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CONTENTS

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BIOTECH STUDIES

RESEARCH PAPER

Assessment of some bread wheat (*Triticum aestivum* L.) genotypes for drought tolerance using SSR and ISSR markers

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Abstract

As a result of the rapid increase in the world population, the need for wheat, which is one of the main nutrition in the human diet, is also rapidly increases. However, due to yield losses caused by abiotic stress factors such as drought, wheat production is not sufficient. Therefore, genetic characterization studies performed on wheat genotypes in terms of drought tolerance are important. In this study, genetic characterization of wheat genotypes regardingdrought tolerance was carried out by using molecular markers associated with drought-tolerance genes. For this purpose, 14 polymorphic markers were used to be able to distinguish between the control groups. Genetic characterization of 27 bread wheat genotypes by using eight ISSR markers revealed a polymorphism rate of 75.8%, and the mean PIC was calculated as 0.55. Based on the results of the genetic characterization performed with six SSR markers, the mean PIC value was 0.77, the mean He was 0.79, and the mean allele number was 6.7. In this study, the characterization of drought-tolerant and sensitive genotypes was carried out, and the potentials of genotypes for breeding studies were revealed. This study also indicates that used SSRs and ISSRs markers are useful in marker-assisted breeding about drought tolerance.

Introduction

The global cereal production is estimated at 2799 million tons in FAO's 2021 forecast. Nowadays, wheat is one of the most important cereal crops cultivated worldwide with a production of approximately 777 million tons (FAOSTAT, 2021). As the world population increases, the need for wheat also increases day by day rapidly. However, current wheat production is not enough mainly due to biotic and abiotic stresses. The main abiotic stress factor causing the greatest damage in wheat production worldwide is drought (Mohammadi & Abdulahi, 2018). Drought caused by changes in climatic variables (i.e., temperature and precipitation) as a result of global warming directly affects agricultural activities such as crop growth, annual crop yield, and crop production (Ates-Sonmezoglu & Terzi, 2018; Mickelbartet al., 2015).

In order to minimize the effects of drought, genetic characterization studies are important in terms of examination of drought tolerance in wheat and determination of drought-tolerant genotypes that have potential to be used in breeding studies. Therefore, scientists have been working to develop wheat genotypes that are tolerant to drought conditions and have high yield for a long time. It is necessary to say that determining genetic diversity is very important for current and future breeding studies in terms of providing preliminary information.

Drought tolerance is a quantitative trait, and the genetic determination of it is complex. Developing superior genotypes through conventional breeding is one of the necessary steps to understand the genetic basis of drought tolerance in wheat (Khaled et al., 2018). Besides morphological analyses, biochemical and molecular techniques have also gained importance in

the determination and evaluation of genetic diversity (Hassan et al., 2020; Iqbal, 2019). Morphological studies are insufficient and not reliable because they are affected by environmental factors during the investigation of drought tolerance. Therefore, drought tolerance studies in plants should be supported by molecular characterization studies. Molecular characterization studies will increase the use of cultivars determined to be tolerant in drought-related breeding programs, and more efficient and faster results will be obtained.

The determination of genetic diversity is also very important for breeding studies. To investigate the genetic diversity of genetic resources and populations, PCR-based markers developed based on differences such as the amount of polymorphism, reproducibility, information content, and the cost are used. Of these markers, Simple Sequence Repeat (SSR) and Inter-Simple Sequence Repeat (ISSR) based markers are used effectively in DNA fingerprinting, linkage analyses, mapping studies, and genetic diversity studies of wheat genotypes (Ates-Sonmezoglu & Terzi, 2018; Khaled et al., 2015). Compared to other markers, ISSR markers are effective DNA markers in terms of revealing genotype identification, genetic-mapping and genetic diversity of wheat due to their superior advantages, such as being highly polymorphic, repeatable, and less plant material requirement (Khaled et al., 2015; Kyrienko et al., 2018). Although they are similar to Random Amplified Polymorphic DNA (RAPD) markers, ISSR markers are more specific and reproducible than RAPD markers due to their longer oligonucleotide primers and higher annealing temperature (Isshiki et al., 2008). In addition, they are fast, easy to apply, reliable and highly informative.

On the other hand, microsatellite markers, also known as SSRs, are one of the most suitable molecular markers for genetic characterization studies of wheat due to their features such as chromosome specificity, locus specificity, co-dominant structures, high polymorphism rate, and wide distribution throughout the wheat genome (Prasad et al., 2009; Yildirim et al., 2009; Dodig et al., 2010; Yildirim et al., 2011; Ates-Sonmezoglu et al., 2012). Thanks to their mentioned advantages, SSR and ISSR markers are successfully used in drought tolerance studies of wheat.

More specifically, Gupta et al. (2017) used 18 SSR markers to understand the genetic mechanism of drought tolerance in wheat cultivation. In another study, Yadav et al. (2018) used 15 ISSR markers for six drought-tolerant and six drought-sensitive wheat varieties, and 14 of markers gave reproducible band results. As a result of the study, it was stated that the genetic diversity of drought-tolerant and drought-sensitive wheat genotypes was determined reliably and successfully by using molecular markers.

In the current study, SSR and ISSR polymorphic markers associated with drought tolerance developed by different researchers were used for the molecular characterization. In the context of the study, 27 bread wheat genotypes were tested through six SSR and eight ISSR markers to evaluate their responses to drought stress variations.

Materials and Methods

Plant materials

In this study, 27 bread wheat genotypes were used as plant material for the characterization of the drought tolerance. Selected four bread wheat varieties, including two drought-tolerant (Mufitbey and Gun 91) and two drought-sensitive (Bezostaja and Aldane), were used as control genotypes. Control varieties were obtained from Tokat Gaziosmanpasa University and the bread wheat genotypes used in the study were collected from the provinces of Amasya, Corum, and Tokat (Table 1).

Table 1. Bread wheat genotypes used in the research

No	Developing Institution	Variety / Line Name	No	Developing Institution	Variety / Line Name
1	TTAE	Aldane	15	ETAEM	TR 63501
2	GKTAE	Bezostaja	16	ETAEM	TR 63575
3	GKTAE	Mufitbey	17	ETAEM	TR 63581
4	TAGEM	Gun 91	18	TAGEM	TGB 000521
5	TIGTHM	Dimenit	19	TAGEM	TGB 000526
6	TIGTHM	Aksunteri	20	TAGEM	TGB 000534
7	TIGTHM	Calibasiran	21	TAGEM	TGB 000543
8	TIGTHM	Ormece	22	TAGEM	TGB 003232
9	TIGTHM	Cambugdayi	23	TAGEM	TGB 003246
10	TIGTHM	Zerun	24	TAGEM	TGB 003247
11	ETAEM	TR 37373	25	TAGEM	TGB 003248
12	ETAEM	TR 44433	26	TAGEM	TGB 003249
13	ETAEM	TR 48371	27	BDUTAE	Dagdas94
14	ETAEM	TR 63497			

BDUTAE: Bahri Dagdas International Agricultural Research Institute
TIGTH: Tokat Directorate of Provincial Agriculture and Forestry
GKTAE: Transitional Zone Agricultural Research Institute
ETAE: Aegean Agricultural Research Institute
TAGEM: Directorate General of Agricultural Research and Policies
TTAE: Thrace Agricultural Research Institute

Molecular screening

For the molecular characterization of wheat genotypes, six SSR and eight ISSR primers that were previously used in drought studies conducted by different researchers were used (Table 2).

A total of 45 SSR and ISSR primers were pre-screened for control genotypes and selected 15 most polymorphic primers were used for molecular characterization. Based on Doyle & Doyle (1990), DNA was extracted from the leaf of bread wheat genotypes by some modifications. PCR reactions were carried out in a 40 µL mixture containing 50–60 ng of genomic DNA, 0.25 µM of each primer, 0.2 µM dNTP mix, 2.5 µM $MgCl_2$, 10x PCR buffer, and 0.1 units of *Taq* DNA polymerase. PCR cycles were performed an initial denaturation step of 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 45 sec at 50-62°C (depending upon the annealing temperature of the primers), extension for 1 min at 72°C, and with a final extension step for 5 min at 72°C. The PCR products were resolved on 2% agarose gels (Figure 1a, 1b). Electrophoresis was performed at constant power of 90 V for 3-4 h. In addition, 100 bp ladder was used for SSR markers, and 1000 bp ladder was used for ISSR markers.

The markers were scored for the presence (1) or absence (0) of amplified bands. Comparison of genotypes and examination of genetic relationships between genotypes were done by the help of Numerical Taxonomy and Multivariate Analysis System software (NTSYSpc, version 2.1) (Rohlf, 1998). To be able to obtain a dendrogram of wheat genotypes, the DendroUPGMA (D-UPGMA) program (http://genomes.urv.cat/UPGMA) was used. The genetic similarity index of wheat genotypes was calculated according to Jaccard (1908). SSR and ISSR marker polymorphism rates were determined using Polymorphism Information Content (PIC) values, which were calculated based on the following formula:

PIC = 1 − ∑ Pij^2'where Pi is the frequency of the i^{th} allele (Anderson et al., 1993). The heterozygosity (He) was calculated according to Liu and Wu (1998).

Results and Discussion

In the study, eight ISSR (UBC 811, UBC 815, UBC 826 UBC 834, UBC 835, UBC 852, UBC 857 and ISSR 827) and six SSR (Xgwm 11, Xbarc 101, Xgwm 165, Xgwm 325, Xgwm 603 and Xgwm 99) markers, which showed polymorphism among control genotypes, were used to determine genetic diversity in 27 bread wheat genotypes in terms of drought tolerance (Figure 1a, 1b).

According to molecular screening (Table 3), a total of 40 alleles were determined by six SSR primers of bread wheat genotypes. The number of alleles in PCR amplification products obtained using SSR primers differed according to the primers and was determined to be between six-eight alleles. The average number of alleles was 6.7. When the allele numbers of the primers were analyzed, it was determined that the Xgwm 11 primer gave the highest allele number with eight alleles, followed by Xbarc 101 with seven alleles. PIC values for SSR primers ranged from 0.74 to 0.81. While the highest PIC value was observed in Xgwm 11 primer with 0.81, the lowest PIC was determined in the Xgwm 603 primer with a value of 0.74 (Table 3). The mean PIC value for all SSR primers was calculated as 0.77. In terms of heterozygosity values, whereas the highest He value was determined in Xgwm 11 primer with 0.84, the lowest He value was determined in Xgwm 603 primer with 0.75. In this study, the mean He value was determined as 0.79 (Table 3).

Table 2. SSR and ISSR primers were used in molecular identification

Primers	Primer Sequence (5' →3')	References
SSR Primers		
Xgwm 99	F-5'AAGATGGACGTATGCATCACA3' R-5' GCCATATTTGATGACGCATA 3'	(Röder et al., 1998)
Xgwm 11	F-5 GGATAGTCAGACAATTCTTGTG 3' R-5' GTGAATTGTGTCTTGTATGCTTCC 3'	(Yang et al., 2002)
Xbarc 101	F-5' GCTCCTCTCACGATCACGCAAAG 3' R-5' GCGAGTCGATCACACTATGAGCCAATG 3'	(Kumar et al., 2018)
Xgwm 165	F-5' TGCAGTGGTCAGATGTTTCC 3' R-5' CTTTTCTTTCAGATTGCGCC 3'	(Iqbal, 2019)
Xgwm 325	F-5' TTTCTTCTGTCGTTCTCTTCCC 3' R-5' TTTTTACGCGTCAACGACG 3'	(Mason et al., 2010)
Xgwm 603	F-5'ACAAACGGTGACAATGCAAGGA3' R-5' CGCCTCTCTCGTAAGCCTCAAC 3'	(Somers et al., 2004)
ISSR Primers		
UBC 811	5'GAGAGAGAGAGAGAGAC 3'	(Khaled et al., 2015)
UBC 815	5'CTCTCTCTCTCTCTCTG 3'	(Khaled et al., 2015)
UBC 826	5' ACACACACACACACACC3'	(Sen et al., 2017)
UBC 834	5' AGAGAGAGAGAGAGAGYT 3'	(Khaled et al., 2015)
UBC 835	5' AGAGAGAGAGAGAGAGY*C 3'	(Sen et al., 2017)
UBC 852	5' TCTCTCTCTCTCTCRA C3'	(Sen et al., 2017)
UBC 857	5' ACACACAC CACACACYG 3'	(Sen et al., 2017)
ISSR 827	5'ACACACACACACACACG 3'	(Barakat et al., 2010)

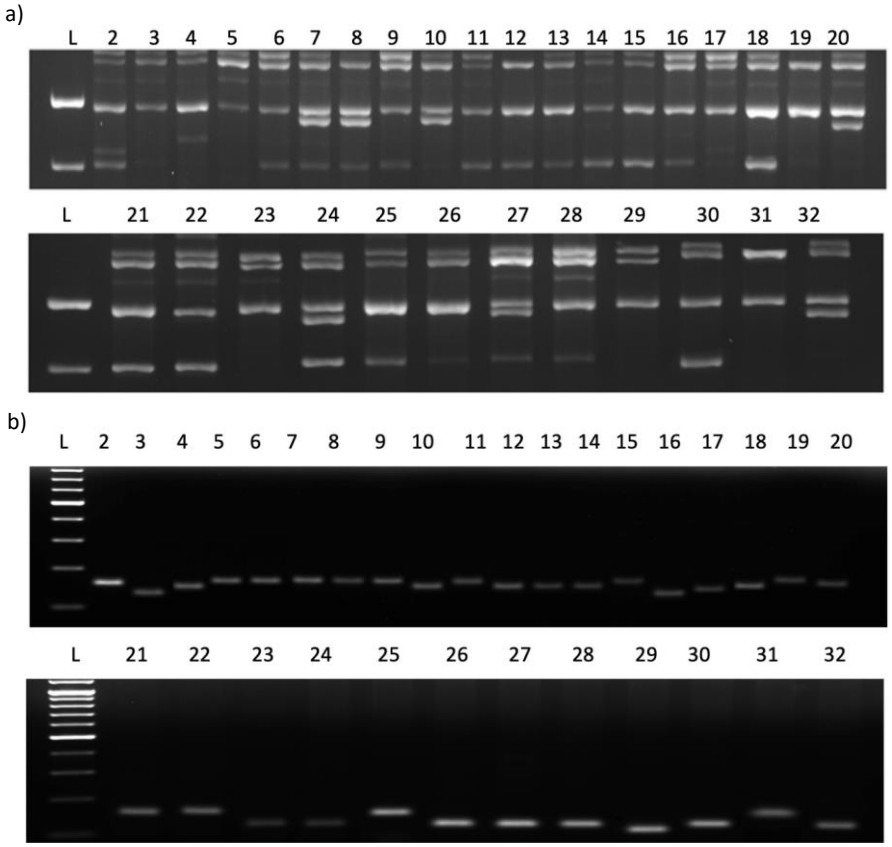

Figure 1. Gel image for molecular characterization of genotypes with a) UBC 811 ISSR primer (L: 1000 bp), b) Xgwm 165 SSR primer (L: 100 bp).
Control Genotypes; Drought-sensitive: Aldane (17, 29) and Bezostaja (18, 30) Drought-tolerant: Mufitbey (19, 31) and Gun 91 (20, 32)

This indicates the presence of significant genetic variation (Mkhabela et al., 2020). For six SSRs, all with more than six alleles, indicated PIC higher than 0.75. Many researchers have suggested that an objective evaluation of genetic diversity in wheat genotypes should be reflected by both PIC values and the number of alleles per locus in combination as in this study (Hao et al., 2006; Hai et al., 2007; Dodig et al., 2010). Another study was conducted to determine the genetic diversity among seven bread wheat genotypes, and phylogenetic relationships of wheat genotypes by using SSR and RAPD markers (Al-Tamimi & Al-Janabi, 2019). Among the DNA markers used, the highest PIC value was produced by SSR marker, and in the genetic diversity study on wheat genotypes, the SSRs were found to be quite informative. It was also stated that such studies would be the basis for the breeders in terms of selecting the appropriate parental genotypes to be able to achieve the highest desired heterosis in wheat populations.

A total of 49 bands were observed in 27 bread wheat genotypes by the alleles obtained from eight ISSR markers, and 25 of these bands were polymorphic (Table 4).

The average number of polymorphic bands was 2.9. While the highest polymorphism was given by UBC 811 and UBC 835 primers with 100%, the lowest polymorphism percentage was obtained by UBC 826 primer with 50%. PIC values for ISSR primers ranged from 0.13 to 0.82 (Table 4). Whereas the highest PIC value (0.82) was determined in UBC 857 primer, the lowest PIC value (0.13) was determined in UBC 826 ISSR primer (Table 4). In another study, the characterization of drought tolerance in three wheat varieties, including sensitive and tolerant, was carried out by using 14 ISSR markers (Eid, 2018). In the study, the number of alleles per locus ranged from 2 to 3, while the PIC value ranged from an average of 0.34 to 0.59. Similar to our study results, they obtained two main clusters (drought-

Table 3. SSR primers, major allele number, allele number, band sizes, heterozygosity ratio (He) and PIC values

Primer Name	Number of Major Alleles	Number of Alleles	Heterozygosity Ratio (He)	PIC	Band Sizes (bp)
Xgwm 11	0.24	8	0.84	0.81	165-195
Xgwm 99	0.36	6	0.78	0.75	115-170
Xbarc101	0.30	7	0.80	0.79	165-200
Xgwm 165	0.31	7	0.80	0.77	115-170
Xgwm 325	0.29	6	0.77	0.76	135-150
Xgwm 603	0.32	6	0.75	0.74	100-130
Total	1.82	40	4.74	4.62	-
Average	0.30	6.7	0.79	0.77	-

Table 4. ISSR primers, total number of bands, percentage of polymorphic bands (P%), PIC values and band sizes

Primer Name	Number of Bands	Number of Polymorphic Bands	P%	PIC	Band Sizes (bp)
UBC 811	5	5	100	0.55	955-2000
UBC 815	3	2	66.7	0.28	650-951
UBC 826	4	2	50.0	0.13	435-750
UBC 834	5	4	80.0	0.75	700-1250
UBC 835	3	3	100	0.50	630-1500
UBC 852	5	3	60.0	0.62	500-1500
UBC 857	4	3	75.0	0.82	600-1000
ISSR 827	4	3	75.0	0.80	500-750
Total	333	25	606.7	4.42	435-2000
Average	4.1	2.9	75.8	0.55	-

tolerant and drought-sensitive genotypes) as a result of the cluster analysis. They emphasized that ISSR markers were a valuable tool for studying genetic diversity in wheat varieties.

In the grouping of wheat genotypes, evaluation of dendrogram and genetic similarity coefficients together is a more accurate approach. The dendrograms are given in Figure 2-4, and genetic similarity values are given in Table S1-S3 Bread wheat genotypes were divided into two main groups based on the SSR markers (Figure 2). While the wheat lines Aldane and Bezostaja (drought-sensitive control varieties), and Dagdas94 were placed in subgroup I, all other genotypes were included in subgroup II with branching. While subgroup II showed branching within itself, the drought-tolerant control varieties (Gun 91 and Mufitbey) were included in the same subgroup.

Based on the ISSR markers (Figure 3), wheat genotypes were divided into two main groups. While the wheat line TGB 003232 was placed in subgroup I, all other genotypes were included in subgroup II with branching. Subgroup II showed branching within itself, while the drought-sensitive control varieties (Aldane and Bezostaja) were included in the subgroup IIa and the drought-tolerant control varieties (Gun 91 and Mufitbey) were included in the subgroup IIb. Ahmad et al. (2019) also reported high level of genetic diversity in the wheat genotypes and grouped the wheat genotypes in four clusters based on the dendrogram results obtained using similar ISSR markers.

The dendrogram generated based on SSR and ISSR markers identified control genotypes as genetically distinct. The use of them in breeding studies is recommended to increase the probability of additive genes that increase yield (Mkhabela et al., 2020).

Based on the dendrogram of combined ISSR and SSR markers (Figure 4), wheat genotypes were divided into two main groups. While the drought-sensitive control varieties Aldane and Bezostaja were placed was placed in same subgroup, the drought-tolerant wheat varieties Mufitbey and Gun 91 were in the other same subgroup.

When the dendrogram obtained by UPGMA analysis and genetic similarity coefficients were evaluated together, reliable results were obtained. According to the data obtained by combined ISSR and SSR markers, drought-sensitive wheat varieties Aldane and Bezostaja gave the high (0.87) genetic similarity

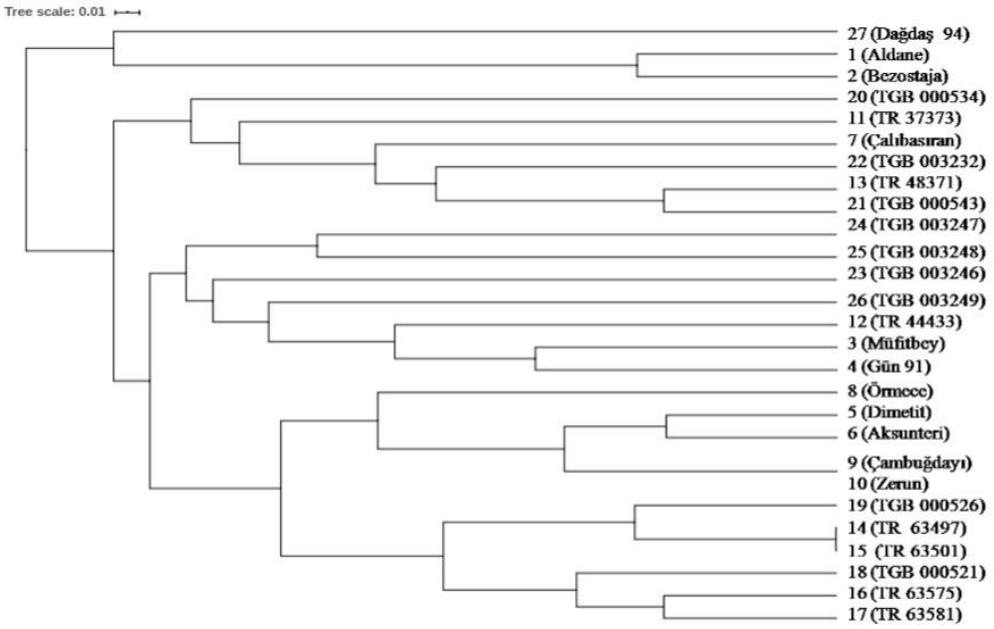

Figure 2. Dendrogram showing the genetic relationship among bread wheat genotypes based on SSR data using UPGMA.
Control Genotypes: Drought-sensitive: Aldane (1) and Bezostaja (2); Drought-tolerant: Mufitbey (3) and Gun 91 (4)

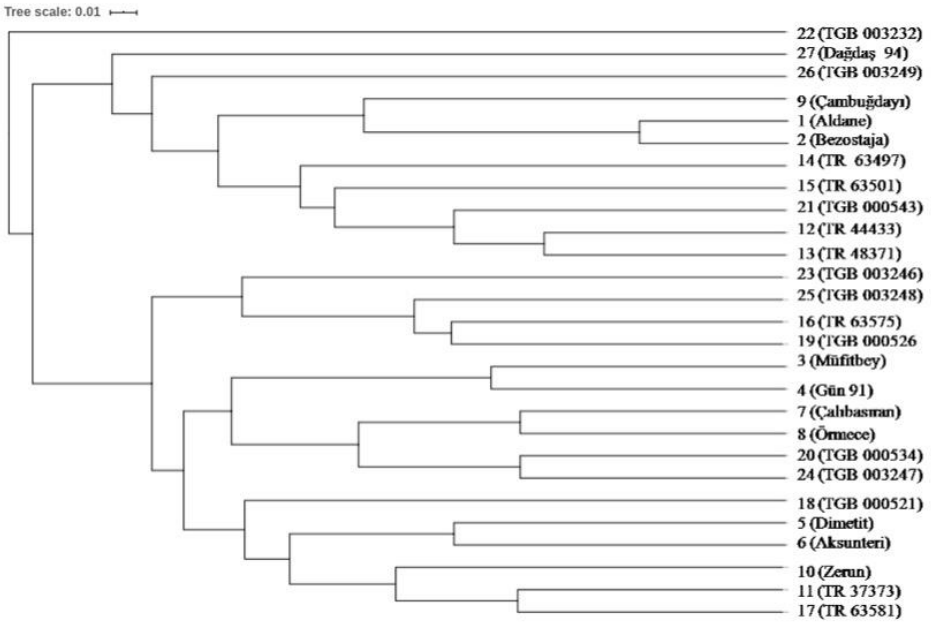

Figure 3. Dendrogram showing the genetic relationship among bread wheat genotypes based on ISSR data using UPGMA.
Control Genotypes: Drought-sensitive: Aldane (1) and Bezostaja (2); Drought-tolerant: Mufitbey (3) and Gun 91 (4)

coefficient (Table S3). Based on the data obtained by SSR markers, drought-tolerant wheat varieties Mufitbey and Gun 91 gave the high (0.75) genetic similarity coefficient (Table S1). The closest genotypes of drought-tolerant wheat variety Mufitbey were Gun 91 and TGB 0003526, while the most distant genotype was Calibasiran. The most distant genotype of drought-tolerant wheat variety Gun91 was line TGB 003232. The closest genotype to the drought-sensitive control Aldane was Bezostaja. On the other hand, the most distant genotypes to the Aldane were TR 37373 and Gun 91. Here, it can be said that cluster analysis is a useful tool in the determination of genotypes based on drought tolerance.

As a result of molecular screenings performed with ISSR markers, drought-sensitive wheat varieties Mufitbey and Gun 91 showed the highest (0.88) genetic similarity (Table S2). Also, the closest genotypes of drought-tolerant wheat variety Gun 91 were Mufitbey and TR 63581, while the most distant genotypes of the Gun 91 variety were TGB 003248 and TGB 003249 wheat genotypes. In addition, the closest genotype to the Aldane, which was a drought-sensitive control type, was TGB 003249. Yadav et al. (2018) used 15 ISSR markers for six drought-tolerant and six drought-sensitive wheat varieties, and 14 of markers gave reproducible band results. As a result of the study, it was stated that the

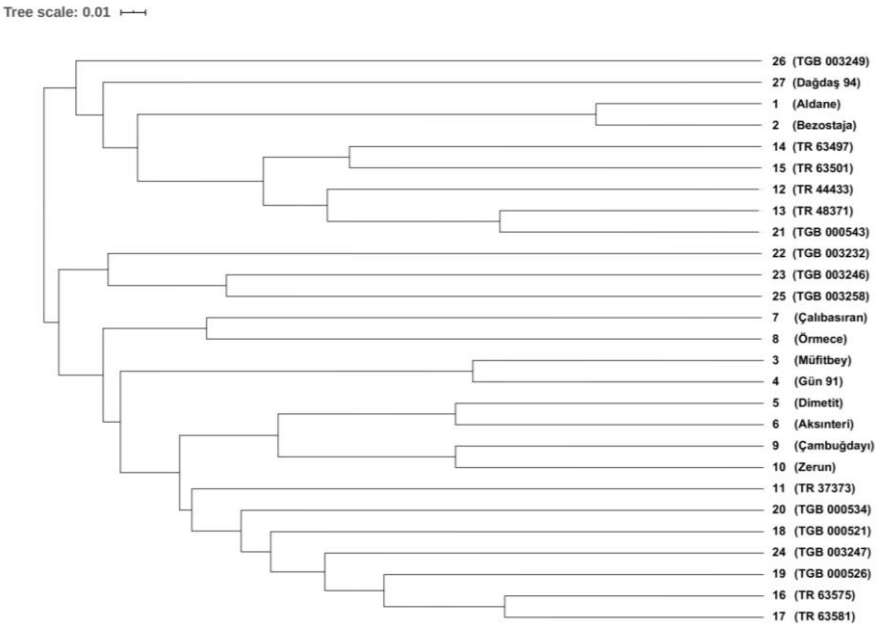

Figure 4. UPGMA dendrogram of combined ISSR and SSR markers for bread wheat genotypes.
Control Genotypes: Drought-sensitive: Aldane (1) and Bezostaja (2); Drought-tolerant: Mufitbey (3) and Gun 91 (4)

genetic diversity of drought-tolerant and drought-sensitive wheat genotypes was determined reliably and successfully by using molecular markers. Tungalag et al. (2018) used 17 ISSR markers to define variants in six Mongolian local wheat varieties. They reported that ISSRs could be used to determine genetic relationships and the fact that these markers did not require target sequence information was an advantage.

Conclusion

In the current study, genetic variations of 27 bread wheat (*Triticum aestivum* L.) genotypes were determined by using 14 SSR and ISSR markers associated with drought-related gene regions. As a result, the mean PIC values were 0.55 for ISSRs and 0.77 for SSRs. Also, all SSR primers gave PIC values higher than 74%. In conclusion, it can be said that the ISSR and SSR markers can be used successfully in genetic diversity, marker-assisted selection, and breeding studies related to drought tolerance in wheat.

When dendrogram results and genetic similarity coefficients were evaluated together, it can be stated that the SSRs and ISSRs used in the study are quite informative for genetic characterization studies related to drought resistance. In this study, based on the results of genetic similarity coefficient and dendrogram distribution, a preliminary data was provided for the use of the examined genotypes in later breeding studies. High genetic diversity was observed among the wheat genotypes, which allowed the identification and selection of drought-related genotypes. The identified genotypes are useful genetic resources for drought-related breeding studies to be conducted on wheat.

Author Contributions

OAS: conceptualization, supervision and designed; EC: experiments, investigation and analyzed the data BTA: writing, review and editing. All contributing authors have read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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References

Ahmad, M. Q., Hassan, M. Qayyum, A., Saleem, M. A., Malik, W., Noor E., & Ul-Allah, S. (2019). Characterization of synthetic wheat germplasm using morphological and molecular markers. *International Journal of Agriculture and Biology*, *22*, 131-138. https://doi.org/10.17957/IJAB/15.1042

Al-Tamimi, A. J. T., & Al-Janabi, A. S. (2019). Genetic diversity among bread wheat genotypes using RAPD and SSR markers. *Sabrao Journal of Breeding and Genetics*, *51*(3).

Anderson, G. A., Churchill, G. A., Autrique, J. E., Tanksley, S. D., & Sorrells, M. E. (1993). Optimizing parental selection for genetic linkage maps. *Genome*, *36*(1), 181-186. https://doi.org/10.1139/g93-024

Ates-Sonmezoglu, Ö. A., Bozmaz, B., Yildirim, A., Kandemir, N., & Aydin, N. (2012). Genetic characterization of Turkish bread wheat landraces based on microsatellite markers and morphological characters. *Turkish Journal of Biology*, *36*(5), 589-597. https://doi.org/10.3906/biy-1202-43

Ates-Sonmezoglu, Ö., & Terzi, B. (2018). Characterization of some bread wheat genotypes using molecular markers for drought tolerance. *Physiology and Molecular Biology of Plants*, *24*(1), 159-166. https://doi.org/10.1007/s12298-017-0492-1

Barakat, M., Al-Doss, A. A., Moustafa, K., & Ibrahim, E. (2010). Morphological and molecular characterization of Saudi wheat genotypes under drought stress. *Journal of Food, Agriculture & Environment*, *8*(1), 220-228.

Ben, H. L. (1998). Statistical genomics: linkage, mapping and QTL analysis. In: Statistical Genomics. CRC Press.

Dodig, D., Zorić, M., Kobiljski, B., Šurlan-Momirović, G., & Quarrie, S. A. (2010). Assessing drought tolerance and regional patterns of genetic diversity among spring and winter bread wheat using simple sequence repeats and phenotypic data. *Crop and Pasture Science*, *61*(10), 812-824. https://doi.org/10.1071/CP10001

Doyle, J. J. (1990). A rapid total DNA preparation procedure for fresh plant tissue. *Focus*, *12*, 13-15.

Eid, M. (2018). Validation of SSR molecular markers linked to drought tolerant in some wheat cultivars. *Journal of Plant Breeding and Genetics*, *6*(3), 95-109. https://doi.org/10.33687/pbg.006.03.2739

FAOSTAT, (2021). Food and Agriculture Organization of the United Nations Statistics Division. http://www.fao.org/worldfoodsituation/csdb/en World Food Situation. (Accessed 12 July 2021)

Gupta, P. K., Balyan, H. S., & Gahlaut, V. (2017). QTL analysis for drought tolerance in wheat: present status and future possibilities. *Agronomy*, *7*(1), 5. https://doi.org/10.3390/agronomy7010005

Hai, L., Wagner, C., & Friedt, W. (2007). Quantitative structure analysis of genetic diversity among spring bread wheats (*Triticum aestivum* L.) from different geographical regions. *Genetica*, *130*(3), 213-225. https://doi.org/10.1007/s10709-006-9008-6

Hao, C. Y., Zhang, X. Y., Wang, L. F., Dong, Y. S., Shang, X. W., & Jia, J. Z. (2006). Genetic diversity and core collection evaluations in common wheat germplasm from the Northwestern Spring Wheat Region in China. *Molecular Breeding*, *17*(1), 69-77. https://doi.org/10.1007/s11032-005-2453-6

Hassan, R., Waheed, M. Q., Shokat, S., Rehman-Arif, M. A., Tariq, R., Arif, M., & Arif, A. (2020). Estimation of genomic diversity using sequence related amplified polymorphism (SRAP) markers in a mini core collection of wheat germplasm from Pakistan. *Cereal Research Communications*, *48*(1), 33-40. https://doi.org/10.1007/s42976-019-00006-y

Iqbal, J. (2019). Morphological, physiological and molecular markers for the adaptation of wheat in drought condition. *Asian Journal of Biotechnology and Genetic Engineering*, *2*(1), 1-13. https://doi.org/10.9734/AJBGE/2019/46253

Isshiki, S., Iwata, N., & Khan, M. M. R. (2008). ISSR variations in eggplant (*Solanum melongena* L.) and related Solanum species. *Scientia Horticulturae*, *117*(3), 186-190. https://doi.org/10.1016/j.scienta.2008.04.003

Jaccard, P. (1908). Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat.*, *44*, 223-270.

Khaled, A. G. A., Motawea, M. H., & Said, A. A. (2015). Identification of ISSR and RAPD markers linked to yield traits in bread wheat under normal and drought conditions. *Journal of Genetic Engineering and Biotechnology*, *13*(2), 243-250. https://doi.org/10.1016/j.jgeb.2015.05.001

Kumar, S., Kumari, J., Bansal, R., Kuri, B. R., Upadhyay, D., Srivastava, A., Rana, B., Yadav, M. K., Sengar, R. S., Singh, A. K., & Singh, R. (2018). Multi-environmental evaluation of wheat genotypes for drought tolerance. *Indian J. Genet*, *78*(1), 26-35. https://doi.org/10.5958/0975-6906.2018.00004.4

Kyrienko, A. V., Rozhkov, R. V., Parii, M. F., & Symonenko, Y. V. (2018). Genotyping of Triticum ssp. hexaploid species samples with ISSR-markers. *Cytology and Genetics*, *52*(4), 276-282. https://doi.org/10.3103/S0095452718040047

Liu, X. C., Wu, J. L. (1998). SSR heterogenic patterns of parents for marking and predicting heterosis in rice breeding. *Molecular Breeding*, 4**,** 263–268. https://doi.org/10.1023/A:1009645908957

Mason, R. E., Mondal, S., Beecher, F. W., Pacheco, A., Jampala, B., Ibrahim, A. M., & Hays, D. B. (2010). QTL associated with heat susceptibility index in wheat (*Triticum aestivum* L.) under short-term reproductive stage heat stress. *Euphytica*, *174*(3), 423-436. https://doi.org/10.1007/s10681-010-0151-x

Mkhabela, S. S., Shimelis, H., & Mashilo, J. (2020). Genetic differentiation of selected drought and heat tolerant wheat genotypes using simple sequence repeat markers and agronomic traits. *South African Journal of Plant and Soil*, *37*(3), 211-219. https://doi.org/10.1080/02571862.2020.1718787

Mickelbart, M. V., Hasegawa, P. M., & Bailey-Serres, J. (2015). Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nature Reviews Genetics*, *16*(4), 237-251. https://doi.org/10.1038/nrg3901

Mohammadi, R., & Abdulahi, A. (2017). Evaluation of durum wheat genotypes based on drought tolerance indices under different levels of drought stress. *Journal of Agricultural Sciences, Belgrad*e, *62*(1), 1-14. https://doi.org/10.2298/JAS1701001M

Prasad, B., Babar, M. A., Xu, X. Y., Bai, G. H., & Klatt, A. R. (2009). Genetic diversity in the US hard red winter wheat cultivars as revealed by microsatellite markers. *Crop and Pasture Science*, *60*(1), 16-24. https://doi.org/10.1071/CP08052

Rohlf, F. J. (1998). NTSYS-pc, numerical taxonomy and multivariate system. version 2.0. Exet Software, New York.

Röder, M. S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M. H., Leroy, P., & Ganal, M. W. (1998). A microsatellite map of wheat. *Genetics*, *149*(4), 2007-2023. https://doi.org/10.1093/genetics/149.4.2007

Tungalag, M., Ariungerel, M., Otgonbayar, B., & Ya, M. (2018). Varietal identification study of six wheat varieties using ISSR markers. *Mongolian Journal of Agricultural Sciences*, *23*(01), 14-17. https://doi.org/10.5564/mjas.v23i01.1014

Sen, A., Ozturk, I., Yaycili, O., & Alikamanoglu, S. (2017). Drought tolerance in irradiated wheat mutants studied by genetic and biochemical markers. *Journal of Plant Growth Regulation*, *36*(3), 669-679. https://doi.org/10.1007/s00344-017-9668-8

Somers, D. J., Isaac, P., & Edwards, K. (2004). A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics*, *109*(6), 1105-1114. https://doi.org/10.1007/s00122-004-1740-7

Yadav, V., Kumar, P., & Goyal, M. (2018). Evaluation of genetic diversity in drought tolerant and sensitive varieties of wheat using ISSR markers. *Electronic Journal of Plant Breeding*, *9*(1), 146-153. https://doi.org/10.5958/0975-928X.2018.00017.0

Yang, J., Sears, R. G., Gill, B. S., & Paulsen, G. M. (2002). Quantitative and molecular characterization of heat tolerance in hexaploid wheat. *Euphytica*, *126*(2), 275-282. https://doi.org/10.1023/A:1016350509689

Yildirim, A., Sönmezoğlu, O. A., Gökmen, S., Kandemir, N., & Aydin, N. (2011). Determination of genetic diversity among Turkish durum wheat landraces by microsatellites. *African Journal of Biotechnology*, *10*(19), 3915-3920. https://doi.org/10.5897/AJB10.2240

Yıldırım, A., Kandemir, N., Sönmezoğlu, Ö. A., & Güleç, T. E. (2009). Transferability of microsatellite markers among cool season cereals. *Biotechnology & Biotechnological Equipment*, *23*(3), 1299-1302. https://doi.org/10.1080/13102818.2009.10817657

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REVIEW PAPER

In food safety control overview of using Real-Time PCR

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Abstract

The identity-determining importance of nucleic acids in living beings is a guide for reaching the desired information about food in quality control analyzes. With these goals, the popularity of Real-time PCR (Polymerase Chain Reaction) methods, which are one of the nucleic acid-based methods, is rapidly increasing due to their high reproducibility, precision and fast result production. It is thought that informative studies about the analysis using the device will provide a basis for researches on the subject. This review contains information about the studies conducted on Real-Time PCR analysis used to detect imitation/adulteration and cheating in foods. General descriptions about the operation of the Real-Time PCR methods are given. The quality control analyzes in which the method was used were classified and explanations were made about each analysis area and examples from the studies in the literature were given.

Introduction

Food safety is a concept that must be followed from the first steps of the production process to reaching the end consumer. Whether the food has its characteristics, the production method, the reliability of the place where it is produced, regional differences, and the composition of the food should be known in terms of food safety. In parallel with the development of food production technologies, new techniques continue to develop in manipulations such as cheating, imitation, and adulteration. It is important that such deceptive practices can be detected before the food reaches the end consumer. Due to the variety of these deceptive applications, the analysis methods to be used in their detection show similar diversity. Spectroscopic, isotopic, chromatographic, immunological, electrophoretic, electronic, and nucleic acid-based analysis can be shown as examples of these methods (Yeşilören & Ekşi, 2014).

As a result of advances in molecular biology and biotechnology, Polymerase Chain Reaction (PCR) and fast and quantitative Real-Time PCR techniques have been used in food quality control analysis (Kesmen et al., 2017; Böhme et al., 2019).

PCR and real-time PCR

Mullis et al. (1986) described the polymerase chain reaction (PCR) in 1986 and won the Nobel Prize in Chemistry in 1990. PCR methods have been used in many areas from clinical analysis to environmental analysis, from criminal tests to food analysis (The Nobel Prize Web Site, 1993; Mullis et al., 1986). The high sensitivity and reliability in the Covid-19 (Corona Virus) epidemic that started to spread from Wuhan City of China in late 2019, the method described by Corman et al. (2020) made the Real-Time PCR method very popular.

The purpose of PCR reactions is to perform the synthesis of Deoxyribonucleic acid (DNA) *in vitro*, which takes place *in vivo*. The reaction takes place in the

presence of single or double DNA strands and oligonucleotide primers and deoxyribonucleoside triphosphates (dNTP) in the presence of a heat-resistant polymerase enzyme and magnesium ions. During the reaction, in reactions where the Taq DNA polymerase enzyme is used, the temperature is raised and lowered between high temperature (95°C) to separate the double-stranded DNA strand into a single strand, and then between the binding temperature (72°C) for the primers in the environment to complete the chain with dNTPs (Kubista et al., 2006; Holland et al., 1991).

At high temperature, breaking of double stranded DNA to single stranded is called denaturation, and elongation of primers with nucleotides with the appropriate DNA polymerase enzyme in the presence of Mg^{2+} ions is called primer extension (Kubista et al., 2006; JRC European Commission, 2006).

Taq DNA Polymerase enzyme produced from *Thermus aquaticus* is widely used due to its high-temperature resistance, high specificity, efficiency, sensitivity, and reproducibility (Holland et al., 1991). The optimum operating temperature of Taq DNA polymerase is 72°C and most PCR protocols use this value as the extension temperature (Kubista et al., 2006). A buffer solution is also needed for PCR. This buffer also contains $MgCl^2$ solution at a concentration between 0.5 and 5 mM. Mg^{2+} ions form a dissolvable mixture with dNTPs, which increases polymerase enzyme activity and primer/target DNA interaction. Free dNTPs are required for DNA replication. It is preferred that the dNTP concentration is in the range of 20-200 µM. To prevent an incorrect match, 4 dNTPs must be used equally.

The design of the primers is critical to increasing PCR success. Even in an environment where all other factors are suitable, an incorrectly designed primer may cause the reaction not to occur. When designing the primer, attention should be paid to primer length, melting temperature, sensitivity, complementary sequences, Guanine/Cytosine (G/C) ratio, polypyrimidine (Thymine and Cytosine) and polypurine (Adenine and Guanine) elongations, and 3' end sequence characteristics (JRC European Commission, 2006; Innis & Gelfand, 1990).

The PCR methods that are widely used today and give the most reliable results are Real-Time PCR methods. Higuchi et al. (1993) designed the PCR technique in which the increase in DNA amount during thermal cycles can be monitored with the help of a CCD camera. In this technique, the amount of DNA accumulated in each PCR could be determined by monitoring the increase in ethidium bromide fluorescence binding to the copied DNA (Higuchi et al., 1993). This study formed the basis of Real-Time PCR. The performance of Real-Time PCR methods depends on the nature of the chemicals that make up the amplification reactions and the device used to maintain and monitor the reaction. Indicator dyes (ethidium bromide, SYBR Green I), hybridization probes (TaqMan probes, FRET probes, molecular beacons, scorpions, etc.) are examples of these factors (JRC European Commission, 2006). Unlike the end product analysis in conventional PCR, the increase in the targeted DNA (amplification) can be observed in the Real-Time PCR method during the reaction. In the Real-Time PCR method, probes and dyes that provide a signal depending on the number of DNA chains that increase during thermal cycles are used. While the signal is weak during the first cycles of the reaction, it rises logarithmically with the increasing number of DNA, and the signal reaches its peak at the end of the reaction. The first starting point of the fluorescent signal is related to the amount of target DNA at the beginning of the reaction (JRC European Commission, 2006; Ahmed, 2002; Zhang et al., 2019).

While the Real-Time PCR method is executed, the fluorescence value is plotted against the number of cycles (Cycle) on a semi-logarithmic scale. It is possible to follow 3 stages in this graph: The lag stage where slight signal fluctuations are seen, the logarithmic phase where increasing fluorescence data is collected and the plateau phase in which the signal value reaches a plateau. When measuring with the Real-Time PCR method, the logarithmic phase in which a rapid increase is observed rather than the signal value in the plateau stage is taken as a basis. The target gene content of the reacted product is the most important factor determining the cycle point where the logarithmic increase begins. This point is called the cycle threshold (Kubista et al., 2006; JRC European Commission, 2006).

Genetically modified organism (GMO) analysis

The rapid developments in gene technology in recent years have made it easy and efficient to change the genetic structure of plant products with commercial concerns. In agricultural production, the expectations to increase the amount and quality of the product resulted in the increased production of genetically modified products that from 1.7 million hectares to 190.4 million hectares from 1997 to 2019 (International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2018). Different approaches of consumers to products containing GMOs has revealed the need to identify foods containing these products. Analysis methods used to identify GMO products can be examined under two main headings, protein-based and DNA-based. Protein-based analysis methods can be listed as the "immunoassay" method, "Western Blot" method, "ELISA" method, and "Lateral Flow Strip" method. DNA-based analysis methods are the "Southern Blot" method and PCR/Real-Time PCR methods (Zel et al., 2012; Fagan, 2004).

In GMO analyses with Real-Time PCR, the presence of regulatory trace genes such as 35S promoter (CaMV 35S) and nos terminator and/or directly the presence of genetically modified crops such as MON87701 Soy, GHB614 Cotton, DAS 40278-9 Maize are investigated. Currently, as new Genetically Modified (GM) types are produced, analysis of these types can be made in the

Real-Time PCR method (Hernandes et al., 2010). There are commercially prepared analysis kits for the detection of these genes, as well as individual primer and probe designs for each gene, and analyzes can be carried out (JRC European Commission, 2006). Suitable forward, reverse and probe sequences for the CaMV 35S promoter are given in the table (Table 1).

Table 1. Primer and Probe Designs for CaMV 35S Promoter Gene (Alary et al., 2002)

Orientation	Sequence (5' → 3')
Forward Primer	CGTCTTCAAAGCAAGTGGATTG
Rewerse Primer	TCTTGCGAAGGATAGTGGGATT
Forward Probe	TCTCCACTGACGTAAGGGATGACGCA

Real-time PCR method allows looking at different gene regions in the same analysis as single, double, triple, and multiple. In this sense, the study of Samson et al. (2012) can be given as an example. In the study, Genetically Modified (GM) maize coded MON810 and GA21 were analyzed. In multiple analyses, detection and identification limit studies were made, and by creating a calibration table, GM corn in the products could be determined as a percentage (Samson et al., 2012).

While more than one gene region can be scanned in the same reaction well in multiple analyzes, single analyzes can also be used to look at more than one gene region in a single reaction using the same plate. Unlike multiple analyses, in a single analysis, the master mix has to be prepared separately for each gene region. This means extra time and labor loss (Alary et al., 2002). In the study of Gerdes et al. (2012), the products coded as NK603, Bt176, MON 810, TC1507, 59122, T25, GA21, Bt11, MON 863, MIR604, 3272, CBH-351, and 40-3-2 GM Soybean were analyzed. In the study where 2 parallel analyzes were performed, 72 wells were filled with positive and negative controls (Gerdes et al., 2012).

Meat species analysis

Meat has an important place in nutrition because it is a high and quality source of protein. It is heavily consumed by people for a balanced diet. While consumers prefer meat products, they benefit from some features such as religious beliefs, usefulness, and allergenic effects. Unlike the animals specified on the label of the product, producers can deceive consumers by using cheap meats in their products. For these reasons, knowing the animal that is source of meat product is very important for food safety (Kaya et al., 2019).

In the analysis of species determination in meat products, protein and DNA-based methods are used depending on the equipment, method, sample preparation costs, and form. Protein-based methods are Electrophoresis, Chromatography, Spectroscopy, and ELISA. DNA-based methods can be listed as Restriction Fragment Length Polymorphism PCR (RFLP-PCR), Multiplex PCR, Real-Time PCR, and Digital PCR. Real-time PCR methods are preferred in routine analysis because they can be applied to mixed meat products, their cost is lower than other DNA-based methods, and they give fast and precise results. It has been reported in the literature that single-copy genes or repeat sequences in the genome are used for animal species and generality in quantification studies. Quantification studies can be carried out by RT-PCR by targeting Mitochondrial tRNA, Mitochondrial rRNA, Mitochondrial cytochrome b, Transferrin, Myostatin, and Mitochondrial D-loop sequences (Alikord et al., 2018; Kaya et al., 2019).

There are many studies in the literature using Real-Time PCR on meat products. Iwobi et al. (2015) conducted a study determining the proportions of beef and pork in mixed minced meat samples. Iwobi et al. (2017) also carried out a study determining the mixing ratios of horse meat in meat products. Thanakiatkrai et al. (2019) performed gene screening for dogs, ducks, buffalo, goats, and sheep in Asian Food Products. Ali et al. (2012) performed pig gene screening in commercially produced hamburger patties. Fang and Zhang, (2016), did mouse gene screening in meat products; while Pegels et al. (Pegels et al., 2015) performed horse gene screening in food and feed samples sold on the Spanish Market.

Allergen analysis

Allergy can be defined as the body's hypersensitivity to the allergen. When explaining the term allergen, it is necessary to mention the antigen. The antigen is the name given to substances that cause antibody production if they enter an organism. If the antigen taken into the body produces antibodies that cause allergic reactions, this antigen is called an allergen. In other words, if the antigen taken into the body activates the body's immunological system against it, this antigen is called an allergen (Karakılıç et al., 2014; Tayfur & Ünlüoğlu, 1996). Allergic reactions caused by food can start from symptoms such as fatigue, weakness, drowsiness, nausea, vomiting, and can reach urticaria and anaphylactic shock (Tayfur & Ünlüoğlu, 1996).

Food allergens appear in a huge range of products. Examples of these products are peanuts, nuts, eggs, cow's milk, soybeans, fish, shellfish, bananas, chicken, chocolate, and wheat. The excess of allergen foods has led to the development of many analysis methods such as ELISA, PCR/Real-Time PCR, and Liquid Chromatography/mass spectrometry combinations to detect them (Tayfur & Ünlüoğlu, 1996; Kizis, 2014). In Real-Time PCR methods, the goal is to determine the specific gene sequence of the food ingredient that causes the allergy. Compared to conventional PCR, it eliminates the need for additional chemical materials such as gel electrophoresis, requires less sample compared to protein-based methods, is more closed to contamination across-contamination and provides high automation in large-scale enterprises, making Real-Time PCR more preferred in allergen analysis. TaqMan probe

methods are used in allergen tests. The target gene sequence to be used in the analysis is derived from the gene sequence encoding the protein related to allergy or a specific gene sequence of the food containing that protein (López-Calleja et al., 2013; Garber et al., 2016). Primers and probes containing these gene sequences can be designed commercially or studies can be performed using analysis kits. Herrero et al. (2014) detected fish-borne allergens in foods containing fish with the help of commercial kits. In another study, Herrero et al. (2012) designed a rapid Real-Time PCR method that can detect allergens in shellfish such as shrimp, crab, lobster and crayfish in a 40-minute analysis time. López-Calleja et al. (2013) devised a method that could detect peanut allergen by Real-Time PCR in processed foods. Primers used in the study were commercially designed, and analysis kit was not used.

Milk origin analysis

Milk and dairy products have an important place in nutrition. It contains a balanced composition to meet nutritional elements such as milk, protein, fat, carbohydrate, minerals, and vitamins. The need to know the animal species from which the food consumed is produced has increased emergence of different research areas in this regard. Labels of dairy products offered for consumption in the market include animal species information. To prevent misinformation and/or fraudulent production, effective analysis management should be used in origin detection (Kara & Demirel, 2016; Narayan Jha et al., 2016).

The method used to detect especially bovine proteins in dairy products works on the isoelectronic basis of ɣ-caseins after plasmolysis. This technique is used for cow's milk but may give erroneous results in heat-treated products and the presence of low contamination. To differentiate between buffalo milk and cow's milk, 13C nuclear magnetic resonance on triacylglycerols, an HPLC technique for β-lactoglobulin analysis on buffalo milk and mozzarella cheese, multivariate regression analysis and capillary electrophoresis and acrylamide gel technique are used. More recently, ELISA methods have been used. However, the negative features of these methods are that they are not suitable for routine use, take a long time, and do not provide absolute certainty (Di Pinto et al., 2017; Bottero et al., 2003; Lopparelli et al., 2007).

Analyzes using Real-Time PCR methods have been extensively applied in numerous tests to identify animal species. These methods are based on the persistence of genomic DNA extracted from somatic cells in both milk and cheese without degradation even in the applied manufacturing processes. The most common assays for species identification are based on PCR analysis of species-specific mitochondrial DNA (mtDNA) sequences. MtDNA can be considered an augmented genetic resource. Apart from these, there are well-known analytical methods for origin identification of dairy products based on nuclear DNA sequences such as β-casein genes and 18S - 28S rRNA multiple copy genes. In Real-Time PCR-based animal origin analyses, mtDNA is preferred because it contains more genes specific to the source and contains more gene sequences compared to nuclear DNA. In addition to Real-Time PCR methods based on the detection of a single species, multiple Real-Time PCR methods that can detect more than one species at the same time provide faster and more economical origin determination (Bottero et al., 2003; Lopparelli et al., 2007; Xue et al., 2017; Kotowicz et al., 2007).

Agrimonti et al. (2015) conducted a study in 2015 on a rapid method design that can detect cow, goat, sheep, and buffalo genes in dairy products with Real-Time PCR. They also developed a method for determining the proportion of cow's milk in products using milk mixtures. Lopez-Calleja et al. (2007) studied a method for determining the proportion of goat milk in sheep milk using a Real-Time PCR method. By comparing the CT values obtained in the reactions, they determined the percentage ratios in the mixed milk. Lopparelli et al. (2007), investigated the determination of cow's milk mixing ratio in buffalo mozzarella cheese using the Real-Time PCR method. In the study conducted on 64 cheese samples, it was determined that the most of the products were contaminated with cow's milk. Kara and Demirel (2016), determined the origin of milk used in the production of Afyon Cream by Real-Time PCR method. In the study, 100 cream samples were analyzed and as a result, it was determined that 13% of these products were produced from buffalo milk, 28% from a mixture of buffalo milk, and 59% from cow milk.

Microbial pathogen analysis

Microbial diseases caused by food are important health problems caused by the consumption of foods contaminated by pathogenic microorganisms. At any stage from the production of food to consume, pathogenic microorganisms that contaminate food cause serious disturbances in the people who consume them. When pathogenic microorganisms such as *Listeria monocytogenes*, *Campylobacter*, *Escherichia coli* O157: H7, and *Salmonella* enter the body with the foods they contaminate, the metabolic products of these microorganisms cause infections. Bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum* can produce toxins. Infections also occur by taking these toxins into the body (Tutar et al., 2015; Rathnayaka et al., 2018; Halkman, 2019).

The methods used to identify pathogenic microorganisms can be listed as colony-based methods, immunological methods, and molecular biological methods. Colony-based methods have disadvantages such as taking a very long time and low reproducibility. In immunological techniques, there are problems such as a high risk of contamination and low sensitivity levels. Molecular biological techniques, on the other hand, have emerged as an alternative to the developing

technology and they have been preferred due to their high sensitivity, high repeatability, and fast results (Tutar et al., 2015; Rathnayaka et al., 2018; Agrimonti et al., 2019).

Kılıç Altun et al. (2017) investigated the presence of *Listeria spp.* by Real-Time PCR in yoghurts sold in Şanlıurfa/Turkey in their study in 2016. As a result of the study performed on 62 yogurt samples, the prevalence of *Listeria spp.* was found to be 3.2%. In another study conducted in Şanlıurfa/Turkey in 2017 Deniz and Kılıç Altun, (2017) tried to detect *Listeria monocytogenes* contamination in regional cheeses produced from raw sheep milk and raw cow milk with Real-Time PCR. As a result of the Listeriolysin gene region scanning in 97 cheese samples, *Listeria monocytogenes* were detected in 3 samples. Liming et al. (2004) conducted a study investigating the presence of *Listeria monocytogenes* in products such as fresh-cut melon and mixed salad using a Real-Time PCR. In the study, the classical colony method, conventional PCR, and Real-Time PCR methods were compared. As a result of the comparison, it was determined that the analysis with the Real-Time PCR method gave results in 26 hours less than the conventional method.

Other analysis

Ferreira et al. (2016) investigated the presence of barley, corn, and rice genes by Real-Time PCR method in their study on soluble coffee products in 2016. While 30 coffee products were collected from different countries and different grains were not found in the products defined as top quality, the grains analyzed were found in lower quality products collected from South America.

Gansbeke et al. (2018) investigated the mixture of apricot kernel paste in almond butter products. Within the scope of the research, apricot kernel paste in different proportions was added to the almond paste and the detection limit study and optimization study were carried out.

Kabacaoğlu and Karakaş Budak, (2019) developed a method for detecting adulteration by Real-Time PCR in the salep product that can be mixed with cornflour or potato starch. They were able to measure up to 2 levels of DNA level with high precision using the primer sets they designed.

Sobrino-Gregorio et al. (2019) conducted a study to determine whether sugar is added from different plant sources in honey with the Real-Time PCR method. In the study, different amounts of rice molasses were added to the natural honey sample and it was aimed to determine the mixing ratios with the developed method.

Mohamad et al. (2018) conducted a study in 2018 on the detection of pig genes in gelatin and gelatin capsules with the Real-Time PCR method. As a result of the study, it has been observed that working with chromosomal DNA in intensely processed products such as gelatin is more efficient than mitochondrial DNA and a method has been developed for the halal food tests of various foods on the market.

Villa et al. (2017) worked on the development of a Real-Time PCR-based method to detect possible adulterations in saffron plant products. As a result of the study, a method has been developed in which the safflower mixture that can be made into saffron products such as stigma, spices, and powders can be determined.

Conclusion

Molecular-based analysis techniques will be used more widely in the future in parallel with biotechnological developments. In the analysis using Real-Time PCR methods, the nucleic acid-containing the targeted gene region must be extracted in sufficient quality and concentration for the analysis to be successful. Correct nucleic acid isolation is the basis of the analysis mentioned in this research. Real-time PCR reactions, starting with the appropriate amount and purity nucleic acid solution and error-free primer-probe sequence, will give results with high repeatability and precision.

In food quality control laboratories, quantitative methods are routinely used in analyzes such as GMO and pathogen microorganism analysis; however, qualitative methods are widely used in analyses such as meat-type determination, milk origin determination, and allergen analysis. On the other hand, an important step can be taken to prevent possible adulteration in food by increasing the frequency of use of biotechnological analysis methods such as Real-Time PCR methods. Although there are studies with quantitation content mentioned in this study, there are no studies on the usability of the methods used in these studies in routine analysis. In such analyzes, it is thought that there is a low level of repeatability in front of the transition to quantitation. On the other hand, it is thought that the efficiency increase studies in nucleic acid extraction will be valuable for the routine quantitation analysis.

Author Contributions

EA: designed, performed, writing; SA: review and editing

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

References

Agrimonti, C., Bottari, B., Sardaro, M. L., & Marmiroli, N. (2019). Application of real-time PCR (qPCR) for characterization of microbial populations and type of milk in dairy food products. *Critical Reviews in Food Science and Nutrition, 59*(3), 423-442. https://doi.org/10.1080/10408398.2017.1375893

Agrimonti, C., Pirondini, A., Marmiroli, M., & Marmiroli, N. (2015). A quadruplex PCR (qxPCR) assay for adulteration in dairy products. *Food Chemistry, 187*, 58-64. https://doi.org/10.1016/j.foodchem.2015.04.017

Ahmed, F. E. (2002). Detection of genetically modified organisms in foods. *Trends in Biotechnology, 20*(5), 215-223. https://doi.org/10.1016/S0167-7799(01)01920-5

Alary, R., Serin, A., Maury, D., Jouira, H. B., Sirven, J.-P., Gautier, M.-F., & Joudrier, P. (2002). Comparison of simplex and duplex real-time PCR for the quantification of GMO in maize and soybean. *Food Control, 13*, 235-244. https://doi.org/10.1016/S0956-7135(02)00015-4

Ali, M. E., Hashim, U., Dhahi, S. T., Mustafa, S., Man, Y., & Latif, M. A. (2012). Analysis of Pork Adulteration in Commercial Burgers Targeting Porcine-Specific Mitochondrial Cytochrome B Gene by TaqMan Probe Real-Time Polymerase Chain Reaction. *Food Analytical Methods,5*, 784-794. http://dx.doi.org/10.1007/s12161-011-9311-4

Alikord, M., Momtaz, H., Keramat, J., Kadivar, M., & Rad, A. H. (2018). Species identification and animal authentication in meat products: a review. *Journal of Food Measurement and Characterization, 12*, 145-155. https://doi.org/10.1007/s11694-017-9625-z

Bottero, M., Civera, T., Nucera, D., Rosati, P., Sacchi, P., & Turi, R. (2003). A multiplex polymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. *International Dairy Journal, 13*, 277-282. http://dx.doi.org/10.1016/S0958-6946(02)00170-X

Böhme, K., Calo-Mata, P., Barros-Velazquez, J., & Ortea, I. (2019). Review of Recent DNA-Based Methods for Main Food-Authentication Topics. *Journal of Agricultural and Food Chemistry, 67*, 3854-3864. https://doi.org/10.1021/acs.jafc.8b07016

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., Drosten, C. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance,25*(3),1-8. https://dx.doi.org/10.2807%2F1560-7917.ES.2020.25.3.2000045

Deniz, H. İ., & Kılıç Altun, S. (2017). The Isolation and Identification of Virulent Listeria monocytogenes Consumption of Local Cheeses in Şanlıurfa Province. *Ataturk University Journal of Veterinary Sciences, 12*(3), 289-295. http://dx.doi.org/10.17094/ataunivbd.368984

Di Pinto, A., Terio, V., Marchetti, P., Bottaro, M., Mottola, A., Bozzo, G., Tantillo, G. (2017). DNA-based approach for species identificaton of goat-milk products. *Food Chemistry,229*,93-97. https://doi.org/10.1016/j.foodchem.2017.02.067

Fagan, J. (2004). DNA Based Methods for Detection and Quantification of GMOs: Principles and Standards. *Testing of Genetically Modified Organisms in Foods* (s. 163). In New York: Food Products Press.

Fang, X., & Zhang, C. (2016). Detection of adulterated murine components in meat products by TaqMan real-time PCR. *Food Chemistry, 192*, 485-490. https://doi.org/10.1016/j.foodchem.2015.07.020

Ferreira, T., Farah, A., Oliveira, T. C., Lima, I. S., Vitorio, F., & Oliveira, E. M. (2016). Using Real-Time PCR as a tool for monitoring the authenticity of commercial coffees. *Food Chemistry,199*,433-438. https://doi.org/10.1016/j.foodchem.2015.12.045

Gansbeke, B., Bény, G., De Loose, M., & Taverniers, I. (2018). ATaqMan Real-Time PCR Assay for Apricot (Prunus armeniaca) as an Authenticity Test for Detection of Traces of Persipan in Marzipan. *Food Anaytical Methods, 11*,62-68. https://link.springer.com/article/10.1007/s12161-017-0964-5

Garber, E., Parker, C., Handy, S., Cho, C., Panda, R., Samadpour, M., Ziobro, G. (2016). Presence of Undeclared Food Allergens in Cumin: The Need for Multiplex Methods. *Journal of Agricultural and Food Chemistry, 64*, 1202-1211. https://doi.org/10.1021/acs.jafc.5b05497

Gerdes, L., Busch, U., & Pecoraro, S. (2012). Parallelised real-time PCR for identification of maize GMO events. *European Food Research and Technology, 234*, 315-322. http://dx.doi.org/10.1007%2Fs00217-011-1634-2

Halkman, K. (2019). Microorganisms in Food. *Food Microbiology* (p. 309-404). In Ankara: Başak Publishing House.

Hernandes, M., Ferrando, A., & Rodriguez-Lazaro, D. (2010). Assessment of Genetically Modifi ed Organisms (GMO) in Meat Products by PCR. *Handbook of Meat Processing* (s. 501-518). In Singapore: Blackwell Publishing. http://dx.doi.org/10.1002/9780813820897.ch29

Herrero, B., Vieites, J. M., & Espineira, M. (2012). Fast Real-Time PCR for the Detection of Crustacean Allergen in Foods. *Journal of Agricultural and Food Chemistry, 60*, 1893-1897. https://doi.org/10.1021/jf2043532

Herrero, B., Vieites, J. M., & Espineira, M. (2014). Development of an in-house fast real-time PCR method for detection of fish allergen in foods and comparison with a commercial kit. *Food Chemistry, 151*, 415-420. https://doi.org/10.1016/j.foodchem.2013.11.042

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions. *Nature Biotechnology, 11*, 1026-1030. https://doi.org/10.1038/nbt0993-1026

Holland, P. M., Abramson, R. D., Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America,88*(16),7276-7280. https://dx.doi.org/10.1073%2Fpnas.88.16.7276

Innis, M. A., & Gelfand, D. H. (1990). Optimization of PCRs. *PCR Protocols* (s. 3-12). In California: Academic Press, Inc.

International Service for the Acquisition of Agri-biotech Applications (ISAAA). (2018). *Global Status of Commercialized biotech/gm crops: 2018.* Accessed From isaaa.org/resources/publications/briefs/54/infographic/pdf/B54-Infographics-English.pdf at 04.11.2020

Iwobi, A., Sebah, D., Kraemer, I., Losher, C., Fischer, G., Busch, U., & Huber, I. (2015). A multiplex real-time PCR method for the quantification of beef and pork fractions in minced meat. *Food Chemistry, 169*, 305-313. https://doi.org/10.1016/j.foodchem.2014.07.139

Iwobi, A., Sebah, D., Spielmann, G., Maggipinto, M., Schrempp, M., Kraemer, I., Huber, I. (2017). A multiplex real-time PCR method for the quantitative determination of equine (horse) fractions in meat products. *Food Control, 74*,89-97. https://doi.org/10.1016/j.foodcont.2016.11.035

JRC European Commission. (2006). Features of Qualitative PCR. *Analysis of Genetically Modified Organisms in Food Samples, Course Handbook* (s. Section 8). In Italy: European Union Official Publications Office.

JRC European Commission. (2006). Polymerase Chain Reaction (PCR). *Analysis of Genetically Modified Organisms in Food Samples, Course Handbook* (s. Section 6). In Italy: European Union Official Publications Office.

Kabacaoğlu, E., & Karakaş Budak, B. (2019). Detection and quantification of salep with real time PCR utilizing the nr-ITS2 region. *Journal of the Science of Food and Agriculture,99*,2447-2454. https://doi.org/10.1002/jsfa.9453

Kara, R., & Demirel, Y. N. (2016). Determination of Milk Species Used in Afyon Kaymak Production with RealTime PCR. *Ataturk University Journal of Veterinary Sciences, 11*(2), 185-190. http://dx.doi.org/10.17094/avbd.77186

Karakılıç, M., Suna, S., Tamer, C. E., & Çopur, Ö. U. (2014). Food Allergy Reactions and Their Formation Mechanism. *Journal of Agricultural Faculty of Uludag University, 28*(1), 73-82.

Kaya, M., Arıkan Asan, A., & Başıaçık Karakoç, Ş. (2019). Determination The Proportion of Chicken and Beef In Meat Products Using Some Commercial Kits. *Süleyman Demirel University Journal of Natural and Applied Sciences,23*(1),52-58. https://doi.org/10.19113/sdufenbed.442199

Kesmen, Z., Büyükkiraz, M. E., Kahraman, N., & Yetim, H. (2017). Detection of Fraudulent Practices Involving Some Plant Derived Compounds In Foods Using Real-Time PCR. *The Journal of Food, 42*(3), 305-314. https://doi.org/10.15237/gida.GD16110

Kılıç Altun, S., Yiğin, A., & Demirci, M. (2017). Investigation of The Presence of *Listeria spp.* in Retailed Yoghurt Samples by Real-Time PCR in Şanlıurfa. *Journal of Faculty of Veterinary Medicine, Erciyes University, 14*(2), 81-86.

Kizis, D. (2014). Detection of plant allergens in foods. *Food Allergen Testing: Molecular, Immunochemical and Chromatographic Techniques* (s. 105-149). In New Jersey: John Wiley & Sons, Ltd. http://dx.doi.org/10.1002/9781118519219

Kotowicz, M., Adamcyzk, E., & Bania, J. (2007). Application of a Duplex-PCR for detection of Cows'milk in Goats' milk. *Annals of Agricultural and Environmental Medicine, 14*, 215-218.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K., Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine,27*(2-3),95-125. https://doi.org/10.1016/j.mam.2005.12.007

Liming, S., Zhang, Y., Meng, J., & Bhagwat, A. (2004). Detection of Listeria monocytogenes in Fresh Produce Using Molecular Beacon-Real-time PCR Technology. *Journal of Food Science, 69*(8), 240-245. https://doi.org/10.1111/j.1750-3841.2004.tb18020.x

Loparelli, R. M., Cardozzo, B., Balzan, S., Giaccone, V., & Novelli, E. (2007). Real-Time TaqMan Polymerase Chain Reaction Detection and Quantification of Cow DNA in Pure Water Buffalo Mozzarella Cheese: Method Validation and Its Application on Commercial Samples. *Journal of Agricultural and Food Chemistry, 55*, 3429-3434. https://doi.org/10.1021/jf0637271

Lopez-Calleja, I., Gonzalez, I., Fajardo, V., Martin, I., Hernandes, P., Garcia, T., & Martin, R. (2007). Quantitative detection of goats' milk in sheep's milk by real-time PCR. *Food Control, 18*, 1466-1473. http://dx.doi.org/10.1016/j.foodcont.2006.11.006

López-Calleja, I., de la Cruz, S., Pegels, N., González, I., García, T., & Martín, R. (2013). Development of a real time PCR assay for detection of allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. *Food Control,30*,480-490. http://dx.doi.org/10.1016/j.foodcont.2012.09.017

Mohamad, N. A., Mustafa, S., Mokhtar, N. K., & El Sheikha, A. (2018). Molecular beacon-based real-time PCR method for detection of porcine DNA in gelatin and gelatin capsules. *Journal of the Science of Food and Agriculture, 98*, 4570-4577. https://doi.org/10.1002/jsfa.8985

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1986). Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction. *Cold Spring Harbor Symposia on Quantitative Biology, 51*, 263-273. https://doi.org/10.1101/sqb.1986.051.01.032

Narayan Jha, S., Jaiswal, P., Grewal, M. K., Gupta, M., & Bhardwaj, R. (2016). Detection of Adulterants and Contaminants in Liquid Foods—A Review. *Critical Reviews in Food Science and Nutrition, 56*, 1662-1684. https://doi.org/10.1080/10408398.2013.798257

Pegels, N., Garcia, T., Martin, R., & Gonzalez, I. (2015). Market Analysis of Food and Feed Products for Detection of Horse DNA by a TaqMan Real-Time PCR. *Food Analytical Methods,8*, 489-498. http://dx.doi.org/10.1007/s12161-014-9914-7

Rathnayaka, R., Devappa, R. K., & Rakshit, S. K. (2018). Rapid Detection of Food Pathogens Using Molecular Methods. *Molecular Techniques in Food Biology* (s. 343-360). In New Jersey: John Wiley & Sons Ltd. https://doi.org/10.1002/9781119374633.ch15

Samson, M. C., Gulli, M., & Marmiroli, N. (2013). Multiplex real-time PCR assays for simultaneous detection of maize MON810 and GA21 in food samples. *Food Control, 30*,518-525. http://dx.doi.org/10.1016/j.foodcont.2012.08.001

Sobrino-Gregorio, L., Vilanova, S., Prohens, J., & Escriche, I. (2019). Detection of honey adulteration by conventional and real-time PCR. *Food Control, 95*, 57-62. http://dx.doi.org/10.1016/j.foodcont.2018.07.037

Tayfur, M., & Ünlüoğlu, İ. (1996). Food Allergy. *The Journal of Food, 21*(4), 293-296.

Thanakiatkrai, P., Dechnakarin, J., Ngasaman, R., & Kitpipit, T. (2019). Direct pentaplex PCR assay: An adjunct panel for meat species identification in Asian food products. *Food Chemistry,271*,767-772. http://dx.doi.org/10.1016/j.foodchem.2018.07.143

The Nobel Prize Web Site. (1993). The Nobel Prize in Chemistry 1993. NobelPrize.org. Nobel Prize Outreach AB 2022. https://www.nobelprize.org/prizes/chemistry/1993/mullis/facts/

Tutar, E., Köksalan, E., & Akyol, İ. (2015). Real-time PCR Technology in Characterzitaion of Foodborne Microbial Pathogens. *Kahramanmaras Sutcu Imam University Journal of Natural Science, 18*(4), 26-29. https://doi.org/10.18016/ksujns.36064

Villa, C., Costa, J., Oliveira, B. M., & Mafra, I. (2017). Novel quantitative real-time PCR approach to determine safflower (Carthamus tinctorius) adulteration in saffron (Crocus sativus). *Food Chemistry, 229*, 680-687. https://doi.org/10.1016/j.foodchem.2017.02.136

Xue, C., Wang, P., Zhao, J., Xu, A., & Guan, F. (2017). Development and validation of a universal primer pair for the simultaneous detection of eight animal species. *Food Chemistry, 221*, 790-796. https://doi.org/10.1016/j.foodchem.2016.11.102

Yeşilören, G., & Ekşi, A. (2014). Food Authenticity and Control Methods. *The Journal of Food, 39*(5), 315-322. http://dx.doi.org/10.15237/gida.GD14017

Zel, J., Milavec, M., Morisset, D., Plan, D., Van den Eede, G., & Gruden, K. (2012). Methods. *How to Reliably Test For GMOs* (s. 29). In London: Springer. http://dx.doi.org/10.1007/978-1-4614-1390-5

Zhang, H., Li, H., Zhu, H., Pekarek, J., Podesva, P., Chang, H., & Neuzil, P. (2019). Revealing the secrets of PCR. *Sensors & Actuators: B. Chemical, 298* (126924), 1-9. http://doi.org/10.1016/j.snb.2019.126924

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BIOTECH STUDIES

REVIEW PAPER

Health promoting benefits of postbiotics produced by lactic acid bacteria: Exopolysaccharide

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Abstract

Exopolysaccharides are high molecular weight polymers of repeated sugar units with diverse chemical structure and unique properties and produced by microorganisms. Lactic acid bacteria are important exopolysaccharide producers. Lactic acid bacteria derived exopolysaccharides, one of the postbiotics, are known to have technological properties such as stabilizing, thickening, emulsifing and also biological activities. Lactic acid bacteria can synthesis exopolysaccharides with large structural variability and this diversity brings these polymers to possess several bioactivities. Bioactivities such as immunomodulatory, antiinflammatory, antitumor and antimutagenicity, antioxidant, antibacterial and antiviral, cholesterol-lowering, antihypertensive activity and gastro-protective activity bring these biopolymers commercial value in the global market and potential to be used in biomedical and pharmaceutical applications. Therefore, to evaluate the availability of these natural exopolysaccharides for new applications extensive understanding of the structure-function relationships will be required. In this review, it is presented a comprehensive overview for the most recent reports on the health benefits of postbiotic lactic acid bacterial exopolysaccharides.

Introduction

Exopolysaccharides (EPSs) are high molecular weight and long chain polymers composed of branched, repeating units of sugars or sugar derivatives. They are surrounding the envelope of most bacteria and are mainly involved in cell adhesion and protection (Sanlibaba & Çakmak, 2016; Taylan et al., 2019; Noroozi et al., 2021). EPS has been widely produced by lactic acid bacteria (LAB). The most noticeable EPS producing LABs are *Lactobacillus, Leuconostoc, Weissella, Lactococcus, Streptococcus, Pediococcus* and *Bifidobacterium* spp. Microbial EPS can be divided into two groups according to their chemical composition: homopolysaccharides (HoPs) which contain a single type of monosaccharides; cellulose, dextran, pullulan, levan, curdlan, etc. and heteropolysaccharides (HePs) which comprise repeating units of different monosaccharides, gellan, galactan, xanthan, kefiran etc. (Laws et al., 2001; Ruas-Madiedo & De Los Reyes-Gavilán, 2005; Chaisuwan et al., 2020; Kavitake et al., 2020).

EPSs can be used in a variety of industrial fields, including biomedical, wastewater treatment, cosmetic, textile, food, and pharmaceutical applications, and are responsible for physicochemical modifications. EPSs mostly serve as stabilizing, thickening, emulsifing agents

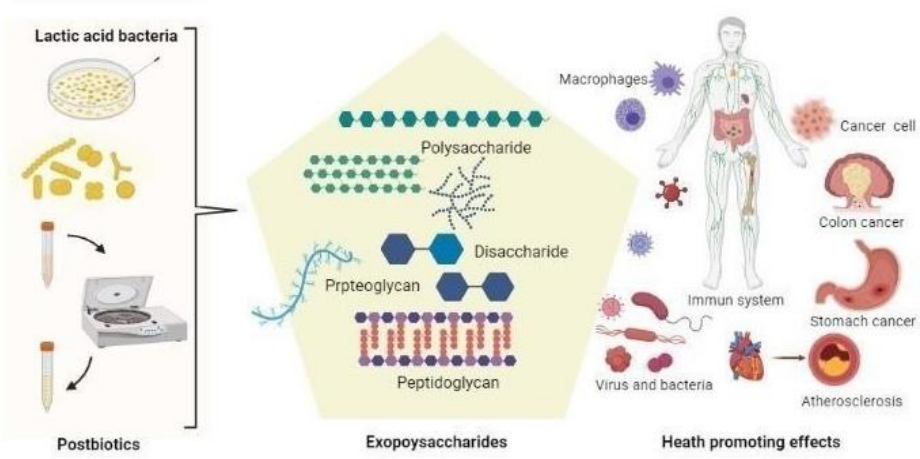

Figure 1. The representation of the health promoting effects of exopolysaccharides, a postbiotic produced by lactic acid bacteria.

and particularly in food industry, it contributes to unique important qualities such as the advanced viscosity and rheology, sensory quality, extended shelf-life, etc. Beside these physicochemical properties, it has been already shown that LAB produced EPSs have numerous physiological functions and potential health benefits (Figure 1). EPS bioactivities are influenced by different factors such as molecular weight, constituent sugars, conformation, glycosidic linkage, and degree of branching (Kumar et al., 2020; Rana & Upadhyay, 2020). The molecule binding and penetration may be related to the size or molecular weight. Lower weight average molecular weight EPS may have stronger binding ability to cell receptors and may penetrate more easily into a cell with better bioactivities than larger weight average molecular weight EPS (Li & Shah, 2016).

The bioactivities of EPS were similarly altered by monosaccharides in their structure. Immunomodulatory activities were observed in EPS fractions isolated from *Lactobacillus reuteri* Mh-001. The antiinflammatory properties of these EPS were regulated by the monosaccharide percentages. For macrophages, the EPS fraction with the highest quantity of galactose had the best antiinflammatory efficacy (Chen et al., 2019). The sugar compositions could be linked to receptor recognition on the immune cell surface (Ren et al., 2016). Sulfate groups (number and position) are also the most important parameters regulating the bioactivities of sulfated EPS. Besides, biological, physical, chemical and biomolecular modifications were reported which can be used to improve the biological activities of microbial EPS (Korcz et al., 2018; Chaisuwan et al., 2020; Schilling et al., 2020).

EPS produced by LAB, including probiotic LAB, has been chosen for a variety of applications due to their biological activity *in vitro* and *in vivo*. Probiotics are living microorganisms that, when ingested in sufficient quantities, have a beneficial effect on the host. Probiotics must be able to grow and persist in the human intestine in order to provide health benefits. The current definition of a probiotic requires that it be alive; thus, it does not apply to dead bacterial cells or cell components. However, recently it is stated that the positive effects of probiotics on health are not only caused by microorganisms but also by the metabolites they produce (Thantsha et al., 2012; Perricone et al., 2014). So, the term 'postbiotic' was invented to describe the health benefits of probiotics that go beyond their inherent viability, adding a new aspect to the probiotic concept (Abbasi et al., 2021). 'Postbiotic' refers to inactivated microbial cells (dead cells), cell fractions; peptidoglycans, polysaccharides, cell surface proteins, teichoic acids or short-chain fatty acids (SCFAs), enzymes, bacteriocins, and organic acids which are also called 'cell-free supernatant (CFS)' made by live cells through the fermentation process. EPSs are also naturally made by live probiotic cells. They can have a variety of physiological health-promoting effects on the consumer if consumed in sufficient quantities (Teame et al., 2020).

Fermented foods can contain probiotic LAB and LAB-derived EPSs with prebiotic attributes which may promote beneficial bacteria to colonize in the gut (Zhou et al., 2019). The production of biofilm by microorganisms induces colonization and ensures population maintenance in the difficult environment of the human gastrointestinal tract (GIT). It has been demonstrated that attaching probiotic LAB to epithelial cells in the GIT prevents pathogenic organism colonization, stimulates the host immune system, and protects epithelial cells from toxic substances (Jurášková et al., 2022). Furthermore, LAB-derived EPS can avoid infectious illnesses by reducing or inhibiting pathogenic bacteria from forming biofilms.

Prebiotics, paraprobiotics and postbiotics have a number of therapeutic properties, including immunomodulatory, antiinflammatory, antigastrointestinal, antiadhesion, antibiofilm, antiviral, antihypertensive, hypocholesterolemic, antiproliferative, antioxidant, and etc. (Teame et al., 2020; Abbasi et al., 2021) (Table 1) (Figure 2).

This article reviews current scientific findings on the beneficial effects of LAB produced EPS as postbiotics, with an emphasis on their health-promoting properties.

Table 1. Biological activities of EPS producing lactic acid bacteria species

LAB Species	Polysaccharide	Biological Acivity	Reference
Lactobacillus plantarum ZDY2013	Sulfated EPS	Antioxidant	Zhang et al., 2016
Lactobacillus plantarum L-14	EPS	Antiinflammatory	Kwon et al., 2020
Lactobacillus plantarum RJF4	EPS	Antioxidant, cholesterol lowering, antiproliferative	Dilna et al., 2015
Lactobacillus plantarum RJF4	EPS	Antiproliferative, antioxidant	Dilna et al., 2015
Lactobacillus plantarum LRCC5310	EPS	Antiviral	Kim et al., 2018
Lactobacillus plantarum SKT109	EPS	Antioxidant	Wang et al., 2018
Lactobacillus plantarum, *Lactobacillus casei*	EPS	Antitumor	Deepak et al., 2016a
Lactobacillus casei 01	EPS	Antiproliferative	Liu et al., 2011
Lactobacillus kefiranofaciens DN1	HePS (Man, Ara, Glc, Gal, Rha)	Antibacterial	Jeong et al., 2017
Lactobacillus kefiranofaciens DN1	EPS	Antibacterial	Jeong et al., 2017
Lactobacillus kefiranofaciens	Kefiran	Cholesterol lowering	Maeda et al., 2004
Lactobacillus kefiranofaciens	Kefiran	Antiinflammatory	Furuno & Nakanishi, 2012
Lactobacillus reuteri Mh-001	EPS fraction containing a high amount of galactose	Immunomodulatory	Chen et al., 2019
Lactobacillus reuteri DSM17938, *Lactobacillus reuteri* L26	EPS	Antigastrointestinal	Kšonžeková et al., 2016
Lactobacillus delbureckii	EPS	Antibacterial	Adebayo-Tayo & Fashogbon, 2020
Lactobacillus delbrueckii subsp. *bulgaricus*	HePS (Gal and Glc)	Antioxidant	Tang et al., 2017
Lactobacillus sanfranciscensis	EPS	Antioxidant	Zhang et al., 2019
Lactobacillus acidophilus	EPS	Antioxidant	Deepak et al., 2016b
Lactobacillus helveticus MB2-1	Cell-bound HePS (Glc, Man, Gal, Rha, Ara)	Anticancer	Li et al., 2015
Streptococcus thermophilus ASCC 1275	Sulfated EPS	Antiinflammatory	Li & Shah, 2016
Streptococcus thermophilus AR333	Polysaccharide	Immunoregulatory	Ren et al., 2016
Streptococcus mutans MTCC 497	EPS	Antiinflammatory	Buddana et al., 2015
Weissella confusa	Dextran	Antifungal	Adesulu-Dahunsi et al., 2018
Weisella confusa	HePS (Gal, Man, Glc, Fru, Rha, Ara, Xyl, Rib)	Antioxidant, immunomodulatory	Adebayo-Tayo et al., 2018
Weissella cibaria 27 (W27)	EPS	Antibacterial	Yu et al., 2018
Leuconostoc mesenteroides S81	HoPS	Antiinflammatory	Taylan et al., 2019
Leuconostoc pseudomesenteroides YB-2	Dextran	Antibacterial	Ye et al., 2019
Pediococcus acidilactici NCDC *252*	EPS	Antioxidant, anticancer	Kumar et al., 2020

Immunomodulatory activities

An immunomodulatory effect of LAB may be attributed to postbiotics; such as exopolysaccharides. EPS have good immunomodulatory and immune-protective functions. Immune stimulating activities of EPS have been already studied both *in vitro* and *in vivo*. Immunomodulator mechanisms may be interconnected to gut microbiota. Most EPS can enhance the diversity and balance of microorganisms in the gut by promoting the growth of the intestinal microbiota. Several EPS derived from LAB, such as *Lactobacillus plantarum*, *Pediococcus pentosaceus, Weissella cibaria,* and *Weissella confusa*, showed prebiotic characteristics and could encourage the growth of a probiotic strain, *Bifidobacterium bifidum* DSM 20456, *in vitro* (Chaisuwan et al., 2020). It was reported that EPS molecules can prevent gastrointestinal tract cancers, inhibit infections, and immunodeficiency induced diseases, such as inflammatory bowel diseases. Two patterns have been proposed to explain EPSs' immunomodulatory capability. Firstly, acidic HePs with phosphate in their composition are good stimulators of the immune response. Secondly, to strengthen the first lines of defense, the mucosal immune system is triggered by increasing host immunoglobulin A (IgA) secretion (Saadat et al., 2019).

Levan (S81), a HoP from *Leuconostoc mesenteroides* S81, had a strong immunomodulatory role, induced the antiinflammatory cytokine IL-4, and had a strong antioxidant capacity with a half maximal

effective concentration (EC_{50}) value of 1.7 mg mL^{-1} as determined by an *in vitro* hydroxyl radical scavenging activity test (Taylan et al., 2019).

Macrophages are known as a major factor in the inflammatory response. And one of the cytokines produced stimulating agents, nitric oxide (NO), is associated with macrophage immunological capabilities. Ren et al. (2016) found that high concentration of EPS333 (≥500 µg/mL) which is gained from *Streptococcus thermophilus* AR333, could promote the NO production in macrophages RAW 264.7. It was suggested that *Streptococcus thermophilus* AR333 could be a potential source of immunoregulatory polysaccharide and could be a potential immunostimulant in dairy products.

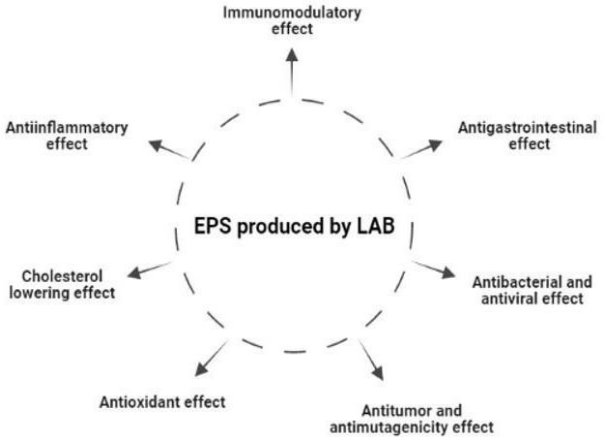

Figure. 2. Biological activities of exopolysaccharides produced by lactic acid bacteria.

EPS can also act as a substrate for other organisms in complex ecosystems (Zannini et al., 2016). In this context, Salazar et al. (2008) stated that intestinal *Bifidobacteria* can produce EPS, which can act as a fermentable substrate for microorganisms in the human gut. They also reported that it promotes alterations in SCFA profiles and changes in relationships between intestinal microbial populations.

The immunomodulating effects and immune-protective functions of EPS that have been reported in both *in vitro* and *in vivo* studies are frequently related to EPS derivatives from various strains of Lactobacilli including *Lb. casei*, *Lb. johnsonii* JCM 2012, *Lb. salivarius* Ls33, *Lb. rhamnosus* CRL1505, *Lb. plantarum* ATCC 14917, *Lb. buchneri* and *Lb. kefiri* (Teame et al., 2020; Rajoka et al., 2020).

Antiinflammatory effects

Inflammation is a complex process initiated by immune system as a response to defend the body from negative stimulation caused by pathogens, toxins, injuries etc. Various cells, metabolic pathways and molecular mediators are activated/induced in inflammation. Upon stimulation, immune cells (e.g. macrophages and mast cells) release inflammatory mediators (e.g. cytokines) that trigger a response from immune system. Cytokines play a key role in inflammation. Pro-inflammatory cytokines (e.g. interleukin-1 (IL-1α, IL-1β), IL-6, tumor necrosis factor (TNF), interferon gamma (IFNγ)) are synthesized and secreted from immune cells and promote inflammation. Antiinflammatory cytokines (e.g. IL-4, IL-10, IL-13) on the other hand inhibit the synthesis of proinflammatory cytokines and suppress the inflammation. Pro-inflammatory cytokines and antiinflammatory components are in a dynamic balance. Besides these proteins, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are also major players in inflammatory reactions, they are rapidly expressed under inflammatory conditions (Dinarello, 2000; Minghetti, 2004; Opal & DePalo, 2000; Venkatesha et al., 2017). Through inflammation, healing process is initiated. Excessive inflammatory responses, on the other hand, can harm host tissues and pose serious risks. Antiinflammatory agents are the compounds used for controlling the inflammation process through altering the metabolic pathways activated in inflammation. Natural products have been constantly investigated for their antiinflammatory activities and LAB EPSs, as a postbiotic, has been the subject of these studies recently (Kwon et al., 2020; Öner et al., 2016; Venkatesha et al., 2017). Antiinflammatory activity of a substance can be tested via different approaches and there are studies reporting LAB EPS's antiinflammatory properties determined by various tests.

Li and Shah (2016) investigated the antiinflammatory activity of EPS derived from *Streptococcus thermophilus* ASCC 1275 via measuring proinflammatory/antiinflammatory cytokine secretion ratios (IL-1β/IL-10, IL-6/IL-10, and TNF-α/IL-10) in RAW 264.7 macrophages stimulated by inflammation initiator lipopolysaccharide (LPS). They found that EPS treatment reduced the pro-/antiinflammatory cytokine secretion ratios of IL-1β/IL-10, IL6/IL-10, and TNF-α/IL-10 in a dose-dependent manner, indicating that EPS had good antiinflammatory activity on LPS stimulated RAW 264.7 macrophages by lowering the pro/antiinflammatory cytokine secretion ratios. They also reported that EPS decreased inflammation inducer NO and reactive oxygen species production in LPS stimulated RAW 264.7 macrophages. In another study, LPS induced RAW 264.7 cells precultured with EPS obtained from *Lactobacillus plantarum* L-14 and proinflammatory cytokine (IL-6, TNF-α, and IL-1β) production in RAW 264.7 cells were analyzed. It was found that IL-6, TNF-α, and IL-1β levels and expression of COX-2 and iNOS genes were suppressed in EPS treated RAW 264.7 cells. It was stated that EPS could be used in natural products in regulating inflammatory reaction (Kwon et al., 2020).

Buddana et al. (2015) determined the antiinflammatory activity of native and sulfated *Streptococcus mutans* MTCC 497 EPS via inhibition of albumin denaturation technique. Compounds that limit protein denaturation or promote protein stability are thought to be antiinflammatory since protein

denaturation is linked to inflammation. In this work, the antiinflammatory effect of EPS was evaluated against heat-mediated protein denaturation and compared to diclofinac sodium, a common antiinflammatory medicine. Researchers reported that at 100 µg/ml concentration, native and sulfated EPS showed antiinflammatory activity of 37% and 45%, respectively. While at 400 µg/mL concentration diclofinac sodium showed complete inhibition, sulfated EPS showed 82% antiinflammatory activity at 500 µg/mL concentration with the half maximal inhibitory concentration (IC_{50}) value of 111.55 µg/mL.

Prado et al., (2016) studied the antiinflammatory activity of kefir polysaccharide extract (ExPP) via hyaluronidase enzyme assay by which the inhibitory activity of ExPP on hyaluronidase enzyme was determined. The enzyme hyaluronidase hydrolyzes hyaluronic acid, which causes extracellular matrix breakdown, which promotes inflammation. Researchers reported that lyophilized (L) ExPP showed 63% antiinflammatory activity compared to dimethyl sulfoxide (DMSO). ExPP (L) inhibited the enzyme with a minimal activity of 2.08 mg/mL and a maximum activity of 2.57 mg/mL, IC_{50} value of lyophilized ExPP was 2.31 mg/mL. They stated that compared with a commercial product (ethanolic extract of propolis), ExPP demonstrated superior action and it have the potential to be used as an antiinflammatory compound. In another kefir related study, kefiran produced by *Lactobacillus kefiranofaciens* was investigated for its antiinflammatory activity on antigen stimulated bone marrow-derived mast cells. Mast cells play a key role in the inflammatory process. Upon stimulation, mast cells degranulate or release compounds that induce inflammation. Researchers found that kefiran treatment significantly inhibited antigen-induced degranulation by suppressing β-hexosaminidase secretion, an indicator of degranulation, completely at 3 mg/mL concentration. Also kefiran suppressed TNF-α, an important inflammatory cytokine, secretion in mast cells completely at 3 mg/mL concentration. Findings of the study indicated the antiinflammatory effect for kefiran in a dose-dependent manner (Furuno & Nakanishi, 2012).

EPS, as one of these postbiotics, were reported to show antiinflammatory effect in the studies mentioned above. Naturally it cannot be argued that all types of LAB EPSs have this effect because different strains can produce EPSs in different structures. However, studies show that LAB EPSs can act as an antiinflammatory agent. Therefore, with the extensive pharmaceutical studies, LAB EPS may be exploited for the development of antiinflammatory drugs as an alternative to synthetic compounds.

Antitumor and antimutagenicity

One of the postbiotics generated by LAB, exopolysaccharide (EPS), can act as ligands for host cells and protect the host by aggregating with pathogens in the intestine (CastroBravo et al., 2018). Furthermore, it has been found that EPS has anticancer properties as well as oxidative stress protection (Saadat et al., 2019; Li et al., 2013).

Microbial EPSs produced from biological, safe sources such as LAB, accepted an alternative to chemotherapy due to its side effcts. Due to their bioactive qualities, LAB have a wide variety of medical applications and have low cytotoxicity and adverse effects, providing them a good substitute for synthetic anticancer drugs (Farag et al., 2020).

The invention and introduction of potential antitumor drugs with low immune system side effects has become a critical goal in many immunopharmacology studies. EPSs derived from safe natural sources, such as LAB, typically have low cytotoxicity and side effects and may be a viable alternative to synthetic antitumor agents (Ismail & Nampoothiri, 2013).

Liu et al. (2011) and Wang et al. (2014) demonstrated the antiproliferative effect of *L. casei* 01 EPS on the HT-29 cell line, as well as the anticancer activity of EPS from *L. plantarum* 70810 on HepG-2, BGC-823, and mainly HT-29 malignant cell. EPSs from *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus acidophilus* have exhibited antitumor effects against a variety of cell lines in a dose-dependent manner (Deepak et al., 2016a; 2016b).

Under both normoxic and hypoxic conditions, the effect of EPS from *Lactobacillus acidophilus* on messenger RNA (mRNA) expression of several genes was investigated using quantitative real-time (RT)-PCR. The hazardous concentration was calculated as 5 mg/mL. EPS has been found to suppress the expression of genes involved in tumor angiogenesis and survival. In two colon cancer cell lines, EPS was also observed to cause cytotoxicity (Deepak et al., 2016b).

Dilna et al., (2015) reported that EPS from *Lb. plantarum* RJF4 had a specific antiproliferative effect to cancer cells and inhibited them. According to the cell viability test results, EPS showed toxic effect to MiaPaCa2-pancreatic cancer cell line in dose dependent manner and remained nontoxic to normal cell line (L6 and L929 fibroblast cells).

Antioxidant activity

Biological systems are exposed to oxidative stress with high levels of reactive oxygen species and free radicals. The presence of oxidative stress is associated with various diseases such as cancer, liver cirrhosis, and fatty liver (Dilna et al., 2015). EPSs from LAB have been discovered to contribute in the elimination of free radicals, so acting as natural strong antioxidants. They have also been found to be harmless, suggesting that they could be used as a replacement for synthetic antioxidants due to their toxicity (Saadat et al., 2019). The ability of LAB-derived EPSs to perform good antioxidant activity has been widely investigated by *in vitro* and *in vivo* studies.

EPS, composed of glucose and mannose, was purified from *Lb. plantarum* RJF4 EPS increased 32% total antioxidant capacity, 23.63% 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability and 50% reduction potential (compared to the ascorbic acid, as a standard) (Dilna et al., 2015).

In DPPH radical-scavenging, reducing power (RP), and ferric reducing antioxidant power (FRAP) assays, EPS from *Lactobacillus acidophilus* showed excellent antioxidative activity on colon cancer cell lines *in vitro*. In all of these assays, increasing the concentration of EPS improved the antioxidative capabilities of the EPS (Deepak et al., 2016b). Similarly EPS from *Lactobacillus plantarum* SKT109 showed superior DPPH-scavenging activity toward hydroxyl (68.52%) radicals at a dose of 5 mg/mL (Wang et al., 2018).

In vitro tests revealed that EPS from *Pediococcus acidilactici* NCDC 252 had antioxidant and profileration reduction potential on HCT116 (colon cancer cells) in a dose-dependent manner. EPS had a total antioxidant potential of 11.9 %, and it inhibited HCT116 cell by 67.1-87.3% at 10 and 100 g/mL, respectively. After *in vivo* experiments, it was indicated that EPS could be employed therapeutically as an antioxidant and anticancer agent (Kumar et al., 2020).

The antioxidant potential of EPSs generated by *Lactobacillus sanfranciscensis*, which was isolated from a Chinese traditional sourdough, was analyzed via free radical ($ABTS^{\bullet+}$) scavenging activity. The scavenging activity rate of polysaccharides were reported to 10.42%, while the EPS concentration was 0.0625 mg/mL. So, certain antioxidant activity even at low EPS concentrations was determined (Zhang et al., 2019).

Antibacterial and antiviral activity

EPS has the ability to have an antagonistic effect on pathogenic bacteria. Exopolysaccharides could actively communicate with Gram-negative and Gram-positive bacteria based on the permeability of their cell membranes, affecting the respiratory chain, cell division machinery, and eventually cell death (Hasheminya & Dehghannya, 2020). Antibiotic use, as is well known, reduces the antagonistic activity of normal microbial flora against pathogenic microorganisms. Probiotic EPSs are used to supplement the treatment of human diseases (Angelin & Kavitha, 2020).

The antilisterial activity of probiotics *Lactobacillus acidophilus* LA5, *Lactobacillus casei* 431, and *Lactobacillus salivarius* was studied *in vitro* and in food concepts. All *Lactobacillus spp.* postbiotics retained more than 50% of their residual antimicrobial activity, implying that *Lb. salivarius* CFS can be used as an effective food additive for controlling *Listeria monocytogenes* (Moradi et al., 2019).

The EPS producing *Lactobacillus kefiranofaciens* DN1 isolated from kefir was investigated for its antibacterial activity against *L. monocytogenes* and *Salmonella enteritidis*. It was discovered that EPS DN1 had bactericidal effects on several pathogens at concentrations of at least 1%. Therefore, the EPS produced by *Lactobacillus kefiranofaciens* DN1 could be used in the food industry to assure food safety or developed as an alternative treatment for foodborne infections (Jeong et al., 2017).

The antibacterial activity of EPS produced by *Lactobacillus delbureckii* against pathogens was researched. It was revealed that EPSs had antibacterial activity with the highest susceptibility against the test pathogens *Bacillus subtilis* and *Staphylococcus aureus* (Adebayo-Tayo & Fashogbon, 2020).

The LAB *Weissella cibaria* 27 (W27) isolated from kimchi was used as an EPS producer to understand the effects of sucrose for improving its EPS productivity. The results showed certain antibacterial activity against *E. coli* BL21, *B. subtilis* and *S. aureus* with or without the addition of sucrose. The inhibition zones for *B. cereus* and *E. coli* increased by the addition of sucrose, however the inhibition zone for *S. aureus* decreased (Yu et al., 2018).

Likewise, *Lactobacillus rhamnosus* isolated from human breastmilk demonstrated strong antibacterial activity against pathogenic *E. coli* and *Salmonella typhimurium in vitro*. HePs from *Lactobacillus gasseri* inhibited *L. monocytogenes* MTCC 657 more effectively. With an initial population of 9 log CFU/mL, EPS-C70 from camel milk exhibited 2 to 3 log reduction against tested food-borne pathogens, with the highest inhibition observed against *S. aureus* and *E. coli* (Alsaadi et al., 2020).

The antiviral effect of *L. plantarum* LRCC5310 isolated from the Korean traditional fermented food kimchi was investigated both *in vitro* and *in vivo*. EPS extracted from *L. plantarum* LRCC5310 were also stated to be effective in the control of rotavirus infection, which is a leading cause of violent diarrhea in newborns and young children worldwide. *L. plantarum* LRCC5310 EPS could help protect the intestinal mucosal barrier from viral shedding and other damages caused by virus infection adjuvant. Moreover, *L. plantarum* LRCC5310 EPS exhibits potent antirotavirus activity *in vitro*, especially against extracellular rotaviruses (Kim et al., 2018).

Cholesterol lowering properties

Excessive cholesterol and high blood pressure could be counted among the risk factors for cardiovascular diseases. High blood pressure levels have been linked to cardiovascular illness, particularly atherosclerosis, and as a result, the incidence of cerebral infarction, cerebral thrombosis, and cardiopathy is on the rise. Furthermore, existing mitigating or curing approaches are limited in intravenous feeding and cannot fundamentally heal diseases, therefore the need for a safe, effective, and side-effect-free cholesterol-lowering agent like EPS is urgent (Chien et al., 2010; Glass & Witztum, 2001).

EPS generated by LAB have been actually able to control cholesterol levels due to the adsorption ability

of polymers to this molecule (Ruas-Madiedo, 2014). A growing number of studies have found that LAB-produced EPS has a hypocholesterolemic effect.

By inhibiting cholesterol absorption, EPS as a postbiotic drug can improve lipid metabolism. In a preclinical animal model, consumption of kefiran by *Lactobacillus kefiranofaciens* delayed the development of atherosclerosis (Khalil et al., 2018; Uchida et al., 2010). Kefiran also reduced blood pressure and stabilized blood glucose levels in rats fed a high cholesterol diet. As a result, EPSs like kefiran have the potential to prevent cardiovascular diseases (Maeda et al., 2004).

Dilna et al., (2015), reported antioxidant activity, α-amylase inhibition, cholesterol lowering, and antiproliferative activities of EPS produced by Lactobacillus *plantarum* RJF4. The variation in health-promotion benefits of EPS depends on species, strain, and EPS type (Ryan et al., 2015). Another research by London et al. (2014) found that dietary intervention with EPS producing probiotics causes lipid metabolism to be modulated in a mouse model of atherosclerosis by lowering blood cholesterol and triglyceride levels. It could lead to positive changes in lipid metabolism.

Gastro-protective activity

Helicobacter pylori infections and long-term use of nonsteroidal antiinflammatory drugs can cause toxic effects on gastric epithelial cells which can lead to gastric ulcers. *Streptococcus thermophilus* CRL1190 and its EPS have been suggested to possess gastroprotective activity. *S. thermophilus* CRL1190 was reported to inhibit the adhesion of *H. pylori* to stomach gastric mucosa, modulate the inflammatory response in gastric epithelial cell line AGS and prevent the gastritis development (Marcial et al., 2017; Rodríguez et al., 2009; Saadat et al., 2019).

Polysaccharides from *L. reuteri* DSM17938 and *L. reuteri* L26 BiocenolTM were determined for their health benefits as an immune protective agent on intestinal porcine epithelial cell line-1 (IPEC-1) cells against haemolytic enterotoxigenic *E. coli* (ETEC) bacteria. They subsequently decreased ETEC-induced gene expression for the proinflammatory cytokines (IL-1, IL-6), which suggesting to role as a prophylactic effect to gastrointestinal infections (Kšonžeková et al., 2016).

Conclusion

Microorganisms are assumed to be protected by EPS from bacteriophages, antibiotics, physical stressors, and toxic compounds. Considering the probiotics, the EPS produced by these microorganisms could also operate as a physical barrier to prevent the toxin from interacting with eukaryotic cells, either by blocking toxin receptors on the cell surface or by serving as toxin-scavengers. LAB group comprises probiotic members also members producing postbiotics with health benefit properties such as EPS. Anticancer, antioxidant, antimicrobial, antiinflammatory, and immunomodulatory activities of LAB EPS have been studied. Studies show that LAB species can synthesis structurally diverse EPSs having different chemical substituents, linkages, and functional groups that brings these compounds the ability to possess biological activities in varying degrees. Information about EPS genetics, biological differences, biosynthetic pathways, metabolic models are used in metabolic engineering studies to modify EPS production and EPS composition. However, studies continue on the basic principles for the production of different EPSs from LAB via metabolic engineering and researches on this subject will probably multiply by diversifying. With aforementioned bioactivities LAB EPSs have the potential to be used in a number of fields including drug delivery, medicine, food, and agriculture and future studies may result in obtaining ready to consume LAB EPSs with outstanding biological activities and EPSs may appear constantly in certain product formulations in the fields of medicine and food.

References

Abbasi, A., Rad, A. H., Ghasempour, Z., Sabahi, S., Kafil, H. S., Hasannezhad, P., Saadat, Y. R., & Shahbazi, N. (2021). The biological activities of postbiotics in gastrointestinal disorders. *Critical Reviews in Food Science and Nutrition*, 1-22. https://doi.org/10.1080/10408398.2021.1895061

Adebayo-Tayo, B., & Fashogbon, R. (2020). In vitro antioxidant, antibacterial, in vivo immunomodulatory, antitumor and hematological potential of exopolysaccharide produced by wild type and mutant *Lactobacillus delbureckii* subsp.*bulgaricus*. *Heliyon*, *6*(2), 1-10. https://doi.org/10.1016/j.heliyon.2020.e03268

Adebayo-Tayo, B., Ishola, R., & Oyewunmi, T. (2018). Characterization, antioxidant and immunomodulatory potential on exopolysaccharide produced by wild type and mutant Weissella confusa strains. *Biotechnology Reports*, *19*, e00271. https://doi.org/10.1016/j.btre.2018.e00271

Adesulu-Dahunsi, A. T., Sanni, A. I., Jeyaram, K., Ojediran, J. O., Ogunsakin, A. O., & Banwo, K. (2018). Extracellular polysaccharide from Weissella confusa OF126: Production, optimization, and characterization. *International Journal of Biological Macromolecules*, *111*, 514-525. https://doi.org/10.1016/j.ijbiomac.2018.01.060

Alsaadi, L. G., Baker, B. A. A., Kadhem, B. M., Mahdi, L. H., & Mater, H. N. (2020). Exopolysaccharide as antiviral, antimicrobial and as immunostimulants: A review. *Plant Archives*, *20*(2), 5859-5875. eISSN: 2581-6063.

Angelin, J., & Kavitha, M. (2020). Exopolysaccharides from probiotic bacteria and their health potential. *International Journal of Biological Macromolecules*, *162*, 853-865. https://doi.org/10.1016/j.ijbiomac.2020.06.190

Buddana, S. K., Venkata Naga Varanasi, Y., & Reddy Shetty, P. (2015). Fibrinolytic, antiinflammatory and antimicrobial properties of α-(1-3)-glucans produced from *Streptococcus mutans* (MTCC 497). *Carbohydrate Polymers*, *15*, 152–159. https://doi.org/10.1016/j.carbpol.2014.08.083

Castro-Bravo, N., Wells, J. M., Margolles, A., & Ruas-Madiedo, P. (2018). Interactions of surface exopolysaccharides from Bifidobacterium and Lactobacillus within the intestinal environment. *Front in Microbiology*, *9*, 2426, 1-15. https://doi.org/10.3389/fmicb.2018.02426

Chaisuwan, W., Jantanasakulwong, K., Wangtueai, S., Phimolsiripol, Y., Chaiyaso, T., Techapun, C., Phongthai, S., You, S., Regenstein, J. M., & Seesuriyachan, P. (2020). Microbial exopolysaccharides for immune enhancement: Fermentation, modifications and bioactivities, *Food Bioscience, 35*, 1-17. https://doi.org/10.1016/j.fbio.2020.100564

Chen, Y. C., Wu, Y. J., & Hu, C. Y. (2019). Monosaccharide composition influence and immunomodulatory effects of probiotic exopolysaccharides. *International Journal of Biological Macromolecules*, *133*, 575-582. https://doi.org/10.1016/j.ijbiomac.2019.04.109

Chien, Y. L., Wu, L. Y., Lee, T. C., & Hwang, L. S. (2010). Cholesterol-lowering effect of phytosterol-containing lactic-fermented milk powder in hamsters. *Food Chemistry*, *119*(3), 1121-1126. https://doi.org/10.1016/j.foodchem.2009.08.023

Deepak, V., Ram Kumar Pandian, S., Sivasubramaniam, S. D., Nellaiah, H., & Sundar, K. (2016a). Optimization of anticancer exopolysaccharide production from probiotic *Lactobacillus acidophilus* by response surface methodology. *Preparative Biochemistry* 396 *and Biotechnology*, *46*(3), 288–297. https://doi.org/10.1080/10826068.2015.1031386

Deepak, V., Ramachandran, S., Balahmar, R. M., Pandian, S. R. K., Sivasubramaniam, S. D., Nellaiah, H., & Sundar, K. (2016b). In vitro evaluation of anticancer properties of exopolysaccharides from *Lactobacillus acidophilus* in colon cancer cell lines. *In Vitro Cellular &Developmental Biology-Animal*, *52*(2),163-173. https://doi.org/10.1007/s11626-015-9970-3

Dilna, S. V., Surya, H., Aswathy, R. G., Varsha, K. K., Sakthikumar, D. N., Pandey, A., & Nampoothiri, K. M. (2015). Characterization of an exopolysaccharide with potential 404 health-benefit properties from a probiotic *Lactobacillus plantarum* RJF4. *LWT-Food Science and Technology*, *64*, 1179-1186. https://doi.org/10.1016/j.lwt.2015.07.040

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest*, *118*(2), 503–508. https://doi.org/10.1378/chest.118.2.503

Farag, M. M., Moghannem, S. A., Shehabeldine, A. M., & Azab, M. S. (2020). Antitumor effect of exopolysaccharide produced by *Bacillus mycoides*. *Microbial pathogenesis*, *140*, 1-10. https://doi.org/10.1016/j.micpath.2019.103947

Furuno, T., & Nakanishi, M. (2012). Kefiran suppresses antigen-induced mast cell activation. *Biological and Pharmaceutical Bulletin*, *35*(2), 178–183. https://doi.org/10.1248/bpb.35.178

Glass, C. K. & Witztum, J. L. (2001). Atherosclerosis: the road ahead. *Cell*, *104*(4), 503-516. https://doi.org/10.1016/s0092-8674(01)00238-0

Hasheminya, S. M., & Dehghannya, J. (2020). Novel ultrasound-assisted extraction of kefiran biomaterial, a prebiotic exopolysaccharide, and investigation of its physicochemical, antioxidant and antimicrobial properties. *Materials Chemistry and Physics*, *243*, 1-8. https://doi.org/ 10.1016/j.matchemphys.2020.122645

Ismail, B., & Nampoothiri, K. M. (2013). Exposition of antitumour activity of a chemically characterized exopolysaccharide from a probiotic *Lactobacillus plantarum* MTCC 9510. *Biologia*, *68*(6), 1041–1047. https://doi.org/10.2478/s11756-013-0275-2

Jurášková, D., Ribeiro, S. C., & Silva, C. C. (2022). Exopolysaccharides produced by lactic acid bacteria: From biosynthesis to health-promoting properties, *Foods*, *11*(2), 156. https://doi.org/10.3390/foods11020156

Jeong, D., Kim, D. H., Kang, I. B., Kim, H., Song, K. Y., Kim, H. S., & Seo, K. H. (2017). Characterization and antibacterial activity of a novel exopolysaccharide produced by Lactobacillus kefiranofaciens DN1 isolated from kefir. *Food Control*, *78*, 436-442. https://doi.org/10.1016/j.foodcont.2017.02.033

Kavitake, D., Kalahasti, K. K., Devi, P. B., Ravi, R., & Shetty, P. H. (2020). Galactan exopolysaccharide based flavour emulsions and their application in improving the texture and sensorial properties of muffin. *Bioactive Carbohydrates and Dietary Fibre*, *24*, 1-8. https://doi.org/10.1016/j.bcdf.2020.100248

Khalil, E. S., Abd Manap, M. Y., Mustafa, S., Alhelli, A. M., & Shokryazdan, P. (2018). Probiotic properties of exopolysaccharide-producing lactobacillus strains ısolated from Tempoyak. *Molecules*, *23*(2), *398*, 1-20. https://doi.org/10.3390/molecules23020398

Kim, K., Lee, G., Thanh, H. D., Kim, J. H., Konkit, M., Yoon, S., Park, M., Yang, S., Park, E., & Kim, W. (2018). Exopolysaccharide from *Lactobacillus plantarum* LRCC5310 offers protection against rotavirus-induced diarrhea and regulates inflammatory response. *Journal of Dairy Science*, *101*(7), 5702-5712. https://doi.org/10.3168/jds.2017-14151

Korcz, E., Kerényi, Z., & Varga, L. (2018). Dietary fibers, prebiotics, and exopolysaccharides produced by lactic acid bacteria: potential health benefits with special regard to cholesterol-lowering effects. *Food & Function*, *9*(6), 3057-3068. https://doi.org/10.1039/c8fo00118a

Kšonžeková, P., Bystrický, P., Vlčková, S., Pätoprstý, V., Pulzová, L., Mudroňová, D., Kubašková, T., Csank, T., & Tkáčiková, Ľ. (2016). Exopolysaccharides of *Lactobacillus reuteri*: Their influence on adherence of *E. coli* to epithelial cells and inflammatory response. *Carbohydrate Polymers*, *141*, 10-19. https://doi.org/10.1016/j.carbpol.2015.12.037

Kumar, R., Bansal, P., Singh, J., & Dhanda, S. (2020). Purification, partial structural characterization and health benefits of exopolysaccharides from potential probiotic *Pediococcus acidilactici* NCDC 252. *Process Biochemistry*, *99*, 79-86. https://doi.org/10.1016/j.procbio.2020.08.028

Kwon, M., Lee, J., Park, S., Kwon, O. H., Seo, J., & Roh, S. (2020). Exopolysaccharide isolated from *Lactobacillus plantarum* l-14 has anti-inflammatory effects via the tolllike receptor 4 pathway in lps-induced raw 264.7 cells. *International Journal of Molecular Sciences*, *21*(23), 1–18. https://doi.org/10.3390/ijms21239283

Laws, A., Gu, Y., & Marshall, V. (2001). Biosynthesis, characterisation, and design of bacterial exopolysaccharides from lactic acid bacteria. *Biotechnology Advances*, *19*(8), 597-625. https://doi.org/10.1016/s0734-9750(01)00084-2

Li, J. Y., Jin, M. M., Meng, J., Gao, S. M., & Lu, R. R. (2013). Exopolysaccharide from *Lactobacillus plantarum* LP6: Antioxidation and the effect on oxidative stress. *Carbohydrate Polymers*, 98, 1147–1152. https://doi.org/10.1016/j.carbpol.2013.07.027

Li, S., & Shah, N. P. (2016). Characterization, anti-Inflammatory and antiproliferative activities of natural and sulfonated exopolysaccharides from *Streptococcus thermophilus* ASCC 1275. *Journal of Food Science*, *81*(5), M1167–M1176. https://doi.org/10.1111/1750-3841.13276

Li, W., Xia, X., Tang, W., Ji, J., Rui, X., Chen, X., Jiang, M., Zhou, J., Zhang, Q., & Dong, M. (2015). Structural characterization and anticancer activity of cell-bound exopolysaccharide from Lactobacillus helveticus MB2-1. *Journal of Agricultural and Food Chemistry*, *63*(13), 3454-3463. https://doi.org/10.1021/acs.jafc.5b01086

Liu, C. T., Chu, F. J., Chou, C. C., & Yu, R. C. (2011). Antiproliferative and anticytotoxic effects of cell fractions and exopolysaccharides from *Lactobacillus casei* 01. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *721*(2), 157–162. https://doi.org/10.1016/j.mrgentox.2011.01.005

London, L. E. E., Kumar, A. H. S., Wall, R., Casey, P. G., O'Sullivan, O., Shanahan, F., Hill, C., Cotter, P. D., Fitzgerald, G. F., Ross, P. R., Caplice, N. M., & Stanton, C. (2014). Exopolysaccharide-producing probiotic Lactobacilli reduce serum cholesterol and modify enteric microbiota in ApoE-deficient mice. *The Journal of Nutrition*, *144*(12), 1956-1962. https://doi.org/10.3945/jn.114.191627

Maeda, H., Zhu X., Omura, K., Suzuki, S., & Kitamura, S. (2004). Effects of an exopolysaccharide (kefiran) on lipids, blood pressure, blood glucose and constipation. *Biofactors*, *22*, 197–200. https://doi.org/10.1002/biof.5520220141

Marcial, G., Villena, J., Faller, G., Hensel, A., & de Valdéz, G. F. (2017). Exopolysaccharide producing *Streptococcus thermophilus* CRL1190 reduces the inflammatory response caused by *Helicobacter pylori*. *Beneficial Microbes*, *8*(3), 451-461. https://doi.org/10.3920/BM2016.0186

Minghetti, L. (2004). Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *Journal of Neuropathology and Experimental Neurology*, *63*(9), 901–910. https://doi.org/10.1093/jnen/63.9.901

Moradi, M., Mardani, K., & Tajik, H. (2019). Characterization and application of postbiotics of Lactobacillus spp. on *Listeria monocytogenes* in vitro and in food models. *LWT-Food Science and Technology*, *111*, 457-464. https://doi.org/10.1016/j.lwt.2019.05.072

Noroozi, E., Tebianian, M., Taghizadeh, M., Dadar, M., & Mojgani, N. (2021). Anticarcinogenic potential of probiotic, postbiotic metabolites and paraprobiotics on human cancer cells. In N. Mojgani & M. Dadar (Eds.), *Probiotic Bacteria and Postbiotic Metabolites: Role in Animal and Human Health* (pp. 166). Springer.

Opal, S. M., & DePalo, V. A. (2000). Anti-inflammatory cytokines. *Chest*, *117*(4), 1162–1172. https://doi.org/10.1378/chest.117.4.1162

Öner, E. T., Hernández, L., & Combie, J. (2016). Review of Levan polysaccharide: From a century of past experiences to future prospects. *Biotechnology Advances*, *34*(5), 827–844. https://doi.org/10.1016/j.biotechadv.2016.05.002

Perricone, M., Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2014). Technological characterization and probiotic traits of yeasts isolated from Altamura sourdough to select promising microorganisms as functional starter cultures for cereal-based products. *Food Microbiology*, *38*, 26-35. https://doi.org/10.1016/j.fm.2013.08.006

Prado, M. R. M., Boller, C., Zibetti, R. G. M., de Souza, D., Pedroso, L. L., & Soccol, C. R. (2016). Anti-inflammatory and angiogenic activity of polysaccharide extract obtained from Tibetan kefir. *Microvascular Research*, *108*, 29–33. https://doi.org/10.1016/j.mvr.2016.07.004

Rana, S., & Upadhyay, L. S. B. (2020). Microbial exopolysaccharides: Synthesis pathways, types and their commercial applications. *International Journal of Biological Macromolecules*, *15*(157), 577-583. https://doi.org/10.1016/j.ijbiomac.2020.04.084

Rajoka, M. S. R., Wu, Y., Mehwish, H. M., Bansal, M., & Zhao, L. (2020). Lactobacillus exopolysaccharides: New perspectives on engineering strategies, physiochemical functions, and immunomodulatory effects on host health. *Trends in Food Science & Technology*, *103*, 36-48. https://doi.org/10.1016/j.tifs.2020.06.003

Ren, W., Xia, Y., Wang, G., Zhang, H., Zhu, S., & Ai, L. (2016). Bioactive exopolysaccharides from a *S. thermophilus* strain: Screening, purification and characterization. *International Journal of Biological Macromolecules*, *86*, 402-407. https://doi.org/10.1016/j.ijbiomac.2016.01.085

Rodríguez, C., Medici, M., Rodríguez, A. V., Mozzi, F., & de Valdez, G. F. (2009). Prevention of chronic gastritis by fermented milks made with exopolysaccharide-producing *Streptococcus thermophilus* strains. *Journal of Dairy Science*, *92*(6), 2423-2434. https://doi.org/10.3168/jds.2008-1724

Ruas-Madiedo, P., & De Los Reyes-Gavilán, C. G. (2005). Invited review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science*, *88*(3), 843-856. https://doi.org/ 10.3168/jds.S0022-0302(05)72750-8

Ruas-Madiedo, P. (2014). Food oligosaccharides: Production, analysis and bioactivity; Biosynthesis and bioactivity of exopolysaccharides produced by probiotic bacteria. In F.L. Moreno & M.L. Sanz (Eds.), *Food oligosaccharides production, analysis and bioactivity* (pp. 118-133). John Wiley & Sons, West Sussex.

Ryan, P., Ross, R., Fitzgerald, G., Caplice, N., & Stanton, C. (2015). Sugar-coated:exopolysaccharide producing lactic acid bacteria for food and human health applications. *Food &Function*, *6*, 679–693. https://doi.org/10.1039/C4fo00529e

Saadat, Y. R., Khosroushahi, A. Y., & Gargari, B. P. A. (2019). A comprehensive review of anticancer, immunomodulatory and health beneficial effects of the lactic acid bacteria exopolysaccharides, *Carbohydrate Polymers*, *217*, 79-89. https://doi.org/10.1016/j.carbpol.2019.04.025

Salazar, N., Gueimonde, M., Hernández-Barranco, A. M., Ruas-Madiedo, P., & de los ReyesGavilán, C. G. (2008). Exopolysaccharides produced by intestinal Bifidobacterium strains act as fermentable substrates for human intestinal bacteria. *Applied and Environmental Microbiology*, *74*(15), 4737–4745. https://doi.org/ 10.1128/AEM.00325-08

Sanlibaba, P., & Çakmak, G. A. (2016). Exopolysaccharides production by lactic acid bacteria. *Applied Microbiology*, *2*(2), 1-5. https://doi.org/10.4172/2471-9315.1000115

Schilling, C., Badri, A., Sieber, V., Koffas, M., & Schmid, J. (2020). Metabolic engineering for production of functional polysaccharides. *Current Opinion in Biotechnology*, *66*, 44-51. https://doi.org/10.1016/j.copbio.2020.06.010

Tang, W., Dong, M., Wang, W., Han, S., Rui, X., Chen, X., Jiang, M., Zhang, Q., Junjun, W., & Li, W. (2017). Structural characterization and antioxidant property of released exopolysaccharides from *Lactobacillus* delbrueckii ssp. *bulgaricus* SRFM-1. *Carbohydrate polymers*, *173*, 654-664. https://doi.org/10.1016/j.carbpol.2017.06.039

Taylan, O., Yilmaz, M. T., & Dertli, E. (2019). Partial characterization of a levan type exopolysaccharide (EPS) produced by *Leuconostoc mesenteroides* showing immunostimulatory and antioxidant activities. *International Journal of Biological Macromolecules*, *136*, 436-444. https://doi.org/10.1016/j.ijbiomac.2019.06.078

Teame, T., Wang, A., Xie, M., Zhang, Z., Yang, Y., Ding, Q., Gao, C., Olsen, R. E., Ran, C., & Zhigang, Z. (2020). Paraprobiotics and postbiotics of probiotic Lactobacilli, their positive effects on the host and action mechanisms: A review. *Frontiers in Nutrition*, *7*, 1-16. https://doi.org/10.3389/fnut.2020.570344

Thantsha, M. S., Mamvura, C. I., & Booyens, J. (2012). Probiotics–what they are, their benefits and challenges. In T. Brzozowski (Ed.). *New Advances in the Basic and Clinical Gastroenterology* (pp. 21-50). InTech: Croatia.

Uchida, M., Ishii, I., Inoue, C., Akisato, Y., Watanabe, K., Hosoyama, S., Toida, T., Ariyoshi, N., & Kitada, M. (2010). Kefiran reduces atherosclerosis in rabbits fed a high cholesterol diet. *Journal of Atherosclerosis and Thrombosis*, *17*, 980–988. https://doi.org/10.5551/jat.4812

Venkatesha, S. H., Acharya, B., & Moudgil, K. D. (2017). Natural Products as Source of AntiInflammatory Drugs. In J.M. Cavaillon & M. Singer (Eds.), *Inflammation - From Molecular and Cellular Mechanisms to the Clinic* (pp. 1661-1690). Wiley.

Wang, K., Li, W., Rui, X., Chen, X., Jiang, M., & Dong, M. (2014). Characterization of a novel exopolysaccharide with antitumor activity from *Lactobacillus plantarum* 70810. *International Journal of Biological Macromolecules*, *63*, 133–139. https://doi.org/10.1016/j.ijbiomac.2013.10.036

Wang, J., Fang, X., Wu, T., Min, W., & Yang, Z. (2018). Exopolysaccharide producing Lactobacillus plantarum SKT109 as adjunct culture in Cheddar cheese production. *LWT-Food Science and Technology*, *97*, 419-426. https://doi.org/10.1016/j.lwt.2018.07.011

Ye, G., Li, G., Wang, C., Ling, B., Yang, R., & Huang, S. (2019). Extraction and characterization of dextran from Leuconostoc pseudomesenteroides YB-2 isolated from mango juice. *Carbohydrate polymers*, *207*, 218-223. https://doi.org/10.1016/j.carbpol.2018.11.092

Yu, Y. J., Chen, Z., Chen, P. T., & Ng, I. S. (2018). Production, characterization and antibacterial activity of exopolysaccharide from a newly isolated *Weissella cibaria* under sucrose effect. *Journal of Bioscience and Bioengineering*, *126*(6), 769-777. https://doi.org/10.1016/j.biosc.2018.05.028

Zannini, E., Waters, D. M., Coffey, A., & Arendt, E. K. (2016). Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. *Applied Microbiology and Biotechnology*,*100*(3),1121-1135.https://doi.org/10.007/s00253-015-7172-2

Zhang, G., Zhang, W., Sun, L., Sadiq, F. A., Yang, Y., Gao, J., & Sang, Y. (2019). Preparation screening, production optimization and characterization of exopolysaccharides produced by *Lactobacillus sanfranciscensis* Ls-1001 isolated from Chinese traditional sourdough. *International Journal of Biological macromolecules*, *139*, 1295-1303. https://doi.org/10.1016/j.ijbiomac.2019.08.077

Zhang, Z., Liu, Z., Tao, X., & Wei, H. (2016). Characterization and sulfated modification of an exopolysaccharide from *Lactobacillus plantarum* ZDY2013 and its biological activities. *Carbohydrate polymers*, *153*, 25-33. https://doi.org/10.1016/j.carbpol.2016.07.084

Zhou, Y., Cui, Y., & Qu, X. (2019). Exopolysaccharides of lactic acid bacteria: Structure, bioactivity and associations: A review. *Carbohydrate Polymers*, *207*, 317-332. https://doi.org/10.1016/j.carbpol.2018.11.093

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RESEARCH PAPER

Appraisal of fungi leaf spots of groundnut (*Arachis hypogea*) and control of *Cylindrocladium* blight disease using biocontrol, botanical, and chemical measures

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Abstract

Groundnut is infected by 55 pathogens which adversely contribute to the low yield of groundnuts. Three sub-trials were conceived to proffer solutions to groundnut *Cylindrocladium parasiticum* disease. These sub-trials (using biocontrol, synthetic and botanical measures) were set up *in vitro* using completely randomized design with each treatment replicated thrice. Firstly, all biocontrol (*Trichoderma*) isolates showed a steady linear increase in the inhibition of the growth of the pathogen. There was no significant difference (P≤0.05) between the isolates of *Trichoderma harzianum* at 96 h after inoculation (HAI). Secondly, the synthetic pesticide Mancozeb® 100% significantly inhibited the pathogen more than all other synthetic chemical treatments, followed by Mancozeb 50%, Tandem® 100% and Tandem 50% (which were at par (at 144 HAI)). Thirdly, the botanical *Parkia* sp. 100% was significantly superior to other botanicals (192 HAI results). Likewise, *Casuarina* sp. 100% and *Parkia* sp. 50% were at par but performed better than other treatments except for *Parkia* sp. 100%. Inhibition by *Casuarina* sp. 50% was at par with *Parkia* sp. 50% but it was significantly superior to other treatments (except for *Parkia* sp. 100%, *Casuarina* sp. 100%, and *Parkia* sp. 50%). Finally, all trials showed that Mancozeb 100% performed better than other treatments.

Introduction

Groundnut (*Arachis hypogaea* L.) is an oil-seed and grain legume crop cultivated in most areas of the tropics and subtropics (latitudes 40°N-40°S) (Pattee & Young, 1982). El-Sherbeny et al. (2020) reported that global annual groundnut yields were up to 45.3 million metric tons per annum. Groundnut seeds contain 50% oil, 25-30% protein, 20% carbohydrate, 5% fibre/ash (Fageria et al., 1997; Naab et al., 2005; Tsigbey et al., 2004). Groundnut seeds are boiled or roasted and consumed as a snack, and soup thickener. While the hay/haulms are fed to livestock. Moreover, this crop fixes atmospheric nitrogen in its nodules thus enhancing soil fertility (Hassain et al., 2005).

Nonetheless, its production and yields are constrained by various pathogens (viruses, bacteria, fungi, and insects) and abiotic factors like drought, salinity, and unfavourable temperature (El-Sherbeny et al., 2020). Globally groundnut is attacked by more than 55 pathogens which is one of the most important factors causing the low yield of groundnuts (Muthukumar et al., 2014).

Some fungi-induced foliar diseases of groundnut include *Macrophomina, Choanephora, Colletotrichum, Cylindrocladium, Drechslera*, *Pestalotiopsis, Phomopsis, Phyllosticta*, zonate, early leaf spot, and late leaf spot.

Besides these, *Alternaria, Botrytis, Myrothecium, Phoma,* and *Phomopsis* leaf blights as well as powdery mildew, rust, web blotch (net blotch), *Alternaria* veinal necrosis, and anthracnose attack the crop (Onyishi, 2010; Porter, 1993; *Shaza* et al., 2004).

Early leaf spots (induced by *Cercospora arachidicola* Hori.: its teleomorph is *Mycosphaerella arachidicola* W.A. Jenkins) and late leaf spot (induced by *Phaeoisariopsis personata* (Berk. & Curt.) V. Arx.: its teleomorph is *Mycosphaerella berkeleyi* W.A. Jenkins) coupled with groundnut rust (*Puccinia arachidis L.*) have been reported to be able to cause severe economic yield loss in groundnut globally (Anco et al., 2016; Damicone, 2017; Ghewande, 2009; Nutsugah et al., 2007; Subramanyam et al., 1991; Waliyar et al., 2000; Woodward et al., 2013). Generally, leaf spots can cause between 50-70% yield losses in West Africa (Naab et al., 2004; Waliyar et al., 2000) and up to 50% yield loss worldwide (Desai & Bagwan, 2005; Koïta et al., 2017; McDonald et al., 1985; Pal et al., 2014; Sangoyomi & Alabi, 2016).

Pal et al. (2014) reported that effective control of fungi diseases in groundnut has not been achievable to date despite the availability of different kinds of fungicides. They pointed out that the situation is going to deteriorate due to climatic change accompanied by the emergence of new virulent strains of pathogens. Grahame (2014) reported that resistance to several classes of fungicides used in groundnut production has been reported for some leaf spot pathogens. For instance, resistance to tebuconazole (triazole fungicides) was reported in the United States. Kishore et al. (2005) reported that host plant resistance to leaf spot diseases is very rare in groundnut cultivars hence use of synthetic pesticides has persisted.

Anco et al. (2016) reported that pest control programs in South Carolina, USA, mainly target late leaf spots which consistently cause economic yield loss. Nutsugah et al. (2007) reported that fungicides (thiophanate methyl, benomyl and tebuconazole) suppressed groundnut leaf spot diseases and increased biomass and pod yields in Ghana. Tebuconazole was the most effective fungicide in reducing leaf spot severity, and increasing biomass and pod yield. Neem seed extract, *Alata samina* extracts, thiophanate-methyl, carbendazim (Bavistin®), and tebuconazole (Folicur®) mixed with azoxystrobin (Abound®) reduced leaf spot severity and increased peanut yield in northern Ghana (Tsigbey et al., 2000, 2001).

So far chlorothalonil is the most reliable pesticide for the control of leaf spots and rust on groundnuts (Andrews, 1992; Kishore et al., 2005; Tsatsia & Grahame, 2013; Woodward et al., 2013). Kishore et al. (2005) reported that at more than 250 μg mL^{-1} chlorothalonil alone or in the presence of *Pseudomonas aeruginosa* GSE 18 and GSE 19, (100 μg mL^{-1}), completely controlled *Phaeoisariopsis personata*. However, Podile and Kishore (2002) revealed that the performance of mycoparasitic fungi (like *Verticillium lecanii*, *Dicyma pulvinata,* and *Acremonium obclavatum)* in the field is highly variable.

Kiran (2012) reported that collar rot or seedling blight or crown rot (by *Aspergillus niger* and *A. pulverulentum*) could be controlled using seed treatment with *Trichoderma viride or T. harzianum* (4 g/kg seeds) preferably in combination with organic amendments such as castor, neem or mustard cakes (500 kg/ha). These researchers confirmed that treatment of the seeds with thiram (75% WP), captan (80% WP), mancozeb (75% WP) or carbendazim (50% WP) effectively controlled seed-borne infections.

Frances et al. (2002) and Buck (2004) stated that another promising direction of research is to utilize fungicide-tolerant biocontrol agents with synthetic fungicides thereby reducing the amount of fungicides required. This form of integration was more effective against damping-off of tomato, *Rhizoctonia* root rot and take-all of spring wheat and postharvest diseases of fruits than using each method of control alone.

Several research findings have proven that plants that contain antimicrobial biochemical metabolites, when applied against microbes are less detrimental to the environment than synthetic pesticides (Hashim & Devi, 2003). Koïta et al. (2017) reported that aqueous extracts of *Lippia multiflora* Moldenke and *Ziziphus mucronata* Wild were most effective and significantly increased groundnut pod yield on a susceptible groundnut variety (TS32-1) in Burkina Faso compared to other treatments.

Awurum and Uwajimgba (2013) reported that plant extracts of *Dennettia tripetala* and benomyl significantly reduced the disease severity of *Fusarium oxysporium* wilt compared to plant extracts of *Spondias mombin* in the screenhouse. Soumya and Bindu (2012) effectively controlled groundnut seed pathogens (*Aspergillus niger*, *A. flavus*, *Penicillium* sp. and *Rhizopus* sp.) using *Capsicum frutescens* extracts.

Asama and Channya (2018) reported the occurrence of *Cylindrocarpon lichenicola, Aspergillus niger (brasilensis), Aspergilus flavus, Penicillium chrysogenum, Rhizopus stolonifer, Paecilomyces lilacinus, Pseudallescheria boydii, and Scedosaporium prolificans* on groundnut seeds in North-eastern Nigeria. *Cylindrocarpon* spp. have been reported to be capable of producing mycotoxins especially afflatoxins, zearalenone and trichothecenes and Deoxynivalenol (i.e. D.O.N.).

Nutsugah et al. (2007) reported that farmers in northern Ghana perceive shoot defoliation as an indicator of plant maturity and not as a disease. This is a common view held by farmers in Nigeria as well. In West Africa use of fungicides in groundnut production is rare due to constraints like lack of credit, low yields, and non-availability of fungicides (McDonald et al., 1985). In this region, the application of fungicides can reduce the severity of leaf spots and increase yields (Kannaiyan & Haciwa, 1990; Waliyar et al., 2000). Based on the foregoing information this research was conceived to

appraise the status quo of leaf spot diseases in groundnuts and proffer some solutions to these plant diseases.

Materials and Methods

Site of the study

This research was carried out at the Faculty of Agriculture Laboratories in Alex Ekwueme Federal University Ndufu-Alike, Ikwo Local Government Area of Ebonyi State. The University is located in Ikwo (6.069°N by 8.199°E) about 21 kilometres from Abakaliki; the State capital. Ebonyi State is in the derived savanna zone of Nigeria with a humid tropical climate.

Isolation and identification of the fungi associated with groundnut leaf spots

The infected groundnut leaves utilized for this research were obtained from farms in Ebonyi State. The fungi were isolated using potato dextrose agar (PDA) medium which was autoclaved at 120°C and 15 psi for 15 min according to the manufacturer's (LifeSave Biotech Inc.) instructions. The isolated fungi were sub-cultured to obtain pure cultures which were used to identify the fungi with the aid of literature on fungi morphology (Barnett & Hunter, 1972). The fungi isolates were stored in the freezer for use later in determining possible management techniques.

Subtrial 1: Effects of *Trichoderma* isolates as a biocontrol agent on *Cylindrocladium parasiticum* associated with groundnut leaf spots

The fungus (*Cylindrocladium parasiticum* Crous, M.J. Wingfield & Alfenas (syn. *C. crotalariae* (Loos) Bell & Sobers) isolate utilized in this sub-trial was isolated from infected groundnut plants as described above. While the *Trichoderma* isolates were isolated from the Bambara groundnut seeds, mushrooms, crop seeds and farmland soils. The experiment was laid out in Petri dishes using completely randomized design and each treatment was replicated three times.

The treatment set consisted of 6 *Trichoderma harzianum* isolates (*T. harzianum* AIBN, *T. harzianum* BGMP, *T. harzianum* ZXMZ, *T. harzianum BGMZ3, T. harzianum* BGMZM, and *T. harzianum* BGMZ4), a check (Mancozeb® at 2000 g/ha) and a control. The control was inoculated with the pathogenic fungi (*C. parasiticum*) isolate alone. The agar medium was inoculated with a 2-mm disc (of the pathogen or biological control agent), placed at the edge of the plate.

Subtrial 2: Effects of synthetic pesticides on *C. parasiticum* associated with groundnut leaf spots

The experiment was carried out using Petri dishes. It was laid out in the laboratory using a completely randomized design (CRD) with 5 treatments. Each treatment was replicated three times. The treatment set included control, Mancozeb 100%, Mancozeb 50%, Tandem 100% and Tandem 50%. Mancozeb® (2000 g/ha - it is a contact fungicide) and Tandem® (wettable powder of Cu (I) O (60%) + Metalaxyl (12%) recommended at 800 g/ha, it is a systemic fungicide) were utilized to compose the treatments. Each treatment consisted of three levels (0.0, 50 and 100% concentrations).

Subtrial 3: Effects of plant extracts on *C. parasiticum* associated with groundnut leaf spots

The experiment was carried out using Petri dishes. It was laid out in the laboratory using a completely randomized design (CRD) with 8 treatments. Each treatment was replicated three times. The treatment set included control, bark of African locust bean (*Parkia biglobosa* (Jacq.) R. Br. ex G. Don), shoot of Australian pine (also called beef-wood, iron-wood, she-oak, whistling pine or horse-tail tree (i.e. *Casuarina equisetifolia*), and shoots/branches of purple-leaved spiderwort (also called Moses-in-the-cradle or oyster plant i.e. *Rhoeo spathacea* (Sw.) Stearn). Each plant tissue was weighed at the rate of 333.3 g tissues/L distilled water for 100% concentration. Each treatment consisted of three levels (0.0, 50 and 100% concentrations).

Data collection used for the subtrials

The radius of *C. parasiticum* colony was measured using a transparent ruler at 24 h intervals starting from day 1 until each sub-trial was terminated. The percentage inhibition of the pathogen was calculated using:

$$PI = ((C - T)/C) \times 100\%$$

Where

PI= Percent inhibition of growth of the fungus

C= Perpendicular radius of fungus colony in the control plate

T= Perpendicular radius of the fungus colony in treated plate

Data analysis used for the subtrials

The data were subjected to analysis of variance (ANOVA) and the means were separated using Student Newmann Keul's (SNK) method. Genstat® 2nd edition discovery statistical package was used to carry out ANOVA test. Descriptive statistics were used to illustrate the trends in the growth of the pathogen and its management.

Results

The results of the trial on inhibition of the growth of *C. parasiticum* from groundnut by three botanical pesticides are presented in Figure 1. It was observed that *Casuarina* and *Rhoeo* spp. (at 50% and 100% concentrations) exhibited a similar pattern of inhibition of *C. parasiticum* whereby the level of inhibition dropped to the right and then resurged as time passed till the trial was terminated. On the contrary, both

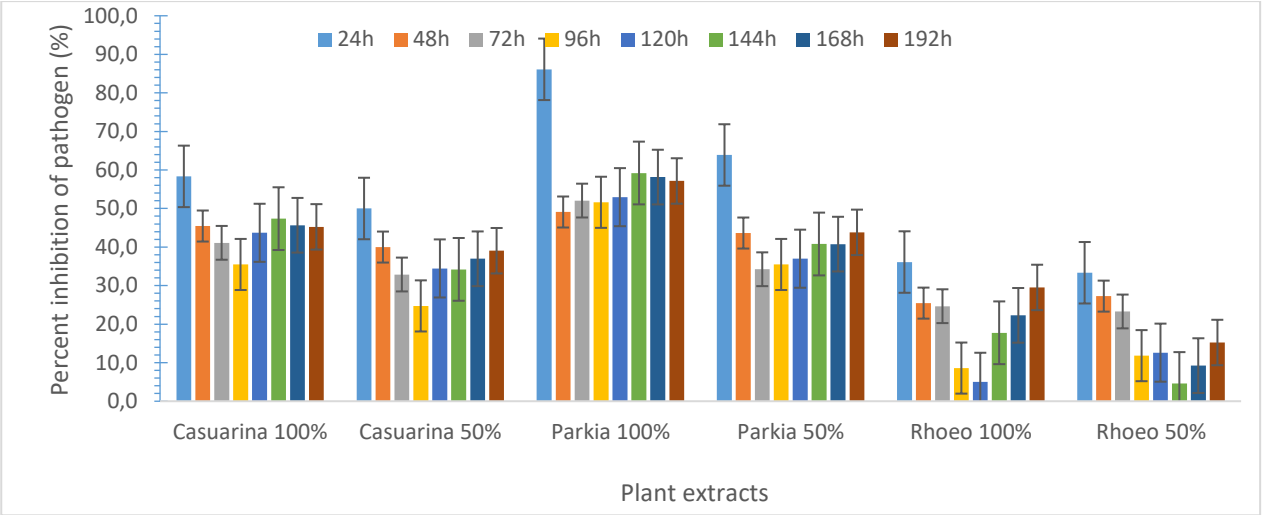

Figure 1. Inhibition of the growth of leaf spot pathogen isolated from groundnut caused by the application of three botanical pesticides.

concentrations of *Parkia* sp. showed an unswerving increase in inhibition of the pathogen all through till the termination of the trial.

The means separation (based on 192 HAI data) showed that the inhibition by *Parkia* 100% was significantly superior ($P \leq 0.05$) to all treatments and control. Means of *Casuarina* 100% and *Parkia* 50% were at par statistically but performed better than all other treatments except *Parkia* 100%. The inhibition by *Casuarina* 50% was at par with *Parkia* 50% but it was significantly superior to all other treatments (excepting *Parkia* 100%, *Casuarina* 100%, and *Parkia* 50%). The inhibition by both extracts of *Rhoeo* sp. was significantly superior to that of the control only. In fact, inhibition by all the plant extracts was significantly higher compared to the control.

The results of the trial on the effects of isolates of *Trichoderma* species against this pathogen (*C. parasiticum*) are presented in Figure 2. All the isolates showed a fairly steady linear increase in inhibition of the pathogen throughout. Mancozeb 100% was consistently superior (24 HAI) compared to the biocontrol agents as time passed. The means separation (96 HAI data) showed no significant difference ($P \leq 0.05$) between the isolates of *T. harzianum* utilized but they were significantly different ($P \leq 0.05$) from the check which was significantly superior to them.

The results of the trial on the effects of synthetic fungicides against *C. parasiticum* are presented in Figure 3. It shows that both concentrations of mancozeb maintained a linear decline in efficiency as time passed. However, both concentrations of tandem had a steady improvement in the efficacy of inhibiting the pathogen as time passed. The means separation showed significant differences ($P \leq 0.05$) between the inhibition caused by chemicals utilized to control the pathogen. Mancozeb 100% was significantly ($P \leq 0.05$) superior to all the treatments and control followed by Mancozeb 50%, Tandem 100% and Tandem 50% (which were at par).

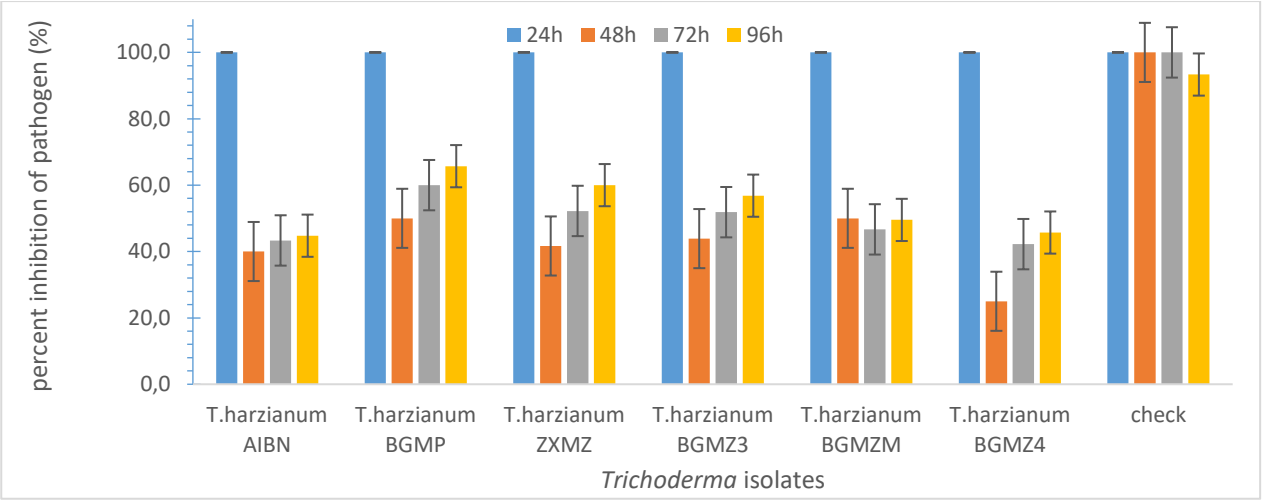

Figure 2. Inhibition of the growth of leaf spot pathogen isolated from groundnut due to the application of six *Trichoderma* isolates.

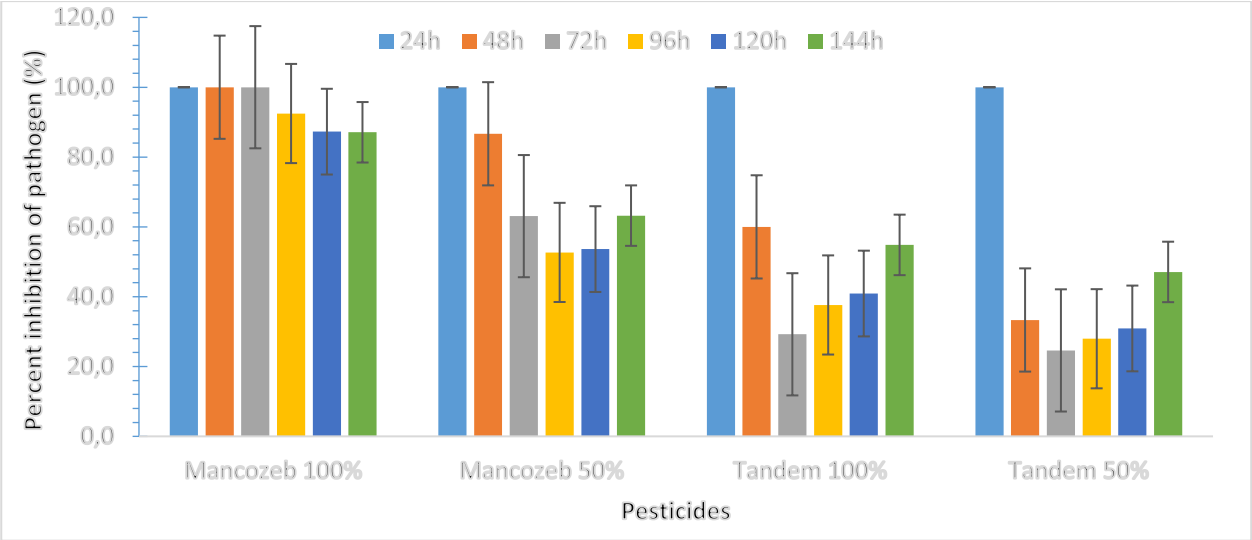

Figure 3. Inhibition of the growth of leaf spot pathogen isolated from groundnut as influenced by the application of two synthetic pesticides.

Discussion

The inhibition of the growth of *C. parasiticum* by *Trichoderma harzianum* isolates corroborated the findings of Kiran (2012) who reported that collar rot or seedling blight or crown rot of groundnut (induced by *Aspergillus niger* and *A. pulverulentum*) could be controlled using seed treatment applications of either *Trichoderma viride* or *T. harzianum* (4 g/kg seeds) preferably in combination with organic amendments such as castor, neem or mustard cakes (500 kg/ha).

Likewise, Ndifon (2022) utilized biocontrol agents (i.e. *Trichoderma* and *Cladosporium* spp.) to inhibit the mycelial growth of *Globisporangium ultimum;* (the causal agent of groundnut pod rot) which corroborated the findings of this present study. Also, Apet et al. (2015) reported that *Ceratocystis paradoxa* was controlled using *Trichoderma viride, T. harzianum,* and *T. harmatum*.

Meanwhile, Zandoná et al. (2019) recommended that fludioxonil combined with *Trichoderma* spp. and Biozyme® may be combined and used for treating soybean seeds. While, Vargas-Inciarte et al. (2019) reported that *T. koningiopsis*, *T. virens, T. harzianum,* and *T. spirale* effectively controlled *Fusarium* wilt *in vivo* in the greenhouse.

Thus the findings of Ndifon (2022), Apet et al. (2015), Zandoná et al. (2019), and Vargas-Inciarte et al. (2019) afirmed the findings in this study based on application of biocontrol agents against *C. parasiticum*.

Mancozeb 100% (contact fungicide) and Tandem® (i.e. Cu (I) O + Metalaxyl, it is a systemic fungicide) both produced significantly higher inhibition of *C. parasiticum* growth than the control. These findings confirmed the findings of Ndifon (2022) who reported that a commercial fungicide (i.e. mancozeb+carbendazim) inhibited the mycelial growth of *Globisporangium ultimum* more than Mancozeb (at 50% and 100% concentrations). These findings also affirmed those of Kiran (2012) reiterated that tikka disease and rust disease should be controlled using foliar sprays of mancozeb. While *Alternaria* leaf spots should be managed using foliar sprays of copper oxychloride and mancozeb.

In the current study, copper (I) oxide plus metalaxyl caused less inhibition of *C. parasiticum* compared to the percentage inhibition of growth caused by mancozeb. This corroborated the findings of Ndifon and Lum (2021) who reported that the level of inhibition of the pathogen's growth was more when Mancozeb® was applied compared to application of Tandem®. Moreover, Apet et al. (2015) reported that *Ceratocystis paradoxa* infecting sugarcane was controlled using systemic fungicides (viz Carbendazim, Propiconazole and Hexaconazole) which caused higher average mycelial growth inhibition followed by non-systemic fungicides (viz Thiram and Captan). These numerous chemical pesticides should be used when farming to delay/avoid development of resistance to over-used chemicals.

Finally, Woodward et al. (2013) reported that the following fungicides: copper dust, sulfur dust, benomyl, chlorothalonil, triazoles, strobilurins, carboximides, propiconazole, cyproconazole, tebuconazole, and propiconazole; have been utilized effectively against leaf spot diseases on groundnuts. While Ziezold et al. (1998) also utilized several fungicides (the best fungicides were Benlate (benomyl), Thiram (thiram), Orbit™ (propiconazole). Crown™ (carbathiin and thiabendazole), fluazinam, and UBI-2584 (tebuconazole) while the less toxic fungicides were UBI-2643 (thiabendazole), UBI-2565 (cyproconazole), and Vitaflo-280 (carbathiin and thiram) to control disappearing root rot of ginseng (*Panax quinquvdius*) caused by *Cylindrocarpon destructans.*

Inhibition by all the plant extracts (in this current study) was significantly higher than that of the control which corroborated the statement by Hashim and Devi

(2003) that plants may contain antimicrobial secondary metabolites that can be used to control pathogens. For instance, Koïta et al. (2017) in Burkina Faso obtained a significant increase in groundnut pod yield. Awurum and Uwajimgba (2013) reduced *Fusarium oxysporium* wilt and Soumya and Bindu (2012) successfully controlled groundnut seed pathogens (*Aspergillus niger*, *A. flavus*, *Penicillium* sp., and *Rhizopus* sp) using plant extracts which agreed with the findings of this current study.

The findings of the present study corroborated those of Ndifon (2022) who successfully utilized plant extracts (i.e. *Parkia biglobosa* (African locust bean tree), mango, shea butter tree, and pawpaw plant tissues) to control *G. ultimum*. Also, this corroborated the findings of Ndifon and Lum (2021) who inhibited the mycelial growth of *Aspergillus niger* using aqueous extracts from five plants (i.e., leaves of *Eucalyptus globulus, Melaleuca cajuputi, Andrographis paniculata* as well as *Azadirachta indica*, and shoots of *Euphorbia hirta* at 50 and 100% concentrations).

To cap it all, we note that initially, Ndifon et al. (2015) utilized *Eucalyptus* sp., cashew, shea butter leaves, *Erythrina* sp., ginger, and garlic plant extracts to control *Fusarium oxysporium f.sp* melongenae. Similarly, Apet et al. (2015) reported that *Ceratocystis paradoxa* was controlled using *Allium sativum, Zingiber officinale,* and *Azadirachta indica,* which is in agreement with the findings reported herein that botanicals effectively controlled *C. parasiticum*.

Still, on botanicals, Niren and Nakul (2019) utilized aqueous extract of eight botanicals including *Eucalyptus globulus* to inhibit the growth of *Thielaviopsis paradoxa* and *Curvularia lunata* from mango and banana. While Rongai et al. (2015) controlled *Fusarium oxysporum* f.sp. lycopersici using 24 plant extracts, among which were *Punica granatum* and *Salvia guaranitica*. Suranjit et al. (2018) used nine botanicals (including garlic) to control *Sclerotium rolfsii*. These three reports above were in complete agreement with the notion that plant extracts can act as alternative pesticides against fungi and other microbes.

Conclusion

The presence of pathogenic fungi on groundnut has a detrimental effect on the production of this important oil-seed and grain legume crop. *In vitro* trials were set up to ascertain the veracity of using *Trichoderma harzianum* isolates, mancozeb and tandem as well as plant extracts from *Parkia biglobosa, Casuarina equisetifolia,* and *Rhoeo spathacea* to manage the *Cylindrocladium parasiticum*. It was proven that *C. parasiticum,* from groundnut can be controlled with this biocontrol, botanical and synthetic pesticides. These pesticides were therefore recommended while work continues to improve the performance of these control agents.

Conflict of Interests

There is no conflict of interests at all.

References

Anco, D., & Jay, W. C. (2016). Peanut disease management. In: South Carolina pest management handbook for field crops. 184-196.

Andrews J. H. (1992). Biological control in the phyllosphere. *Annu Rev Phytopathol*, 30:603-635. https://doi.org/10.1146/annurev.py.30.090192.003131

Apet, K. T., Sayyad, A. S., Wagh, S. S., & Chavan, P. G. (2015). Bioefficacy of Fungicides, Bioagents and Phytoextracts against Ceratocystis paradoxa, Causing Pineapple Disease of Sugarcane. http://doi.org/2739-0106-2015-319

Asama, P., & Channya, F. K. (2018). Fungal pathogens of postharvest rot of groundnut (*Arachys hypogaea* L.) in Hong Local Government Area of Adamawa State, Nigeria. *International Journal of Science and Research*, *7*(10), 303-311. http://doi.org/ 10.21275/ART20191348

Awurum, A. N., & Uwajimgba, J. (2013). Varietal screening and comparative toxicity of some plant extracts for control of *Fusarium* wilt of groundnut (*Arachis hypogaea L.*). *Continental Journal of Agricultural Science*, *7*(1), 11–16. http://doi.org:10.5707/cjagricsci.2013.7.1.11.16

Barnett, H. L., & Hunter, B.B. (1972). Illustrated genera of imperfect fungi. 3rd Edition, Burgess Publishing Co., Minneapolis. pp. 241.

Buck, J. W. (2004). Combinations of fungicides with phylloplane yeasts for improved control of Botrytis cinerea on geranium seedlings. P*hytopathology,* 94, 196-202. https://doi.org/10.1094/PHYTO.2004.94.2.196

Damicone, J. P. (2017). Foliar diseases of peanuts. Id: EPP-7655**.** OSU Extension.

Desai, S., & Bagwan, N. B. (2005). Fungal diseases of rapeseed-mustard. In: Diseases of oilseed crops. Industrial Publishing Co., New Delhi, India. (pp. 108-149).

El-Sherbeny, S. N., El-Maghraby, O. M. O., Samia, A. S., & Ibrahim, Y. M. M. (2020). Pathogenic and/or saprophytic fungi of wilted peanut seedling and their role in pathogenicity of peanut seeds. *Journal of Basic and Applied Mycology* (*Egypt*), *11*, 99-105. p-ISSN 2090-7583, e-ISSN 2357-1047. http://www.aun.edu.eg/aumc/Journal/

Fageria, U. K., Baligar, V. C., & Jones, C. (1997). Growth and mineral nutrition of field crops. (2nd Edition, 494 pp.). Marcel Derker.

Frances, J., Vilardell, P., Bonaterra, A., Badosa, E. & Mantesinos, E. (2002). Combination of Pseudomonas fluorescens EPS288 and reduced fungicide dose for control of *Penicillium* rot during post harvest storage of pear. *Acta Horticulture*, 596, 883- 886. http://doi.org/10.17660/ActaHortic.2002.596.154

Ghewande, M. P. (2009). Early and late leaf spots of groundnut and their management. (pp. 326-334). In: Integrated pests and disease management. Mukerji, R. K. G., Upadhyay, H., Chamola, B. P., & Dueby, O. P. (Eds.). APH Publishing Corporation.

Grahame, J. (2014). Stem and pod rot of groundnut by *Athelia rolfsii.* Contamination in Peanut under Terminal Drought. *Agronomy, 9*(8), 419.

Hashim, M. S., & Devi, K. S. (2003). Insecticidal action of the polyphenolic rich fraction from the stem barks of *Sterlus asper* on *Dysdercus cingulatus*. *Fitoterapia*, *74*:670-676. http://doi.org/10.1016/s0367-326x(03)00186-2

Hassain, M. A., Hamid, S., & Nasreen, S. (2005). Effect of nitrogen, phosphorus fertilizer on up-take and yield performance of groundnut. *Research Guide Institute*, *2*, 45 pages. ISSN: 0368-1157

Kannaiyan, J., & Haciwa, H.C. (1990). Economic benefits of spraying fungicides to control groundnut foliar disease in Zambia. *Tropical Pest Management*, *36*, 21-22. https://doi.org/10.1080/09670879009371429

Kishore, G. K., Pande, S., & Podile, A. R. (2005). Management of late leaf spot of groundnut (*Arachis hypogaea*) with chlorothalonil-tolerant isolates of *Pseudomonas aeruginosa. Plant Pathology*, *54*(3), 401-408. http://doi.org/10.1111/j.1365-3059.2005.01160.x

Kiran, Y. (2012). Integrated disease management in groundnut. *Agropedia.*

Koïta, K., M'bi Zagre, B., & Sankara, P. (2017). Aqueous plant extracts for control of groundnut leaf spot in Burkina Faso. *African Crop Science Journal*, *25*(3), 311–319. http://doi.org/10.4314/acsj.v25i3.5

McDonald, D., Subramanyam, P., Gibbons, R. W., & Smith, D. H. (1985). Early and late leaf spots of groundnut. Information Bulletin No. 21. ICRISAT, Patancheru, AP 502324, pp: 19.

Muthukumar, A., Naveenkunar, R., & Venkatesh, A. (2014). Efficacy of water extracts of some mangrove plants for eco-friendly management of root rot disease of groundnut. *Journal of Plant Pathology and Microbiology*, *5,* 243. http://doi.org/10.4172/2157-7471.1000243

Naab, J. B., Singh, P., Boote, K. J., Jones, J.W., & Marfo, K. O. (2004). Using the CROPGRO-peanut model to quantify yield gaps of peanut in the Guinean savanna zone of Ghana. *Agronomy Journal*, *96*, 1231-1242. https://doi.org/10.2134/agronj2004.1231

Naab, J. B., Tsigbey, F. K., Prasad, P. V. V., Boote, K. J., Bailey, J. E., & Brandenburg, R. L. (2005). Effects of sowing date and fungicide application on yield of early and late maturing peanut cultivars grown under rainfed conditions in Ghana. *Crop Protection*, *24*, 325-332. https://doi.org/10.1016/j.cropro.2004.09.002

Ndifon E. M. (2022). Management of *Globisporangium ultimum* infecting groundnut and Bambara groundnut pods using diverse methods. *New Countryside*, *1*(1), 13-20. DOI: https://doi.org/10.55121/nc.v1i1.27

Ndifon, E. M., Ekefan, E. J., Oluwatayo, O. J., & Okoro, J. O. (2015). Management of *Fusarium oxysporium* f.sp. melongenae using plant extracts *in vitro* in Makurdi, Nigeria. *Advances in Agriculture, Sciences and Engineering Research*, *5*(10), 1857 – 1865.

Ndifon, E. M., & Lum, A. F. (2021). Assessment of White Yam Tuber Rot Disease and in vitro Management of Aspergillus niger in Ebonyi State, Nigeria. *International Journal of Biosciences*, *19*(4), 32-40. ISSN: 2220-6655. http://doi.org/10.12692/ijb/19

Niren, M., & Nakul, C. M. (2019). Screening of different botanicals extract on two polyphagous postharvest pathogens from mango and banana. *Journal of Pharmacognosy and Phytochemistry*, *8*(3), 4253-4256.

Nutsugah, S. K., Abudulai, M., Oti-Boateng, C., Brandenburg, R. L., & Jordan, D. L. (2007). Management of leaf spot diseases of peanut with fungicides and local detergents in Ghana. *Plant Pathology Journal*, *6*, 248-253.http://doi.org/10.3923/ppj.2007.248.253

Onyishi, L. (2010). Web blotch disease of groundnut (*Arachis hypogaea*) in Nsukka. *Bio-Research, 8*(2), 694–696. http://doi.org/10.4314/br.v8i2.66892

Pal, K. K., Dey, R., & Tilak, K. V. B. R. (2014). Fungal diseases of groundnut: control and future challenges. In: Future challenges in crop protection against fungal pathogens. (pp.1-29). http://doi.org/10.1007/978-1-4939-1188-2_1

Pattee, H. E., & Young, C. Y. (1982). Peanut science and technology. American Peanut Research and Education Society Inc., Yoakum. (pp. 655-687).

Podile, A. R., & Kishore, G. K. (2002). Biological control of peanut diseases. In Gnanamanickam, S. S. (Eds) Biological Control of Major Crop Plant Diseases. Marcel & Dekker, New York. pp. 131–160.

Porter, D. M. (1993). Diseases of peanut (*Arachis hypogaea* L.). ©The American Phytopathological Society (APS).

Rongai, D., Pulcini, P., Pesce, B., & Milano, F. (2015). Antifungal activity of some botanical extracts on *Fusarium oxysporum. Open Life Science*, *10*, 409–416. http://doi.org/10.1515/biol-2015-0040

Sangoyomi, T. E., & Alabi, O. (2016). Foliar disease assessment of groundnut (*Arachis hypogea* L.) varieties in forest/savanna transition agro-ecology of Osun State, Nigeria. *Bowen Journal of Agriculture*, *6*(1).

Shaza, R., Shlnae, P., & Abudula, A. (2004). Location of fungi in groundnut seed. *Pakistan Journal of Botany*, *136*(3), 663–668.

Soumya, S. L., & Bindu, R. N. (2012). Antifungal efficacy of *Capsicum frutescens* L. extracts against some prevalent fungal strains associated with groundnut storage. *Journal of Agricultural Technology*, *8*(2), 739-750. https://www.researchgate.net/publication/216899563

Suranjit, S., Sanatombi, D. R. K., Beenakumari, D. N. & Shashikumar, S. O. (2018). Antagonistic activities of plant extracts against the phytopathogen *sclerotium rolfsii,* during pre-harvest horticultural practice - a component of integrated disease management. *International Journal of Current Research in Life Sciences*, *7*(11), 2813-2815.

Subramanyam, P., Greenberg, D. C., Savary, S., & Bosc, J. P. (1991). Diseases of groundnut in West Africa and their management: Research priorities and strategies. *Tropical Pest Management*, *37*, 259-269. https://doi.org/10.1080/09670879109371596

Tsatsia, H., & Grahame, J. (2013). *Passalora personata.* PaDIL - Australian Centre for International Agricultural Research under project PC/2010/090.

Tsigbey, F. K., Nutsugah, S. K., & Bailey, J. E. (2000). Evaluation of neem, soap and Topsin-M in groundnut leaf spots control in northern Ghana. Proceedings of the 6th Regional Groundnut Meeting for Western and Central Africa, October 5-8, 2000, Bamako, Mali. (35 pp.)

Tsigbey, F. K., Bailey, J. E., & Nutsugah, S. K. (2001). Managing groundnut leaf diseases in northern Ghana with fungicides, neem seed extract and local soap. Proceedings of the American Peanut Research. Education Society, July 17-20, 2001, Oklahoma City, Oklahoma, USA. (pp: 38-45).

Tsigbey F. K., Brandenburg R. L., & Clottey V. A. (2004). Peanut production methods in Northern Ghana and some disease perspectives. World Geography of the Peanut Knowledge Base Website. 9 pp.

Vargas-Inciarte, L., Fuenmayor-Arrieta, Y., Luzardo-Méndez, M., Da Costa-Jardin, M., Vera M., Carmona, D., Homen-Pereira, M., Da Costa-Jardin, P., & San-Blas, E. (2019). Use of different *Trichoderma* species in cherry type tomatoes (*Solanum lycopersicum* L.) against *Fusarium oxysporum* wilt in tropical greenhouses. *Agronomía Costarricense*, *43*(1). http://doi.org/10.15517/rac.v43i1.35671

Waliyar, F., Adomou, M., & Traore, A. (2000). Rational use of fungicide applications to maximize peanut yield under foliar disease pressure in West Africa. *Plant Disease*, *84*, 1203-1211. http://doi.org/10.1094/PDIS.2000.84.11.1203

Woodward, J. E., Brenneman, T. B., & Kemerait, R. C. Jr. (2013). Chemical control of peanut diseases: targeting leaves, stems, roots, and pods with foliar-applied fungicides, fungicides - showcases of integrated plant disease management from around the world, Mizuho Nita, IntechOpen, Intech open book series. http://doi.org/10.5772/51064

Zandoná, R. R., Pazdiora, P. C., Pazini, J., & Seide, E. J. (2019). Chemical and biological seed treatment and their effect on soybean development and yield. *Revista Caatinga*, *32*(2), 559-565. http://doi.org/10.1590/1983-21252019v32n229rc

Ziezold, A. M., Hall, R., Reeleder, R. D., & Proctor, J. T. A. (1998). Toxicity of Fungicides in vitro to Cylindrocarpon destructans. *Journal of Ginseng Research*, *22*(4).

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BIOTECH STUDIES

RESEARCH PAPER

Development of homozygous maize lines differing in oil and zein content using *in-vivo* maternal haploid technique

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Abstract

This study was carried out in order to develop homozygous lines that differ in grain quality from the local maize population. Twelve different local maize landraces were used as donor materials in the study. These populations were subjected to induction crossing under greenhouse conditions in September 2020 with the ADAIL-I inducer line. In September 2021, a total of twelve haploid lines were grown in greenhouse conditions. Some plant traits and some grain quality characteristics were examined. Zein protein fractions were also analyzed with SDS-PAGE analysis. The haploid induction rates (HIR) of donor materials ranged from 6.08% to 11.71%. The average HIR value of the ADAIL-I inducer line was determined as 8.20%. The average value of plant height of developed lines varied between 123 cm and 250 cm; first ear height between 54 cm and 120 cm; stem diameter between 0.7 cm and 1.2 cm; crude oil content between 2.39% and 7.54%; oleic acid content between 15.34% and %30.98; linoleic acid content between 50.4% and 67.8%; protein content between 6.75% and 13.74%; and zein content between 4.58% and 5.04%. Some the homozygous lines carry the desired protein bands in terms of zein fractions.

Introduction

Maize is one of the most common cereal species, and its cultivation is becoming more widespread every year. Among the staple cereal species, maize has reached the second place in the world in terms of cultivation areas and the first place in terms of the amount of production (Erenstein et al., 2021). The use of this type of grain used in human and animal nutrition in different industries has also become widespread. Considering the cereal cultivation in our country, the maize has had a significant increase in the planting area and production amount in the last 10 years (TUIK, 2020). However, one of the biggest shortcomings in terms of marketing and converting, maize products to commercial value is that the production of high-value-added raw materials from maize has not become widespread enough. Raw materials that are used in the production of products with high added value are imported raw or processed.

Zein proteins and crude oil are important products with high added values obtained from maize kernels. Of the raw materials required in the production of maize oil are mostly imported items in Turkey. The primary reason for this situation is due to the lack of Turkish maize varieties suitable for oil production. The oil content varies between 3-5.5% in normal maize genotypes (Lambert, 2001). Although it is not included in the oil plants, maize is used as a source of vegetable oil both in the world and in Turkey in this regard. The most important advantage of maize in comparison with the species classified as oil plants is that it is a high-

yielding plant (TUIK, 2020). In addition, maize oil has desirable properties both in terms of both health and nutrition. For this reason, a significant effort is being made worldwide to develop high-oil maize lines and hybrids (Singh et al., 2014). There is more than 6% oil in the genotypes described as "high-oil maize" (Lambert, 2001). The development of high oil maize lines has reached such an advanced point that today there are lines that contain about 20% oil in their seed, have been developed with the support of tissue culture methods and are the subject of patents (Foley, 2009; Patent no: US007495155B2). Development of high oil maize studies is important for seed breeding targets in Turkey and it is an issue that lags behind other countries. Raw zein extracts, which are used as protein-based film raw materials in the textile, food and pharmaceutical industries in recent years, are among the raw materials with high added value that can be obtained from maize. The price per kilogram of raw zein obtained from maize is about 10-40 US dollars (Anderson & Lamsal, 2011). There are decisions taken at the international level to focus on the production of biodegradable plastics to reduce environmental pollution. These decisions will effect on the increasing the production of raw materials used in biodegradable plastics or films in the near future It is expected that the sectors for maize-derived products are developing around the world and will have significant commercial potential in the coming years (Anonymous, 2019). To take part in the zein market, it is important to increase the activities aimed at this market and to meet the needs of raw materials. The most important aspect in this regard is that maize lines and varieties suitable for the production of high added value raw materials have not been developed yet in our country. It is possible to say that there is a significant gap in this area in Turkey.

An important time is allocated for the work of developing pure lines by classical methods in breeding programs, and this is undesirable for breeders (Eder & Chalyk, 2002). In this regard, it becomes possible to develop 100% homozygous lines in a short time with the help of *in vivo* or *in vitro* methods as an alternative to traditional breeding methods (Chalyk, 1994, Chidzanga et al., 2017). The *in vivo* doubled haploid method is widely preferred in practice by researchers in maize breeding studies due to its high success rate and ease of use (Ren et al., 2017). In this context, various studies have been conducted in different countries on the development of homozygous lines from maize germplasms and the use of these lines in variety of development programs such as Germany, (Schmidt, 2003), USA (Seitz, 2005), Croatia (Mazur et al., 2019), Turkey (Cerit et al., 2016; Erdal et al., 2019). The *in vivo* doubled haploid technique has proven its validity as a practical method of developing parental lines in maize and has become a widely used technique in practice. The *in vivo* doubled haploid technique can also be used for the development of parental lines in terms of grain quality characteristics. So far, it is known that there is no study on the development of homozygous lines for the oil and zein content in Turkey.

The aims of this study are, to develop homozygous maize lines from 12 different donor materials, mostly consisting of local maize populations, ii) and to study the agronomic characteristics of the developed lines, in addition to zein and oil contents, as well as the variations in zein fractions. With the current study, it was aimed to give a new direction to launch seed breeding programs suitable for the production of high added value raw materials from the seeds of developed varieties.

Materials and Methods

Plant Material

Twelve local maize landraces were used as donor material which were collected from different regions of Turkey and previously screened for zein and oil content. These populations are obtained from the Faculty of Agriculture of Ordu University. The local populations have no restrictions on their commercial and research use. The ADAIL-1 inducer line was used as a male parent to obtain haploid seeds from donor materials. This inducer was developed by the Sakarya Maize Research Institute in Türkiye.

Field-Greenhouse Experiments and Laboratory Studies

The study was carried out within an 18-month plan covering field and greenhouse trials between 2020-2022 (Figure 1). First of all, the donor materials and the inducer line were germinated in the growth chamber. Once the plants reached the third to fourth leaf stage, they were transplanted into 30-cm-diameter plastic pots and grown under greenhouse conditions in the winter period of 2018 for induction crossing. In this context, donor materials were pollinated by the ADAIL-I inducer line according to the method proposed by Prasanna et al. (2012). Controlled pollination method was used and the pollen collected from different plants belonging to the inducer line were brought together and transferred to the previously protected ears of the donor materials. Heating and lighting conditions under the greenhouse were provided at 20°C and 16/8-h photoperiod (day/night), respectively. Irrigation was carried out according to the water requirements of the plants with the drip irrigation method. Fertigation was

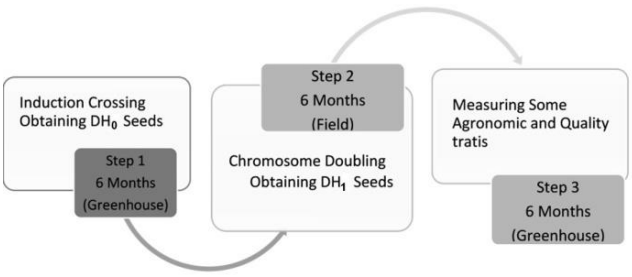

Figure 1. The diagram showing the steps of current study.

applied using drip irrigation fertilizer containing NPK and an equal amount of fertilizer was made with irrigation. At harvest, the ears were collected by hand and transported to the laboratory for the separation of haploid/diploid seed samples. Kernels from each ear sample were seperated according to the classification method, taking into account R1-nj (Navajo) gene expression, proposed by Prassana et al. (2012). The haploid induction ratios according to the donors were determined by the formula below, based on the number of seeds total and putative haploid kernels. These calculations were made at the cob level and then the average values were calculated according to the populations and the haploid induction rate (HIR) values of the donor materials were determined.

HIR(%) = (putative haploid/ total number of seeds) × 100

Colchicine treatment was applied as a stem injection method proposed by Zabirova et al. (1996) according to the method. For this purpose, haploid seeds were planted into the germination trays and moved to a controlled environment. When the plants reached the 3-4 leaf stage, 100 µL colchicine solution (0.125% colchicine 0.5% DMSO) was injected into the stem of haploid plants using a sterile injector (Figure 2). The plants were not watered for one day after the injection to ensure the expected effect of colchicine treatment. After several days, double haploid seedlings were transplanted to the field with 70 × 20 cm row spacing in May 2021 (Figure 2). This trial was set up in a single block and side by side rows and irrigated with a drip irrigation system. Fertilization was carried out with the drip irrigation system with the account of 8 kg of pure nitrogen and 4 kg of pure phosphorus per decare. When the plants reached the generative stage, they were pollinated in a controlled pollination method recommended by Kahriman (2016). For this purpose, field controls were made every morning between 8:00 and 10:00 A.M and pollen samples collected from doubled haploid lines were transferred to the ear of the same plant. Pollen collection was performed using a special vacuum system (Kahriman, 2021), due to the fact that doubled haploid plants could have limited pollen production. When the plants reached harvest maturity, ears were harvested by hand and DH_0 seed samples were obtained by the threshing of the ear samples.

Twelve doubled haploid lines were obtained from twenty donor materials. Some of the donors had sterility and no emergence of doubled haploid seeds. Therefore, the rest of the study was conducted using twelve doubled haploid lines. In September 2021, DH_0 seeds of twelve doubled haploid lines from the previous field trial were planted in germination viols. Emerging plants were grown in greenhouse conditions by transplanting in the 18 liter pots containing field soil and manure with 3:1 ratio (Figure 2). Applications and growth conditions in this step were kept the same as those specified in the induction crossing experiment. Each plant was selfed with the controlled pollination method recommended by Kahriman (2016) and kernel samples were obtained for further analyses. In the greenhouse experiment where double haploids were tested, observations and measurements were made regarding the following traits.

Figure 2. Colchicine treatment, transplanting DH0 plants into the field and growing DH1 plants in the greenhouse.

Agronomic Traits: Plant height, first ear height, stem diameter measurements were carried out according to the maize technical instruction of the Seed Registration Certification Directorate (TTSM, 2018).

Crude Oil Content: It was determined using a Near Infrared Spectroscopy (NIR) device with a previously developed local calibration model (Kahriman et al., 2021).

Oleic and Linoleic Acid Content: The oleic and linoleic acid contents of the lines was determined using a local calibration model (Kahriman et al., 2021) developed on the Near Infrared Spectroscopy (NIR) device.

Zein Content: Extraction of zein fractions was carried out using 70% ethanol and 2% ß-mercaptoethanol (Yau et al., 1999). The defeated flour samples were weighed 100 mg into the eppendorf tube. One mL extraction solution was added and the tubes were incubated at 22°C for 1 hour after vortexing. Then, the samples were centrifuged at 10,000 x g for 10 min. The supernatants were taken into a new tube for further steps and preserved at + 4°C. The Bradford method was used for the quantitative content of zein proteins from the obtained extracts. In this context, 200 µL of Bradford solution was added on 50 µL samples within a 96-well microplate, and the absorbance values were recorded at 595 nm with a microplate reader (Biotek Instruments) after waiting for 45 min at 10°C. Using the same method, the standard curve was created with the BSA (Bovine Serum Albumin) standard and the zein content of the samples was determined with the help of this curve (Bradford, 1976).

Separation of Zein Fractions: Zein fractions were separated using the SDS-polyacrylamide gel electrophoresis technique in a vertical electrophoresis device. SDS-PAGE gel by 12% concentration (12.35 mL distilled water, 14.1 mL 30% stock acrylamide solution, 18.8 mL 4X Tris solution, 350 µL 10% ammonium persulfate, 35 µL TEMED) was prepared (Yau et al., 1999). The prepared gel solution was filled between the gel plates. To load samples onto the gel, the samples were kept in a hot water bath at 95°C for 5 min. Subsequently, it was centrifuged at 3000×g. After

centrifugation, 8 µL was taken from each sample and molecular standards were loaded with the samples. To complete separation, the samples were run at 100 V until blue dye reached the bottom of the gel. After the samples were separated, the gels taken from the gel tank were left in the shaker overnight with a mixture solution of 60 g TCA, 1 g Coomassie Brilliant Blue and 25 mL of ethanol made up to 500 mL with distilled water. The gel image was taken and scored according to the presence and absence of bands. The molecular weights of the bands were determined using the MW (Molecular Weights) standard (Catalog number: 26617, Sigma, USA).

Statistical Analysis

The data obtained from the study were analyzed in the R statistical package program (R Core Team, 2019). The data for the examined features were carried out with one-way analysis of variance. The Predictive PCA-Biplot graphical method was used to show the differences of genotypes in terms of the investigated traits (La Grange et al., 2009). While the vectors on these graphs show the numerical values for the examined traits, the angles between the vectors and the direction of the values are used to evaluate the correlations between the related traits. If the angle between the vectors is small and the numerical values on the vector change in the same direction, it is understood that there is a positive correlation between the traits related to these vectors, and if the vector values are in the opposite direction, there is a negative correlation. The gel images obtained from SDS-PAGE analysis were transferred to the GelAnalyzer program to encode the band values appearing in gel analyze as present/absent (1/0), and the molecular weights of the bands appearing in the samples were determined according to the band positions of the molecular standard. Genotype profiles were scored according to molecular weights and genotypic evaluation was made according to variation of zein band fractions.

Results and Discussion

Development of Homozygous Lines by In-vivo Maternal Haploid Technique

The HIR values of donor materials according to the number of seeds obtained from induction crossing and number of putative haploid seeds are presented in Table 1. According to the donor materials, haploid induction rates varied between 6.08% and 11.61%. Considering these values of the donors, the average HIR of the ADAIL-1 inducer line was calculated as 8.20%. In different studies, it is seen that HIR values vary in a wide range in the induction crossing of different inducer lines and different donors. HIR values were found between 7.1% and 12.8% in 7 donor materials by Zararsız et al. (2019). In another study, five donor materials and two inducers were used and HIR values were found between 9.20% and 16.10%. Although there is no study on the change in HIR value of the same inducer line in different locations or conditions, it has been emphasized in previous studies that the HIR values of the inducer lines may change depending on environmental conditions (Rotarenco et al., 2010). If the donor materials carry some inhibitory genes that prevent the effect of the Navajo genes, some unexpected situations may be encountered in the calculations of the HIR value (Chaikam et al., 2015) observed that HIR value of some donor materials (population 9) were significantly low, while others were found to be high, which may be a result of this situation. On the other hand, the ADAIL-1 inducer line was never tested under greenhouse conditions during its breeding process. It was stated in the paper on the development that this inducer had an HIR value of 11-12% under field conditions (Cengiz & Esmeray, 2021). The HIR value of the ADAIL-1 inducer in greenhouse conditions was tested for the first time in our study. The fact that this inducer line has a lower HIR value under greenhouse than field conditions could be attributed to the fact that it was not tested for HIR values under greenhouse during the development process.

Table 1. The number of seeds obtained induction crossing, the number of putative haploids and HIR values by source materials in this study

Code	Number of Seeds of Induction Crossing	Putative Haploids according to R1-nj Marker	Haploid Induction Rate (%)
Pop 1	616	43	7.00
Pop 2	220	21	9.43
Pop 3	331	25	7.51
Pop 6	286	21	7.28
Pop 8	301	30	9.85
Pop 9	507	41	8.09
Pop 10	430	50	11.61
Pop 15	305	23	7.69
Pop 16	362	22	6.08
Pop 17	510	42	8.16
Pop 18	217	20	9.07
Pop 19	469	31	6.64
	Total:4554	Total:368	Mean:8.20

Table 2. Descriptive statistics and results of analysis of variance about the investigated traits

	Descriptive Statistics				Analysis of Variance
Trait	Mean	Minimum	Maximum	Std. Dev.	Mean of Squares
Plant Height (cm)	179.0	124.0	250.0	29.9	9720.7**
First Ear Height (cm)	77.9	54.3	120.0	17.8	3453.5**
Stem Diameter (cm)	1.58	0.70	2.10	0.421	1.93**
Protein (%)	9.74	6.75	13.70	1.94	41.03**
Oil Content (%)	4.15	2.39	7.54	1.4	21.27**
Oleic Acid (%)	22.2	15.3	31.0	4.8	251.04**
Linoleic Acid (%)	60.7	50.4	67.8	5.43	321.7**
Zein (%)	4.77	4.58	5.04	0.172	0.32**

** statistically significant at the p<0.01 level.

Agronomic and Kernel Quality Characteristics of the Doubled Haploid Lines

Descriptive statistics and variance analysis results related to the traits examined in the study are shown in Table 2. Among the agronomic traits examined, the plant height was found between 124 cm and 250 cm, the first ear heights were between 54 cm and 120 cm, and the stem diameter was between 0.70 and 2.10 cm. In terms of kernel quality characteristics, the oil contents of the lines were found between 2.39% and 7.54%, oleic acid contents were found between 15.34% and 30.98%, and linoleic acid contents were found between 40-50% and 67.8%. While the protein contents of the lines were varied between 6.75% and 13.70%, the minimum and maximum values of the lines in terms of zein content were determined as 4.58% and 5.04%, respectively. The results of the analysis of variance showed that there were significant differences between the lines for all the traits examined (Table 2).

The results of some agronomic traits and kernel quality characteristics of the lines in the experiment carried out with these seeds under greenhouse conditions are shown in Figure 3a and Figure 3b. According to the Predictive Principal Component Analysis-Biplot (P-PCA-Biplot) method, it is seen that the lines have a remarkable variation in terms of agronomic and kernel quality characteristics. The lines numbered 15, 16, 18, and 19 were above the average of lines in terms of plant height (PH) and first ear height (FEH). In terms of stem diameter, it was determined that lines 1, 10, 15, 16, 17, 18, 19 had values above the average (Figure 3a). In the study by Bayhan et al. (2021), in which homozygous pure lines were tested under greenhouse conditions, the plant height was found between 103.75 cm-190.00 cm, the first ear height was between 29.00 cm and 63.00 cm, and the stem diameter between 6.54 mm and 8.45 mm. Although the results obtained in our study were within the limits specified values, it was observed that some genotypes were out of the limits than the reported ranges. It has been emphasized that environmental factors such as lighting and temperature, may affect plant growth in studies carried out under controlled conditions and plant growth differed from field conditions (Poorter et al., 2016). Although greenhouse conditions are controlled environments, differences may arise between greenhouse trials carried out in different regions due to the climatic characteristics of the region where the greenhouse is located and the characteristics of the light source used (LED, Halogen, etc.). On the other hand, the materials used in these studies are homozygous lines obtained from different donor materials. There may be

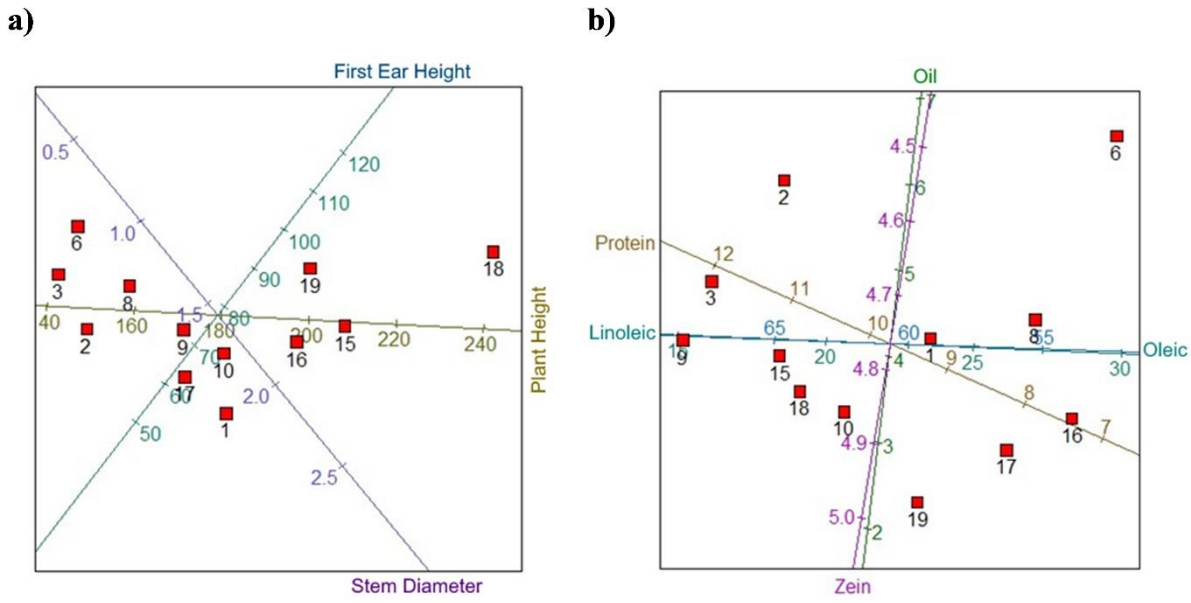

Figure 3. The results of Predictive PCA-Biplot Analysis for agronomic (a) and grain quality (b) traits.

differences in the results obtained between studies due to genotypic effects.

It was determined that genotypes 2 and 6 were genotypes with higher oil content compared to the others. They could be considered "High Oil Maize" given the fact that their average for oil contents was higher than the previously mentioned limit (>6%). In terms of zein content, lines 16, 17 and 19 were found to have values above the average. In terms of protein content, lines 2, 3 and 9 had higher protein content than the other lines. It was determined that the lines with high oleic acid content were lines 6, 8 and 16, while lines 3 and 9 were prominent in terms of linoleic acid content (Figure 3b). The number of studies examining kernel quality traits in studies conducted under greenhouse conditions is limited. On the other hand, it was determined that there was a significant variation for kernel quality characteristics in maize inbred lines in studies carried out under field conditions. In a study conducted by Kahriman et al. (2016), the inbred lines with different genetic backgrounds showed a considerable variation for protein (9.6% to 21.7%), oil contents (3.33% to 14.5%), oleic acid contents (22.1% to 47.1%), and linoleic acid contents (40.7% to 63.8%). While the results obtained in our research were between the values specified for protein and oil, it was noted that there were genotypes outside the specified limits in terms of other characteristics. As in the agronomic traits, these differences could be attributed to the growing conditions and genotypic effects.

When the values on the vectors of agronomic traits and the angles between the vectors are taken into account in PPCA-Biplot graphics, it can be interpreted as there is a positive correlation between stem diameter, first ear height and plant height (Figure 3a). In addition to studies reporting a positive correlation between plant height and stem diameter in maize (Mousavi & Nagi, 2021), positive correlations are also reported between plant height and first ear height (Sadek et al. 2006). According to the PPCA-Biplot for quality traits (Figure 3b), it was observed that there was a negative correlation between zein and oil contents. Ray et al. (2019) attributed the negative correlation between oil and protein content in maize to the differences in the proportional weights of embryo and endosperm in the maize kernel. They suggested that the increase in embryo size increases the oil content, and this situation decreases the protein content because it decreases the proportional share of the endosperm. Zein proteins are the predominant type of protein in maize and constitute 50-60% of the total protein. Therefore, the negative correlation between oil and zein content in our study was also found and it could be related to the situation described by Ray et al. (2019). There was a negative correlation between oleic and linoleic acid (Figure 3b). High and negative correlations between two fatty acids in maize were also determined in previous studies (Baldin et al., 2018, Ray et al., 2019). This relationship has been associated with the activity of the n-6 desaturase enzyme and is based on the fact that linoleic acid synthesis occurs from oleic acid.

Protein Band Analysis

According to the results of protein band analysis, it was determined that there were some differences in zein fractions in doubled haploid lines. SDS-PAGE gel analysis showed that 19 kDa and 22 kDa bands are present in all lines (Figure 4) In addition to these bands, 27 kDa zein fractions and 15 kDa fractions were also observed in some lines. On the other hand, 27 kDa zein bands were not found in lines 2, 6 and 8. These lines are genotypes with higher values in terms of oil content than the others. Although zein band analyzes were performed for qualitative discrimination in current study, it was noted that 27 kDa band intensities of some genotypes (3 and 9) were higher than the others despite the same amount of sample loaded to SDS-PAGE gel.

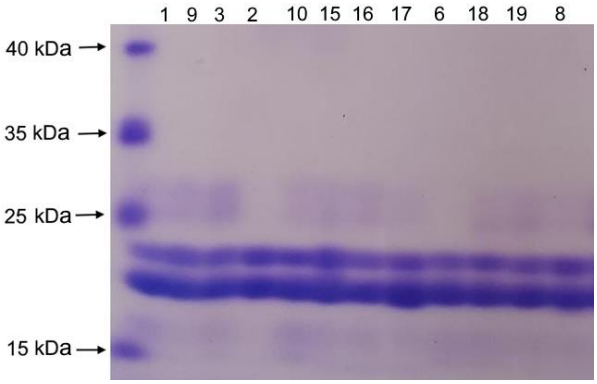

Figure 4. Distribution of zein fractions in SDS-PAGE gel analysis.

Although zeins are the dominant fraction in maize proteins, band variation may vary according to different genotypes. Zein proteins are divided into two subgroups, prolamins and prolamin-like proteins. These groups are mainly separated depending on the differences in the disulfide bonds. Zeins classified as prolamin group are 19 and 22 kDa α-zeins, while prolamin-like zein differences include γ (50, 27 and 16 kDa), β (15 kDa) and δ (18, 10 kDa) zein fractions (Feng et al., 2009). The densities of the sub-fractions vary according to the formation stages of the zein structures in the maize kernel. While zein protein structures consist of γ and β fractions in the early stage, α and δ fractions begin to form in the middle developmental stage, and α-zein fractions become dominant in the last stage (Holding, 2014). While α-zein fractions with a size of 19-22 kDa are located in the inner part of the zein structure, whose formation is completed, β and γ fractions are localized in the outer layers. The higher amount of α-zein in maize genotypes compared to other fractions is related to this formation process. In most of the lines developed in our study, bands belonging to the prolamin group fractions were observed to be intense. γ-zein fractions were not found in lines with high oil content.

Conclusion

As a result of the study, twelve doubled haploid lines from local maize populations were developed. It was noted that the inducer line used in terms of HIR value had a relatively lower value in greenhouse conditions than its previously reported HIR value. It was determined that there was a remarkable variation between the lines in terms of the oil and zein content. However, in SDS-PAGE analysis, a similar profile was observed in most of the lines in terms of subfractions of zein proteins. Although the agronomic performances of the developed lines are considered sufficient under greenhouse conditions, they should also be tested under field experiments. Among the developed homozygous materials, two lines were high-oil (>6%) maize (numbered 2 and 6). In addition, it was determined that three lines (16, 17, and 19) had higher zein content than other lines. However, there is a need to test these genotypes both under field conditions and to evaluate their combination abilities with other lines. Registration of parent lines and hybrids may be possible if promising hybrid combinations are available.

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Author Contributions

FK: Designed, Performed, Analyzed, Writing - review and editing; AK: Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing - original draft; AMG: Investigation, Methodology, Writing -review and editing; and NNY: Data Curation, Formal Analysis, Investigation, Methodology.

References

Anderson, T.J., & Lamsal, B.P. (2011). Zein extraction from maize, maize products, and coproducts and modifications for various applications: A Review. *Cereal Chemistry,* (88), 159-173. https://doi.org/10.1094/CCHEM-06-10-0091

Anonymous, (2019). Zein protein market forecast, trend analysis & competition tracking - global Review 2018 to 2028. https://www.factmr.com/report/654/zein-protein market

Baldin, M., Ying, Y., Fan, Y., Roth, G., Casper, D. P., & Harvatine, K. J. (2018). Characterization of linoleic acid (C18:2) concentration in commercial corn silage and grain hybrids. *Journal of Dairy Science, 101*(1),222-232. https://doi.org/10.3168/jds.2017-12972

Bayhan, M., Özkan, R., Albayrak, Ö., Yıldırım, M., & Akıncı, P. D. C. (2021). İn vivo double haploid tekniği ile yerel çeşitlerden elde edilen haploid bitkilerin saf hat olarak kullanilabilirliğinin belirlenmesi. *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi*, *24*(5), 1029-1036. https://doi.org/10.18016/ksutarimdoga.vi.825121

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemestry, (*722), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Cengiz, R., & Esmeray, M. (2021). Development of late temperate *in vivo* haploid inducers, *Genetika*, Vol. (53), No1, 51-64. https://doi.org/10.2298/GENSR2101051C

Cerit, İ., Cömertpay, G., Oyucu, R., Çakır, B., Hatıpoğlu, R. & Özkan, H. (2016). Melez Mısır Islahında In-vivo Katlanmış Haploid Tekniğinde Kullanılan Farklı Inducer Genotiplerin Haploid İndirgeme Oranların Belirlenmesi "Tarla Bitkileri Merkez Araştırma Enstitüsü Dergisi" 52-57. https://doi.org/10.21566/tarbitderg.280162

Chaikam, V., Nair, S. K., Babu, R., Martinez, L., Tejomurtula, J., & Boddupalli, P. M. (2015). Analysis of effectiveness of R1-nj anthocyanin marker for *in vivo* haploid identification in maize and molecular markers for predicting the inhibition of R1-nj expression. *Theorotical Applied Genetics 128*(1),159–171. https://doi.org/10.1007/s00122-014-2419-3

Chalyk, S. T. (1994). Properties of maternal haploid maize plants and potential application to maize breeding. *Euphytica 79,* 13–18. https://doi.org/10.1007/BF00023571

Chidzanga, C., Muzawazi, F., Midzi, J. & Hove, T. (2017). Production and use of haploids and doubled haploid in maize breeding: A review. *African Journal of Plant Breeding* 4: 201-213.

Eder, J., & Chalyk, S. (2002). In vivo haploid induction in maize. *Theor. Appl. Genet, 104(*4), 703-708. https://doi:10.1007/s00122-001-0773-4

Erdal, S., Cengiz, R., & Ozturk, A. (2019). Breeding doubled haploid maize inbred lines for methionine and lysine amino acid composition. Maydica, *64*(2), 1-10.

Erenstein, O., Chamberlin, J., & Sonder, K. (2021). Estimating the global number and distribution of maize and wheat farms. *Global Food Security*, *30*, 100558. https://doi.org/10.1016/j.gfs.2021.100558

Feng, L., Zhu, J., Wang, G., Tang, Y., Chen, H., Jin, W., Wang, F., Mei, B., Xu, Z., & Song, R. (2009). Expressional profiling study revealed unique expressional patterns and dramatic expressional divergence of maize alpha- zein super gene family. *Plant Molecular Biology*, (69), 649–659. https://doi.org/10.1007/s11103-008-9444-z

Foley, T. (2009). Plants and seeds of high oil corn variety HOOO1, *US Patents,* US007495155B2.

Holding, D. R. (2014). Recent advances in the study of prolamin storage protein organization and function. *Frontiers in Plant Science*, (*5)*, 276. https://doi.org/10.3389/fpls.2014.00276

Kahrıman F. (2016). Mısırda polen etkisi ve bu etkinin kontrolünde uygulanan yöntemler, Lambert Academic Publishing, Saarbrükhen.

Kahrıman, F., Songur, U., Dişbudak, A., Kızık, S. & Vural, B. (2022). Effects of donor x inducer interaction on the success of haploid induction and comparison of haploid seed identification methods in the *in vivo* maternal haploid technique in maize. *Journal of Agricultural Sciences, 28*(3), 385-395, 22-22. https://doi.org/10.15832/ankutbd.824114

Kahrıman, F., (2021). Polen toplama ve filtreleme sistemi, Ulusal Faydalı Model, Başvuru Numarası: 2021/018211.

Kahrıman, F., Sütal, A., Topçakıl, M. & Gezer, Ö. (2021) Prototype near-infrared (NIR) reflectance spectrometer for the analysis of maize flour, *Instrumentation Science & Technology*, *49*(5), 521-531. https://doi.org/ 10.1080/10739149.2021.1890611

Kahriman, F., Egesel, C. Ö., Orhun, G. E., Alaca, B., & Avci, F. (2016). Comparison of graphical analyses for maize genetic experiments: Application of biplots and polar plot to line x tester design. *Chilean Journal of Agricultural Research*, *76*(3), 285-293. https://doi.org/10.4067/S0718-58392016000300004

La Grange, A., Le Roux, S., & Gardner-Lubbe, S. (2009). BiplotGUI: Interactive biplots in R. *Journal of Statistical Software 30*(1), 1-37. https://doi.org/10.18637/jss.v030,i12

Lambert, R., (2001). High-oil corn hybrids. In: Zallauer A (ed) specialty maizes, 2nd edn. *CRC Press, Boca Raton, pp* 131–154.

Mazur, M., Vila, S., Brkić, I., Jambrović, A. & Šimić, D. (2019). The development of homozygous maize lines using an in vivo haploid induction in the Croatian germplasm. *Poljoprivreda, 25*(1), 19-25. https://doi.org/10.18047/poljo.25.1.3

Mousavi, S. M. N., & Nagy, J. (2021). Evaluation of plant characteristics related to grain yield of FAO410 and FAO340 hybrids using regression models. *Cereal Research Communications,* (*49*)**,** 161–169. https://doi.org/10.1007/s42976-020-00076-3

Poorter, H., Fiorani, F., Pieruschka, R., Wojciechowski, T., van der Putten, W.H., Kleyer, M., Schurr, U., & Postma, J., (2016). Pampered inside, pestered outside, differences and similarities between plants growing in controlled conditions and in the field. *New Phytologist*, (*212*), 838-855. https://doi.org/10.1111/nph.14243

Prasanna, B. M., Chaikam, V., & Mahuku, G. (2012*).* Doubled haploid technology in maize breeding: *Theory and practice*. Mexico, D. F., CIMMYT.

R Core Team. (2019) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Ray, K., Banerjee, H., Dutta, S., Hazra, A. K., & Majumdar, K., (2019). Macronutrients influence yield and oil quality of hybrid maize (*Zea mays* L.*). Plos One, 14(5), e0216939.* https://doi.org/10.1371/journal.pone.0216939

Ren, J., Wu, P., Trampe, B., Tian, X., Lübberstedt, T., & Chen, S. (2017). Novel technologies in doubled haploid line development. *Plant biotechnology journal*, *15*(11), 1361–1370. https://doi.org/10.1111/pbi.12805

Rotarenco, V., Dicu, G., & Fuia, S. (2010). New inducers of maternal haploids in maize. *Maize Genetics Cooperation Newsletter*, 84, 21-22.

Sadek, S. E., Ahmed, M. A., & Abd El-Ghaney, H. M. (2006). Correlation and path coefficient analysis in five parents inbred lines and their six white maize (Zea mays L.) single crosses developed and grown in Egypt. *Journal of Applied Sciences Research*, *2*(3), 159-167.

Schmidt W. (2003) Hybrid maize breeding at KWS SAAT AG. In: Bericht über die Arbeitstagung der Vereinigung der Pflanzenz üchter und Saatgutkaufleute Österreichs, Gumpenstein, Österreich, 25–27 November, pp. 1–6.

Seitz G. (2005) The use of doubled haploids in corn breeding. In: Proc. of the 41th Annual Illinois Corn Breeders' School 2005. Urbana-Champaign, Il, USA, p. 1–7.

Singh, N., Vasudev, S., Yadava, D., Chaudhary, D., & Prabhu, K. (2014). Oil Improvement in Maize: Potential and Prospects. In Maize*: Nutrition Dynamics and Novel Uses*. Springer, Indian, 77-82. https://doi.org/10.1007/978-81-322-1623-0_6

TTSM, (2018). Mısır Teknik Talimatı. https://www.tarimorman.gov.tr/BUGEM/TTSM

TUIK, (2020). Türkiye İstatistik Kurumu Verileri. www.tuik.gov.tr

Yau, J. C., Bockholt, A. J., Smith, J. D., Rooney, L. W. &Waniska, R. D. (1999). Maize endosperm proteins that contribute to endosperm lysine content. *Ceral Chemistry*, (76), 668-672. https://doi.org/10.1094/CCHEM.1999.76.5.668

Zabirova, E. R, Chumak, M. V., Shatskaia, O. A. & Scherbak, V. S. (1996). Technology of the mass accelerated production of homozygous lines (in Russian). Kukuruza Sorgo 4: 17-19

Zararsız, D., Yanıkoğlu, S., Öztürk, L., Turgut, İ., Kızık, S., & Bilgin, B. (2019). Production of double haploid plants using *in vivo* haploid techniques in maize. *Journal of Agricultural Sciences*, *25*(1), 62-69. https://doi.org/0.15832/ankutbd.539000

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RESEARCH PAPER

Juglans regia kernel meal; A prospective nutraceutical feed supplement

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Abstract

This study aims to characterize the proximate composition, antioxidant activity, phytochemical profile, anti-diabetic, and anti-inflammatory properties of *Juglans regia* kernel meal (JKM). The examination of the proximate composition reveals that JKM contains moisture (7.74%), ash (4.46%), crude fat (31.26%), crude fiber (8.41%), crude protein (8.99%) and nitrogen-free extract (39.14%). The analysis of JKM for antioxidant properties shows lipid peroxidation inhibition (63.78%), ferric ion reducing antioxidant power (103.44 mg/g), 2,2-diphenyl-1-picrylhydrazyl hydrate (57.91%), and vitamin C (152.87 mg/g). The phytochemical compositional analysis shows that JKM has alkaloids (12.08%), saponins (43.49 mg/g), steroids (4.84 mg/g), flavonoids (14.74 mg/g), tannins (1.69 mg/g) and phenol (35.93 mg/g). The JKM also demonstrated alpha-amylase inhibition and alpha-glucosidase inhibition activities of 61.06% and 67.76%, respectively; while 62.71% and 79.17% were reported for the albumin denaturation inhibition and antiproteinase activity of JKM, respectively. JKM dietary supplementation may enhance the animals' welfare. It is advised to employ it in an animal model, though.

Introduction

The term "nutraceutical," which combines the words "nutrition" and "pharmaceutical," refers to either a food or a component thereof that has the ability to both prevent and/or treat diseases (Alagawany et al., 2021). Numerous bioactive substances included in nutraceuticals frequently have a variety of pharmacological actions, including anti-inflammatory, antibacterial, adaptogenic, free radical scavenging and antioxidative, immunomodulatory, and sedative properties (Gupta 2016). Due to the current limitation on the use of antibiotics in feed supplements for animal production and the growing need to improve production performance in both animals and humans, nutraceuticals have recently acquired relevance (Alagawany et al., 2021).

A significant portion of the nutraceuticals category consists of botanicals, which are whole, fragmented, or cut plants, algae, fungi, lichens, and botanical preparations made from these materials through extraction, distillation, expression, fractionation, purification, concentration, and fermentation (Gulati et al., 2014).

Juglans regia commonly known as walnuts is from the Juglandaceae family and most widespread tree nut in the world (Aryapak & Ziarati, 2014). When the plant reaches reproductive maturity, it becomes a woody perennial climber or climbing shrub that can grow to be between 6 and 18 meters tall. When young, the stem is

green and glabrous, but as it ages, it can girth up to 16 cm (Nwachoko & Jack 2015). Nearly all parts of walnuts are employed in ethnomedicine, including the leaves, seeds, stem barks, and roots. Specifically, the stem bark, seed kernel, and leaf are used as a mild laxative (Janick & Paul, 2008), aphrodisiac and tonic (Aiyeloja & Bello, 2006), fertility enhancer in women (Nwauzoma & Dappa, 2013), respectively.

The chemical and bioactive makeup of botanicals may affect how therapeutically effective they are (Adeyeye et al., 2020; Oloruntola & Ayodele, 2022). Comparatively to other botanicals such as sunflower, goat weed (Adeyeye et al., 2020), ginger (Anwar et al., 2020), *Corchorus capsularis* and *Corchorus olitorius* (Biswas et al., 2020), *Moringa oleifera* (Iqbal & Bhanger, 2006), *Anacardium occidentale* (Oloruntola, 2021), nettle (Otles & Yalcin, 2012), *Niebuhria apetala* (Rajesh et al., 2019), etc.; *Juglans regia* kernel chemical composition has received less research. Given that feed additives are a variety of groups of chemicals, substances, or organisms that support growth as well as alter physiological processes like stress resistance, reproduction, and immunological function. Additionally, using plant components as phytogenic additions is growing in popularity lately. Consequently, regular testing and characterisation of plant parts for phytochemical composition, antioxidant properties, antibiotic properties, and anti-cholesterol properties, among others are also necessary to support the use of plant parts as nutraceutical feed additives and supplements (Oloruntola, 2021; Oloruntola & Ayodele, 2022). Therefore, the objective of this study is to investigate the proximate, and phytochemical composition, antioxidant, alpha-amylase inhibitory, alpha-glucosidase inhibitory, albumin denaturation inhibitory and antiproteinase activities of *Juglans regia* kernel meal.

Materials and Methods

Processing of *Juglans regia* kernel

Juglans regia fruits were obtained from villages in Akungba Akoko, Nigeria. Raw kernels were extracted from the *Juglans regia* fruits, finely chopped, sparingly scattered, allowed to air dry in the shade for 14 days, milled and referred to as *Juglans regia* kernel meal (JKM). The JKM was thereafter stored for laboratory analysis. Four hundred grams (400 g) of JKM was added to 2000 ml of 70% ethanol, shaken for 6 hours, allowed to stand still for an additional 48 hours, and then filtered through Whatman No 1 filter paper. A rotary evaporator was used to vacuum condense the JKM ethanolic extract at 35–40°C.

Chemical analysis

The analytical reagent grade reagents used for chemical analysis were purchased from Sigma-Aldrich.

Proximate and vitamin C composition determination

The AOAC (2010) method was used to examine JKM for ash, crude fiber, crude fat, crude protein, and nitrogen-free extract. Vitamin C (Benderitter et al., 1998) was determined in JKM and the procedures were earlier reported by Oloruntola (2021).

Lipid peroxidation inhibition

The Bajpai et al., (2015) method was used to evaluate the JKM extracts' ability to inhibit lipid peroxidation. In both the absence and addition of JKM extract (50-250 g/mL) or reference compound, the reaction mixture of 1 mM $FeCl_3$, 50 µL of bovine brain phospholipids (5 mg/L), and 1 mM ascorbic acid in 20 mM phosphate buffer was incubated at 37°C for 60 minutes. Malondialdehyde (MDA), which was measured by the 2-thiobarbituric acid (TBA) reaction, was created as a byproduct of the process as hydroxyl radicals, which led to lipid peroxidation. The percentage of inhibitory activity was calculated.

$$\text{Percent inhibition } (\%) = \frac{(A\ control - A\ test)}{(A\ control)} x100$$

A control: Absorption of the controlling reaction

A test: Test reaction absorbency.

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH)

The DPPH radical degradation activity method was used to assess the JKM's DPPH antioxidant activity (Otles & Yalcin, 2012). Pure methanol ($6x10^{-5}$ M), sample extract or standard solution (100 liters), and methanolic DPPH solution (2 liters) were used to form the DPPH radical. The DPPH radical was kept in the dark for 20 minutes. The sample absorbance was calculated at 515 nm. As a control, a blank solution made entirely of methanol was employed. 100 µL of pure water were substituted for 100 µL of extract in the control solution. To examine the antioxidant capacities of JKM extracts, a calibration curve with various gallic acid solution concentrations (10-100 ppm) was created.

Ferric-reducing antioxidant power (FRAP)

Using the Benzie and Strain (1996) method, the spectrophotometric antioxidant capacity of the JKM was calculated. To make the FRAP reagent, 300 mM acetate buffer was combined with 10 mL TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ at 37°C in the ratio of 10:1:1. Using a 1–5 mL variable micropipette, 3.995 mL freshly made functioning FRAP reagent was pipetted and fully blended with 5 µL of the properly diluted JKM. When the ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was converted to a ferrous (Fe^{2+}) form, a strong blue color complex was created. The absorbance at 593 nm was measured in comparison to a reagent blank (3.995 mL FRAP reagent and 5 µl distilled water) after 30 minutes of 37°C incubation. Plotting the absorbance at 593 nm vs various $FeSO_4$ concentrations produced the calibration curve. The levels of $FeSO_4$ were then compared to the levels of the common antioxidant Trolox. The FRAP values, which are reported as mg of

Trolox equivalent per gram of material, were calculated by comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe^{3+}.

Phytochemical analysis

The detailed outlines for determining the phytochemicals (alkaloids, saponins, steroids, flavonoids, tannins, and phenol) have been reported by Oloruntola & Ayodele (2022). The JKM was analyzed for alkaloids using the gravimetric method (Adeniyi et al, 2009), total saponins with vanillin and concentrated sulfuric acid colorimetric technique (He et al., 2014); steroids (Madhu et al., 2016); total flavonoids (Surana et al., 2016); total tannins (Biswas et al., 2020) and total phenol (Otles & Yalcin, 2012).

Alpha-amylase inhibitory activity

The α-amylase inhibition study was carried out using the 3,5-Dinitrosalicylic acid (DNSA) method (Wickramaratne et al., 2016). After being treated with at least 10% Dimethylsulfoxide, the JKM extract was diluted in buffer ((Na_2HPO_4/NaH_2PO_4 (0.02 M, at pH 6.9), (NaCl (0.006 M)) to provide concentrations between 10 and 1000 g/mL. 200 mL of extract and 2 mL of α-amylase solution were mixed and incubated at 30°C for 10 minutes. After that, each tube received 200 µL of the starch solution (1 percent in water (w/v)) and was incubated for 3 minutes. The process or reaction was halted by adding 200 µL DNSA reagent (12 g sodium potassium tartrate tetrahydrate in 8.0 mL 2 M NaOH and 20 mL 96 mM 3,5-Dinitrosalicylic acid solution) boiled for ten minutes in a water bath between 85 and 90°C. After being brought to room temperature, the mixture was diluted with 5 mL of distilled water and then subjected to a UV-Visible spectrophotometer analysis at 540 nm. The JKM extract was swapped out for 200 L of buffer, yielding a blank with 100% enzyme activity. A blank reaction was produced using the JKM extract at each concentration in the absence of the enzyme solution. As a positive control sample, acarbose (100–200 µg/mL) was employed, and the reaction was conducted in the same manner as the JKM extract reaction. Using the equation below, the inhibitory activity of α-amylase was calculated and reported as a percentage of inhibition. By graphing the percentage of α-amylase inhibition versus the extract concentration, the IC_{50} values were determined.

$$\text{Percentage } \alpha - \text{amylase inhibition} = 100\ X \frac{Abs\ 100\%\ Control - Abs\ Sample}{Abs 100\%\ Control}$$

Alpha-glucosidase inhibitory activity

An assay for assessing α-glucosidase inhibitory activity was described by Dej-adisai and Pitakbut (2015). The glucosidase enzyme converts the substrate, p-nitrophenol-D-glucopyranoside (pNPG), into the yellow product, p-nitrophenol (pNP), which was used to analyze the α-glucosidase reaction. 50 µL of a 10 mM phosphate buffer solution (pH 7) containing 0.2 mg/mL sodium azide and 2 mg/mL bovine serum albumin were added to a well plate. One unit/mL of Saccharomyces cerevisiae α-glucosidase (Type I, lyophilized powder, Sigma, EC 3.2.1.20) and 50 µL of an 8 mg/mL sample solution were added to the phosphate buffer solution. The solvent control was a 5% DMSO solution, and the positive control was 8 mg/mL of acarbose in each well. The mixes were incubated at 37°C for 2 minutes. 50 µL of 4 mM pNPG were then put into the well. The mixture has to incubate for a further five minutes in the same circumstances. For 5 minutes, the pNP was carried out and timed using a microplate reader at 405 nm every 30 seconds. The mixture has to incubate for a further five minutes in the same circumstances. For 5 minutes, the pNP was carried out and timed using a microplate reader at 405 nm every 30 seconds. The following linear relationship equation between absorbance and time was used to calculate the velocity (V).

$$\text{Velocity} = \frac{\Delta Absorbance\ at\ 405\ nm}{\Delta\ Time}$$

Each sample's initial reaction's highest velocity was gathered, and the equation below was used to calculate the percentage of inhibition.

$$\%\ \text{Inhibition} = \frac{\text{V control} - \text{V sample}}{V\ control} X\ 100$$

Anti-inflammatory activities

Albumin denaturation inhibition

The assay was performed as specified by Osman et al., (2016). Ibuprofen and diclofenac, two positive standards, were prepared at a concentration of 0.1 percent each (1.0 mg/mL), along with the JKM extract. The reaction vessel for each mixture contained 200 µL of egg albumin, 1400 µL of phosphate-buffered saline, and 1000 µL of JKM extract. Instead of the JKM extract, distilled water was used as a negative control. After 15 minutes of incubation at 37°C, the mixtures were then heated for 5 minutes at 70°C. After cooling, their absorbances at 660 nm were assessed. The percentage of protein denaturation inhibition was calculated using the following formula:

% Denaturation inhibition=(1-(Absorbance reading of the test sample)/(Absorbance reading without test sample (-ve control)))*100%

Antiproteinase activity

Rajesh et al., (2019) guidelines for the antiproteinase activity experiment were followed. The reaction mixture (2 mL) contained 1 mL of the test sample at various concentrations (100-500 g/mL), 0.06 mg of trypsin, and 20 mM Tris-HCl buffer (pH 7.4). The mixture was maintained warm at 37°C for 5 minutes. The mixture was then given 1 mL of casein which was 0.8 percent (w/v). The combination was maintained at a high temperature for another 20 minutes. 2 mL of 70% perchloric acid was added to the mixture to halt the

reaction. After that, the murky suspension was centrifuged. Then, using a buffer as a blank, the absorbance of the supernatant was measured at 210 nm. The investigation was carried out three times. Proteinase inhibitory activity was calculated using the formula shown below:

% inhibition= (Abs control-Abs sample)*100/Abs control

Statistical analysis

Three times each of the assays were run, and the average mean of the results was given. Descriptive statistics were adopted to analyze the data. Excel was used to create bar graphs for a better understanding of the average mean.

Results and Discussion

It is crucial to quantify the approximate amounts of a common feed/food ingredient or supplement to show its nutritional profile and choose the appropriate amount to add to a compounded feed/food (Oloruntola, 2021). Figure 1 depicts the proximate composition of *Juglans regia* kernel meal. *Juglans regia* kernel meal (JKM) proximate profile are nitrogen-free extract (39.14%), crude fat (31.26%), crude protein (8.99%), crude fiber (8.41%), moisture (7.74%) and ash (4.46%) in decreasing level of concentration. The values for various aspects of proximate composition in this study differ from the values previously reported for carbohydrates (24%), crude fat (52-77.5%), crude protein (11-25%), and ash (1.3-2.5%) (Ozkan & Koyuncu, 2005; Martinez & Maestri, 2008; Savage, 2001). This variance may result from various factors, such as processing techniques (Osum et al., 2013), geographic location (Khattak & Rahman, 2015), or developmental stage (Kiskini et al., 2016).

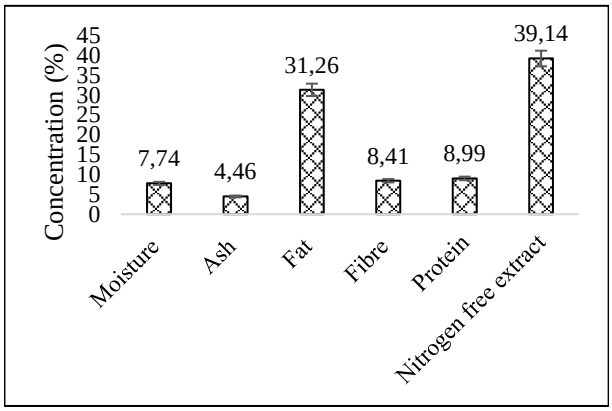

Figure 1. Proximate compositon of *Juglans regia* kernel meal.

Figure 2 depicts the antioxidant activity of JKM. There is evidence that plants are abundant sources of natural antioxidants such as vitamin C, flavonoids, carotenoids, tocopherols, and other phenolic compounds (Iqbal & Bhanger, 2006).

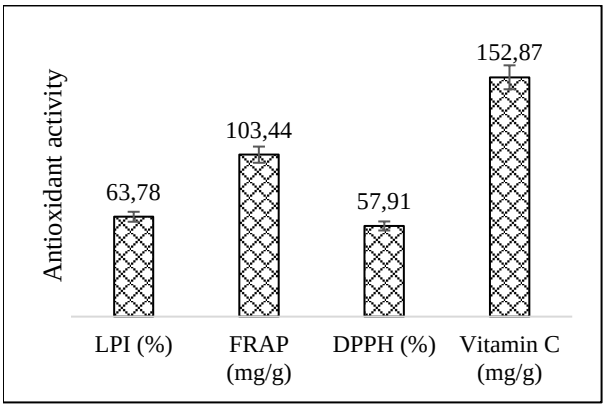

Figure 2. Vitamin C concentration and antioxidant activities of *Juglans regia* kernel meal.

LPI: Lipid peroxidation inhibition; FRAP: Ferric ion reducing antioxidant power; DPPH: 2,2-diphenyl-1-picrylhydrazyl hydrate.

Atherosclerosis, rheumatoid arthritis, atherosclerotic disease, and neurological illnesses have all been linked to lipid peroxidation. Therefore, the function of antioxidants in preventing the production of active oxidants, quenching and removing active oxidants, scavenging, repairing damage, excreting toxic oxidants, and promoting adaptive responses has garnered considerable attention (Niki et al., 2005). This study's discovery of JKM's significant lipid peroxidation inhibition properties of 63.78% (Figure 2) reveals a crucial nutraceutical property of health significance. This corroborated an earlier discovery that some foods and feeds include different antioxidants that scavenge free radicals (Niki & Noguchi, 2000). Additionally, the study's detection of JKM's FRAP (103.44 mg/g), DPPH (57.91%), and vitamin C (152.87mg/g) shows that the phytogens have bioactive compounds that exhibit antioxidant activities. The DPPH and FRAP assays evaluate the primary (an activity that scavenges free radicals) and secondary (mitigation of radical production and defence against oxidative damage) antioxidant abilities of phytogens, respectively (Lim et al., 2007; Yin et al., 2016). The DPPH value observed in this study is less than the 75.02–85.96% reported by Kabiri et al (2019). The JKM's DPPH concentration in this study was greater than 42.24% reported for *Anacardium occidentale* L. leaf powder (Oloruntola, 2021), but lower than 67.32%, 87.15% and 69.17% reported for *Ficus carica, F. exasperata* and *F. thonningii* leaf meals, respectively (Osowe et al., 2021). Furthermore, the JKM's vitamin C concentration in this study was greater than 7.74 mg/g reported for *Anacardium occidentale* L. leaf powder (Oloruntola, 2021) and 28.07 mg/g, 7.15 mg/g and 5.91 mg/g reported for *Ficus carica, F. exasperata* and *F. thonningii* leaf meals, respectively (Osowe et al., 2021).

The antioxidant capacity of JKM is demonstrated by its phytochemical makeup, which includes alkaloids (120.80 mg/g), saponins (43.49 mg/g), steroids (4.84 mg/g), flavonoids (14.72 mg/g), tannins (1.69 mg/g), and phenol (35.93 mg/g) (Figure 3). According to reports, phytochemicals (such as carotenoids, flavonoids, and alkyl sulphide) have antioxidant action

and lower the risk of a variety of diseases (Agbafor & Nwachukwu, 2011). Total phenolic content and overall antioxidant activity in phytogens are directly correlated (Sun et al., 2002). The essential enzyme for the cellular generation of reactive oxygen species, NADPH-oxidase, has also been shown to be inhibited by alkaloids. This inhibition can occur by preventing the synthesis, activation, or translocation of NADPH-oxidase subunits (Macakova et al., 2019). Xu and Yu (2021) also reported that saponins exhibit a wide range of pharmacological actions, such as antioxidant, anti-inflammatory, antiviral, anticancer, antifungal, antibacterial, and immunomodulatory properties. Tannins have been reported to be active in the modulation of immunological response, increasing blood flow, lowering blood pressure and serum cholesterol concentration, and producing antimicrobial, anti-allergic, anti-cancerous and anti-inflammatory properties, even though they have depressing effects on feed intake, digestibility and metabolism in experimental animals (Sharma et al., 2019; Oloruntola, 2021).

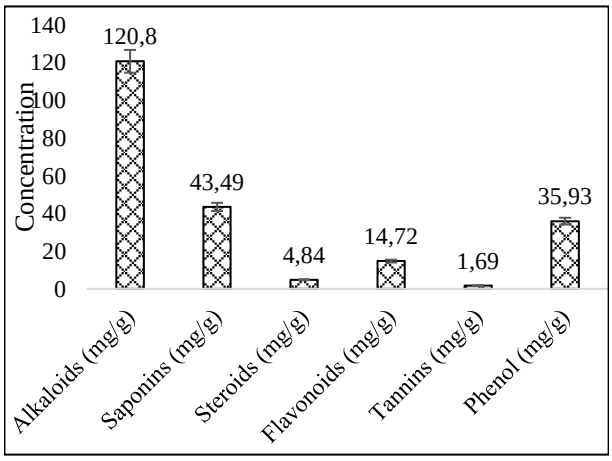

Figure 3. Phytochemical composition of *Juglans regia* kernel meal.

In this study, the JKM had an alkaloid content that was higher than the amounts reported for leaf meals from *Ficus carica, F. exasperata*, and *F. thonningii*, which were 103 mg/g, 81 mg/g, and 59.50 mg/g, respectively (Osowe et al., 2021); While JKM's saponin concentration was less than that of *F. carica, F. exasperata*, and *F. thonningii* leaf meals (60.17 mg/g, 80.72 mg/g, and 51.42 mg/g, respectively) (Osowe et al., 2021). The flavonoid concentration of JKM in this study is at variance with 0.80-1.10 g/100g reported by Mo et al., (2022) and 12.59-62.11 mg/100g by Kabiri et al., (2019). Additionally, this study's JKM phenol content differed from the 1017–3739 mg/100g reported by Kabiri et al (2019). This variation might be brought on by processing methods (Osum et al., 2013), place (Khattak & Rahman, 2015), or developmental stage (Kiskini et al., 2016).

The chemical compounds known as alpha-glucosidase inhibitors prevent the enzymes glucoamylase, sucrase, maltase, and isomaltase from converting complex, non-absorbable carbohydrates into simple, absorbable carbohydrates. This delays the absorption of carbohydrates and lowers the rise in postprandial blood glucose levels (Kumar et al., 2011; Derosa & Maffioli, 2012); while the alpha-amylase inhibitors' function as carbohydrate blockers, they restrict the gastrointestinal tract's ability to digest and absorb carbohydrates. They can be used to stop disorders including obesity, diabetes, hyperglycemia, and hyperlipemia (Gong et al., 2020). The nutraceutical properties of JKM, when used as food/feed supplements or ingredients, are revealed by the alpha-amylase inhibition (61.06%) and alpha-glucosidase inhibition (67.76%) capacities of JKM, as reported in this study (Figure 4). Previous research showed that phytogens had alpha-amylase and alpha-glucosidase inhibitory activities (Boulfia et al., 2021; Oloruntola & Ayodele, 2022). The phytochemical makeup of JKM may be responsible for its alpha-amylase and alpha-glucosidase inhibitory capabilities; for example, peptides derived from cereals, phenolic compounds, non-starch polysaccharides, and lipids were confirmed to inhibit alpha-glucosidase and alpha-amylase activities (Gong et al., 2020).

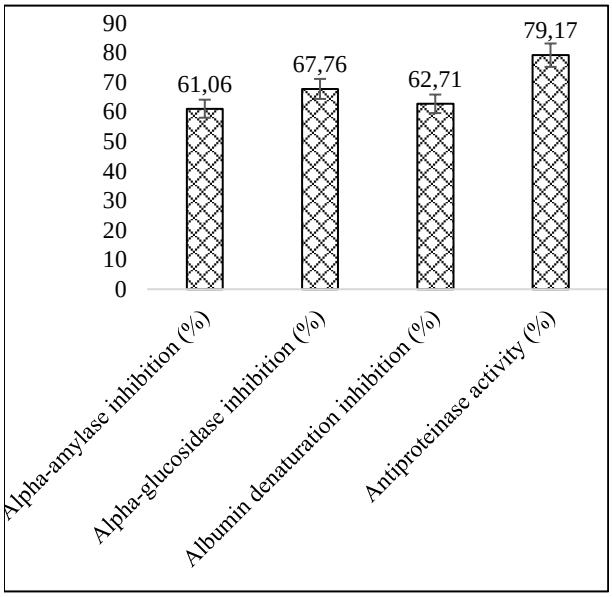

Figure 4. The alpha-amylase inhibition, alpha-glucosidase inhibition, albumin denaturation inhibition and antiproteinase activity of *Juglans regia* kernel meal.

The complex process of inflammation, which commonly entails pain, includes things like increased vascular permeability, increased protein denaturation, and membrane modification (Ruiz-Ruiz et al., 2017). Anti-proteinase activity is also thought to be involved in controlling inflammatory disorders because of its capacity to protect against proteinase-induced tissue damage. Protein denaturation may be a significant contributor to inflammation because it may result in the production of auto-antigens in certain inflammatory conditions (Anwar et al., 2020). Inhibition of albumin denaturation (62.71%) and antiproteinase activity

(79.17%) by JKM in this study demonstrate the potential for JKM to have anti-inflammatory effects.

Conclusions

These findings, therefore, showed that JKM has anti-inflammatory, antioxidant, and anti-diabetic properties. In feeding studies using an animal model, the JKM is suggested as a dietary supplement to validate its usefulness as a nutraceutical feed supplement.

Conflict of Interest

The author declares that he has no known competition for financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

References

Adeniyi, S. A., Orjiekwe, C. L., & Ehiagbonare, J. E. (2009). Determination of alkaloids and oxalates in some selected food samples in Nigeria. *African Journal of Biotechnology*, *8*(1),110-112.

Adeyeye, S. A., Oloruntola, O. D., Ayodele, S. O., Falowo, A. B., & Agbede, J. O. (2020). Wild sunflower and goat weed composite-mix supplementation in broiler chicken: effects on performance, health status and meat. *Acta Fytotechnica et Zootechnica*, *23*(4), 205-212. http://dx.doi.org/10.15414/afz.2020.23.04.205-212

Agbafor, K. N., & Nwachukwu, N. (2011). Phytochemical Analysis and Antioxidant Property of Leaf Extracts of *Vitex doniana* and *Mucuna pruriens*, *Biochemistry Research International,* 1-4. https://doi.org/10.1155/2011/459839

Aiyeloja, A. A. & Bello, O. A. (2006). Ethnobotanical potentials of common herbs in Nigeria: A case study of Enugu State, *Educational Research and Review*, *1*(1), 16–22. http://www.academicjournals.org/ERR

Alagawany, M., Elnesr, S. S., Farag, M. R., Abd El-Hack, M. E., Barkat, R. A., Gabr, A. A., Foda, M. A., Noreldin, A. E., Khafaga, A. F., El-Sabrout, K., Elwan, H. A. M., Tiwari, R., Yatoo, M. I, Michalak I, Cerbo A. D., & Dhama, K. (2021). Potential role of important nutraceuticals in poultry performance and health - A comprehensive review, *Research in Veterinary Science*, *137*(2021), 9-29, https://doi.org/10.1016/j.rvsc.2021.04.009

Anwar. S., Almatroudi, A., Allemailem, K. S., Joseph, R. J., Khan, A. A., & Rahmani, A. H. (2020). protective effects of ginger extract against glycation and oxidative stress-induced health complications: an in vitro study. *Processes*, 8, 448-468. https://doi.org/10.3390/pr8040468

AOAC. (2010). Official Methods of Analysis of Association of Offical Analytical Chemists. 18th Edition, Washington DC.

Aryapak, S., & Ziarati, P. (2014). Nutritive value of Persian walnut (*Juglans regia* L.) Orchards. *American-Eurasian Journal of Agriculture and Environmental Science*, *14*(11), 1228-1235. https://doi.org/ 10.5829/idosi.aejaes.2014.14.11.12438

Bajpai, V. K., Park Y., & Agrawal, P. (2015). Studies on phytochemical analysis, antioxidant and lipid peroxidation inhibitory effects of a medicinal plants, *Coleus forskohlii*. *Frontiers in Life Science, 8*(2), 139-147. https://doi.org/10.1080/21553769.2014.998777

Benderitter, M., Maupoli, V., Vergely, C., Dalloz, F., Briot, F., & Rochette, L. (1998). Studies by electron paramagnetic resonance of the importance of iron in the hydroxyl scavenging properties of ascorbic acid in plasma: Effects of iron chelators. *Fundamental Clinical Pharmacology*, *12*(5),510-516. https://doi.org/10.1111/j.1472-8206.1998.tb00979.x

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as measurement of "antioxidant power" The FRAP assay. *Analytical Biochemistry*, 239, 70-76. https://doi.org/10.1006/abio.1996.0292

Biswas, A., Dey, S., Li, D., Yiu, L., Zhang, J., Huang, S., Pan, G., & Deng, Y. (2020). Comparison of Phytochemical Profile, Mineral Content, and in Vitro Antioxidant Activities of *Corchorus capsularis* and *Corchorus olitorius* Leaf Extracts from Different Populations. *Journal of Food Quality*, 2020, *2020*(9). https://doi.org/10.1155/2020/2931097

Boulfia, M., Lamchouri, F., & Toufik, H. (2021). Mineral analysis, in vitro evaluation of alpha-amylase, alpha-glucosidase, and beta-galactosidase inhibition, and antibacterial activities of *Juglans regia* L. bark extracts, *BioMed Research International*, 2021,1-14. https://doi.org/10.1155/2021/1585692

Dej-adisai, S., & Pitakbut, T. (2015). Determination of α-glucosidase inhibitory activity from selected Fabaceae plants. *Pakistan Journal of Pharmacological Science*, *28*(5), 1679-1683. https://pubmed.ncbi.nlm.nih.gov/26408887

Derosa, G., & Maffioli, P. (2012). α-Glucosidase inhibitors and their use in clinical practice. *Archives of Medical Science*, *8*(5), 899-906. https://doi.org/10.5114/aoms.2012.31621

Gong, L., Feng, D., Wang, T., Ren, Y., Liu, Y., & Wang, J. (2020). Inhibitors of α-amylase and α-glucosidase: Potential linkage for whole cereal foods on prevention of hyperglycemia. *Food Science & Nutrition*, *8*(12),6320-6337. https://doi.org/10.1002/fsn3.1987

Gulati, O. P., Ottaway, P. B., & Coppens, P. (2014). Chapter 14 - Botanical Nutraceuticals, (Food Supplements, Fortified and Functional Foods) in the European Union with Main Focus on Nutrition and Health Claims Regulation, Editor(s): Debasis Bagchi, In Food Science and Technology, Nutraceutical and Functional Food Regulations in the United States and Around the World (Second Edition), Academic Press, Pp. 221-256, https://doi.org/10.1016/B978-0-12-405870-5.00014-1

Gupta, R. C. (2016). Nutraceuticals: efficacy, safety and toxicity. In: Gupta RC (ed) Academic Press/Elsevier, Amsterdam, p 1022. https://doi.org/10.1016/C2014-0-01870-9

He, J., Wu, Z. Y., Zhang, S., Zhou, Y., Zhao, F., Peng, Z. Q., Hu, Z. W. (2014). Optimisation of microwave-assisted extraction of tea saponin and its application on cleaning of historic silks. *Journal of Surfactants and Detergents*, *17*(5), 919-928. https://doi.org/10.1007/s11743-013-1523-8

Iqbal, S., & Bhanger, M. (2006). Effect of season and production location on antioxidant activity of Moringa oleifera leaves grown in Pakistan. *Journal of Food Composition and Analysis,* 19, 544–551. https://doi.org/10.1016/j.jfca.2005.05.001

Janick, J. & Paul, R. E. (2008). The encyclopedia of fruits and nuts, Cab International England, Oxfordshire. http://dx.doi.org/10.1079/9780851996387.0000

Kabiri, G., Bouda, S., Elhansali, M., & Haddioui, A. (2019). Biochemical characterization and antioxidant activity of walnut kernel (*Juglans regia* L.) of accessions from Middle and High Atlas in Morocco. *Acta Scientiarum. Biological Sciences*, 41, e46411. https://doi.org/10.4025/actascibiolsci.v41i1.46411

Khattak, K. F., & Rahman, T. R. (2015). Effect of geographical distributions on the nutrient composition, phytochemical profile and antioxidant activity of Morus nigra. *Pakistan Journal of Pharmaceutical Science*, *28*(5), 1671-1678.

Kiskini, A., Vissers, A., Vincken, J. P., Gruppen, H., & Wierenga, P. A. (2016). Effect of Plant Age on the Quantity and Quality of Proteins Extracted from Sugar Beet (*Beta vulgaris* L.) Leaves. *Journal of Agricultural and Food Chemistry*, *64*(44), 8305-8314. https://doi: 10.1021/acs.jafc.6b03095

Kumar, S., Narwal, S., Kumar, V., & Prakash, O. (2011). α-glucosidase inhibitors from plants: A natural approach to treat diabetes. *Pharmacognosy Reviews*, 5(9),19-29. https://doi.org/10.4103/0973-7847.79096

Lim, Y. Y., Lim, T. T, & Tee, J. J. (2007). Antioxidant properties of several tropical fruits: A comparative study, *Food Chemistry*, *103*(3), 1003-1008. https://doi.org/10.1016/j.foodchem.2006.08.038

Macakova, K., Afonso, R., Saso, L. & Mladenka, P. (2019). The influence of alkaloids on oxidative stress and related signaling pathways. *Free Radical Biology and Medicine*, 134,429-444. https://doi.org/10.1016/j.freeradbiomed.2019.01.026

Madhu, M., Sailaja, V., Satyadev, T. N. V. S. S., & Satyanarayana, M. V. (2016). Quantitative phytochemical analysis of selected medicinal plant species by using various organic solvent. *Journal of Pharmacognosy and Phytochemistry*, 5(2), 25-29. https://www.phytojournal.com/archives/2016/vol5issue2/PartA/5-1-31.pdf

Martinez, M. L., & Maestri, D. (2008). Oil chemical variation in walnut (*Juglans regia* L.) genotypes grown in Argentina. *European Journal of Lipid Science and Technology*, 110, 1183–1189. https://doi.org/10.1002/ejlt.200800121

Mo, R., Zheng, Y., Ni, Z., Shen, D & Liu, Y. (2022). The phytochemical components of walnuts and their application for geographical origin based on chemical markers. *Food Quality and Safety*. 6(1), fyac052, https://doi.org/10.1093/fqsafe/fyac052

Niki, E., & Noguchi, N. (2000). Evaluation of antioxidant capacity. What capacity is being measured by which method? *IUBMB Life*, *50*(2000), 323–329. https://doi.org/10.1080/713803736

Niki, E., Yoshida, Y., Saito, Y., & Noguchi, N. (2005). Lipid peroxidation: Mechanisms, inhibition, and biological effects. *Biochemical and Biophysical Research Communications*, *338*(1), 668-676. https://doi.org/10.1016/j.bbrc.2005.08.072

Nwachoko, N. & Jack, I. R. (2015). 'Phytochemical screening and anti-diarrhea activities of *Tetracarpidium conophorum* induced in albino rats', *Sky Journal of Biochemistry Research, 4*(4), 21–24. http://dx.doi.org/10.33545/26174693.2022.v6.i2b.139

Nwauzoma, A. B., & Dappa, M. S. (2013). 'Ethnobotanicals studies of Port Harcourt metropolis, Nigeria', ISRN Botany 1–5. https://doi.org/10.1155/2013/829424

Oloruntola, O. D. (2021). Proximate, phytochemical, mineral composition and antioxidant activity of *Anacardium occidentale* L. leaf powder. *DYSONA - Life Science*, 2 (2021),39-49. https://doi.org/10.30493/DLS.2021.290718

Oloruntola, O. D., & Ayodele, S. O. (2022). Phytochemical, proximate and mineral composition, antioxidant and antidiabetic properties evaluation and comparison of mistletoe leaves from moringa and kolanut trees. *Turkish Journal of Agriculture - Food Science and Technology*, *10*(8), 1524-1531. https://doi.org/10.24925/turjaf.v10i8.1524-1531.5134

Osman, N. I. Sidik, N. J., Awal, A., Adam, N. A. & Rezali, N. I. (2016). In vitro xanthine oxidase and albumin denaturation inhibition assay of *Barringtonia racemosa* L. and total phenolic content analysis for potential anti-inflammatory use in gouty arthritis. *Journal of Intercultural Ethnopharmacology*, *5*(4), 343-349. https://doi.org/10.5455/jice.20160731025522

Osowe, C. O., Olowu O. P. A., Adu O. A., Oloruntola O. D., & Chineke C. A. (2021). Proximate and mineral composition, phytochemical analysis, and antioxidant activity of fig trees (*Ficus spp*.) leaf powder. *Asian Journal of Biochemistry, Genetics and Molecular Biology*. *9*(1): 19-29.

Osum, F. I., Okonkwo, T. M., & Okafor, G. I. (2013). Effect of processing methods on the chemical composition of *Vitex doniana* leaf and leaf products. *Food Science and Nutrition*, *1*(3), 241-245. https://doi: 10.1002/fsn3.31

Otles, S., & Yalcin, B. (2012). Phenolic compounds analysis of root, stalk, and leaves of nettle. *Scientific World Journal*, 2012, 564367. https://doi: 10.1100/2012/564367

Ozkan, G., & Koyuncu, M. A. (2005). Physical and chemical composition of some walnut (*Juglans regia* L.) genotypes grown in Turkey. *Grasas Aceites*, 56, 141–146. https://doi.org/10.3989/gya.2005.v56.i2.122

Rajesh, A., Dossa, A., Tresina, P. S., Mohan, V. R. (2019). Anti-inflammatory activity of methanol extract of *Niebuhria apetala* (Roth) Dunn – in vitro models. *Asian Journal of Pharmaceutical and Clinical Research*, *12*(5), 278-281. https://doi.org/10.22159/ajpcr.2019.v12i5.32512

Ruiz-Ruiz, J. C., Matus-Basto, A. J., Acereto-Escoffié, P. & Segura-Campos, M. R. (2017) Antioxidant and anti-inflammatory activities of phenolic compounds isolated from Melipona beecheii honey. Food and Agricultural Immunology, 28:6, 1424-1437. https://doi.org/ 10.1080/09540105.2017.1347148

Savage, G. P. (2001). Chemical composition of walnut (*Juglans regis* L) grown in New Zealand. *Plant Foods for Human Nutrition,* 2001, 56, 75–82. https://doi: 10.1023/a:1008175606698

Sharma, K., Kumar, V., Kaur, J., Tanwar, B., Goyal, A., Sharma, R., Gat, S., & Kumar, A. (2019). Health effects, sources, utilisation and safety of tannins: a critical review. *Toxin Reviews*, *40*(4), 432-444. https://doi.org/10.1080/15569543.2019.1662813

Sun, J., Chu, Y. F., Wu, X. Z., & Liu, R. H. (2002). Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry,* 50,7449–7454. https://doi.org/ 10.1021/jf0207530

Surana, A. R., Kumbhare, M. R., & Wagh, R. D. (2016). Estimation of total phenolic and total flavonoid content and assessment of in vitro antioxidant activity of extracts of *Hamelia patens* Jacq. stems. *Research Journal of Phytochemistry*, *10*(2), 67-74. http://dx.doi.org/10.3923/rjphyto.2016.67.74

Wickramaratne, M. N., Punchihewa, J. C., & Wickramaratne, D. B. (2016). In-vitro alpha-amylase inhibitory activity of the leaf extracts of *Adenanthera pavonina*. *BMC Complementary Alternative Medicine*, *16*(1), 466. https://doi.org/10.1186/s12906-016-1452-y

Xu, P. & Yu, B. (2021). Chapter One - Chemical synthesis of saponins: An update, Editor(s): David C. Baker, Advances in Carbohydrate Chemistry and Biochemistry, Academic Press. 79:1-62. https://doi.org/10.1016/bs.accb.2021.11.001

Yin, T., Cai, L., Xing, Y., Yu, J., Li, X., Mei, R., & Ding, Z. (2016). Alkaloids with antioxidant activities from *Aconitum handelianum*. *Journal of Asian Natural Products Research*, *18*(6):603-610. http://doi.org/ 10.1080/10286020.2015.1114473

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CONTENTS