Corzo GA. Novel Insecticidal Spider Peptide that Affects the Mammalian Voltage-Gated Ion Channel hKv1.5. Frontiers in Pharmacology. 2021; 11. DOI:10.3389/fphar.2020.563858
[27] Santos AGD, Cruz GS, Rádis-Baptista G. Anti-inflammatory activities of arthropod peptides: a systematic review. Journal of Venomous Animals and Toxins Including Tropical Diseases. 2021; 27. DOI:10.1590/1678-9199-jvatitd-2020-0152
[28] Bucciarelli GM, Lechner M, Fontes A, Kats LB, Eisthen HL, Shaffer HB. From Poison to Promise: The Evolution of Tetrodotoxin and Its Potential as a Therapeutic. Toxins. 2021; 13(8), 517. DOI:10.3390/toxins13080517
[29] Wulff H, Christophersen P, Colussi PA, Chandy KG, Yarov-Yarovoy V. Antibodies and venom peptides: new modalities for ion channels. Nature Reviews Drug Discovery. 2019; 18(5), 339–357. DOI:10.1038/s41573-019-0013-8
[30] De Souza JS, Gonçalves B, Gomez MV, Vieira LB, Ribeiro FM. Animal Toxins as Therapeutic Tools to Treat Neurodegenerative Diseases. Frontiers in Pharmacology. 2018; 9. DOI:10.3389/fphar.2018.00145
[31] Trim CM, Byrne LJ, Trim SA. Utilisation of compounds from venoms in drug discovery. Prog Med Chem. 2021; 60:1-66. DOI: 10.1016/bs.pmch.2021.01.001
[32] Koh CY, Kini RM. From snake venom toxins to therapeutics – Cardiovascular examples. Toxicon. 2012; 59(4), 497–506. DOI: 10.1016/j.toxicon.2011.03.017
[33] Safavi-Hemami H, Brogan S, Olivera BM. Pain therapeutics from cone snail venoms: From Ziconotide to novel non-opioid pathways. Journal of Proteomics. 2019; 190, 12–20. DOI:10.1016/j.jprot.2018.05.009
[34] Bhavsar S, Mudaliar S, Cherrington AD. Evolution of Exenatide as a Diabetes Therapeutic. Current Diabetes Reviews. 2013; 9(2), 161–193. DOI:10.2174/1573399811309020007
[35] Aramadhaka LR, Prorock A, Dragulev B, Bao Y, Fox JW. Connectivity maps for biosimilar drug discovery in venoms: The case of Gila Monster Venom and the anti-diabetes drug Byetta®. Toxicon. 2013; 69, 160–167. DOI: 10.1016/j.toxicon.2013.03.018

[36] Abd El-Aziz TM, Soares AG, and Stockand JD. Snake Venoms in Drug Discovery: Valuable Therapeutic Tools for Life Saving. Toxins. 2019; 11, 564. DOI:10.3390/toxins11100564
[37] Vetter I, Davis JL, Rash LD, Anangi R, Mobli M, Alewood PF, Lewis RJ, King GF. Venomics: a new paradigm for natural producs-based drug discovery, Amino Acids. 2011; 40:15-28. DOI: 10.1007/s00726-010-0516-4
[38] Bordon KCF, Takeno Cologna C, Fornari-Baldo CE, Pinheiro-Junior EL, Cerni FA, Amorim FG, Pino Anjolette FA, Cordeiro FA, Wiezel GA, Cardoso IA, Ferreira IG, de Oliveira IS, Boldrini-Franca J, Berto Pucca M, Baldo MA, and Arantes EC. From Animal Poisons and Venoms to Medicines: Achievements, Challenges and Perspectives in Drug Discovery. Frontiers in Pharmacology. 2020; 11: 1132. DOI: 10.3389/fphar.2020.01132
[39] Coulter-Parkhill A, McClean S, Gault VA, and Irwin N. Therapeutic Potential of Peptides Derived from Animal Venoms: Current Views and Emerging Drugs for Diabetes. Clinical Medicine Insights: Endocrinology and Diabetes. 2021; 14: 1–13. DOI:10.1177/11795514211006071
[40] Doley R, Kini R. M. Protein complexes in snake venom. Cellular and molecular life sciences. 2009; 66(17), 2851–2871. DOI:10.1007/s00018-009-0050-2
[41] Roy A, Bharadvaja N. Venom-Derived Bioactive Compounds as Potential Anticancer Agents: A Review. International Journal of Peptide Research and Therapeutics. 2021; 27, 129–147. DOI:10.1007/s10989-020-10073-z
[42] Fischer T, Riedl R. Paracelsus' legacy in the faunal realm: Drugs deriving from animal toxins. Drug discovery today. 2022; 27(2), 567–575. DOI: 10.1016/j.drudis.2021.10.003
[43] Zhijian C, Feng L, Yingliang W, Xin M, Wenxin L. Genetic mechanisms of scorpion venom peptide diversification. Toxicon: official journal of the International Society on Toxinology. 2006; 47(3), 348–355. DOI: 10.1016/j.toxicon.2005.11.013
[44] Uzair B, Bint-E-Irshad S, Khan BA, Azad B, Mahmood T, Rehman MU, Braga VA. Scorpion Venom Peptides as a

Potential Source for Human Drug Candidates. Protein and peptide letters. 2018; 25(7), 702–708. DOI:10.2174/0929866525666180614114307
[45] Ojeda PG, Wang CK, Craik DJ. Chlorotoxin: Structure, activity, and potential uses in cancer therapy. Peptide Science. 2016; 106: 25-36. DOI: 10.1002/bip.22748
[46] Skinner WS, Adams ME, Quistad GB, Kataoka H, Cesarin BJ, Enderlin FE, Schooley DA. Purification and characterization of two classes of neurotoxins from the funnel web spider, Agelenopsis aperta. J Biol Chem. 1989; 264(4):2150-2155.
[47] Estrada G, Villegas E, Corzo G. Spider venoms: a rich source of acylpolyamines and peptides as new leads for CNS drugs. Nat Prod Rep. 2007 Feb;24(1):145-61. DOI:10.1039/b603083c.
[48] Escoubas P, Diochot S, Corzo G. Structure and pharmacology of spider venom neurotoxins. Biochimie. 2000; 82(9-10):893-907. DOI: 10.1016/s0300-9084(00)01166-4.
[49] Rady I, Siddiqui IA, Rady M, Mukhtar H. Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. Cancer letters. 2017; 402, 16–31. DOI: 10.1016/j.canlet.2017.05.010
[50] Möller C, Marí F. 9.3 KDa components of the injected venom of Conus purpurascens define a new five-disulfide conotoxin framework. Biopolymers. 2011; 96(2), 158–165. DOI:10.1002/bip.21406
[51] Bordon KCF, Cologna CT, Fornari-Baldo EC, Pinheiro-Ju´ nior EL, Cerni FA, Amorim FG, Anjolette FAP, Cordeiro FA, Wiezel GA, Cardoso IA, Ferreira IG, Oliveira IS, Boldrini-Franc¸a J, Pucca MB, Baldo MA and Arantes EC. From Animal Poisons and Venoms to Medicines: Achievements, Challenges and Perspectives in Drug Discovery. Front. Pharmacol. 2020; 11:1132. DOI:10.3389/fphar.2020.01132
[52] Beeton C. Targets and Therapeutic Properties. In: Kastin A, editor. Handbook of Biologically Active Peptides. 2nd. ed. Elsevier 2013. p. 473-482. DOI:10.1016/B978-0-12-385095-9.00064-6.
[53] National Center for Biotechnology Information. PubChem Compound Summary for CID 16135415,

Ziconotide. [internet]. 2023. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Ziconotide. [Accessed: 2023-04-27]
[54] US Food and Drug Administration, Drugs FDA: FDA approved drug products. [Internet]. 2021. Available from: https://www.accessdata.fda.gov/scripts/cder/daf/ [Accessed: 2023-04-24]
[55] Zhang Y. Why do we study animal toxins? Science Press Zoological Research. 2015; 36: 183-222. DOI: 10.13918/j.issn.2095-8137.2015.4.183
[56] Lewis R, Garcia M. Therapeutic potential of venom peptides. Nat Rev Drug Discov. 2003; 2:790-802. DOI:10.1038/nrd1197
[57] Nguyen T, Guo R, Chai J, Wu J, Liu, J, Chen X, Abdel-Rahman MA, Xia H, Xu X. Smp24, a Scorpion-Venom Peptide, Exhibits Potent Antitumor Effects against Hepatoma HepG2 Cells via Multi-Mechanisms In Vivo and In Vitro. Toxins. 2022; 14, 717. DOI:10.3390/toxins14100717
[58] Conde R, Zamudio FZ, Rodríguez MH, Possani LD. Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom. FEBS letters. 2000; 471, 165–168. DOI:10.1016/s0014-5793(00)01384-3
[59] Jridi I, Catacchio I, Majdoub H, Shahbazeddah D, El Ayeb M, Frassanito MA, Ribatti D, Vacca A, Borchani L. Hemilipin, a novel Hemiscorpius lepturus venom heterodimeric phospholipase A2, which inhibits angiogenesis in vitro and in vivo. Toxicon, official journal of the International Society on Toxinology. 2015; 105, 34–44. DOI: 10.1016/j.toxicon.2015.08.022
[60] Redd MA, Scheuer SE, Saez N J, Yoshikawa Y, Chiu H, Gao L, Hicks M, Villanueva JE, Joshi, Chow, CB, Cuellar-Partida G, Peart JNJ, Hoe LES, Chen , Sun, Y, Suen JY, Hatch RL, Rollo B, Xia D, Alzubaidi MAH, , Maljevic S, Quaife-Ryan GA, Hudson JE, Porrello ER, White MY, Cordwell SJ, Fraser JF, Petrou S, Reichelt ME, Thomas WG, King GF, Macdonald PS, Palpant NJ. Therapeutic Inhibition of Acid-Sensing Ion Channel 1a Recovers Heart Function After Ischemia–Reperfusion Injury. Circulation, 2021; 144, 947–960. DOI: 10.1161/circulationaha.121.054360

[61] Oldrati V, Bianchi E, Stöcklin R. Spider Venom Components as Drug Candidates. In: Nentwig W. ed. Spider Ecophysiology. Springe; Berlin, Heidelberg; 2013. p. 491-503. DOI:10.1007/978-3-642-33989-9_37
[62] US National Library of Medicine (2021). ClinicalTrials.gov [internet]. 2023. Available from: https://clinicaltrials.gov/ [Accessed 2023-04-20].

