ISSN: 2667-4203

ESKİŞEHİR TECHNICAL UNIVERSITY JOURNAL OF SCIENCE AND TECHNOLOGY
C– Life Sciences and Biotechnology

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C – Yaşam Bilimleri ve Biyoteknoloji

Volume/Cilt 13 Number/Sayı 1 January / Ocak - 2024

ESKİŞEHİR TECHNICAL UNIVERSITY JOURNAL OF SCIENCE AND TECHNOLOGY
C- Life Sciences and Biotechnology
Eskişehir Teknik Üniversitesi Bilim ve Teknoloji Dergisi C – Yaşam Bilimleri ve Biyoteknoloji

Volume: 13 / Number: 1 / January - 2024

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Indexed by **ULAKBIM TR Dizin**

ISSN: 2667-4203

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Volume: 13 / Number: 1 / January – 2024

Owner / Publisher: Prof. Dr. Adnan ÖZCAN for Eskişehir Technical University

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Books
Haupt R.L. and Haupt S.E. Practical Genetic Algorithms. 2nd ed. New York, NY, USA: Wiley, 2004.

Kennedy J. and Eberhart R. Swarm Intelligence. San Diego, CA, USA: Academic Press, 2001.

Chapters in boks
Poore JH, Lin L, Eschbach R and Bauer T. Automated statistical testing for embedded systems. In: Zander J, Schieferdecker I, Mosterman PJ, editors. Model-Based Testing for Embedded Systems. Boca Raton, FL, USA: CRC Press, 2012. pp. 111-146.

Conference proceedings
Li RTH. and Chung SH. Digital boundary controller for single-phase grid-connected CSI. In: IEEE 2008 Power Electronics Specialists Conference; 15–19 June 2008; Rhodes, Greece. New York, NY, USA: IEEE. pp. 4562-4568.

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Volume/ Cilt**: 13 / Number/**Sayı**: 1 / January /** Ocak**- 2024**

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A- APPLIED SCIENCES AND ENGINEERING

2024, 13(1), pp. 1-7, DOI: 10.18036/estubtdc.1224531

RESEARCH ARTICLE

COMPARATIVE RESPONSE OF TWO WHEAT VARIETIES TO BASAL AND SPLIT POTASSIUM NUTRITION UNDER FIELD CONDITIONS

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[1] Department of Soil Science, Sindh Agriculture Unviersity, 70060, Pakistan.

ABSTRACT

Potassium (K) is an essential plant nutrient. Several research studies are available on the basal application of K concerned with yield and quality of wheat (*Triticum aestivum* L.). Conversely, very limited studies are available on the split application of K. During 2018-2019, a field experiment was performed at the Southern Wheat Research Station, Agriculture Research Institute, Tandojam, Pakistan to determine the importance of the right time for K fertilization in wheat. The experiment included 18 plots, each having an area of 12m^2 (4m × 3m) involving two cultivars of winter wheat, viz. *Benazir* and *Sindhu*, sown in two-factor Randomized Complete Block Design, arranged in a split pattern (main plots = varieties, sub plots = treatments). Three K fertilization levels were tested, i.e. T_1 = No K fertilization, T_2 = 50 kg K_2O ha^{-1} applied at the time of sowing, T_3 = two splits of K, i.e. 25 kg K_2O ha^{-1} applied at sowing and 25 kg K_2O ha^{-1} applied at grain filling stage (top dressing). According to results, the yield components showed a positive enhancement upon split application of K as compared to basal application in terms of significantly higher ($p<0.05$) number of tillers (10.1 against 8.18), number of grains per plant (548.0 against 374.2), 1000 grain weight (44.7 against 41.9 g), grain yield (4.5 against 4.2 Mg ha^{-1}) and straw yield (8.5 and 8.3 Mg ha^{-1}), and K concentration in grain (0.38 against 0.32%) and straw (0.44 against 0.40%) was recorded for *Sindhu* and *Benazir*, respectively. The varietal interaction revealed that *Sindhu* was significantly different as compared to *Benazir*. These results advocate that the split application of K is better for obtaining the higher yield of wheat, especially *Sindhu*.

Keywords: Cereal, Fertilization methodology, Modern cultivars, yield constraints, K-splits

1. INTRODUCTION

Potassium (K) is an essential plant nutrient which plays pivotal roles in plant growth, metabolism and contributes remarkably to the survival of plants. There are various stresses that plants are subjected to both biotically and abiotically [1]. It improves crop resistance to stress in low-moisture soils by inducing deeper rooting, higher absorption surfaces and increased water maintenance in plant tissues. The use of K-fertilizer worldwide is increasing, but its efficiency is declining due to the introduction of high-yielding varieties under intensive farming. The soil K reserves had already begun to deplete, which in turn resulted in reduced crop yields and high economic losses for farmers [2]. In general, crops take more K from soils than the amount available in it, as it is a water soluble and highly exchangeable element [3]. Potassium deficiency may result in more damaging physiological stress for plants in terms of frost damage. When plants are drought-stricken, they remain flaccid while moisture-logged conditions cause them to wilt for longer periods of time. Moreover, crops will become more prone to diseases and pests, especially where nitrogen (N) and K imbalances exit, leading to weaker growth. Interestingly, the amount of K taken up by crops from the soil is almost equal or even greater to than that of N [4]. Applied K significantly affected the uptake of N and P in straw and wheat grain [5]. In plants, the high intake of nitrate ions (NO_3^-) during vegetative growth is usually matched by an equivalent consumption of positively charged potassium ions (K^+), which maintains electrical neutrality Therefore, sufficient potash is crucial for the quality production of wheat [6]. The yield increment of wheat is directly related to kernel weight which is increased with K fertilization [7]. There are several

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Received: 26.12.2022 Published:30. 01.2024

studies reported to advocate the positive impacts of K nutrition on wheat yield as a basal application, nonetheless, the results vary from one study to another [8,9].

The use of a single dose of K at sowing time increases the risk of its fixation and consequently the plant is unable to absorb it properly [10]. During the growth period, split applications of K have been found to be beneficial in that they simultaneously reduce the loss of K due to leaching and boost the efficient use of K fertilizers [11]. In addition, K applications in splits together with improved yield can also improve wheat crop quality [12]. Prevention of K losses through split application of K fertilizer can increase the sucrose supply, encourage starch accumulation in grains and consequently increase grain yield [13]. Additionally, split applications of K increase protein and wet gluten content in wheat grains more than basal applications alone [14]. Scientific community carries a difference of opinion regarding the method of K application. Very little is known about the single and split application of K fertilizers. We conducted this study to investigate the comparative response of two newly developed wheat cultivars, viz. *Benazir* and *Sindhu* (indigenous high-yielding modern wheat varieties of Sindh, Pakistan) in terms of growth and yield under basal and split K nutrition with these specific objectives; 1) to examine the comparative response of two wheat varieties under basal and split fertilization of K nutrition in terms of growth and yield and 2) to evaluate K accumulation by grain and straw of two wheat varieties under basal and split K nutrition.

2. MATERIALS AND METHODS

2.1. Experimental Setup and Treatment Details

This field experiment was conducted using a two-factor RCBD, with split-plot arrangement (main plot=varieties, sub plots=treatments), in three replications. The experimental site was located at 25°25′12"N 68°32′49"E Sindh, Pakistan, during winter 2018-19. The treatments involved were: T_1 = No K application, T_2 = K application at 50 kg K_2O ha^{-1} at the time of sowing, T_3 = K applications in two splits, i.e., 25 kg K_2O ha^{-1} at sowing and 25 kg K_2O ha^{-1} at the grain filling stage (same three treatments for each variety). Each treatment also received a blanket recommended dose of P_2O_5 in the form of single super phosphate [$Ca(H_2PO_4)_2$] applied at the rate of 90 kg ha^{-1} and N in the form of Urea [$CO(NH_2)_2$] at the rate of 120 kg ha^{-1} [15]. A piece of 216m^2 land was selected for this experiment. The area was divided into 18 equal experimental units of 12m^2 (4m ×*3m).

2.2. Sowing and Harvesting of Wheat

To prevent winter injuries, wheat seeds were sown using drills at the recommended seed rate of 125 kg ha^{-1}. It is imperative to seed wheat in a moist, firm seedbed prior to planting. All the recommended cultural and management practices were followed during the early growth stages. Canal water from the River Indus was used for irrigation. Total six irrigations were recommended; initial irrigation was applied after 20 days of sowing and the subsequent irrigations were set as 5 irrigations at 15 days intervals. Precise agronomic observations were recorded at the time of maturity, number of tillers $plant^{-1,}$ number of grain $plant^{-1}$, 1000 grain weight (g), grain yield (kg ha^{-1}), and straw yield (kg ha^{-1}). Wheat with its two local genotypes was sown on the 3rd of December 2018 and harvested at maturity after 140 days.

2.3. Soil and Plant Analysis

Soil was analyzed (samples collected from 0-30 cm depth randomly to produce representative soil samples). Soil was silty clay containing sand 0.38%, silt 41.37% & clay 58.32%, deficient in N (0.40 g kg^{-1}) and organic matter (6.98 g kg^{-1}). The extractable K was found to be 115 mg kg^{-1} determined by the AB-DTPA method. The soil under study was alkaline in reaction (pH 7.89), non-saline (EC 0.14 dS cm^{-1}) and moderately calcareous in nature ($CaCO_3$-74.0 g kg^{-1}) [16]. The plant analysis was done for K concentration in grains and straw. Plant (grain and straw) samples were collected as per treatment (five

samples per replication), washed with tap water followed by deionized water, and then oven dried at 65°C for 24 hours. Samples were ground in a Wiley Mill and analyzed by the wet acid digestion method [16]. The concentration of K^+ in plant samples was calculated by the following formulae:

Potassium = GR X volume made/weight of plant material

2.4. Statistical Analysis

This field study was conducted in a Randomized Complete Block Design (RCBD) involving two factors, viz. three K application treatments with two varieties, and three replications. Based on this design the Analysis of Variance was done using Statistix ver. 8.1. The treatment means were separated using the Least Significant Difference (LSD) test at alpha 0.05.

3. RESULTS AND DISCUSSION

3.1 Growth Attributes

The results in relation to growth attributes of wheat and varietal differences as affected by K mode of application are shown in Table-1&2. The number of tillers due to different K modes and its interaction on two varieties was found significant ($p \leq 0.05$). The maximum numbers of tillers per plant were recorded in plants which received 50 kg K_2O ha^{-1} in two splits followed by 50 kg K_2O ha^{-1} as a basal application once only while the minimum numbers of tillers were observed in control. According to varietal differences the maximum number of tillers per plant in two splits, were measured in variety *Sindhu* (12.13) while (10.31) in *Benazir*, followed by 50 kg K_2O ha^{-1}. However, the minimum number of tillers per plant (7.71 and 6.10) was recorded at 0 kg K_2O ha^{-1} (control) in *Sindhu* and *Benazir* respectively. The same trend was seen in the number of grains per plant. The maximum number of grains per plant was noted in plants that received K in two doses followed by basal application. In contrast, the minimum number of grains per plant was recorded in the control. Varietal difference was significant ($p \leq 0.05$) as *Sindhu* (611.75) and *Benazir* (475.30) responded by producing more grains after split K fertilization, in comparison to basal K application. It was found that both cultivars responded positively where K was applied in two equal splits, at planting time (25) and during active tillering (25) as compared to K applied as a basal. The application of K in 2 equal splits is an indication of more K availability and less transformation into a non-exchangeable pool, which regulates cells and tissue growth in a right manner with a continuous process [17]. In addition, K application at once may result in cation imbalances, in particular Ca and Mg, due to the long lifecycle of the crop (120 days). It has been highlighted in the literature that the single application of K fertilizers may not necessarily be helpful for plants to complete their growth and development properly [18]. The role of K in plants is sugar translocation and starch biosynthesis [19]. Generally, K is associated with membrane permeability, opening and closing of stomata (as K is low, plants would be unable to open stomata). Stomatal closing causes a lower input of carbon dioxide and thereby lowers photosynthetic activity [20]. The split K application provides better results than the one-go and less available K (basal application). Several biochemical and physiological processes rely on K as a "quality element" and K is required at higher concentrations for better growth [21].

Table 1. Growth attributes of two wheat cultivars towards different K applications time.

Treatments (kg K_2O ha^{-1})	***Sındhu* No. of tillers $plant^{-1}$**	***Benazir* No. of tillers $plant^{-1}$**	***Sindhu* No. of grains $plant^{-1}$**	***Benazir* No. of grains $plant^{-1}$**
Control (0)	7.71 c	6.10 c	466.30 c	306.70 c
K1 (50 as basal)	10.33 b	8.13 b	566.00 b	340.70 b
K2 (50 two splits)	12.13 a	10.31 a	611.75 a	475.30 a
SE±	0.65		5.8	
LSD 0.05	0.29		2.6	

Means with different letters are significantly different from each other at LSD <0.05

Table 2. Response of Two wheat cultivars towards different K applications time

Growth parameters	**Interaction of varieties**		**SE± (T*V)**	**LSD 0.05 (T*V)**
	Sındhu	***Benazir***		
No. of tiller $plant^{-1}$	10.06 A	8.18 B	0.91	0.41
No. of grains $plant^{-1}$	548.02 A	374.23 B	8.21	3.68

3.2. Yield Parameters

The yield parameters affected by different K application timings were significant (*p<0.05*). Likewise, the interaction of two wheat cultivars was significant too (Table 3-4). The maximum seed index (1000 grain weight) was recorded in plants that received 50 kg K_2O ha^{-1} in two splits. In both varieties, *Sindhu* (51.60 g) and *Benazir* (48.30 g), split K application performed better than basal K application; however, a minimum seed index was observed in the control. According to the findings, maximum grain yields of 4.8 and 4.0 Mg ha^{-1} were received in *Sindhu* and *Benazir* respectively in plants using 50 kg K_2O applied in two splits. Straw yields had a similar pattern. Potassium enhances physiological activities, which was reflected in an increase in yield attributes. As a result of physiological activities, including photosynthesis, translocation, and assimilation of photosynthates, and spikelet initiation, spikelet numbers increased. In addition, abundant K availability resulted in a faster photosynthetic rate and improved material transition in the phloem, which culminated in a substantial number of grains filled. Results are in line with Zhao et al. [22], who have noted similar enhancement by applying K in different modes. These results confirm the findings of Lu et al. (2014), who observed that K application in splits can improve the yield parameters significantly. Since K is its own element and can contribute in many uncertainties (as mentioned earlier), its application in parts eliminates the risk of K losses. Increasing wheat yield under current research is consistent with Corrêa et al. [23]. They obtained the most effective results when K is applied 50% at planting and 50% at topdressing.

Table 3. Yield attributes of two wheat cultivars towards different K applications time

Treatments (kg K_2O ha^{-1})	***Sindhu* Grain yield (Mg ha^{-1})**	***Benazır* Grain yield (Mg ha^{-1})**	***Sindhu* Straw yield (Mg ha^{-1})**	***Benazir* Straw yield (Mg ha^{-1})**	***Sindhu* Seed index (g)**	***Benazir* Seed index (g)**
Control (0)	3.5 c	3.1 c	8.9 a	8.5 a	37.10 c	35.70 c
K1 (50 as basal)	4.1 b	3.6 b	8.4 b	8.1 b	45.30 b	41.70 b
K2 (50 two splits)	4.8 a	4.0 a	8.2 c	8.3 c	51.60 a	48.30 a
SE±	0.4		0.3		0.08	
LSD 0.05	0.02		0.23		0.03	

Means with different letters are significantly different from each other at LSD <0.05

Table 4. Response of Two wheat cultivars towards different K applications time

Growth parameters	Interaction of varieties *Sındhu*	*Benazir*	SE± (T*V)	LSD 0.05 (T*V)
No. of tiller plant^{-1}	10.06 A	8.18 B	0.91	0.41
No. of grains plant^{-1}	548.02 A	374.23 B	8.21	3.68

3.3. Potassium Concentration in Grain and Straw

The data in Table 5 display the results in relation to the average K concentration (%) in grains and straw. Moreover, the data in Table 6 display the results in relation to the interaction between varieties in grains and straw both. The different methods used for applying K to grains and straw resulted in a significant difference in K concentrations ($p \leq 0.05$). It was found that split K application was associated with a significant difference in the K concentration in grains (*Sindhu* 0.54%; *Benazir* 0.51%) and straw (*Sindhu* 0.65%; *Benazir* 0.63%) in plots where K was applied in two splits followed by basal K application. Control grain and straw were also found to contain the lowest K concentration.

Table 5. K-concentration in grains and straw towards different K applications time

Treatments (kg K_2O ha^{-1})	*Sindhu* Grains %	*Benazır* Grains %	*Sindhu* Straw %	*Benazir* Straw %
Control (0)	0.20 c	0.16 c	0.23 c	0.21 c
K1 (50 as basal)	0.41 b	0.40 b	0.43 b	0.40 b
K2 (50 two splits)	0.54 a	0.51 a	0.65 a	0.63 a
SE±	0.01		0.16	
LSD 0.05	7.42		0.07	

Means with different letters are significantly different from each other at LSD <0.05

Table 6. Response of Two wheat cultivars towards different K applications time

K concentration	Interaction of varieties *Sındhu*	*Benazir*	SE± (T*V)	LSD 0.05 (T*V)
K concentration in Grains	0.38 A	0.32 B	0.02	0.01
K concentration in Straw	0.44 A	0.40 B	0.22	0.1

There was a substantial difference in response between *Sindhu* and *Benazir*. Therefore, topdressing with 50% of the seed and planting with 50% of the seed gave the most promising results. According to Kang et al. [24], excessive K can lead to soil salinization and imbalance in the absorption of other nutrients, such as N, Ca, and Mg; thus, impairing the formation of roots. Thus, it is believed that the supply of K only at planting allows leaching losses [23].

4. CONCLUSION

The study concluded that K application in two equal splits, each at planting and topdressing, was significantly better than the single application of K. Moreover, the wheat variety *Sindhu* performed significantly better as compared to *Benazir* in terms of growth and yield attributes. Further research is warranted on this subject to reduce the luxury consumption of K and the input cost of farmers on K fertilizers.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

AUTHORSHIP CONTRIBUTIONS

Saima Kalsoom Babar: Writing original draft, conceptulization, supervision. **Tarique Ali Jatoi:** Formal analysis, Investigation, visualization**. Zia-ul-Hassan Shah:** supervision, conceptulization.

REFERENCES

[1] Shabala S, Pottosin I. Regulation of potassium transport in plants under hostile conditions: implications for abiotic and biotic stress tolerance. Physiol. Plant 2014; 151(3): 257-279.

[2] Dhillon JS, Eickhoff EM, Mullen RW, Raun WR. World potassium use efficiency in cereal crops. Agron J. 2019; 111(2): 889-96.

[3] Wani JA, Malik MA, Dar MA, Akhter F, Raina SK. Impact of method of application and concentration of potassium on yield of wheat. J. Environ Bio 2014; 1;35(4):623.

[4] Van Duivenbooden N, De Wit CT, Van Keulen H. Nitrogen, phosphorus and potassium relations in five major cereals reviewed in respect to fertilizer recommendations using simulation modelling. Fert res 1995; 44(1): 37-49.

[5] Saifullah A, Ranjha M, Yaseen M, Akhtar MF. Response of wheat to potassium fertilization under field conditions. Pak. J. Agric. Sci. 2002;39(4):269-72.

[6] Hossain A, da Silva JA, Bodruzzaman M. Rate and application methods of potassium in light soil for irrigated spring wheat. Songklanakarin J Sci Tech. 2015; 1;37(6)635-642.

[7] Sharma S, Duveiller E, Basnet R, Karki CB, Sharma RC. Effect of potash fertilization on Helminthosporium leaf blight severity in wheat, and associated increases in grain yield and kernel weight. Field Crops Res. 2005; 14;93(2-3):142-50.

[8] Sweeney DW, Granade GV, Eversmeyer MG, Whitney DA. Phosphorus, potassium, chloride, and fungicide effects on wheat yield and leaf rust severity. J Plant Nutr. 2000; 1;23(9):1267-81.

[9] Kolar JS, Grewal HS. Effect of split application of potassium on growth, yield and potassium accumulation by soybean. Fert res. 1994;39(3):217-22.

[10] Ali I, Khan AA, Munsif F, He L, Khan A, Ullah S, Saeed W, Iqbal A, Adnan M, Ligeng J. Optimizing rates and application time of potassium fertilizer for improving growth, grain nutrients content and yield of wheat crop. Open Agriculture 2019; 1;4(1):500-8.

[11] Römheld V, Kirkby EA. Research on potassium in agriculture: needs and prospects. Plant soil 2010;335(1):155-80.

[12] Wang Y, Zhang Z, Liang Y, Han Y, Han Y, Tan J. High potassium application rate increased grain yield of shading-stressed winter wheat by improving photosynthesis and photosynthate translocation. Front Plant Sci 2020; 28;11:134.

[13] Liang X, Yu Z. Effect of potassium application stage on photosynthetic characteristics of winter wheat flag leaves and on starch accumulation in wheat grains. Ying Yong Sheng tai xue bao= The J Appl Eco 2004; 1;15(8):1349-52.

[14] Yu ZW, Liang XF, Li YQ, Wang X. Effects of potassium application rate and time on the uptake and utilization of nitrogen and potassium by winter wheat. Ying Yong Sheng tai xue bao= The J App Eco 2007; 1;18(1):69-74.

[15] Chandio AA, Jiang Y, Joyo MA, Rehman A. Impact of area under cultivation, water availability, credit disbursement, and fertilizer off-take on wheat production in Pakistan. Journal of Applied Environ Bio Sci 2016; 6(10):10-8.

[16] Ryan J, Estefan G, Rashid A. Soil and plant analysis laboratory manual. ICARDA; 2001.

[17] Mathukia RK, Kapadiya JK, Panara DM. Scheduling of nitrogen and potash application in irrigated wheat (Triticum aestivum L.). J Wheat Res 2014;2:171-2.

[18] Akhter S, Kotru R, Lone BA, Jan R. Effect of split application of potassium and nitrogen on wheat (Triticum aestivum) growth and yield under temperate Kashmir. Indian J Agron 2017; 62(1):49-53.

[19] Kumar P, Pandey SK, Singh BP, Singh SV, Kumar D. Influence of source and time of potassium application on potato growth, yield, economics and crisp quality. Potato Res 2007; 50(1):1-3.

[20] Marschner H. Mineral Nutrition of Higher Plants Second Edition Academic Press Edition London.

[21] Khan AA, TARIQ JAN MU. Impact of various nitrogen and potassium levels and application methods on grain yield and yield attributes of wheat. Sarhad J Agri 2014; 30(1):35-46.

[22] Zhao X, Gao S, Lu D, Chen X, Yuan G, Wang H. Grain yield and soil potassium fertility changes arising from different potassium-bearing materials in rice–wheat rotation. Nutr Cycl Agroeco 2022;124(1):117-29.

[23] Corrêa CV, Gouveia AM, Lanna ND, Tavares AE, Mendonça VZ, Campos FG, Silva JO, Cardoso AI, Evangelista RM. The split application of potassium influence the production, nutrients extraction, and quality of sweet potatoes. J Plant Nutri 2018; 2;41(16):2048-56.

[24] Kang W, Fan M, Ma Z, Shi X, Zheng H. Luxury absorption of potassium by potato plants. American Journal of Potato Research. 2014 Oct;91(5):573-8.

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology C- Life Sciences and Biotechnology

2024, 13(1), pp. 08-17, DOI: 10.18036/estubtdc.1265111

RESEARCH ARTICLE

EXOPOLYSACCHARIDE (EPS) ISOLATED FROM *ENTEROCOCCUS FAECIUM* D36 SHOWS ANTI-CANCER AND ANTI-INVASIVE ACTIVITY POTENTIAL VIA DOWN-REGULATION OF MUC5AC GENE ON HUMAN COLORECTAL ADENOCARCINOMA (CACO-2) CELLS

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ABSTRACT

To investigate the antiproliferative properties of exopolysaccharides (EPS) on human colorectal adenocarcinoma cell line (Caco-2) and the regulation of MUC5AC gene expression, the antiproliferative effect of EPS isolated from D36 strain was determined by MTT test and the regulation of MUC5AC gene expression was examined using Real-Time PCR. *Enterococcus faecium* D36 (*E. faecium* D36) were characterized by Ribotyping analysis. Some biochemical methodologies were preliminarily used to characterize the probiotic potential of *E. faecium* D36, including morphological, cultural, and physiological characteristics. EPS isolated from *E. faecium* D36 strain has an antiproliferative effect on Caco-2 cell line, and mucin gene (MUC5AC) expression levels decreased. These results suggest that EPS isolated from *E. faecium* D36 strain might be a source for a novel anticancer agent. Based on our results, it is believed that EPS obtained from *E. faecium* D36 can be used as a protective and therapeutic substance during the early stages of cancer, especially colon cancer. EPS affects colon cancer by reducing the invasion ability of cancer by decreasing MUC5AC expression. These findings are thought to shed light on future *in vivo* studies.

Keywords: Colorectal cancer, Lactic acid bacteria, *Enterococcus faecium*, Exopolysaccharide, MUC5AC

1. INTRODUCTION

Probiotics are described as beneficial microorganisms. Lactic acid bacteria (LAB) are one of these. LABs benefit human health and have become necessary due to their efficiency in secreting exopolysaccharides (EPS). EPS are long-chain and water-soluble polysaccharides that consist of branched and repeating units of sugars or sugar derivatives [1,2]. EPSs obtained from LAB have antitumor, antioxidant, antibiofilm, immune system support, and cholesterol-reducing properties [3,4]. Probiotics also exhibit anticancer activities against different carcinoma cells, such as the colon, bladder, and breast. Most studies have focused on the anticancer effect of probiotics on colon carcinoma cells [5]. Because of these probiotics characteristics, the food industry has expressed interest in them and their daily growth rate. Of the many probiotic microorganisms available, LABs are widely known and used.

Cancer is one of the leading causes of death. Colorectal cancer is one of the most seen types, and overall morbidity is high worldwide due to its high metastatic capacity. Humans consume a diet rich in proteins and carbohydrates but lack fibre, which results in various gastrointestinal problems, diseases, and cancer, especially colorectal cancer, related to these problems [6]. The high incidence of such problems

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Received: 14.03.2023 Published: 30.01.2024

leads humans to search for natural ways to overcome them. Foods containing probiotics are one of these ways [7].

The members of the genus Enterococcus are among the LABs found in many food products, the human gut, and the urogenital tract [8]. Several genes of Enterococcus are also used as probiotics in some countries [9,10,11]. Previous studies have shown the anticancer effects of LABs [12]. LAB strains produce microbicidal substances that act against gastric and bowel pathogens and other microbes or compete with them to bind to the cell surface and mucin-binding domains [13]. Changes have been shown in the location of mucin expression and expression levels, especially in cancer cells, as well as in the glycosylation of mucin glycoprotein. Some cancer cells developed invasion abilities because of these changes. For example, changing mucin expression and glycosylation changes in colon cancer cells increased the invasion ability of the cells. As a result, they gained the ability to metastasis [14,15,16]. Serine threonine O-glycosylation sites are abundant in glycoproteins bound to the cell surface or that secrete mucin. Mucins, highly glycosylated proteins, are the significant components of mucus. MUC5AC, MUC5B, and MUC8 are representative secretory mucins. However, O-glycans are widely found around cells that have been depolarized. This results in the loss of regular cell organization [17, 18, 19]. MUC5AC, a member of the group of mucins including MUC2, MUC5B, and MUC6, is located within the 11p15 chromosomal locus [20]. MUC5AC is not synthesized in normal colon epithelia but is *de novo* by colonic cancer cells [21, 22]. Pothuraju et al. (2020) reported that Overexpression of MUC5AC is observed in CRC (Colorectal cancer) patient tissues and cell lines. MUC5AC expression enhanced cell invasion and migration and decreased apoptosis of CRC cells [23]. This study investigated the effects of EPS isolated from LABs on cell proliferation of Caco-2 cell line and regulation of MUC5AC gene expression.

2. MATERIAL AND METHODS

2.1. Bacterial Culture and Identification

Enterococcus faecium was isolated from Turkish milk samples cultured on de Man, Rogosa, and Sharpe (MRS) Agar (Sigma-Aldrich, Steinheim, Germany), as well as on M-17 Agar (Sigma-Aldrich, Steinheim, Germany) plates for isolation of LAB. The plates were anaerobically incubated at 37 °C for 48 h, and isolates were examined by microscope. Cell morphology and Gram-staining reactions were determined. Next, isolates were tested for oxidase and catalase activities. Then, sugar fermentation patterns of the isolates were determined using API 20 STREP organisms according to manufacturer guidelines (BioMerieux, France). Growth at different temperatures (4, 15, and 45 °C) and pH values (3.9 and 9.6) was observed. Ammonia production from arginine was then studied. Next, growth was examined at different NaCl concentrations (6, 7.5 & 10%) [24, 25]. Finally, isolates were identified using an EcoRI automated RiboPrinter® microbial characterisation system according to manufacturer instructions. Cultures were maintained at -80 C in 20% glycerol.

2.2. Production of EPS

MRS Agar was inoculated into the broth and incubated for 24-48 hours under optimal conditions. At the end of the incubation period, the bacteria were centrifuged at 6000 rpm for 20 min at +4 °C. The supernatant that formed after was then transferred to another tube. Twenty % trichloroacetic acid was added to the tube and left overnight at +4 °C. Next, the samples were centrifuged at 10.000 rpm for 30 min at +4 °C. Then, the resultant supernatant was transferred to another tube, and an equal volume of chilled ethanol was added to the tube and left overnight at -20 °C. Finally, samples were centrifuged at 10.000 rpm for 30 min at +4 °C. The resultant supernatant was discarded. The hot distilled water was poured onto the pellet that had formed at the bottom of the tube, after which the pellet dissolved. The ensuing EPS solution was used.

2.3. Analysis of Partially Purified EPS Contents

Analyse the calibration standards, calibration verification standard CVS, and samples by HPLC using a Biorad Aminex HPX-87H column. 0.005 M sulfuric acid was used as mobile phase, which is 0.2 µm filtered and degassed. Flow rate and column temperature were adjusted to 0.6 mL/minute and 60 °C, respectively. Refractive Index Detector (RID) was used [26]. After pH adjustment, the sample was filtered by a 0,45µm syringe filter. Contents of the samples were calculated from areas of the obtained peaks.

2.4. Cells and Cell Culture

Caco-2 cell lines (HÜKÜK No: 98052301) were obtained from Şap Enstitüsü (Ankara, Turkey). DAPI staining was performed to check mycoplasma presence or not. Cells were cultured in minimum essential medium (MEM) (Sigma-Aldrich, Steinheim, Germany) containing 10% fetal bovine serum (Sigma-Aldrich, Steinheim, Germany), 1% nonessential amino acids (Sigma-Aldrich, Steinheim, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich, Steinheim, Germany). Cells were maintained at 37 °C in a humidified incubator (Thermo Scientific Heracell, USA) containing 5% CO_2.

2.5. Cell viability assay

Two strategies were used to investigate EPS's effect on cell viability and protection.
Strategy 1: The aim was to study EPS's protective effect during early diagnosis. An experiment was designed that reduced the cell number to $5x10^3$, and the concentration-dependent effects of EPS at 24 and 48 h were subsequently analysed.
Strategy 2: Colon cancer cells form a tumour density by proliferation. To obtain an *in vitro* model, 20.000 and 30.000 cells were seeded into each well of a 96-well plate. A 24-h experiment was designed, and the appropriate concentration of EPS was studied.

The effect of EPS on cell viability was studied using the MTT protocol, an assay based on the reduction of the yellow dye, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide, to purple formazan crystals by mitochondrial dehydrogenase activity [27]. Briefly, as part of the continuous treatment procedure, 5000-20.000-30.000 cells (Caco-2) were seeded in each well a 96-well microplate at a final volume of 100 µL. After 48 h of seeding, cells were treated with 4-8-10-12.5-15-18-20 (mg/mL) EPS for 24 and 48 hours. Eight replicate wells per concentration were used, and the experiments were repeated in triplicate at different intervals. Untreated medium controls (blank) and solvent controls (ultra-pure water) were also assayed in parallel. After treatment with various concentrations of test samples for 24 and 48 h, the liquid media containing the relevant samples from each well was replaced with 100 µL fresh medium containing 0.5 mg/mL MTT dissolved in phosphate buffer saline (PBS). Samples were added to culture wells and incubated for 2 h at 37 °C. The supernatant solution was removed, 100 µL/well DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Steinheim, Germany) was added, and samples were shaken for 5 min. Absorbance was measured at 570 nm with a Bio-Tek ELx808 microplate reader (http://www.biotek.com/).

2.6. mRNA isolation and Real-Time PCR

Total RNA was isolated using a RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Total RNA quantity was measured by NanoVue™ Plus Spectrophotometer (GE Healthcare, Buckinghamshire, UK). For quantitative RT-PCR, mRNA was converted to cDNA using a QuantiTect Reverse Transcription cDNA kit (QIAGEN, Valencia, CA, USA) and amplified on a Palm Cycler using a Taqman® Universal PCR Master Mix (QIAGEN) following manufacturer instructions.

DD-Hu-600 MUC5AC gene (Primerdesign, Ltd, Southampton, UK) primer and double-dye assay (Taqman-style) was used as target gene. HK-DD-Hu-18S rRNA (Primerdesign, Ltd, Southampton, UK) primer and double-dye assay were used as a housekeeping gene, and fluorescent signals generated during PCR amplifications were monitored by a QIAGEN Corbett Rotor-Gene® PCR (Corbett Life Science, USA). To determine the efficiency of each Taqman-style gene expression assay, standard curves were generated by dilution of cDNA, and quantitative evaluations of target and housekeeping gene levels were analysed by Rotor-Gene 6000 software.

Statistical analysis

The SPSS® Statistical Package for Social Sciences software was used to analyse all data. Data were evaluated using one-way ANOVA (Analysis of Variance) followed by Tukey's test. A value of $p < 0.05$ was considered significant. All data were expressed as means and standard deviations of triplicate measures determined in 3 independent experiments.

3. RESULTS

3.1. Properties of EPS

The results of different temperatures and salt concentrations on cell morphology are shown in Table 1. The isolates were Gram-positive as well as catalase- and oxidase-negative cocci. Sugar fermentation of the strains was performed using the API ID 32 STREP system. According to phenotypic tests, the strains were identified as E. faecium. Similarly, the automated EcoRI ribotyping results (Fig. 1) showed that phenotypic characterisation was confirmed for E. faecium. Partially purified EPS contents were analysed by HPLC (Table 2).

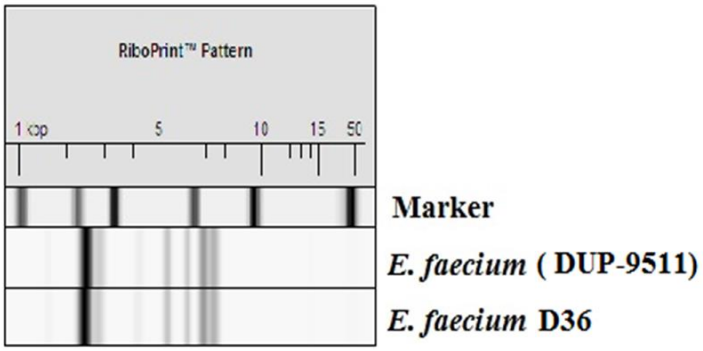

Figure. 1. Ribotyping profiles of *E. faecium* D36 and standards DUP-9511

Table 1 Morphological, cultural and physiological characteristics of the *E. faecium* D36

Gram reaction	Morphology	Catalase	Oxidase	+4 °C	20°C	45°C	%6 NaCl	%7.5 NaCl
+	coc	-	-	+	+	+	+	+
%10 NaCl	NH_3 - Arginine	pH 3.9	pH 9.6	Glucose	Lactose	Sucrose	Fructose	
-	+	+	+	+	+++	+	+	

Table 2 EPS Contents

Glucose (mg/mL)	Xylose (mg/mL)	Ethanol (mg/mL)
0.276	0.051	9.121

3.2. MTT Assay

According to MTT results, there was a decrease in cell viability of Caco-2 cells after EPS application in a concentration and time-dependent manner.

Strategy 1: The EPS's protective effect for early diagnosis was studied. An experiment was designed to reduce cell number to 5000. The concentration-dependent effect of EPS at 24 and 48 h were studied. It was shown that EPS isolated from *Enterococcus faecium* D36 strain decreased cancer cells by 25% at 24 h at a 20 mg/mL concentration and by 35% at 48 h at a 12.5 mg/mL concentration (Figure 2).

Strategy 2: EPS concentrations were applied to different cell numbers. An *in vitro* density forming model was required. To create this *in vitro* model, 20.000 and 30.000 cells were seeded into each well of a 96-well plate, and a 24h experiment was designed. Then 4-8-10 mg/mL EPS concentrations were explored. There was no effect of 4-8-10 mg/mL EPS concentrations on Caco-2 cells, but increased cell viability (Figure 3).

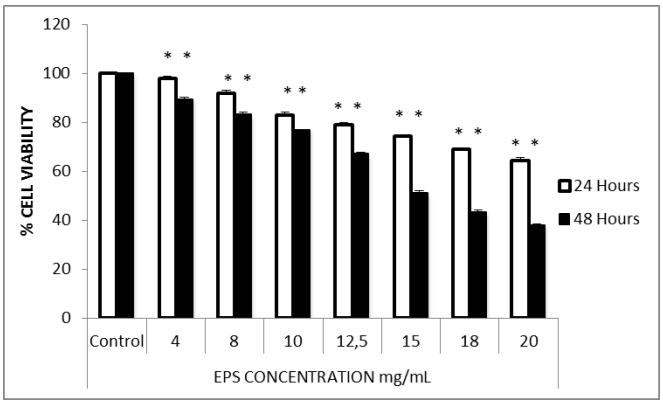

Figure 2. Cytotoxic activity of EPS obtained from *Enterococus faecium* D-36 strain isolated from milk on Caco-2 cell line as determined in the MTT assay. Concentration-response graph of MTT assay of the antiproliferative effect of EPS after 24 and 48 hours of treatment. Results are expressed as mean SD. *indicates a significant difference compared to the control group by the Tukey test ($p < 0.05$)

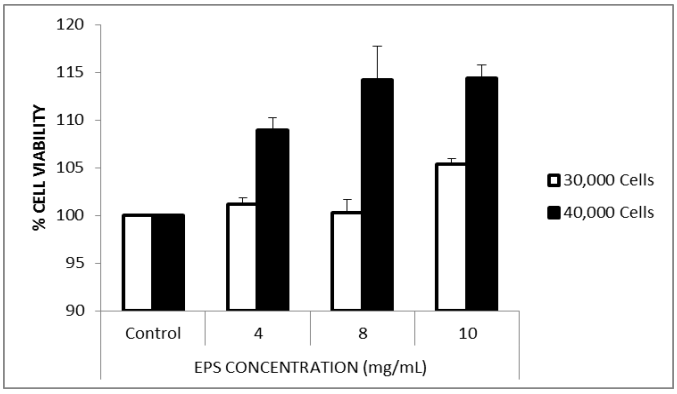

Figure 3 Cytotoxic activity of EPS obtained from *Enterococus faecium* D-36 strain isolated from milk on Caco-2 cell line as determined in the MTT assay. Concentration-response curves of the anti-proliferative effect of EPS for MTT assays performed after 24 h treatment. The results are expressed as the mean_SD.

3.3. Gene Expression

For Real-Time PCR, 220.000 cells were seeded in a T25 flask. EPS obtained from *Enterococcus faecium* D36 strain isolated from milk was applied to Caco-2 cells at dosages of 15, 18, and 20 mg/mL, respectively. At the end of 24 h, exposure and its effect on the MUC5AC gene expression increased on Caco-2 cells. Expression levels was evaluated by Real-Time PCR. Compared to the control group, there was a concentration-dependent decrease in MUC5AC gene expression levels (Figure 4).

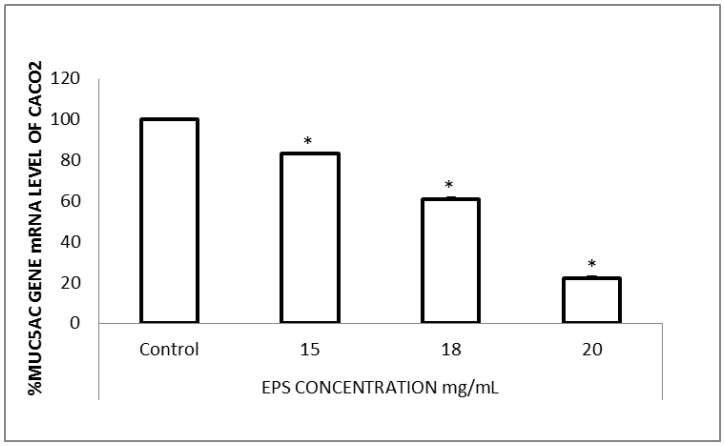

Figure 4 Effect of EPS obtained from *Enterococus faecium* D-36 strain isolated from milk regulation of MUC5AC gene expression level on Caco-2 cell line as determined by Real Time PCR. Concentration-response curves of MUC5AC gene expression level of EPS for Real Time PCR performed after 24 h treatment. Results are expressed as mean SD. *indicates a significant difference compared to the control group by the Tukey test ($p < 0.05$)

4. DISCUSSION and CONCLUSION

EPSs are used in the food industry as stabilisers and anticoagulants. Also, medicinal biochemistry has shown that they have tumorigenesis inhibition properties, support the immune system, macrophage and lymphocyte activation, and lower cholesterol levels [19, 28, 29]. Enterococcus species are Gram-positive bacteria [30]. These bacteria have also been utilised as probiotics to improve the intestine's microbial balance and treat gastroenteritis in humans and animals [30, 31]. Name et al. (2014) showed that secreted metabolites of Enterococcus faecalis strain showed high anticancer activity against the cancer cell lines HeLa, AGS, MCF-7 and HT-29.

In this study, EPS isolated from *Enterococcus faecium* D36 strain isolated from milk which affected cell proliferation of Caco-2 cell line, a human colon cancer cell line and investigated expression levels of the MUC5AC gene that expresses mucin glycoprotein in Caco-2 cell line.

Two different approaches were used to study the effect of EPS's isolated from *Enterococcus faecium* D36 strain isolated from milk on cell viability of Caco-2 cell line. First, 5000 cells were used and exposed to EPS for 24 and 48 h. In the second approach, cells were exposed to EPS for 24 h using a density of 20.000-30.000 cells. Strategy 1: According to the results obtained, a decrease in cell viability was observed after 24 and 48 hours. Cell viability was particularly evident in the results of the 48-h experiment. While there was a decrease depending on the concentration, a 60% decrease was observed at the highest concentration.

Strategy 2: It was found that the changing concentration did not cause a decrease in cell viability, but an increase in cell viability was observed in cells exposed to EPSs for 24 hours.

In colon cancer, changes occur in mucin glycoprotein expression and glycosylation, similarly to many other proteins. This change in mucin glycosylation enables cells to develop invasive properties, which means cells move toward blood vessels and become metastatic [14, 32, 33]. As mucin gene expression levels increase, mucin protein increases and continue with faulty glycosylation [32]. An effective concentration of EPS obtained from *Enterococcus faecium* D36 isolated from Milk was determined by MTT analysis. Following this, the MUC5AC gene responsible for mucin expression in Caco-2 cell line was detected using Real-Time PCR.

This experiment has shown that MUC5AC gene expression was decreased compared to the control group in a concentration-dependent manner. EPS from *Enterococcus faecium* D36 reduced MUC5AC gene expression levels in a concentration-dependent manner. This observation suggests that this substance has the effect of decreasing mucin expression in colon cancer cells and that it can reduce invasion ability and metastatic ability.

Probiotics protect the gut against the formation of precancerous lesions by suppressing the activity of carcinogen enzymes such as azoreductase [34, 35]. It has been observed that EPS obtained from *Enterococcus faecium* D36 was not effective in high cell numbers but low cell numbers. This reminds us of the importance of early diagnosis since colon cancer can form a tumoral mass from a single cell [36, 37]. Based on these results, it is believed that EPS obtained from *Enterococcus faecium* D36 can be used as a protective and therapeutic substance during the early stages of cancer, especially colon cancer.

Cancer patients have a high demand for a diet containing natural products. Although probiotic foods are among these natural products, the microorganism content is a disadvantage. Since chemotherapy drugs and radiotherapies are taken during cancer treatment weaken the immune system, foods containing probiotic microorganisms cause a danger to cancer patients. It has been suggested that if the EPS (D36) obtained can be added to foods as a pure additive (eg EPS containing yogurt) and made into food, it can be used as a food supplement in patients receiving chemotherapy and radiotherapy who consume these foods, and can play a protective role in healthy individuals.

Finally, our findings showed that EPS (D36) might have protective and possible therapeutic effects on early-stage colon cancer and on reducing the invasion ability of cancer by decreasing mucin expression. However, further *in vivo* studies are required to support these findings

ACKNOWLEDGEMENTS

This study was supported by Anadolu University Scientific Research Projects Commission under grant no: 1201F014

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

AUTHORSHIP CONTRIBUTIONS

Burcugül Altuğ-Tasa: Literature search, Writing – Original draft preparation, Analysis, Investigation, Visualization, Conceptualization. **Merih Kıvanç:** Supervision, Review and editing, **Ayşe Tansu Koparal;** Supervision**,** Review and editing.

REFERENCES

[1] Welman AD. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 2003; Jun 269-274.

[2] Li L, Jiang YJ, Yang XY, Liu Y, Wang JY, Man CX. Immunoregulatory effects on caco-2 cells and mice of exopolysaccharides isolated from Lactobacillus acidophilus NCFM. Food Funct. 2014;5, 3261 -3268.

[3] Lollo PCB, Moura S, Morato PN, Cruz AG, Castro FW, Betim CB, Nisishima L, Faria JAF, Junior MM, Fernandes CO, Amaya-Farfan J. Probiotic yogurt offers higher immune protection than probiotic whey beverage. Food Res. Int. 2013;54, 118–124.

[4] Wang K, Li W, Rui X, Chen X, Jiang M., & Dong M. Structural characterization and bioactivity of released exopolysaccharides from Lactobacillus plantarum 70810. Int. J. Biol. Macromol. 2014; 67, 71-78.

[5] Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, Klinder A, O'Riordan M. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr. 2007;85, 488-496.

[6] McIntosh GH, Le Leu RK. The influence of dietary proteins on colon cancer risk. Nutr. Res. 2001;21, 1053–1066.

[7] Rafter J. Probiotics and colon cancer. Best Pract. Res. Clin. Gastroenterol. 2003; Vol. 17, No. 5, pp. 849–859.

[8] Nami Y, Abdullah N, Haghsheneas B, Radiah D, Rosli R, Yari Khosroushahi A. A newly isolated probiotic Enterococcus faecalis strain from vagina microbiota enhances apoptosis of human cancer cells. J. Appl. Microbiol. 2014;1364-5072

[9] Devriese LA, Van de Kerckhove A, Kilpper-balz IR, Schleifer KH. Schleifer characterization and identification of enterococcus species isolated from the intestines of animals. Int. J. Syst. Bacteriol. 1987;37,257-259.

[10] Franz CMAP, Stiles ME, Schleifer KH, Holzapfel WH. Enterocci in foods-a connundrum for food safety. Int. J. Food. Microbiol. 2003;88, 105-122.

[11] Foulquie Moreno MR., Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of enterococci in food and health. Int J. Food Microbiol. 2006;88, 105-122

[12] Rafter J. Lactic acid bacteria and cancer: mechanistic perspective. Br. J. Nutr. 2002;88, 89-94.

[13] Ljungh A & Wadström T. Lactic acid bacteria as probiotics. Curr Issues Intest Microbiol. 2006;7(2) 73-90.

[14] Varki A, Kannagi R, Toole BP. Glycosylation Changes in Cancer, Essentials of Glycobiology, Chapter 44, 2nd edition, 2009; Cold Spring Harbor Press.

[15] Stowell SR, Ju T, Cummings RD. Protein glycosylation in cancer. Annu Rev Pathol. 2015;10:473–510

[16] Venkitachalam S & Guda K. Altered glycosyltransferases in colorectal cancer. Expert Rev Gastroenterol Hepatol. 2017;11:1, 5-7, DOI: 10.1080/17474124.2017.1253474

[17] Suzaki I, Kawano S, Komiya K, Tanabe T, Akaba T, Asano K, ... & Rubin BK. Inhibition of IL-13-induced periostin in airway epithelium attenuates cellular protein expression of MUC5AC. Respirology. 2017; 22(1), 93-100.

[18] Bae CH, Jeon BS, Choi YS, Song SY, Kim YD. Delphinidin Inhibits LPS-Induced MUC8 and MUC5B Expression Through Toll-like Receptor 4-Mediated ERK1/2 and p38 MAPK in Human Airway Epithelial Cells. Clin. Exp. Otorhinolaryngol. 2014; Vol. 7, No. 3: 198-204

[19] Chugh S, Gnanapragassam VS, Jain M, Rachagani S, Ponnusamy MP, Batra SK. Pathobiological implications of mucin glycans in cancer: Sweet poison and novel targets. Biochimica et Biophysica Acta. 1856, 2015; 211–225

[20] Ringel J & Löhr M. The MUC gene family: Their role in diagnosis and early detection of pancreatic cancer. Mol cancer. 2003;2(1), 1-5.

[21] Bu XD, Li N, Tian XO, Huang PL. Caco-2 and LS174T cell lines provide different models for studying mucin expression in colon cancer. Tissue and Cell. 2011;43, 201–206.

[22] Porter GA, Urquhart R, Bu J, Johnson P, Grunfeld E. The impact of audit and feedback on nodal harvest in colorectal cancer. BMC Cancer. 2011; Jan3;11:2 doi: 10.1186/1471-2407-11-2.

[23] Pothuraju R, Rachagani S, Krishnan SR, Chaudhary S, Nimmakayala RK, Siddiqui JA, & Batra SK. Molecular implications of MUC5AC-CD44 axis in colorectal cancer progression and chemoresistance. Mol. Cancer. 2020; 19(1), 1-14.

[24] Schillinger U & Lücke FK. Antibacterial activity of Lactobacillus sake isolated from meat Appl. Environ. Microbiol. 1989;55:1901-1906.

[25] Stiles ME, & Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol. 1997;Apr 29;36(1):1-29.

[26] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J & Templeton D. Determination of sugars, by products, and degradation products in liquid fraction process samples. NREL, Golden, CO. 2006.

[27] Mossman T. Rapid Colorimetric Assay For Cellular Growth And Survival: Application To Proliferation And Cytotoxicity Assays. J. Immunol. Methods. 1983; 65:55–63.

[28] Meulen RV, Grosu-Tudor S, Mozzi F, Vaningelgem F, Zamfir M., Valdez GF, Vuyst L. Screening of Lactic Acid Isolates From Dairy And Cereal Products For Exopolysaccharide Production And Genes Involved. Int. J. Food Microbiol. 2007;118, 250–258.

[29] Lin TY & Chien MF. Exopolysaccharides Production as Affected by Lactic Acid Bacteria and Fermentation Time. Food Chem. 2007;100, 1419-1423.

[30] Banwo K, Sanni A, Tan H. Technological Properties and Probiotics Potential of *Enterococcus faecium* Strains Isolated from Cow Milk. J. Appl. Microbiol. 2012;114, 229-241

[31] Kayser FH. Safety Aspects of Enterococci From the Medical Point of View. Int J Food Microbiol. 2003;88, 255–262.

[32] Li M, Song L, Qin X. Glycan Changes: Cancer Metastasis and Anticancer Vaccines. J. Biosci. 2010;35665–673.

[33] Kufe DW. Mucins in Cancer: Function, Prognosis and Therapy. Nat. Rev. Cancer. 2009; Dec;9(12):874-85.

[34] Dallal MM.S, Mojarrad M, Baghbani F, Raoofin R, Mardaneh J, Salehipour Z. Effetcs of Probiotic Lactobacillus acidophilus and Lactobacillus casei on Colorectal Tumor Cells Activity (Caco-2). Arch. Iran. Med. 2015;18(3), 167-172

[35] Burns AJ & Rowland IR. Anti-Carcinogenicity of Probiotics and Prebiotics. Curr Issues Intest Microbiol. 2000;1(1). 13-24.

[36] O'Connell JB, Maggard MA, Ko CY. Colon Cancer Survival Rates with the New American Joint Committee on Cancer, Sixth Edition Staging. J. Natl. Cancer. Inst. 2004; Vol. 96, No. 19.

[37] Roig AI, Wright WE, Shay JW. Is Telomerase a Novel Target for Metastatic Colon Cancer?. Curr. Colorectal Cancer Rep. 2009;5, 203–208

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology C- Life Sciences and Biotechnology

2024, 13(1), pp. 18-22, DOI: 10.18036/estubtdc.1282765

RESEARCH ARTICLE

THE MIXTURE OF CARVACROL AND ESSENTIAL OILS FOR ROOM AIR DISINFECTION

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ABSTRACT

The purpose of this study is to explore the effect of essential oils and carvacrol combination for room air disinfection. Combination of essential oils and carvacrol was homogeneously sprayed to a room containing 44.3 m3 of air. Petri dishes containing Nutrient Agar medium were opened for 60 min in ambient air for sampling from ambient air before and after disinfection at the same four different regions of the room. The incubation was performed at 25 °C for 10 days. After incubation period, the colonies were counted. Microbial load was decreased by essential oils and carvacrol combination approximately 50% for the room air. Essential oils and carvacrol combination may be beneficial in decreasing the microbial load of ambient air. This combination should be a green alternative to chemical disinfectants.

Keywords: Essential oils, Carvacrol, Air Borne Pathogens, Disinfection

1. INTRODUCTION

Ambient air can be composed of large numbers of microbial agents which is called bioaerosols. The risks of epidemics and pandemics increase with airborne transmission of respiratory illness dramatically. Hence, the microbial quality of air is essential [1].

Sick people, an infected/polluted HVAC system, an infected building materials or a terrorist attack may be sources of the floating pathogens in air. There are techniques to keep airborne pathogens away from resident in buildings, or levels low enough not to cause a disease. One of these techniques is using essential oils. Essential oils are used in cosmetics, food, pharmaceutical and beverage industries have strong antimicrobial properties, and they could be applied in the airing industry [2].

Plants have been used in traditional medicine for centuries [3]. Essential oils are the volatile and aromatic products [4] extracted from different plant parts like flowers, herbs, roots, seeds, buds, leaves, twigs, bark, wood, and fruits [5]. Essential oils are the volatile constituent of plants. They usually extracted by steam distillation using a Clevenger type apparatus [6]. Plant essential oils are composed of polar and non-polar natural compounds mixtures. They are well-known for their medicinal feature such as spasmolytic, antiseptic, analgesic, local anesthetic, sedative, anti-inflammatory, anti-carcinogenic [7]. Therefore, the composition and antimicrobial activities of essential oils have been thoroughly and systematically examined in the literature [8].

Plant essential oils have been used for different aims for many years, especially scientific and commercially in many fields. These usages include cosmetics, sanitary, agricultural, pharmaceutical industry, food industry, aromatherapy and phytotherapy [9,10]. In addition to their usage, they can be used as a natural control source like insecticide, fungicide, herbicide and nematocide. It has recently found that they can be used in animal production, poultry, and beekeeping [11].

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Received: 04.05.2023 Published: 30.01.2024

The purpose of this study is to explore the effect of essential oils and carvacrol combination on decreasing microbial load of room ambient air.

2. MATERIALS and METHODS

2.1. Materials

The mixture (combination of essential oils and carvacrol) was purchased from DVL İlaç Sağlık Hiz. ve Gıda Takviyeleri San. ve Dış Tic. Ltd. Şti. The mixture contains oregano oil (*Thymus* sp), carvacrol, aromatic essentials oils of mint (*Mentha piperita*), eucalyptus (*Eucalyptus* sp) lavandula (*Lavandula angustifolia* Miller), clove (*Eugenia caryophyllata*), rosmarinus (*Rosmarinus officinalis*). Nutrient Agar was purchased from Merck.

2.2. Experimental Studies

Combination of essential oils and carvacrol was diluted by distilled water with 1:20 ratio. 20 mL of prepared solution was homogeneously sprayed to a room containing 44.3 m^3 of air to determine the disinfection effect of essential oils and carvacrol combination on ambient air. The room, where the experiments were done, is without air conditioning, without sunlight, at 22 °C and containing 44.3 m^3 of air. After 5 min, petri dishes containing Nutrient Agar medium were opened for 60 min in ambient air at room temperature for the plate method. These four petri dishes were placed at the same four different regions in the room. Then, they were incubated at 25 °C for 10 days. After the incubation period, the colonies were counted. Experimental study was carried out 2 times at 15 days apart.

Spraying of the prepared solution and opening closing of petri dishes procedures were performed by same person wearing facial mask. During the spraying and opening-closing procedures, nobody was not in the room except performer.

As a control, petri dishes containing Nutrient Agar medium were opened for 60 min in ambient air for sampling from ambient air before disinfection at room temperature. This procedure was applied at four different regions in the room. Then, they were incubated at 25 °C for 10 days. After the incubation period, the colonies were counted.

2.3. Statistical Analysis

In statistical analysis, two-way repeated measures ANOVA and non-parametric Mann-Whitney U test was found out as mean ± standard deviation for different comparisons using SPSS version 3.6. software ($p<0.05$).

3. RESULTS and DISCUSSION

When all the literature up to now is reviewed, there have been no studies that show the disinfection effects of combination of essential oils and carvacrol on ambient air except this study. Therefore, this study is the first study investigating room air disinfection by combination of essential oils and carvacrol (oregano oil, carvacrol, aromatic essentials oils of mint, eucalyptus, lavandula, clove, rosmarinus).

This experiment was performed two times. The results of first experiment were given in Table 1. The number of colonies were decreased considerably after disinfection for all regions in the first experiment. The total number of colonies for four regions before disinfection was 71. After disinfection, the total

number of colonies for four regions was 30. The number of microorganisms was decreased approximately 58%.

Table 1. The number of colonies before and after disinfection for the first experiment.

Regions that Nutrient Agar Medium Placed	Number of Colonies Before Disinfection (Control)	Number of Colonies After Disinfection
1.region	22	7
2.region	13	7
3.region	20	7
4.region	16	9

The results of second experiment were given in Table 2. The number of colonies were decreased considerably after disinfection for all regions in the second experiment. The number of colonies for four regions before disinfection was 96. After disinfection, the number of colonies for four regions was 53. The number of microorganisms was decreased approximately 45%.

Table 2. The number of colonies before and after disinfection for the second experiment.

Regions that Nutrient Agar Medium Placed	Number of Colonies Before Disinfection (Control)	Number of Colonies After Disinfection
1.region	32	14
2.region	24	14
3.region	18	12
4.region	22	13

The number of colonies were decreased considerably after disinfection as it can be seen in Figure 1.

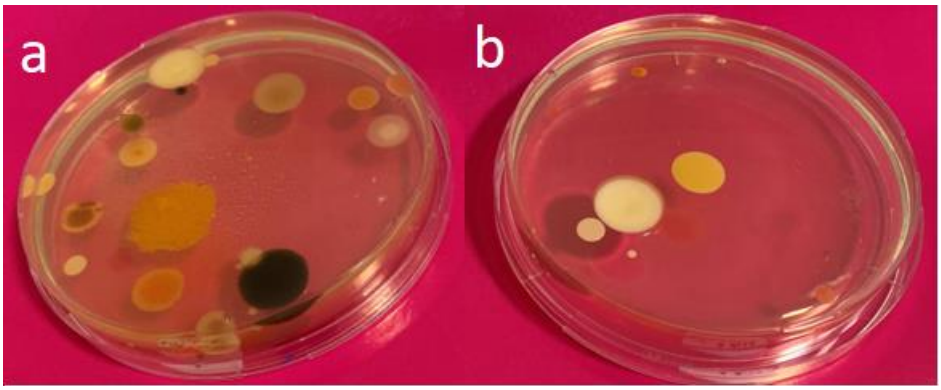

Figure 1. Colonies a) before disinfection, b) after disinfection

Two repeated experiments were analyzed by Mann-Whitney U test. Table 3 was obtained by evaluation of Table 1 and Table 2. It was demonstrated that the reduction in the number of colonies in the first and second regions were statistically significant ($p<0.05$). There were reductions in the third and fourth regions, however these were not significant ($p>0.05$) (Table 3).

Table 3. The statistical results of reduction in the number of colonies before and after disinfection.

Regions that Nutrient Agar Medium Placed	**Number of Colonies Before Disinfection (Control)**	**Number of Colonies After Disinfection**
1.region	27.0±7.1[&]	10.5±4.9[&]
2.region	18.5±7.8[#]	10.5±4.9[#]
3.region	19.0±1.4	9.5±3.5
4.region	19.0±4.2	11.0±2.8

Common disinfectant and cleaning products contain alcohol, chlorine dioxide, organic acids, hydrogen peroxide, iodophor, carboxylic acids, phenolic compounds, ozone, peracetic acid, peroxyacid mixtures, sodium hypochlorite and quaternary ammonium compounds. Some of these agents can lead to the formation of potentially carcinogenic compounds. Unfortunately, potential carcinogenic compounds are still used in laboratory disinfection and/or on food contact surfaces. However antimicrobial products must be efficient against varied pathogens, cannot be toxic to personnel or the environment, must not be potential for antibiotic/biocide cross-resistance, must not interact with matter on surfaces, and/or must not cause harm to the surface. Using essential oils (such as peppermint, oregano**,** cinnamon, and clove) are environmentally friendly and safe for using on various surfaces. Also, the disinfectant properties of natural solutions, which is based essential oil, was mostly equal to potentially carcinogenic compounds or better than them [1].

The microbial load of the room ambient air was decreased by essential oils and carvacrol combination as nearly 50%. So that, this combination can be useful in decreasing the microbial load of air. As a result, it may be a green alternative to chemical disinfectants. Further research is warranted to fulfill the gap in this field to investigate the disinfection effect well.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

AUTHORSHIP CONTRIBUTIONS

Ayşegül HOŞ: Conceptualization, Investigation, Formal analysis, Visualization, Writing-original draft
Ayşe İNCİ: Conceptualization, Investigation, Visualization, Writing-original draft
Ozan Emre EYUPOGLU: Conceptualization, Formal analysis, Writing-original draft
Çağlar MACİT: Conceptualization, Visualization, Writing-original draft
Hasan Hüseyin ÜNAL: Investigation, Visualization, Writing-original draft

REFERENCES

[1] Sengun IY, Senturk S, Gul S, Kilic G. Potential of essential oil combinations for surface and air disinfection. Letters in Applied Microbiology 2020; 72:526-34.

[2] Bolashikov ZD, Melikov AK. Methods for air cleaning and protection of building occupants from airborne pathogens. Building and Environment 2009; 44:1378-85.

[3] Kürekci C, Sakin F. Essential oils: Essential oils as antimicrobials: In-vitro and in-vivo studies. J Anim Nutr&Nutr Dis-Special Topics 2017; 3(1):15-20.

[4] Bajpai VK, Baek K-H. Biological efficacy and application of essential oils in foods-A review. Journal of Essential Oil-Bearing Plants 2016; 19(1):1-19.

[5] Seow YX, Yeo CR, Chung HL, Yuk H-G. Plant essential oils as active antimicrobial agents. Critical Reviews in Food Science and Nutrition 2014; 54:625-44.

[6] Williams LAD, Porter RB, Junor GO. Biological activities of selected essential oils. Natural Product Communications 2007; 2(12):1295-96.

[7] Nazzaro F, Fratianni F, Coppola R, Feo VD. Essential oils and antifungal activity. Pharmaceuticals 2017; 10(86):1-20.

[8] Kalemba D, Kunicka A. Antibacterial and antifungal properties of essential oils. Current Medicinal Chemistry 2003; 10:813-29.

[9] Çelik E, Çelik GY. Antimicrobial properties of plant essential oils. Orlab On-Line Mikrobiyoloji Dergisi 2007; 5(2):1-6.

[10] Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils – A review. Food and Chemical Toxicology 2008; 46:446-75.

[11] Karık Ü, Öztürk M. Current situation of essential oil sector in Turkey, problems and solution recommendations. Alatarım 2010; 9(2):30-37.

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology C- Life Sciences and Biotechnology

2024, 13(1), pp. 23-30, DOI: 10.18036/estubtdc.1289435

RESEARCH ARTICLE

Pistacia terebinthus FRUIT: AN ALTERNATIVE TO PREVENT FOOD SPOILAGE

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ABSTRACT

The aim of this work was to investigate the usage possibilities of *P. terebinthus* fruit, which has a limited usage area, in the food industry. Antimicrobial activity of hexane extract obtained from *P. terebinthus* fruits from Adıyaman (Turkey) was determined by disc diffusion and micro-dilution assays against test microorganisms. In addition, the antibacterial activity of the extract on *Escherichia coli* O157:H7, one of the most important food-borne pathogens, was determined by viable cell count using a macro-dilution assay. The potential for use of the extract with probiotic candidate lactic acid bacteria (LAB) strains was also investigated. The hexane extract presented antimicrobial activity against all the tested microorganisms, with good inhibition zone diameters between 10.51 mm and 18.02 mm. MIC and MFC or MBC values of *P. terebinthus* fruit extract were determined as 5-80 µg/µL against all tested microorganisms. The lowest MIC and MBC values (5 µg/µL) of the extract were obtained against *E. coli* O157:H7. Macro-dilution assay results indicated that the *P. terebinthus* extract at various concentrations (5-10-20 mg/mL) inhibited the growth of *E. coli* O157:H7 more than the control group after all of the incubation hours. No viable *E. coli* O157:H7 cells were detected after 48 hours at all concentrations. The extract showed low antimicrobial activity, and relatively high bactericidal concentration on probiotic candidate LAB strains. This shows that *P. terebinthus* hexane extract at appropriate concentrations can be used together with probiotic strains as a natural preservative and biopreservative to prevent food spoilage and extend shelf life.

Keywords: *Pistacia terebinthus*, Lactic Acid Bacteria, Extract, Antimicrobial, Biopreservative

1. INTRODUCTION

Pistacia terebinthus L., belonging *Anacardiaceae*, is known by different names such as menengiç, bıttım and çedene in some regions of Turkey. *P. terebinthus*, a symbolic plant of the Mediterranean and West Asia, is predominantly found in the southern and western parts of Turkey [1]. *P. terebinthus* is a tree species that grows to a height of 10 meters and grows in the hills and rocks of coastal areas. The fruit, which is pink before ripening, turns green and blue as it matures [2].

P. terebinthus fruit is rich in oil, protein, and dietary fiber and has long been known for its unique taste and aroma properties [2]. The fruits have been the focus of many research due to their anti-inflammatory [3], antimicrobial [4] and antioxidant [5,6] properties. The World Health Organization (WHO) reported in 2018 that 88% of its 194 member states accepted the use of traditional and alternative medicine [7]. In recent years, research has been directed towards finding natural food preservatives due to the potential harms of synthetic preservatives to health [8, 9]. As consumers become more conscious, they are concerned about consuming foods containing these chemical additives. Plants are considered relatively safe for human use and the environment as a source of antimicrobial compounds [10].

Biopreservation refers to the controlled use of microorganisms or their metabolites to prevent microbial spoilage, the growth of pathogenic microorganisms, extend the shelf life of foods, and ensure their microbial safety [11]. The ability of lactic acid bacteria (LAB) to inhibit the growth of pathogenic bacteria and their alternative use in the food industry have been reported in the literature [12, 13]. LAB

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Received: 04.05.2023 Published: 30.01.2024

are commonly used as probiotic cultures in various processes that are generally considered safe and have therapeutic effects on the host [14]. LAB and herbal extracts can be used together as natural preservatives to prevent microbial spoilage of food.

In the study, the alternative potential usage of *P. terebinthus* fruit extract to prevent food spoilage and extend shelf life was investigated. Therefore, the antimicrobial activity of the hexane extract against test microorganisms was tested to determine its potential usage as a natural preservative additive in the food industry. It is also aimed at evaluating the potential usage of *P. terebinthus* fruit hexane extract together with LAB in the food industry as a natural bioadditive.

2. MATERIALS AND METHODS

2.1. Preparation of Extract

P. terebinthus fruits were obtained from a herbalist in Adıyaman (Turkey). After the fruit material was washed, it was air-dried at room condition. The fruits were ground and then the powdered sample was extracted with hexane using the soxhlet system. The solvent was then evaporated from the extract by using a rotary evaporator. After dissolving the hexane fruit extract with dimethyl sulfoxide (DMSO) at the concentration of 100 µg/µl, it was sterilized with a 0.45 µm filter.

2.2. Determination of Antimicrobial Activity

The inhibitory activity of the extract was tested with the disc diffusion method. *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218, *E. coli* O157:H7, *Bacillus cereus* RSKK 863, *Shigella sonnei* Mu:57, *Salmonella enteritidis* ATCC 13076, *Yersinia enterocolitica* ATCC 11175, *Micrococcus luteus* B-4375 and *Staphylococcus aureus* ATCC 25923 were cultured in Nutrient Broth (NB)/Agar. *L. monocytogenes* ATCC 7644 was grown in Tryptic Soy Broth (TSB)/Agar. *Candida glabrata* RSKK 04019 and *C. albicans* ATCC 10231 were cultured in Yeast Extract Peptone Dextrose (YPD)/Agar. De Man, Rogosa and Sharpe (MRS)/Agar was used for *Lactobacillus delbrueckii* MA-9, *Lactobacillus fermentum* MA-8, *L. gasseri* MA-2, *L. gasseri* MA-3, *L. gasseri* MA-4, *L. gasseri* MA-5, *L. fermentum* MA-7, *Lactobacillus vaginalis* MA-10, and *Lactobacillus plantarum* RSKK 1062 as growth medium.

The tested LAB were isolated from human milk in our previous studies and then characterized for probiotic potential [15-17]. *L. plantarum* RSKK 1062 is a commercial LAB strain used as a control.

The test microorganisms (adjusted to 0.5 McFarland, ~$1x10^8$ CFU/mL) were inoculated, and sterile discs (6 mm in diameter) were placed on the agar medium. Then, the extract (20 µL, 2000 µg/disc) was dropped onto the discs and incubated for 24 h at 37°C for bacteria and at 30°C for yeast. After the incubation period, the inhibition zone around the discs was recorded [18]. The solvent (DMSO) of the extract and the antibiotic Gentamicin (CN, 10 µg/disc) were used as negative and positive control groups, respectively. The assays were done in triplicate.

2.3. Micro-Dilution Method

Micro-dilution assay was used to determine the MIC (Minimal Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) or MFC (Minimum Fungicidal Concentration) values of the hexane extract against the tested microorganisms [19]. The microorganisms (0.5 McFarland) were added to a tube containing growth media and extract. After incubation, the concentration of the hexane extract (80, 40, 20, 10, 5 and 2.5 µg/µL) in the liquid medium without growth was recorded as MIC values. The samples from broth media were then inoculated on the agar medium. After the incubation, extract

concentrations in which microbial growth was not observed on solid medium were recorded as MFC or MBC values.

2.4. Macro-Dilution Method

Macro-dilution assay was also performed to determine the antimicrobial activity of *P. terebinthus* fruit extract. The antibacterial activity of the hexane extract on *E. coli* O157:H7 was determined by counting viable cells using the method of Sousa et al. [20] with some modifications. *E. coli* O157:H7 culture (adjusted to 0.5 McFarland) was added into the fruit extract (5-10-20 mg/mL concentrations) and growth medium mixture (total volume 10 mL). The cell suspension without extract was used as a control group. Then, the control group and the bacterial suspensions containing the extract were incubated at 37°C. The samples from the mixture were then diluted and inoculated onto NA medium. After incubation, the viable cells were counted and recorded as $\log_{10}$ CFU/mL.

2.5. Statistical Analysis

Data were analyzed using GNU SPSS version software, and analysis was performed using One Way ANOVA at $p<0.05$. In addition, differences in antimicrobial activity between the tested microorganisms were evaluated using the post-hoc Tukey test. The differences in inhibition zone diameter means were evaluated as statistically significant (*p-value* < 0.05). The analyzed data are reported as the mean and standard deviation (SD) of three replicate results.

3. RESULTS AND DISCUSSION

The antimicrobial activity of the hexane extract from *P. terebinthus* fruit was investigated against test microorganisms. The disc diffusion assay results indicated that the highest two inhibition zone diameters among the test bacteria were 18.02 mm against *L. monocytogenes* ATCC 7644 and 16.38 mm against *P. aueroginosa* ATCC 27853. The highest antifungal activity was obtained against *C. glabrata* RSKK 04019 with an inhibition zone diameter of 17.19 mm. MIC and MFC or MBC values of *P. terebinthus* fruit extract were determined as 5-80 µg/µL against all tested microorganisms (Table 1).

Table 1. Antimicrobial activity of *P. terebinthus* fruit hexane extract

Test Microorganisms	Inhibition Zone Diameters (mm±SD)		MIC (µg/µL)	MBC or MFC (µg/µL)
	Extract	Antibiotic disc CN		
E. coli O157:H7	12.51±0.66[a]	14.07±0.01	5	5
E. coli ATCC 35218	10.52±0.66[c]	10.19±0.02	20	40
L. monocytogenes ATCC 7644	18.02±5.73[b,d]	19.38±0.02	10	10
B. cereus RSKK 863	13.86±0.86	12.97±0.30	40	80
S. sonnei Mu:57	15.39±0.29	11.08±0.80	40	80
S. enteritidis ATCC 13076	13.91±0.63	10.51±0.02	20	40
Y. enterocolitica ATCC 11175	12.76±1.05	19.92±0.01	10	20
M. luteus B-4375	15.55±1.17	10.93±0.01	20	20
P. aueroginosa ATCC 27853	16.38±0.68[e]	16.31±0.02	40	40
S. aureus ATCC 25923	15.78±0.12	13.05±0.02	40	40
E. faecalis ATCC 29212	15.36±0.46	13.48±1.44	40	80
C. albicans ATCC 10231	16.55±1.44[f]	NA	20	20
C. glabrata RSKK 04019	17.19±1.29[f]	NA	40	80

Different successive superscript values in columns differ significantly ($p<0.05$) by one-way ANOVA followed by Tukey's post-hoc test.
NA: No activity

Hacıbekiroğlu et al. [21] investigated the antimicrobial activity of *P. terebinthus* fruit hexane extract (100 mg/mL) using the broth dilution method, however, the hexane extracts did not show any antimicrobial activity against tested microorganisms such as *E. coli*. Durak & Uçak [22] extracted *P. terebinthus* fruit with an acetone-water solvent and investigated its antimicrobial activity using disc diffusion method. The inhibition zone diameters of the extract (0.5 mg/disc) against *S. aureus*, *L. monocytogenes*, *S. typhimurium* and *E. coli* O157:H7 were 9-13 mm, 11.25-14.25 mm, 11.67-15.25 mm and 8.75-11.5 mm, respectively. In current study, the inhibition zone diameters of hexane extract were higher against *L. monocytogenes* ATCC 7644 and *E. coli* O157:H7, and close to the other tested bacteria. In the study of Doğan [23], it was determined that water, ethanol and methanol extracts of *P. terebinthus* fruit did not show antifungal activity on *C. albicans* ATCC 87392 and antibacterial activity against *E. coli* ATCC 1213. The differences among the results may be due to the solvent used in the extraction, the extract concentration differences used, cultivation conditions such as climate and soil characteristics [24].

Today, diseases caused by food-borne pathogens pose a great problem. *E. coli* O157:H7 is a food-borne pathogen frequently associated with disease cases characterized by bloody diarrhea, hemolytic uremic syndrome, and hemorrhagic colitis outbreaks [25]. The hexane extract was used to determine the antimicrobial activity by counting viable cells because it showed the lowest MIC and MBC value (5 µg/µL) on *E. coli* O157:H7 among tested microorganisms. In our study, it was found that the extract of *P. terebinthus* prepared at various concentrations inhibited the growth of *E. coli* O157:H7 more than the control group after all of the incubation hours. As the extract concentration increased, a decrease in the viable cell of *E. coli* O157:H7 was obtained after incubation periods. No viable cells were determined after 48 hours at all tested concentrations (5-10-20 mg/mL) (Figure 1). These results indicate that *P. terebinthus* hexane extract, which has a cidal effect against food-borne *E. coli* O157:H7, may have the potential to prevent food contamination as a natural biopreservative.

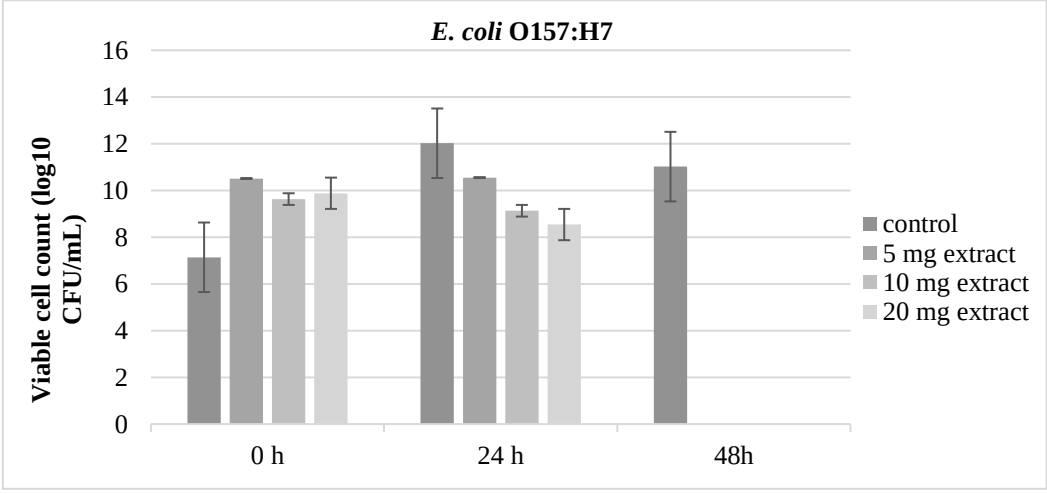

Figure 1. Viable cell counts of *E. coli* 0157:H7

In a study, the antimicrobial activity of *P. terebinthus* seed essential oil (MEO) against *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 11778, and *Staphylococcus aureus* ATCC 29213 was investigated. The inhibition zone diameters of MEO were recorded as 2.0 mm, 3.5 mm, and 3.5 mm, respectively [26]. In a study conducted by Çoban et al. (2017), it was reported that *P. terebinthus* methanol, ethyl acetate, water, pure methanol, pure ethyl acetate, distilled water fruit extracts did not show antimicrobial

activity on *Klebsiella pneumoniae* ATCC 13882, *C. albicans* ATCC 10231 and *C. glabrata*. In our study, *P. terebinthus* fruit hexane extract exhibited antifungal activity with good inhibition zones on *C. albicans* ATCC 10231 and *C. glabrata* RSKK 04019 [27]. In another study, the inhibition zone diameters of *P. terebinthus* fruit methanol extract were determined as 9 mm for *E. coli* ATCC 25922, 13 mm for *S. aureus* ATCC 25923 and 12 mm for *P. aereginosa* ATCC 9027. The inhibition zone diameters obtained as a result of this study were found to be higher for these three bacteria [28]. This difference may be due to the difference in the solvent and method used in extraction.

LAB, the most common probiotic microorganism, is found in human milk, the gastrointestinal tract, or the urogenital tract [29]. The antimicrobial activity of the hexane extract against LAB obtained from breast milk is presented in Table 2. The results indicated that the hexane extract had inhibitory activity on all the tested LAB strains. The data of the disc diffusion test showed the lowest inhibition zone diameters of 10.48 mm on *L. delbrueckii* MA-9. The MIC values of the hexane extract on the tested LAB were determined as 20-40 µg/µL, and the MBC values were determined as 20-80 µg/µL. The extract inhibited all the tested LAB, however, with high MIC and MBC values. Therefore, appropriate concentrations of the hexane extract together with the probiotic candidate LAB strains may be used as natural bioadditives in the food industry.

Table 2. Inhibitory activity of *P. terebinthus* fruit hexane extract on probiotic candidate LAB originated from human milk

Test Microorganisms	**Inhibition Zone Diameter (mm±SD)**	**MIC (µg/µL)**	**MBC (µg/µL)**
L. gasseri MA-2	$11.82\pm0.49^{a,n}$	20	20
L. gasseri MA-3	$10.96\pm0.65^{c,f,h,j,l}$	20	20
L. gasseri MA-4	$12.77\pm0.30^{d,e,n}$	40	40
L. gasseri MA-5	$12.89\pm0.39^{d,g,n,s}$	20	20
L. fermentum MA-7	$12.47\pm0.23^{d,I,n}$	20	40
L. fermentum MA-8	$12.33\pm0.54^{d,k,n}$	40	40
L. delbrueckii MA-9	$10.48\pm0.65^{b,f,h,j,l,m,p}$	20	40
L. vaginalis MA-10	$11.85\pm0.35^{n,o}$	20	20
L. plantarum RSKK 1062	$11.59\pm0.17^{h,r}$	40	80

Different successive superscript values in columns differ significantly ($p<0.05$) by one-way ANOVA followed by Tukey's post-hoc test.

The statistical analysis results indicated a statistically significant variation in inhibition zone diameter averages of the extract for test microorganisms as well as LAB ($p<0.05$). Multiple comparison analysis using by the Tukey test was performed to determine the microbial strains that caused the difference and is presented in Table 1 and Table 2.

4. CONCLUSION

Synthetic additives, the use of which has increased with the development of technology, have many side effects on health. In addition, it is an undeniable fact that microorganisms form resistance to synthetic antimicrobials that are used unconsciously. For such reasons, the use of plant extracts has come to the fore again, and studies on the development of the use of these products in many areas have been accelerated. The study demonstrated the antimicrobial activity of the hexane extract of *P. terebinthus* fruit. The extract with high antimicrobial activity may have potential use as a natural antimicrobial agent. The study revealed the promising potential of using *P. terebinthus* fruit, which has a very limited usage area, in the food industry to prevent food spoilage and extend shelf life.

ACKNOWLEDGEMENTS

This work (Project number 2019/022) was supported by Research Fund of Aksaray University. I would like to thank Songul Tacer for her help in the study.

CONFLICT OF INTEREST

The author stated that there are no conflicts of interest regarding the publication of this article.

REFERENCES

[1] Özçelik MM. Some functional foods of plant origin. Biyoloji Bilimleri Araştırma Dergisi 2016; 9(1): 57-68.

[2] Dalgıç L, Sermet OS, Özkan G. Effect of Roasting Temperatures on Quality Parameters of Turpentine Oil. Academic Food Journal/Akademik GIDA 2011; 9(3): 26-36.

[3] Giner-Larza EM, Máñez S, Giner-Pons RM, Recio MC, Ríos JL. On the anti-inflammatory and anti-phospholipase A2 activity of extracts from lanostane-rich species. Journal of Ethnopharmacology 2000; 73(1-2): 61-69.

[4] Iauk L, Ragusa S, Rapisarda A, Franco S, Nicolosi VM. In vitro antimicrobial activity of *Pistacia lentiscus* L. extracts: preliminary report. Journal of Chemotherapy 1996; 8(3): 207-209.

[5] Topçu G, Ay M, Bilici A, Sarıkürkcü C, Öztürk M, Ulubelen A. A new flavone from antioxidant extracts of *Pistacia terebinthus*. Food Chemistry 2007; 103(3): 816-822.

[6] Matthäus B, Özcan MM. Quantitation of Fatty Acids, Sterols, and Tocopherols in Turpentine (*Pistacia terebinthus* Chia) Growing Wild in Turkey. Journal of Agricultural and Food Chemistry 2006; 54(20): 7667-7671.

[7] World Health Organization. Global report on traditional and complementary medicine. Geneva: World Health Organization. 2019.

[8] Shah MA, Mir SA. Plant extracts as food preservatives. Plant Extracts: Applications in the food industry. Chapter 6. 2022; 127-141.

[9] Tran VT, Nguyen TB, Nguyen HC, Do NHN, Le PK. Recent applications of natural bioactive compounds from *Piper betle (*L.*)* leaves in food preservation. Food Control 2023; 154: 110026.

[10] Aygün A, Gülbağça F, Nas MS, Alma MH, Çalımlı MH, Ustaoglu B, Altunoglu YC, Baloglu MC, Cellat K, Sen F. Biological synthesis of silver nanoparticles using *Rheum ribes* and evaluation of their anticarcinogenic and antimicrobial potential: A novel approach in phytonanotechnology. Journal of Pharmacuetical and Biomedical Analysis 2020; 179:113012.

[11] Oliveira M, Ferreira V, Magalhaes R, Teixeira P. Biocontrol strategies for Mediterranean- style fermented sausages. Food Research International 2018; 103: 438–449.

[12] Azizi F, Habibi Najafi MB, Edalatian Dovom MR. The biodiversity of *Lactobacillus* spp. from Iranian raw milk Motal cheese and antibacterial evaluation based on bacteriocin-encoding genes. AMB Express 2017; 7(1): 176.

[13] Jabbari V, Khiabani MS, Mokarram RR, Hassanzadeh AM, Ahmadi E, Gharenaghadeh S, Karimi N, Kafil HS. *Lactobacillus plantarum* as a probiotic potential from Kouzeh cheese (traditional Iranian cheese) and its antimicrobial activity. Probiotics and Antimicrobial Proteins 2017; 9(2): 189–193.

[14] Gerez CL, Torres MJ, De Valdez GF, Rollán G. Control of spoilage fungi by lactic acid bacteria. Biological Control 2013; 64(3): 231-237.

[15] Asan-Ozusaglam M, Gunyakti A. *Lactobacillus fermentum* strains from human breast milk with probiotic properties and cholesterol-lowering effects. Food Science and Biotechnology 2019; 28(2): 501–509.

[16] Asan-Ozusaglam M, Gunyakti A. A new probiotic candidate bacterium from human milk: *Limosilactobacillus vaginalis* MA-10. Acta Alimentaria 2021; 50(1): 13–21.

[17] Asan-Ozusaglam M, Gunyakti A. Investigation of a new *Lactobacillus delbrueckii* strain from human milk as a probiotic candidate. Journal of Food Safety and Food Quality 2022; 73(2): 58-66.

[18] Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA. Manual of Clinical Microbiology. 9th ed. Volume 2: ASM press, 2007.

[19] Chandrasekaran M, Venkatesalu V. Antibacterial and antifungal activity of *Syzygium jambolanum* seeds. Journal of Ethnopharmacology 2004; 91(1): 105-108.

[20] Sousa A, Ferreira ICFR, Calhelha R, Andrade PB, Valentão P, Seabra R, Estevinho L, Bento A, Pereira JA. Phenolics and antimicrobial activity of traditional stoned table olives 'alcaparra'. Bioorganic & Medicinal Chemistry 2016; 14(24): 8533-8538.

[21] Hacıbekiroğlu I, Köseoğlu Yılmaz P, Haşimi N, Kılınç E, Tolan V, Kolak U. In vitro biological activities and fatty acid profiles of *Pistacia terebinthus* fruits and *Pistacia khinjuk* seeds. Natural Product Research 2015; 29(5): 444-446.

[22] Durak MZ, Uçak G. Solvent optimization and characterization of fatty acid profile and antimicrobial and antioxidant activities of Turkish *Pistacia terebinthus* L. Extracts. Turkish Journal of Agriculture and Forestry 2015; 39(1): 10-19.

[23] Doğan C., Determination of menengiç and some nuts husk's antimicrobial and antioxidant properties and use in fruit yogurt productions. Ph.D. Thesis, Harran University Institute of Natural and Applied Sciences Department of Food Engineering, Şanlıurfa, Türkiye, 2016.

[24] Pereira AP, Ferreira ICFR, Marcelino F, Valentão P, Andrade PB, Seabra R, Estevinho L, Bento A, Pereira JA. Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. cv. Cobrançosa) leaves. Molecules 2007; 12:1153-1162.

[25] Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, et al,. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. New England Journal of Medicine 1983; 308(12): 681-685.

[26] Dhifi W, Mnif W, Ouerhani B, Ghrissi K. Chemical composition and antibacterial activity of essential oil from the seeds of *Pistacia terebinthus* grown in Tunisia. Journal of Essential Oil Bearing Plants 2012; 15(4): 582-588.

[27] Poyrazoğlu Çoban E, Bıyık HH, Törün B. Investigation of Antimicrobial Effects in Different Solvents of *Pistacia terebenthus* L. Fruits Collected from Aydın. Journal of Applied Biological Sciences 2017; 11 (3): 20-22.

[28] Nazim B, Houssem F, Ismail B, Yassine M, Derouicha M. Antimicrobial Activity of Leaf, Fruit, and Gall Extract of *Pistacia terebinthus* Growing in Tessala. Current Perspectives on Medicinal and Aromatic Plants 2021; 4(2): 87-92.

[29] Anandharaj M, Sivasankari B. Isolation of potential probiotic *Lactobacillus oris* HMI68 from mother's milk with cholesterol-reducing property. *Journal of Bioscience and Bioengineering* 2014; 118(2): 153-159.

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology C- Life Sciences and Biotechnology

2024, 13(1), pp. 31-51, DOI: 10.18036/estubtdc.1378676

RESEARCH ARTICLE

PROTEIN STRUCTURE PREDICTION: AN IN-DEPTH COMPARISON OF APPROACHES AND TOOLS

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ABSTRACT

Proteins play crucial roles, including biocatalysis, transportation, and receptor activity, in living organisms. Moreover, their functional efficacy is influenced by their structural properties. Determining the three-dimensional structure of a protein is crucial to comprehending its catalytic mechanism, identifying potentially beneficial mutations for industrial applications, and enhancing its properties, including stability, activity, and substrate affinity. Although X-ray crystallography, nuclear magnetic resonance (NMR), and electron microscopy are employed to ascertain protein structures, many researchers have turned to bioinformatics modeling tools because of the high cost and time demands of these techniques. For structure prediction, there are three basic methods: ab initio (de novo), homology-based, and threading-based modeling techniques.

In this study, 11 modeling tools belong to different approaches were compared through modeling of various proteins; *Geobacillus kaustophilus* ksilan alpha-1,2-glucuronidase, *Actinosynnema pretiosum* bifunctional cytochrome P450/NADPH-P450 reductase, human high affinity cationic amino acid transporter 1 (SLC7A), human proton-coupled zinc antiporter (SLC30A) and *Bacillus subtilis* RNA polymerase sigma factor (sigY). Generated models were validated through QMEAN, QMEANDisCo, ProSA, ERRAT and PROCHECK tools. All of the studied proteins could be successfully modeled using homology modeling techniques, while some of the proteins could not be effectively modeled using threading or ab initio-based methods. YASARA generated reliable models for proteins that contain heteroatoms, such as P450 monooxygenases, because other tools exclude heteroatoms in their produced structures. Among approaches for modeling without templates, AlphaFold is a potent tool. On the other side, well-known template-based tools like YASARA, Robetta, and SWISS-MODEL have arisen. These results will help scientists choose the best protein modeling strategy and tool to guarantee high-quality structures.

Keywords: Protein structure prediction, Homology modeling, Alphafold, YASARA, Ab initio modeling

1. INTRODUCTION

The interatomic angles, folding-loop motives, and ultimately the three-dimensional structures of the proteins are determined by their sequences and complex interactions with amino acids, which are the building blocks of proteins. [1] While being synthesized in the ribosomes of cells, enzymes acquire three-dimensional structures with distinct folding patterns. These three-dimensional structures are critical for proteins to perform their biological functions, and misfolding can result in enzyme dysfunction or structural disorders in the organism. [2] Predicting the three-dimensional structures of proteins with known sequences and revealing their patterns is critical for understanding their functional mechanisms and improving the properties such as activity and stability. [3–5] Although traditional methods such as X-ray crystallography, nuclear magnetic resonance (NMR), and electron microscopy can be used to determine protein structures, their high cost and time requirements necessitated the development of alternative methods. [6] Various approaches for protein structure prediction in bioinformatics studies have been developed, ranging from databases using basic statistical methods to artificial intelligence and deep learning algorithms. [7] The first examples of computational protein structure prediction were based on detecting amino acid affinities for folding. In the following years, more successful results in structure estimation were obtained using techniques such as the calculation of free energy levels and the use of multiple alignment methods. [8] There are three main approaches

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Received: 20.10.2023 Published:30. 01.2024

for structure prediction: ab initio (de novo), homology-based, and threading-based modeling methods. They may also be divided into template-based modeling and template-free modeling categories. [9] With the aid of computer-assisted protein structure prediction and design techniques, which are widely used today, it is possible to understand the secondary and tertiary structures of proteins based on their amino acid sequences.

De novo or ab initio modeling is based on estimating the most likely low-energy conformation that the amino acid sequence may have. [10] Although the accuracy of the models obtained is low in ab initio modeling, which is a basic approach in which protein structure is estimated based on physicochemical properties, successful results can be obtained in modeling proteins with shorter than 100 amino acid sequences. [11] To reduce computation time, probabilistic and predictive approaches have led to the use of ab initio methods as techniques for revealing folding patterns of small proteins rather than exact structure prediction. [12] In the future, particle-based methods that provide coarse-grained predictions, such as Monte Carlo simulation, have developed to overcome these constraints. [11] In a study by Liwo et al. on optimization of potential energy functions, <6Å models were created with root mean square deviations of protein fragments up to 61 amino acids. [13] Simon et al. successfully modeled 73 of 172 target proteins up to 150 amino acid sequences using ROSETTA. [14] TOUCHSTONE II software was used to generate folding patterns for 83 of 125 target proteins with up to 174 amino acid sequences. [15] Bradley et al. used atomic ROSETTA to predict high-resolution models on sequences of less than 85 amino acids, employing a combination of structural sampling methods, methods that calculate the packaging of protein nuclei in detail, and high-resolution structure prediction. [16] In another study, the I-TASSER method was developed, which allows the accurate detection of folding regions in small proteins by iterative application of the TASSER method. [17] Different algorithms designed to improve the accuracy of methods for estimating the spatial structure with the lowest free energy allow for greater accuracy in predicting the three-dimensional structures of larger proteins. Rashid et al.'s random-start strand method, developed in 2013, greatly improved the results of single-point searches using a three-dimensional 100-center cubic lattice. [18] Studies have also been conducted to improve the quality of the fragment libraries based on the ratio of the number of segments close to the main backbone of the protein to the accuracy of the structure prediction. In a study that targeted particles recorded on templates with similar structural information in order to reduce the size of the conformational search space, the accuracy rate of the models obtained after classification was found to be 7% higher than that of standard piece-based estimators. [19] End-to-end learning and attention-based networks are used by AlphaFold; therefore, models with high confidence can be created. A study using AlphaFold2, an ab initio modeling tool, by Akdel et al. demonstrated that this tool can provide models with accuracy close to experimental data. [20] The accuracy of ab initio tools is limited and they require considerable computing resources to explore the wide range of protein conformations. This is why, up to now, only the structures of small proteins have been successfully predicted with this approach. [21]

In homology-based approaches, also known as comparative modeling, experimentally determined structures that are topologically equivalent to the target protein are used. [22] With the development of remote homology detection methods based on pairwise comparison of protein sequences, the use of tools that can obtain reliable and sensitive results in protein structure prediction has become widespread. [23] Finding the best pattern is the first step in homology modeling. Then the target and template sequences are aligned, the framework is built, and finally the model is evaluated. [24] The accuracy of homology models based on the structure of distantly related or spatially equivalent proteins is proportional to the alignment quality. [22]

Threading-based approaches are based on solvent availability and the secondary structure of proteins. These methods use the effect of different amino acid alternatives on structural coiling motives and align the questioned protein sequence with previously resolved similar template protein sequences and model them according to statistical probabilities and energy calculations. [25] Proteins acquire their three-dimensional natural structure according to distant interactions between amino acids. In threading-based

approaches, the data of these interactions are transferred to a scoring system and three-dimensional models are created by overlapping the structure information with the existing template models. [26,27] These approaches can be thought of as an intersection of ab initio and homology-based approaches. Just like in ab initio design, threading-based methods use energy minimization. [13] Threading methods, like homology-based modeling, aim to create a model by predicting the curling of the query protein sequence based on previously defined patterns. However, both methods ignore the possibility of the protein folding in a random conformation with a previously unknown template. [15] The main distinction between threading-based methods and comparative methods is that threading-based methods can model without requiring structure knowledge of homologous sequences. Threading uses sequence-to-structure alignment, whereas comparative methods require sequence-to-sequence alignment. [12] Since threading-based techniques focus on structural similarity, they are successful in recognizing sequence-structure pairs with similarity in folding motifs, but may be insufficient in recognizing homologous pairs. [28] In this study, six proteins from different protein classes were modeled using eleven different modeling tools belonging to three approaches. The generated models were then evaluated and compared in terms of model quality, RMSD and visually.

2. MATERIALS AND METHODS

Geobacillus kaustophilus ksilan alpha-1,2-glucuronidase (Accession Number: KJE27682), *Actinosynnema pretiosum* subsp. auranticum bifunctional cytochrome P450/NADPH-P450 reductase (Accession Number: Q8KUI0), human high affinity cationic amino acid transporter 1 (Accession Number: P30825) (SLC7A), human proton-coupled zinc antiporter (Accession Number: Q9Y6M5) (SLC30A) and *Bacillus subtilis* RNA polymerase sigma factor (sigY) protein sequences were used for modeling.

2.1. Ab Initio (De novo) Based Modeling Tools

The Alphafold, BhageerathH+, and RaptorX tools were used for *ab initio*-based modeling of target proteins. AlphaFold is a neural network-based structure prediction tool that enables modeling based on physical and biological data when data on similar protein structures are not available. [29] AlphaFold, an artificial intelligence tool based on deep learning principles, produces modeling results that are very close to experimental studies. It predicts structure by constructing new neural network architectures within the framework of the evolutionary, physical, and geometric rules of protein structure. [30] Another prediction tool, BhageerathH+, is an energy-based application for structure prediction of small globular proteins. [31] BhageerathH+ provides modeling results based on *ab initio* modeling. [32] The sequence in FASTA format is converted to PDB format, trial models are created, energy minimization is performed after steric mismatches are eliminated by passing through biophysical filters, the models with the lowest energy are selected, filtered, and the results are ordered. [31] RaptorX is a tool that focuses on solving the therading problem with the linear integer programming method, using *ab initio* methods to generate the unaligned loop regions and the final model. [12,33] RaptorX can estimate structural elements as well as the amino acid ratios that contribute to the disordered conformational randomness of the secondary structure. [34] The model is generated by energy optimization after obtaining the set of all applicable solutions. [25]

2.2. Homology Based Modeling Tools

SWISS-MODEL, IntFOLD, Phyre^2, Robetta, ModWeb and YASARA tools were used for homology based modeling of proteins. SWISS-MODEL was the first online modeling tool. [35] The SWISS-MODEL, which performs homology-based modeling; consists of five basic steps: amino acid sequence input, pattern search, pattern selection, model building, and quality estimation. [36] Protein sequences or UniProt accession code can be used directly , as well as target-pattern sequence alignment via a manually or automatically selected template. [37]

Another homology modeling tool, Robetta, generates several alignment alternatives based on features such as the requirement of a region in the protein sequence for folding. The best models are selected by combining criteria such as alignment compromise, hydrophobic embedding measure, low and high resolution energy functions. [22] IntFOLD, which allows the estimation of amino acids in the binding sites as well as access to the structure information from the protein sequence, is one of the alternative tools that provides the revealing of the relationship between the structure and function of the protein. [38] IntFOLD generates folding libraries during analysis by using multiple templates during model building. [32,39] Phyre^2, another online application for predicting the secondary and tertiary structures of proteins using a homology-based analysis method, provides users with both *ab initio* modeling from amino acid sequences and manual pattern generation. The tool's modeling method consists of collecting homologous sequences, scanning the folding library for known folding patterns, modeling loops and side chain placement. [40]

ModWeb, yet another comparative protein structure modeling tool, works on the principle of aligning the PSI-BLAST sequence profile of the target sequence with template sequences extracted from the Protein Data Bank and comparing them to select the best model. [26] Models are created by aligning one or more FASTA-formatted sequences to the best patterns using ModPipe. In addition, ModWeb allows users to define and model all homologous sequences in the UniProtKB database by using a protein structure and sequence profile as input. [41]

YASARA, generates hybrid models, is a molecular modeling tool with a visualization algorithm that performs a lattice-based neighbour search in conjuction with unbound force calculations at each step of the simulation without generating pair lists. [42,43] YASARA; is used to display models created by other methods as well as molecular models in appropriate formats [44] and for docking to understand the protein-ligand interaction. [45]

2.3. Threading Based Modeling Tools

The C-I-TASSER and LOMETS tools were used for threading based modeling of proteins. The C-I-TASSER tool was developed by combining I-TASSER's fragment assembly simulations with inter-aminoacid contact maps from deep neural network learning for modeling the folding motifs of non-homologous proteins. [46] C-I-TASSER, which offers the opportunity to make successful models in the structure prediction of proteins -especially when there is not any template models are available- finds structure patterns through LOMETS using contact maps and atomic models collected with Monte Carlo simulations, and creates the final model. [9,46] Another threading-based application, LOMETS, is a local meta-threading application combining nine different threading servers. LOMETS scans a library of patterns at various resolutions obtained by different methods such as X-ray crystallography, electron microscopy and NMR spectroscopy. The resulting patterns are evaluated based on query-sequence-to-pattern alignment scores using threading methods. [9] LOMETS also includes C_α atom and side chain contact distance maps assembled as a result of threading alignments and guides applications such as MODELLER, ROSETTA, TASSER. [47]

2.4. Model Quality Determination

QMEAN, QMEANDisCo, ProSA, ERRAT and PROCHECK tools were used to determine the quality of the protein models. QMEAN (Qualitative Model Energy Analysis - Qualitative Model Energy Analysis) model quality detection tool of the SWISS-MODEL server is an application that compares and scores the geometric properties of the model (dual atomic distances, rotation angles, all atom interaction, solvent accessibility, etc.) with statistical data obtained from experimental structures. With QMEAN, it is possible to both measure the overall reliability of the model and determine the local quality per amino acid. [48] In QMEAN, each amino acid is scored between 0 and 1 by calculating the statistical values of the potential mean strength in terms of similarity to the natural structure. The higher

the similarity, the higher the model reliability and score. [36,37] The Z-score is calculated by comparing the QMEAN score to the distributions obtained from the high-resolution structures resolved by X-ray chromatography. QMEANDisCo, another quality detection application developed over QMEAN calculations, is a tool that evaluates the distance localization of experimentally determined protein structures homologous to the model under consideration. The accuracy of the results obtained is proportional to the number of homologs of the query model. QMEANDisCo scoring cannot provide reliable results in protein models with few or no homologs. [49]

ProSA-web (Protein Structure Analysis) is another online tool used to validate protein models. It compares the query model to results from X-ray analysis, NMR spectroscopy, or theoretical calculations. The tool computes the model's structural energy and displays it as a Z-score and an amino acid energy graph. [50] The Z-score indicates the overall model quality and measures the deviation of the total energy of the structure according to an energy distribution derived from random conformations. [51,52] Positive values of the Z-score may indicate that the model is problematic or inaccurate. [50]

Model errors are caused by three major factors: misdirection of amino acids due to backbone linkages, errors in alignment or misregistration of amino acids, and side chain misplacement. [53] To detect faulty areas, various techniques are used. Ramachandran analysis of peptide dihedral angles is the first of these methods, and it is based on the classification of allowed and disallowed conformations. [54] Protein folding is defined by the φ (phi), ψ (psi) and ω (omega) angles of the backbone loops. Among them, the allowed loop options of angle ω are quite limited. [55] Ramachandran analysis is based on the principle of constructing two-dimensional scatter plots of other φ and ψ angles and comparing them with a predicted distribution. [56] By analyzing the statistics of unbound interactions between different types of atoms, ERRAT calculates the quality factor by plotting the data obtained as a result of the calculations, the value of the error function against a sliding window position of 9 amino acids. [53]

The SAVES developed by UCLA-DOE-LAB is an online verification tool that includes PROCHECK and ERRAT calculates Ramachandran plots and scores model quality respectively. The PROCHECK tool generates the graphs based on the comparison of stereochemical parameters of the given protein against similar patterns of known structure. [57] These parameters are stereochemical criteria used to determine the quality of a structure. [58] The obtained Ramachandran graphs show φ and ψ twist angles for all amino acids in the query protein structure except the chain ends. Because glycine amino acids are incompatible with other side chain types, they are depicted as independent triangles. The dark red regions shown in Ramachandran plots are identified as "nuclei". In these regions, amino acids with optimal angles are marked, and more than 90% of the amino acid sequence of an ideal model would be expected to be found. [58]

3. RESULTS AND DISCUSSION

Geobacillus kaustophilus ksilan alpha-1,2-glucuronidase, complete sequence and heme domain of *Actinosynnema pretiosum subsp. auranticum* bifunctional cytochrome P450/NADPH-P450 reductase, human high affinity cationic amino acid transporter 1 (SLC7A1), human proton-coupled zinc antiporter (SLC30A1), and *Bacillus subtilis* RNA polymerase sigma factor (sigY) protein models were created using the AlphaFold, BhageerathH+, C-I-TASSER, IntFOLD, LOMETS, ModWeb, Phyre^2, RaptorX, Robetta, SWISS-MODEL and YASARA modeling tools. The aforementioned proteins are members of different protein families. While *Geobacillus kaustophilus* ksilan alpha-1,2-glucuronidase and *Actinosynnema pretiosum subsp. auranticum* bifunctional cytochrome P450/NADPH-P450 reductase are enzymes, SLC7A1 and SLC30A1 are membrane proteins and sigY is a regulatory protein that controls the transcription. Since AlphaFold models are accessed through the database, AlphaFold models for all proteins were downloaded from UniProt except the heme domain (the catalytic domain of the enzyme) of the bifunctional cytochrome P450/NADPH-P450 reductase since the model of the full protein exist in the database. BhageerathH+ failed during fragment assembly and *ab initio* loop sampling

of high affinity cationic amino acid transporter 1 and proton-coupled zinc antiporter proteins. The RaptorX built model for only the *Geobacillus kaustophilus* ksilan alpha-1,2-glucuronidase.

The models were validated using the ERRAT, PROVE, ProSA, SWISS-MODEL QMEAN, and QMEANDisCo tools. In order to compare the modeling tools, the models with the highest accuracy of the tools that output more than one model were selected based on the results of the validation. The models with the highest ERRAT quality score were selected and compared with the models obtained from the other tools. In addition, RMSD values were calculated from the model-to-model comparisons. The ERRAT quality scores of all generated models, local quality estimation tables of QMEAN and QMEANDisCo tools, Z-PLOTs, and Ramachandran plots of the best models of each tool are shown in the Supporting Information.

3.1. 3D Modeling of *Geobacillus kaustophilus* xylan alpha-1,2-glucuronidase

From the modeling of the xylan alpha-1,2-glucuronidase protein, composed of 679 amino acids, using various tools, one structure prediction was obtained from AlphaFold, ModWeb, Phyre2, and YASARA, while two were obtained from the SWISS-MODEL tool. Additionally, five structure predictions were acquired through the use of BhageerathH+, C-I-TASSER, IntFOLD, LOMETS, RaptorX, and Robetta tools. All other tools generated models with 679 residues, while the Phyre2 generated model with 677, ModWeb 675, and SWISS-MODEL 677 residues (Table 1). All generated models were visualized using PyMOL and shown in Figure 2. Additionally, Table 1 shows the ERRAT quality scores, QMEAN, QMEANDisCo values, and Z-Scores. The YASARA tool generated the best model in terms of ERRAT Quality Score (98.36), while the SWISS-MODEL tool generated the best model in terms of QMEAN and QMEANDisCo scores (0.06 and 0.94 respectively). Furthermore, most of the models have ERRAT score above 90 and all models except RaptorX have QMEANDisCo score close to 1. All models have similar Z-values and Z-Plots (Figure S3) which are within the range of scores for similarly sized native proteins. Upon examination of the QMEAN graphs, it is evident that the low confidence regions among all models are quite similar. Analysis of Ramachandran plots (Figure S4) and statistics reveals that the number of amino acids residing in disallowed regions vary across the AlphaFold, LOMETS and YASARA models was 1, IntFOLD was 2 and the model obtained by SWISS-MODEL was 4. The ratio of the residues located in the most favored regions varies between 79.8 to 93.4. All of the protein models exhibit nearly identical structures based on their topology. This is associated with the RMSD values obtained from comparing the structural characteristics of each model. Notably, the RaptorX model displays a high RMSD in comparison to the other models, which indicates that there are some notable differences in the RaptorX model.

Table 1. ERRAT Quality Scores, QMEAN, QMEANDisCo Values, and Z-Scores of *Geobacillus kaustophilus* ksilan alpha-1,2-glucuronidase modeling.

Approach	Tool	Amino acid number in model	ERRAT Quality Score	QMEAN	QMEANDisCo	Z-SCORE
Ab initio	***AlphaFold***	679	96.42	0.40	0.93	-11.90
	BhageerathH+	679	92.85	-1.18	0.90	-11.55
	RaptorX	679	87.16	-2.56	0.61	-12.07
Threading	***C-I-Tasser***	679	95.37	-2.42	0.92	-11.91
	LOMETS	679	93.89	-0.72	0.93	-11.96
Homology based	***IntFOLD***	679	91.21	-0.86	0.91	-11.62
	ModWeb	675	89.51	-0.56	0.92	-11.79
	Phyre²	677	90.28	-0.18	0.91	-11.53
	Robetta	679	96.42	0.72	0.93	N.C
	SWISS-MODEL	676	93.69	0.06	0.94	-11.95
	YASARA	679	98.36	-0.26	0.89	-11.70

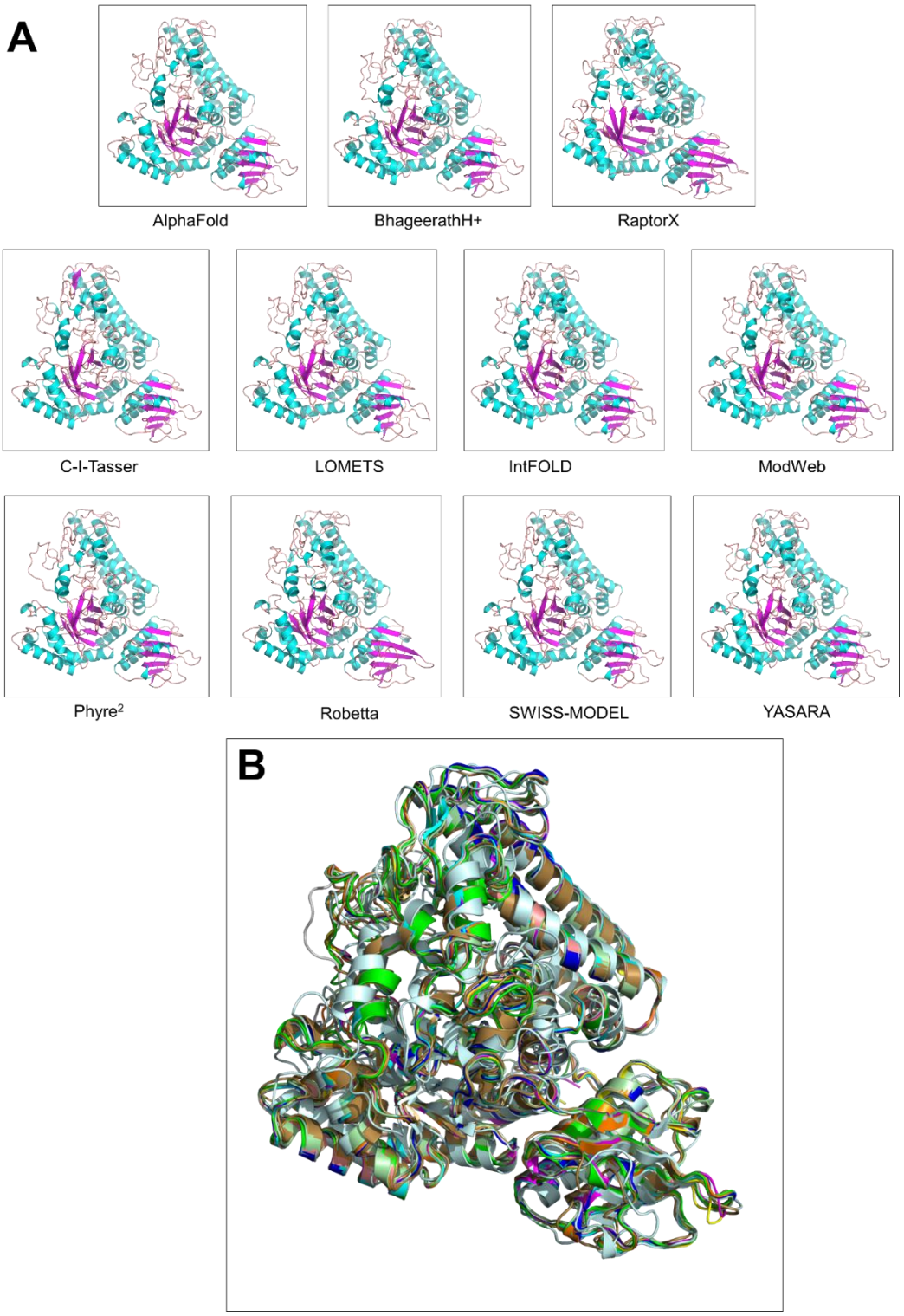

Figure 1. 3D models of *Geobacillus kaustophilus* ksilan alpha-1,2-glucuronidase protein. A. Cartoon representation of models generated by different tools (Cyan shows a-helix's, magenta shows b-sheets, and salmon color shows loops). B. Overlaid view of all generated models. Blue; AlphaFold, green; BhageerathH+, pale cyan; RaptorX, cyan; C-I-Tasser, magenta; LOMETS, yellow; IntFOLD, salmon color; ModWeb, grey; Phyre2, sand color; Robetta, orange; SWISS-MODEL, pale green; YASARA.

Table 2. RMSD values of model-to-model comparisons for *Geobacillus kaustophilus* ksilan alpha-1,2-glucuronidase protein.

Approach	RMSD	*AlphaFold*	*BHAGEERATHH+*	*RaptorX*	*C-I-Tasser*	*LOMETS*	*IntFOLD*	*ModWeb*	*Phyre²*	*Robetta*	*SWISS-MODEL*	*YASARA*
Ab initio	***AlphaFold***											
	BhageerathH+	1.51										
	RaptorX	4.79	5.09									
Threading	***C-I-Tasser***	1.46	2.25	5.05								
	LOMETS	1.19	2.00	5.02	1.78							
Homology based	***IntFOLD***	1.09	1.49	4.77	1.59	1.25						
	ModWeb	0.97	1.40	4.75	1.51	1.28	0.86					
	Phyre²	1.51	1.74	4.85	1.65	1.48	1.57	1.57				
	Robetta	2.10	2.69	4.90	2.40	2.38	2.15	2.07	2.23			
	SWISS-MODEL	0.84	1.29	4.77	1.48	1.15	1.02	1.00	1.41	2.13		
	YASARA	1.46	2.22	5.08	2.04	1.90	1.60	1.43	1.70	2.50	1.37	

3.2. Bifunctional Cytochrome P450/NADPH-P450 Reductase Models

Since the heme domain of P450 monooxygenases refers to the catalytic domain, both the heme domain and full protein were modeled separately. First, we modeled the heme domain of the bifunctional cytochrome P450/NADPH-P450 reductase, which consists of 482 amino acids, using 11 different tools. We retrieved the AlphaFold structures from the UniProt database. However, since the heme domain is part of the full sequence, the AlphaFold structure was not available in the database. Additionally, the RaptorX tool failed for modeling. On the contrary, one structure was derived from the utilization of Phyre2, SWISS-MODEL, and YASARA tools, whereas three were obtained from the ModWeb tool. Additionally, the remaining tools each yielded five structure predictions. All other tools output models comprising 482 amino acid sequences, while the ModWeb models comprise 466, Phyre^2 457 and SWISS-MODEL 462 residues. The models generated by C-I-Tasser, Robetta, and YASARA exhibited the highest ERRAT quality scores (Table 3). Based on the ERRAT, QMEAN and QMEANDisCo scores, homology based methods have higher quality scores compared to ab-initio and threading based methods. All models' Z-Plots are in the range of scores for similarly sized native proteins except for the model generated by BhageerathH+ (Figure S7). The Z-Score and Z-Plot for the models obtained using the Robetta tool were not calculable. The SWISS-MODEL tool produced the model with the highest QMEANDisCo score. When the QMEAN Local Quality Estimation plots (Figure S5 and S6) were examined, it was discovered that the positions of the heme domain's beginning and ending amino acids were the least reliable regions of all models. Based on the Ramachandran Plots (Figure S8), models generated by BhageerathH+, C-I-Tasser, and LOMETS possessed the lowest quality, with residues located in the most favored regions ranging between 70-85 %. When visualizing the generated models in Pymol, it was clearly seen that BhageerathH+ generated the least favorable model due to numerous secondary structure elements, including α-helix and β-sheets, not being modeled (Figure 2). However, the rest of the models share similar folding and low RMSD values except BhageerathH+ and LOMETS. Furthermore, P450 monooxygenases are heme containing enzymes and thus, structure should have heme molecule in the structure. Only the model generated by YASARA has the heme molecule and the remaining models lack the heteroatom in their final structure. As a result, YASARA yielded the best model for the heme domain of the bifunctional cytochrome P450/NADPH-P450 reductase, based on quality scores and heteroatom feature.

Table 3. ERRAT Quality Scores, QMEAN, QMEANDisCo Values, and Z-Scores of bifunctional Cytochrome P450/NADPH-P450 reductase heme domain modeling.

Approach	Tool	Amino acid number in model	ERRAT Quality Score	QMEAN	QMEANDisCo	Z-SCORE
Ab initio	***AlphaFold***	-	-	-	-	-
	BhageerathH+	482	46.51	-11.15	0.35	-4.26
	RaptorX	-	-	-	-	-
Threading	***C-I-Tasser***	482	96.19	-5.33	0.74	-10.54
	LOMETS	482	82.87	-5.72	0.63	-11.02
Homology based	***IntFOLD***	482	77.92	-2.59	0.73	-10.62
	ModWeb	458	77.11	-2.75	0.72	-10.66
	Phyre²	457	65.70	-3.63	0.69	-10.79
	Robetta	482	95.98	0.58	0.75	NA
	SWISS-MODEL	462	91.69	-1.94	0.76	-10.98
	YASARA	482	95.72	-1.53	0.72	-10.59

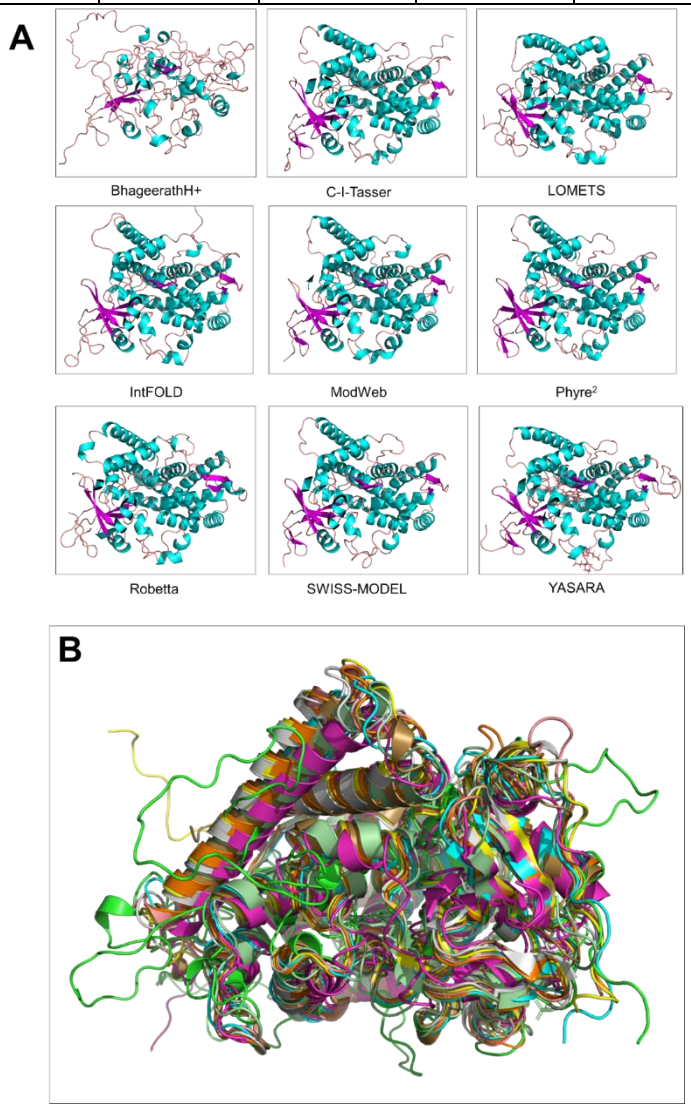

Figure 2. 3D models of bifunctional cytochrome P450/NADPH-P450 reductase heme domain protein. A. Cartoon representation of models generated by different tools (Cyan shows a-helix's, magenta shows b-sheets, and salmon color shows loops). B. Overlaid view of all generated models. Green; BhageerathH+, cyan; C-I-Tasser, magenta; LOMETS, yellow; IntFOLD, salmon color; ModWeb, grey; Phyre², sand color; Robetta, orange; SWISS-MODEL, pale green; YASARA.

Table 4. RMSD values of model-to-model comparisons for bifunctional cytochrome P450/NADPH-P450 reductase heme domain protein.

Approach	*RMSD*	*AlphaFold*	*BHAGEERATHH+*	*RaptorX*	*C-I-Tasser*	*LOMETS*	*IntFOLD*	*ModWeb*	*Phyre2*	*Robetta*	*SWISS-MODEL*	*YASARA*
Ab initio	***AlphaFold***											
	BhageerathH+											
	RaptorX											
Threading	***C-I-Tasser***		21.79									
	LOMETS		21.25		5.96							
Homology based	***IntFOLD***		21.44		3.95	6.43						
	ModWeb		21.48		2.69	4.19	2.24					
	Phyre²		21.46		2.93	4.16	2.27	2.63				
	Robetta		21.55		4.35	5.18	3.99	2.87	2.95			
	SWISS-MODEL		21.53		2.41	4.24	2.16	2.31	2.43	2.78		
	YASARA		21.85		4.78	6.08	6.62	3.14	3.46	5.02	3.23	

The full sequence of the bifunctional Cytochrome P450/NADPH-P450 reductase enzyme containing 1005 residues was also modeled. Out of eleven tools, six generated models for the target sequence. However, all ab-initio and threading based tools, which are template independent tools, except AlphaFold, failed to generate a model. Usually, these tools have sequence length limitations. When analyzing the obtained six structural models, the models generated by AlphaFold, Phyre^2, Robetta and SWISSMODEL comprised the almost entire sequence on the structure. Nevertheless, ModWeb generated the model with 522 residues and YASARA generated the model with 642 residues which contains the heme and FMN binding domain. AlphaFold, Robetta, SWISSMODEL, and YASARA models have higher quality scores (Table 5) compared to ModWeb and Phyre^2. When evaluating the QMEAN scores, Phyre^2 has below -4 which indicates low quality. And similarly, ModWeb has -3,56 which is close to -4 and it has also low quality. The QMEAN score for YASARA is -1,17 this is lowered because of the lower quality of some residues around 480 which lie in the linker region between heme and FMN domain. However, aside from this linker region, the rest of the model's QMEAN score is better. However, the model obtained from AlphaFold is the only model which is out of the range in the Z-Plots (Figure S11). All generated models have high percentage of residues located in the most favored regions on their Ramachandran plots (Figure S12). Visual inspection of the models reveals low folding similarities among the models, resulting in high RMSD values. Regarding heteroatom composition, the model generated by YASARA contains both heme and FMN molecules, while SWISSMODEL generated model contains only the heme molecule. The remaining four models lack heteroatoms as observed in the heme domain modeling.

Table 5. ERRAT Quality Scores, QMEAN, QMEANDisCo Values, and Z-Scores of bifunctional Cytochrome P450/NADPH-P450 reductase full sequence modeling.

Approach	**Tool**	**Amino acid number in model**	**ERRAT Quality Score**	**QMEAN**	**QMEANDisCo**	**Z-SCORE**
Ab initio	***AlphaFold***	1005	93.139	0.40	0.71	-16.22
	BhageerathH+	-	-	-	-	-
	RaptorX	-	-	-	-	-
Threading	***C-I-Tasser***	-	-	-	-	-
	LOMETS	-	-	-	-	-
Homology based	***IntFOLD***	-	-	-	-	-
	ModWeb	522	62.840	-3.56	0.63	-10.5
	Phyre²	1000	69.596	-4.20	0.69	-14.61
	Robetta	1005	96.3	1.04	0.70	-16.31
	SWISS-MODEL	1000	93.598	-2.57	0.73	-15.01
	YASARA	642	97.078	-1.17	0.71	-12.83

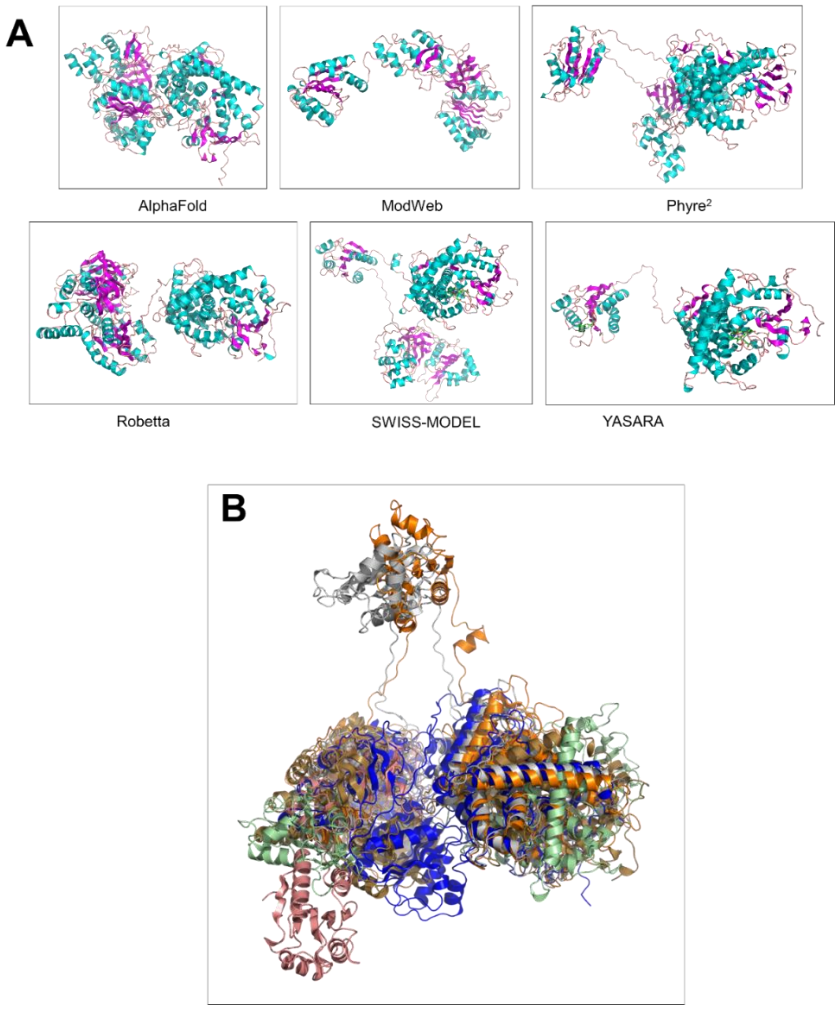

Figure 3. 3D models of bifunctional cytochrome P450/NADPH-P450 reductase full sequence protein. A. Cartoon representation of models generated by different tools (Cyan shows a-helix's, magenta shows b-sheets, and salmon color shows loops). B. Overlaid view of all generated models. Blue; AlphaFold, salmon color; ModWeb, grey; Phyre^2, sand color; Robetta, orange; SWISS-MODEL, pale green; YASARA.

Table 6. RMSD values of model-to-model comparisons for bifunctional cytochrome P450/NADPH-P450 reductase full sequence protein.

Approach	**RMSD**	***AlphaFold***	***BHAGEERATHH+***	***RaptorX***	***C-I-Tasser***	***LOMETS***	***IntFOLD***	***ModWeb***	***Phyre²***	***Robetta***	***SWISS-MODEL***	***YASARA***
Ab initio	***AlphaFold***											
	BhageerathH+											
	RaptorX											
Threading	***C-I-Tasser***											
	LOMETS											
Homology based	***IntFOLD***											
	ModWeb	57.97										
	Phyre²	78.83						89.57				
	Robetta	54.88						79.12	69.86			
	SWISS-MODEL	64.02						62.77	74.50	96.38		
	YASARA	63.78						6.38	67.54	99.59	8.04	

3.3. High Affinity Cationic Amino Acid Transporter 1 Models (SLC7A1)

Ten tools successfully generated models for the high affinity cationic amino acid transporter protein, which consists of 629 residues. It is difficult to determine membrane proteins structure experimentally, thus computational prediction is a promising approach. [21] While, one structure prediction was obtained from AlphaFold, Phyre^2, and YASARA tools. 2 from SWISS-MODEL, 3 from the ModWeb tool and 5 structure predictions were obtained from the remaining tools. ModWeb, Phyre^2, and SWISS-MODEL generated models consisting of 247, 451, and 589 residues, respectively. The remaining tools generated models with entire sequence. ERRAT quality scores, QMEAN, QMEANDisCo values, and Z-Scores of all models are shown in Table 7. Of the models, Robetta, YASARA, and AlphaFold had ERRAT quality scores exceeding 90 and their QMEAN values were higher than -4. Conversely, the remaining tools had QMEAN values below -4 indicating a low quality model. Furthermore, Robetta, YASARA, and AlphaFold had the highest QMEANDisCo scores which were approximately 0.6. According to Z-Plots, the scores for models generated by YASARA, and AlphaFold are out of the range of typically observed for native proteins of similar size determined by X-Ray and NMR. Additionally, the Z-Score and Z-Plot for Robetta model was not calculated. Based on Ramachandran Plots (Figure S16), more than 90% of residues in models generated by AlphaFold and YASARA are in the most favored regions. Generated ten models showed varying topology, but, AlphaFold, LOMTES, Phyre^2, Robetta, and SWISS-MODEL share more or less similar folding structures (Figure 4). This similarity was also confirmed through RMSD calculation and AlphaFold, Phyre^2, and Robetta have lowest RMSD values which indicates the folding similarity (Table 8). As a conclusion, all tested tools did not generate reliable 3D model for the SLC7A1, which is a membrane transporter protein, based on different quality parameters.

Table 7. ERRAT Quality Scores. QMEAN. QMEANDisCo Values. and Z-Scores of High Affinity Cationic Amino Acid Transporter 1 modeling.

Approach	Tool	Amino acid number in model	ERRAT Quality Score	QMEAN	QMEANDisCo	Z-SCORE
Ab initio	***AlphaFold***	629	94.79	-2.37	0.64	-6.78
	BhageerathH+	629	15.58	-14.23	0.22	0.91
	RaptorX	-	-	-	-	-
Threading	***C-I-Tasser***	629	81.48	-11.68	0.57	-3.56
	LOMETS	629	84.33	-5.93	0.55	-6.23
Homology based	***IntFOLD***	629	73.29	-9.18	0.58	-4.58
	ModWeb	247	79.83	-7.98	0.28	-1.96
	Phyre^2	451	88.18	-5.80	0.71	-3.51
	Robetta	629	99.19	-2.08	0.61	N.C.
	SWISS-MODEL	589	85.38	-7.50	0.61	-4.65
	YASARA	629	96.93	-3.83	0.60	-5.30

Table 8. RMSD values of model-to-model comparisons for High Affinity Cationic Amino Acid Transporter 1 protein.

Approach	RMSD	*AlphaFold*	*BHAGEERATHH+*	*RaptorX*	*C-I-Tasser*	*LOMETS*	*IntFOLD*	*ModWeb*	*Phyre^2*	*Robetta*	*SWISS-MODEL*	*YASARA*
Ab initio	***AlphaFold***											
	BhageerathH+	30.88										
	RaptorX											
Threading	***C-I-Tasser***	15.75	36.02									
	LOMETS	10.40	31.13		16.20							
Homology based	***IntFOLD***	19.30	28.09		25.98	18.61						
	ModWeb	28.32	28.62		27.69	28.83	28.40					
	Phyre^2	4.00	23.70		2.50	4.73	3.07	23.05				
	Robetta	4.66	30.75		15.63	10.66	19.13	28.16	3.95			
	SWISS-MODEL	19.58	26.07		23.80	19.09	19.40	29.85	2.55	19.81		
	YASARA	27.56	35.30		33.34	29.74	34.31	29.42	4.96	28.18	23.77	

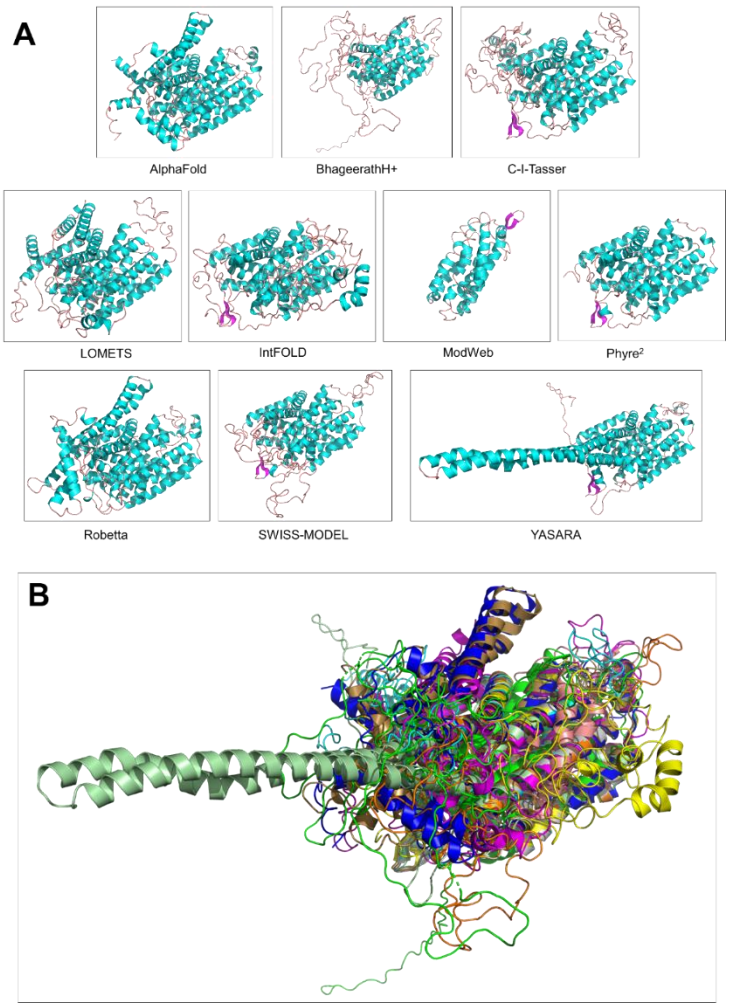

Figure 4. 3D models of High Affinity Cationic Amino Acid Transporter 1 protein. A. Cartoon representation of models generated by different tools (Cyan shows a-helix's. magenta shows b-sheets. and salmon color shows loops). B. Overlaid view of all generated models. Blue; AlphaFold. green; BhageerathH+. cyan; C-I-Tasser. magenta; LOMETS. yellow; IntFOLD. salmon color; ModWeb. grey; Phyre^2. sand color; Robetta. orange; SWISS-MODEL. pale green; YASARA.

3.4. Proton-Coupled Zinc Antiporter Models (SLC-30A)

Nine tools, apart from BhageerathH+ and RaptorX, successfully generated models of the human Proton-Coupled Zinc Antiporter, comprised of 507 amino acids. AlphaFold, C-I-TASSER, LOMETS, IntFOLD, and Robetta generated full sequence models. However, the remaining tools were unsuccessful in generating models with entire sequence (Table 9). AlphaFold, Robetta, and YASARA had the higher ERRAT quality scores of 93.38, 95.82, and 89.87, respectively, compared to other full sequence models. Only, the model generated by Robetta had the QMEAN value higher than –4, while all other models had QMEAN values below -4 which is an indicator of a model with low quality. Comparing the QMEANDisCo scores of AlphaFold, Robetta, and YASARA, Robetta had the highest score of 0.6. Moreover, 86.1 % of the residues in the model generated by Robetta were in the most favored regions on the Ramachandran Plot. On visual inspection, AlphaFold and Robetta exhibited structural similarities. The Robetta tool generated the model with the top scores for ERRAT, QMEAN, and QMEANDisCo. After analyzing the QMEAN Local Quality Estimation graphs (Figure S17-18), it was found that there were frequently occurring low confidence regions in all models. Further examination revealed that the region spanning from the 140th to 220th amino acids served as the common region

with the lowest confidence score in all models. Hence, this could be a contributing factor to the varying folding patterns observed in the models. Robetta, a homology-based tool, generated a more reliable model for the SLC-30A based on the evaluations mentioned earlier. Moreover, Robetta generated better models for membrane proteins. However, it takes days to build models in the Robetta server.

Table 9. ERRAT Quality Scores. QMEAN. QMEANDisCo Values. and Z-Scores of Proton-Coupled Zinc Antiporter modeling.

Approach	Tool	Amino acid number in model	ERRAT Quality Score	QMEAN	QMEANDisCo	Z-SCORE
Ab initio	***AlphaFold***	507	93.38	-6.74	0.44	-5.87
	BhageerathH+	-	-	-	-	-
	RaptorX	-	-	-	-	-
Threading	***C-I-Tasser***	507	88.94	-12.00	0.36	-5.84
	LOMETS	507	81.19	-5.68	0.50	-6.16
Homology based	***IntFOLD***	507	59.957	-9.65	0.47	-5.63
	ModWeb	110	87.25	-4.23	0.36	-2.93
	Phyre²	288	84.64	-6.41	0.60	-4.04
	Robetta	506	95.82	-1.26	0.60	N.C.
	SWISS-MODEL	421	70.3	-6.86	0.46	-4.99
	YASARA	507	89.87	-6.72	0.27	-4.06

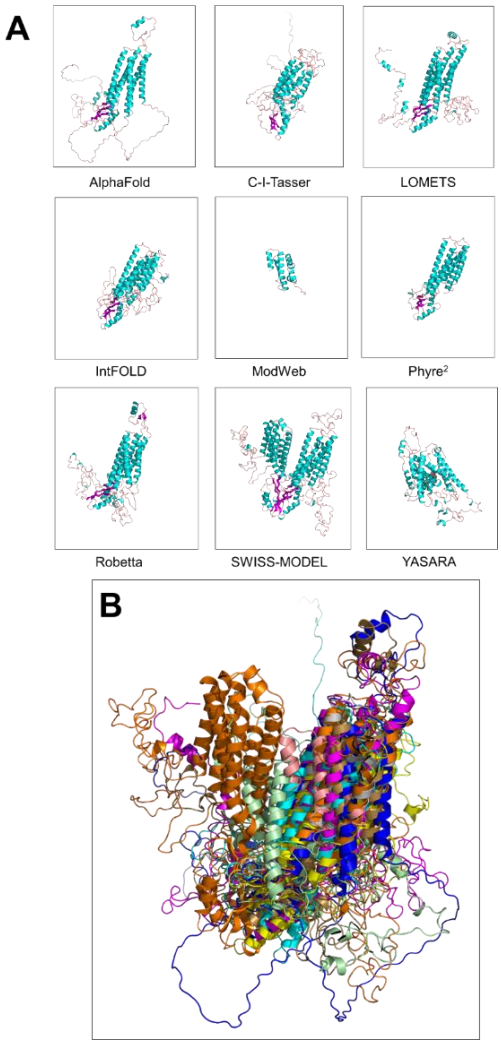

Figure 5. 3D models of Proton-Coupled Zinc Antiporter protein. A. Cartoon representation of models generated by different tools (Cyan shows a-helix's. magenta shows b-sheets. and salmon color shows loops). B. Overlaid view of all generated models. Blue; AlphaFold. cyan; C-I-Tasser. magenta; LOMETS. yellow; IntFOLD. salmon color; ModWeb. grey; Phyre². sand color; Robetta. orange; SWISS-MODEL. pale green; YASARA.

Table 10. RMSD values of model-to-model comparisons for Proton-Coupled Zinc Antiporter protein.

Approach	RMSD	*AlphaFold*	*BHAGEERATHH+*	*RaptorX*	*C-I-Tasser*	*LOMETS*	*IntFOLD*	*ModWeb*	*Phyre²*	*Robetta*	*SWISS-MODEL*	*YASARA*
Ab initio	***AlphaFold***											
	BhageerathH+											
	RaptorX											
Threading	***C-I-Tasser***	34.90										
	LOMETS	22.66			28.27							
Homology based	***IntFOLD***	27.55			28.40	21.81						
	ModWeb	17.30			17.35	16.92	18.07					
	Phyre²	6.11			6.74	7.15	3.58	18.06				
	Robetta	18.51			32.76	16.59	22.20	16.63	6.59			
	SWISS-MODEL	15.90			24.83	14.62	18.41	17.90	3.49	13.01		
	YASARA	38.56			40.07	34.29	30.84	16.50	28.17	34.25	32.38	

3.5 *Bacillus subtilis* RNA Polymerase Sigma Factor (sigY)

The sigma factor protein of *Bacillus subtilis* RNA polymerase comprises 178 residues and is the shortest and final protein utilized for modeling. All tools except RaptorX generated model for sigY. Phyre^2, ModWeb, and SWISS-MODEL generated models of 154, 171, and 175 residues respectively (Table 11). It is worth noting that the model generated by Phyre2 lacks the region spanning between residues 90 and 109. Moreover, ModWeb and SWISS-MODEL lack the residues present at the beginning of the sequence. Alphafold, YASARA, and Robetta achieved perfect ERRAT scores of 100, while threading-based methods surpassed 95. The worst QMEAN scores were seen in BhageerathH+ and C-I-Tasser, with -10.28 and -3.78, respectively. On the other hand, IntFOLD had the highest QMEAN score, reaching 0.31, which was closest to 1.0. Except for BhageerathH+ (0.26), all models scored above 0.6 in terms of QMEANDisCo. All models had Z-scores similar to those of native proteins of similar size. Ramachandran plots indicated that over 95% of the residues in the protein models produced by AlphaFold, Robetta, IntFOLD, ModWeb, and YASARA are situated in the most favored regions. BhageerathH+, C-I-Tasser, and ModWeb generated models with differing structures from the other tools, as observed from Figure 6 and confirmed by RMSD calculations in Table 12.

Table 11. ERRAT Quality Scores. QMEAN. QMEANDisCo Values. and Z-Scores of *Bacillus subtilis* RNA polymerase sigma factor modeling.

Approach	Tool	Amino acid number in model	ERRAT Quality Score	QMEAN	QMEANDisCo	Z-SCORE
Ab initio	***AlphaFold***	178	100	-0.81	0.66	-6.88
	BhageerathH+	178	50.74	-10.26	0.26	-1.77
	RaptorX	-	-	-	-	-
Threading	***C-I-Tasser***	178	98.24	-3.78	0.63	-5.75
	LOMETS	178	95.88	-1.12	0.67	-6.52
Homology based	***IntFOLD***	178	98.23	0.31	0.67	-6.98
	ModWeb	171	76	-2.13	0.63	-5.9
	Phyre²	154	87.67	-1.99	0.70	-6.54
	Robetta	178	100	1.46	0.67	-6.86
	SWISS-MODEL	175	88.62	-2.17	0.66	-6.2
	YASARA	178	100	-1.05	0.66	-6.13

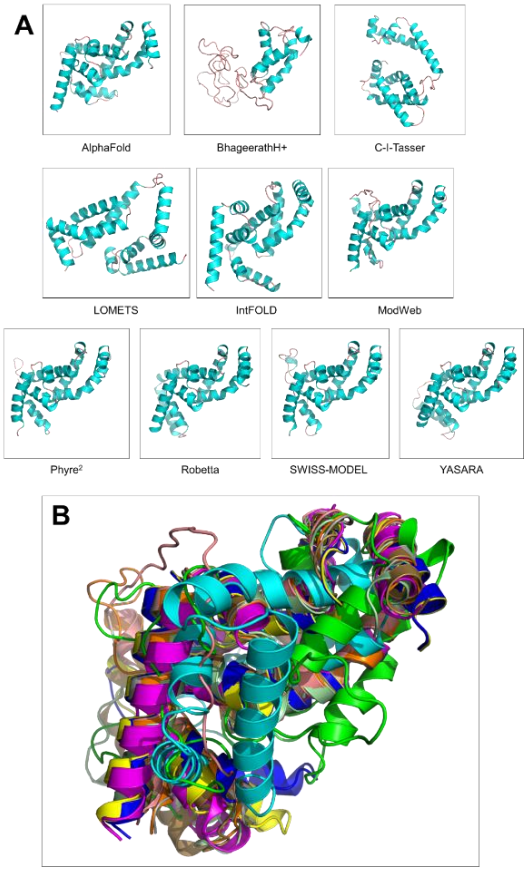

Figure 6. 3D models of *Bacillus subtilis* RNA polymerase sigma factor protein. A. Cartoon representation of models generated by different tools (Cyan shows a-helix's. magenta shows b-sheets. and salmon color shows loops). B. Overlaid view of all generated models. Blue; AlphaFold. green; BhageerathH+. cyan; C-I-Tasser. magenta; LOMETS. yellow; IntFOLD. salmon color; ModWeb. grey; Phyre^2. sand color; Robetta. orange; SWISS-MODEL. pale green; YASARA.

Table 12. RMSD values of model-to-model comparisons for *Bacillus subtilis* RNA polymerase sigma factor protein.

Approach	**RMSD**	***AlphaFold***	***BHAGEERATHH+***	***RaptorX***	***C-I-Tasser***	***LOMETS***	***IntFOLD***	***ModWeb***	***Phyre²***	***Robetta***	***SWISS-MODEL***	***YASARA***
Ab initio	***AlphaFold***											
	BhageerathH+	16.56										
	RaptorX											
Threading	***C-I-Tasser***	14.88	19.08									
	LOMETS	5.29	17.99		13.32							
Homology based	***IntFOLD***	2.24	16.52		14.84	4.62						
	ModWeb	11.25	17.74		16.62	12.06	11.10					
	Phyre²	5.82	13.77		14.22	11.04	6.08	6.48				
	Robetta	4.52	17.15		14.48	3.96	3.71	11.04	5.47			
	SWISS-MODEL	7.17	16.85		14.40	7.38	6.69	7.69	4.60	5.86		
	YASARA	4.71	17.06		14.47	4.82	4.47	11.51	5.67	3.88	6.79	

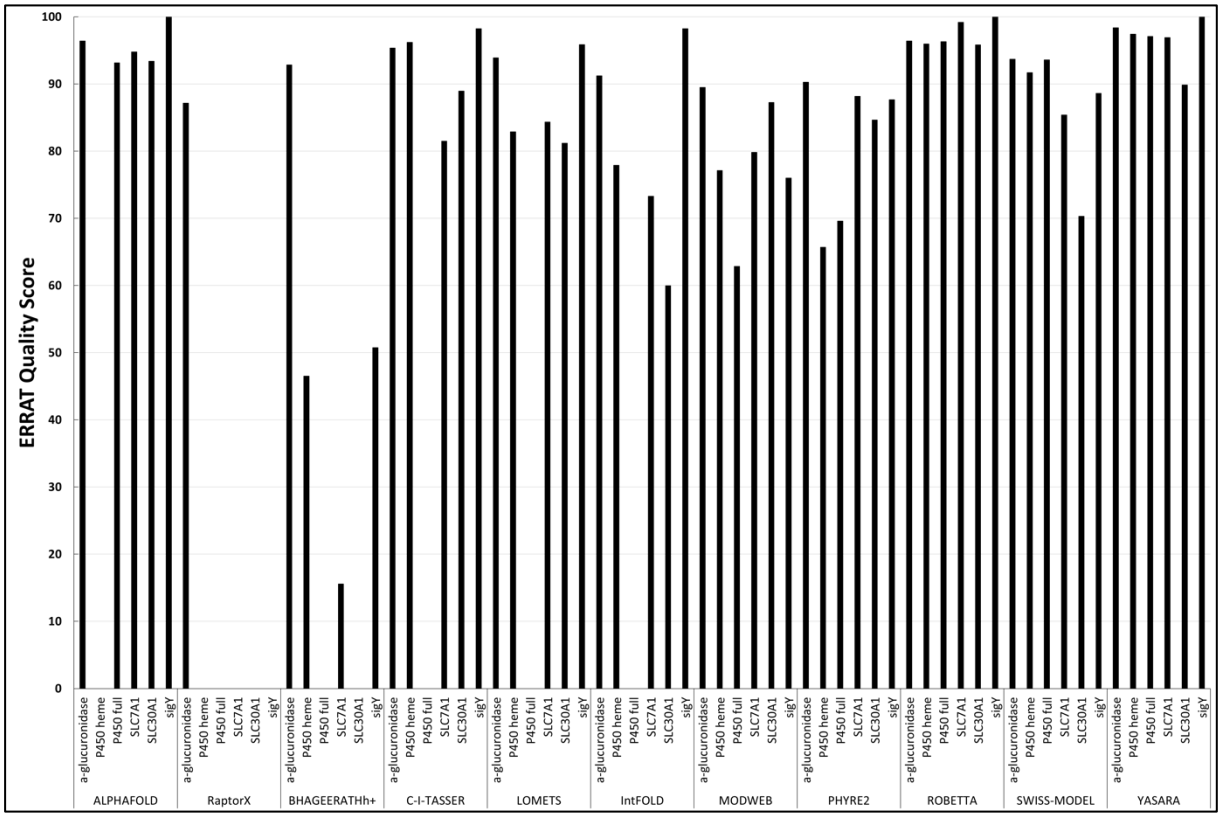

Figure 7. ERRAT quality scores of all generated models with tested eleven different tools.

Elucidating functions and mechanism of proteins is one of the primary questions in biochemistry. The structure of proteins has a major influence on the variety of activities they can perform. [59] Thus, one of the main goals of structural biology is to determine the three-dimensional structure of proteins. [21] Limited research has been conducted thus far to compare various model building tools, with a strong focus on homology modeling tools. Nikolaev and colleagues compared three homology modeling tools (Modeller, I-TASSER, and Rosetta) for predicting membrane proteins. Their findings indicate that successful modeling requires a target-template sequence identity of at least 40%. [21] Jang et al., conducted a study in order to compare multiple alignment tools and two template based model building program. SWISS-MODEL built models with better accuracy compared to Modeller. Because RMSD values of models generated both tools were below 1 and thus there is no significant quality difference between two tested programs for the modeling of soluble proteins. [60]

In our study, we have compared various tools from three different modeling approach. In addition to above mentioned results, YASARA, AlphaFold and SWISS-MODEL are the fastest tools among the all tested tools. On the other hand, other tools also had server problems and thus, they were not available time to time. Furthermore, YASARA generated high quality models since it generates hybrid models through performing hybrid modeling by separating the query protein into different units and selecting separate patterns for each unit. Major drawback of YASARA is that it is a paid tool, while all tested other tools are free of charge.

4. CONCLUSIONS

In this study, we conducted a comparison between ab-initio, threading, and homology modeling protein approaches. We tested 11 modeling tools to build models of six proteins. Template-based homology

modeling tools, in particular, successfully built models for all of the tested proteins; however, threading and ab initio-based tools were unsuccessful in building models for some of the proteins. For example, ab initio and threading-based methods were unsuccessful in generating a model for the complete sequence of the Bifunctional Cytochrome P450/NADPH-P450 Reductase protein. Furthermore, RaptorX could only produce a model for *Geobacillus kaustophilus* ksilan alpha-1,2-glucuronidase. YASARA is suitable for proteins that contain heteroatoms, such as P450 monooxygenases, since most other tools do not include heteroatoms in their produced structures. AlphaFold is a powerful tool among template-free modeling methods. On the other hand, YASARA, Robetta, and SWISS-MODEL have emerged as prominent template-based tools. These findings will aid researchers in selecting the suitable protein modeling approach and tool for ensuring high-quality structures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Elif Altunkülah; performed the analysis, collected the data, analyzed the data, wrote the manuscript draft. **Yunus Ensari**; conceptialization, design of study, supervised the research, wrote and edited the manuscript.

REFERENCES

[1] Smith GM. The Nature of Enzymes. In: Biotechnology. 1995. p. 4–72.

[2] Benítez CMV, Lopes HS. Protein structure prediction with the 3D-HP side-chain model using a master–slave parallel genetic algorithm. J Brazilian Comput Soc. 2010;16(1):69–78.

[3] Divya M, Jain SJMN, Phadke SR, Kishore R, Kamate M, Gupta N, et al. Protein structure prediction for novel mutations in Arylsulfatase-A gene. Mol Cytogenet. 2014;7(1):P62.

[4] Alford RF, Fleming PJ, Fleming KG, Gray JJ. Protein Structure Prediction and Design in a Biologically Realistic Implicit Membrane. Biophys J. 2020 Apr;118(8):2042–55.

[5] Batbat T, Öztürk C. Ayrık Yapay Arı Kolonisi Algoritması İle Protein Yapısı Tahmini. Bilişim Teknol Derg. 2016 Sep 30;9(3):260–3.

[6] Li X, Hu C, Liang J. Simplicial edge representation of protein structures and alpha contact potential with confidence measure. Proteins. 2003 Dec;53(4):792–805.

[7] Torrisi M, Pollastri G, Le Q. Deep learning methods in protein structure prediction. Comput Struct Biotechnol J. 2020;18:1301–10.

[8] Aydin Z, Singh A, Bilmes J, Noble WS. Learning sparse models for a dynamic Bayesian network classifier of protein secondary structure. BMC Bioinformatics. 2011;12(1):154.

[9] Pearce R, Zhang Y. Toward the solution of the protein structure prediction problem. J Biol Chem. 2021;297(1):100870.

[10] ANFINSEN CB, HABER E, SELA M, WHITE FHJ. The kinetics of formation of native ribonuclease during oxidation of the reduced polypeptide chain. Proc Natl Acad Sci U S A. 1961 Sep;47(9):1309–14.

[11] Lee J, Wu S, Zhang Y. Ab Initio Protein Structure Prediction. In: From Protein Structure to Function with Bioinformatics. Dordrecht: Springer Netherlands; 2009. p. 3–25.

[12] Abbass J, Nebel JC, Mansour N. Ab Initio Protein Structure Prediction: Methods and challenges. In: Biological Knowledge Discovery Handbook. 2013. p. 703–24.

[13] Liwo A, Lee J, Ripoll DR, Pillardy J, Scheraga HA. Protein structure prediction by global optimization of a potential energy function. Proc Natl Acad Sci U S A. 1999 May;96(10):5482–5.

[14] Simons KT, Strauss C, Baker D. Prospects for ab initio protein structural genomics. J Mol Biol. 2001 Mar;306(5):1191–9.

[15] Zhang Y, Kolinski A, Skolnick J. TOUCHSTONE II: A New Approach to Ab Initio Protein Structure Prediction. Biophys J. 2003;85(2):1145–64.

[16] Bradley P, Misura KMS, Baker D. Toward high-resolution de novo structure prediction for small proteins. Science. 2005 Sep;309(5742):1868–71.

[17] Wu D, Wu T, Liu Q, Yang Z. The SARS-CoV-2 outbreak: What we know. Int J Infect Dis IJID Off Publ Int Soc Infect Dis. 2020 May;94:44–8.

[18] Rashid MA, Shatabda S, Newton MAH, Hoque MT, Sattar A. A Parallel Framework for Multipoint Spiral Search in ab Initio Protein Structure Prediction. Adv Bioinformatics. 2014;2014:985968.

[19] Abbass J, Nebel JC. Customised fragments libraries for protein structure prediction based on structural class annotations. BMC Bioinformatics. 2015;16(1):136.

[20] Akdel M, Pires DE V, Pardo EP, Jänes J, Zalevsky AO, Mészáros B, et al. A structural biology community assessment of AlphaFold2 applications. Nat Struct Mol Biol. 2022;29(11):1056–67.

[21] Nikolaev DM, Shtyrov AA, Panov MS, Jamal A, Chakchir OB, Kochemirovsky VA, et al. A Comparative Study of Modern Homology Modeling Algorithms for Rhodopsin Structure Prediction. ACS Omega. 2018;3(7):7555–66.

[22] Chivian D, Baker D. Homology modeling using parametric alignment ensemble generation with consensus and energy-based model selection. Nucleic Acids Res. 2006;34(17):e112.

[23] Battey JND, Kopp J, Bordoli L, Read RJ, Clarke ND, Schwede T. Automated server predictions in CASP7. Proteins. 2007;69 Suppl 8:68–82.

[24] Heneghan MN, McLoughlin L, Murray PG, Tuohy MG. Cloning, characterisation and expression analysis of α-glucuronidase from the thermophilic fungus Talaromyces emersonii. Enzyme Microb Technol. 2007;41(6):677–82.

[25] Xu Y, Liu Z, Cai L, Xu D. Protein Structure Prediction by Protein Threading BT - Computational Methods for Protein Structure Prediction and Modeling: Volume 2: Structure Prediction. In: Xu Y, Xu D, Liang J, editors. New York, NY: Springer New York; 2007. p. 1–42.

[26] Eswar N, John B, Mirkovic N, Fiser A, Ilyin VA, Pieper U, et al. Tools for comparative protein

structure modeling and analysis. Nucleic Acids Res. 2003 Jul;31(13):3375–80.

[27] Shao M, Wang S, Wang C, Yuan X, Li SC, Zheng W, et al. Incorporating Ab Initio energy into threading approaches for protein structure prediction. BMC Bioinformatics. 2011 Feb;12 Suppl 1(Suppl 1):S54.

[28] Shi J, Blundell TL, Mizuguchi K. FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. J Mol Biol. 2001 Jun;310(1):243–57.

[29] Varadi M, Anyango S, Deshpande M, Nair S, Natassia C, Yordanova G, et al. AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. Nucleic Acids Res. 2022 Jan;50(D1):D439–44.

[30] Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596(7873):583–9.

[31] Jayaram B, Bhushan K, Shenoy SR, Narang P, Bose S, Agrawal P, et al. Bhageerath: an energy based web enabled computer software suite for limiting the search space of tertiary structures of small globular proteins. Nucleic Acids Res. 2006;34(21):6195–204.

[32] Jabeen A, Mohamedali A, Ranganathan S. Protocol for Protein Structure Modelling. In: Ranganathan S, Gribskov M, Nakai K, Schönbach CBTE of B and CB, editors. Oxford: Academic Press; 2019. p. 252–72.

[33] Chen CC, Hwang JK, Yang JM. (PS)2-v2: template-based protein structure prediction server. BMC Bioinformatics. 2009;10(1):366.

[34] Chandra Sekhar Mukhopadhyay, Ratan Kumar Choudhary MAI. Basic Applied Bioinformatics. Wiley-Blackwell; 2017. 472 p.

[35] Guex N, Peitsch MC, Schwede T. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. Electrophoresis. 2009 Jun;30 Suppl 1:S162-73.

[36] Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 2018 Jul;46(W1):W296–303.

[37] Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. 2014 Jul;42(Web Server issue):W252-8.

[38] Roche DB, Buenavista MT, Tetchner SJ, McGuffin LJ. The IntFOLD server: an integrated web resource for protein fold recognition, 3D model quality assessment, intrinsic disorder prediction, domain prediction and ligand binding site prediction. Nucleic Acids Res. 2011 Jul;39(Web Server issue):W171-6.

[39] Roche DB, Tetchner SJ, McGuffin LJ. FunFOLD: an improved automated method for the prediction of ligand binding residues using 3D models of proteins. BMC Bioinformatics. 2011;12(1):160.

[40] Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 2015;10(6):845–58.

[41] Pieper U, Webb BM, Dong GQ, Schneidman-Duhovny D, Fan H, Kim SJ, et al. ModBase, a database of annotated comparative protein structure models and associated resources. Nucleic Acids Res. 2014 Jan;42(Database issue):D336-46.

[42] Krieger E, Vriend G. YASARA View - molecular graphics for all devices - from smartphones to workstations. Bioinformatics. 2014;

[43] Krieger E, Vriend G. New ways to boost molecular dynamics simulations. J Comput Chem. 2015 May;36(13):996–1007.

[44] Joosten RP, te Beek TAH, Krieger E, Hekkelman ML, Hooft RWW, Schneider R, et al. A series of PDB related databases for everyday needs. Nucleic Acids Res. 2011 Jan 1;39(suppl_1):D411–9.

[45] Krieger E, Vriend G. Models@Home: distributed computing in bioinformatics using a screensaver based approach. Bioinformatics. 2002 Feb;18(2):315–8.

[46] Zheng W, Zhang C, Li Y, Pearce R, Bell EW, Zhang Y. Folding non-homologous proteins by coupling deep-learning contact maps with I-TASSER assembly simulations. Cell reports methods. 2021 Jul;1(3).

[47] Wu S, Zhang Y. LOMETS: A local meta-threading-server for protein structure prediction. Nucleic Acids Res. 2007 May 15;35(10):3375–82.

[48] Bienert S, Waterhouse A, de Beer TAP, Tauriello G, Studer G, Bordoli L, et al. The SWISS-MODEL Repository-new features and functionality. Nucleic Acids Res. 2017 Jan;45(D1):D313–9.

[49] Studer G, Rempfer C, Waterhouse AM, Gumienny R, Haas J, Schwede T. QMEANDisCo—distance constraints applied on model quality estimation. Bioinformatics. 2020 Mar 15;36(6):1765–71.

[50] Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 2007 Jul;35(Web Server issue):W407-10.

[51] Sippl MJ. Recognition of errors in three-dimensional structures of proteins. Proteins. 1993 Dec;17(4):355–62.

[52] Sippl MJ. Knowledge-based potentials for proteins. Curr Opin Struct Biol. 1995 Apr;5(2):229–35.

[53] Colovos C, Yeates TO. Verification of protein structures: Patterns of nonbonded atomic interactions. Protein Sci. 1993 Sep 1;2(9):1511–9.

[54] Ramachandran GN, Sasisekharan V. Conformation of Polypeptides and Proteins In: Anfinsen CB, Anson ML, Edsall JT, Richards FMBTA in PC, editors. Academic Press; 1968. p. 283–437.

[55] MacArthur MW, Thornton JM. Deviations from planarity of the peptide bond in peptides and

proteins. J Mol Biol. 1996 Dec;264(5):1180–95.

[56] Hooft RWW, Sander C, Vriend G. Objectively judging the quality of a protein structure from a Ramachandran plot. Bioinformatics. 1997 Aug 1;13(4):425–30.

[57] Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Crystallogr. 1993 Apr 1;26(2):283–91.

[58] Morris AL, MacArthur MW, Hutchinson EG, Thornton JM. Stereochemical quality of protein structure coordinates. Proteins. 1992 Apr;12(4):345–64.

[59] Gligorijević V, Renfrew PD, Kosciolek T, Leman JK, Berenberg D, Vatanen T, et al. Structure-based protein function prediction using graph convolutional networks. Nat Commun. 2021;12(1).

[60] Jang WD, Lee SM, Kim HU, Lee SY. Systematic and Comparative Evaluation of Software Programs for Template-Based Modeling of Protein Structures. Biotechnol J. 2020;1–21.

ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology C- Life Sciences and Biotechnology

2024, 13(1), pp. 52-62, DOI: 10.18036/estubtdc.1236273

REVIEW

RELATIONSHIP OF LEAD WITH FREE RADICALS, REACTIVE OXYGEN SPECIES, OXIDATIVE STRESS AND ANTIOXIDANT ENZYMES

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ABSTRACT

Heavy metals, which are high in the environment, are substances that have a high toxic effect even at low concentrations. Heavy metals taken into the organism through mouth, respiration and skin cannot be eliminated by the body's excretory pathways. In addition, since they have a durable structure, they participate in the food chain and accumulate in various body tissues. With the increase in industrial activities, heavy metal pollution has also emerged and has increased day by day. Lead is an element that is widely used in industry because it has a low melting temperature. However, it is known that lead, like other heavy metals, has an effect on problems such as environmental pollution and health problems. Occupational diseases such as lead poisoning occur as a result of direct exposure to lead. Direct exposure in this way can also cause death. Lead causes undesirable conditions such as increase of reactive oxygen species (ROS), emergence of oxidative stress and weakening of antioxidant system. Lead disrupts the prooxidant/antioxidant ratio. As a result, effects such as an increase in ROS and thus damage to the basic components of the cell such as lipid, protein and nucleic acid are observed. In particular, lead inhibits enzymes and prevents some enzymes from acting as antioxidants. As a result of exposure to lead, there is a decrease in the defense abilities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and ascorbic acid in blood and tissues. Lead increases lipid peroxidation and thus causes oxidative damage. Evaluation of the effect of lead at the cellular level is important in terms of developing solutions for the toxic effects of lead. In this study, the effect of lead on the cellular level in the organism and its effects on free radicals, ROS and oxidative stress were evaluated.

Keywords: Antioxidant enzymes, Free radical, Heavy metals, Lead, Oxidative stress

1. INTRODUCTION

Due to industrial development, waste materials are discharged to the environment, primarily soil and water. This situation causes heavy metal accumulation especially in urban areas. Unconsciously releasing heavy metals to the environment creates long-term toxicity in the ecosystem. It also causes health problems in terms of human health. Heavy metals can have a toxic effect on metabolism by changing enzymatic and structural functions when taken by the organism [1]. Most heavy metals can be toxic even at low concentrations. Heavy metals such as arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, silver, zinc is carcinogenic and mutagenic [2]. Toxicity from heavy metals is generally associated with the formation of reactive oxygen species (ROS). ROS increase primarily leads to oxidative stress. Oxidative stress causes changes in the electron transport chain, disruption of macromolecules and finally cellular damage in different tissues and organs [3]. All biological systems are affected because of exposure to lead through air, water and food sources. In this regard, lead is characterized as a dangerous and cumulative environmental pollutant. Regardless of the way it is taken into the body, all chemical forms of lead have a toxic effect [4]. Lead is dispersed into the environment in three forms: metallic lead, lead salts, and organic lead containing carbon [5].

The sources of lead exposure can be listed as leaded gasoline, lead-based paints, lead-containing food cans, ceramic glasses and batteries [4, 6]. Health problems caused by lead have been seen as a public health problem especially in recent years. Therefore, health problems that may arise with lead contact

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Received: 01.02.2023 Published: 30.01.2024

are controlled [7]. The widespread presence of lead in industry and the environment makes lead exposure occupationally important [8]. In addition, exposure to lead through ways such as domestic tap water, food contamination and house dust causes environmental pollution. It can cause behavioral, cognitive, physiological, and biochemical abnormalities on living things [7, 9, 10].

During normal biochemical reactions, compounds called reactive oxygen species (ROS) are formed [11]. Oxidative stress may occur when ROS reaches a high concentration, and the antioxidant mechanism cannot tolerate this situation. In this case, cellular structures may be damaged [12,13]. Macromolecules such as lipid, protein and nucleic acid can be oxidized by ROS. Lipid peroxidation reactions occur as a result of the oxidation of unsaturated fatty acids in the cell membrane structure. Thus, intermediate products are formed with lipid peroxides. The most common of these intermediates is malondialdehyde (MDA). MDA affects the cell membrane and physiological functions of the cell [12, 14].

Reactive oxygen species (ROS) produced in living organisms are detoxified by enzymatic and non-enzymatic antioxidant defense systems [11, 15]. Antioxidant enzymes are generally superoxide dismutases, catalases and glutathione (GSH) peroxidases. Non-enzymatic antioxidants are glutathione (GSH), α-tocopherol, carotenoids, and vitamin C [16, 17].

Lead disrupts the prooxidant/antioxidant balance, causing an increase in ROS. Thus, it damages cellular structures such as lipids, proteins, and nucleic acids [11]. In this review, it is aimed to evaluate the scientific data about the effect of lead at the cellular level, the formation of free radicals, ROS and oxidative stress, and their relationship with lead.

2. FREE RADICALS

Free radicals are formed during physiological and pathological reactions. They have one or more unpaired electrons in their orbitals. They are unstable, low molecular weight and highly reactive molecules that can act independently [12, 17, 18, 19, 20].

Free radicals formed in cells consist of reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is produced against degenerative conditions and various physical-chemical stimuli in the organism [21]. The presence of free radicals in medium or low amounts in the environment provides benefits in signaling and immune mechanisms. The presence of excess free radicals causes lipid peroxidation, inhibition of enzyme activity, and apoptosis. In addition, it causes events such as the destruction of the DNA molecule, which results in undesirable situations such as mutagenesis and carcinogenesis. It causes events such as the destruction of the DNA molecule resulting in undesirable situations such as mutagenesis and carcinogenesis [21, 22, 23].

3. REACTIVE OXYGEN SPECIES (ROS)

Oxygen-derived free radicals are very important for living things [14, 24]. Oxygen radicals are formed when an unstable oxygen uses the electrons of another oxygen together. When oxygen is reduced, short-lived but strong oxidant free oxygen radicals are formed. With the reduction of oxygen, free oxygen radicals with short-lived but strong oxidant properties emerge [24].

ROS is formed as a result of the biochemical reactions of the cell and shows toxic properties. Molecules such as lipid, protein and nucleic acid can be oxidized by ROS. Peroxidation reactions start with the oxidation of unsaturated fatty acids in the membrane structure. As a result of lipid peroxidation, lipid peroxides and intermediate products are formed. Malondialdehyde (MDA), one of these intermediates, affects cell membrane and physiological functions [11, 14, 25].

Major reactive oxygen species; superoxide anion (O^{2-}); hydrogen peroxide (H_2O_2); hydroxyl radical ($\cdot OH$); and singlet oxygen ($^1O_2$), which constitutes a large part of the biologically important free radicals [13]. Reactive oxygen species are detoxified by antioxidants. Thus, the cell keeps the amount of ROS under control. The intracellular ROS concentration is usually around 8-10 molar [26, 27]. ROS, which are formed as a result of oxidative stress, damage the membrane lipids and thus the cell through lipid peroxidation. In addition to lipid peroxidation, lead also causes erythrocyte (RBC) hemolysis by directly causing hemoglobin oxidation. This is due to inhibition of the enzyme Delta-aminolevulinic acid dehydratase (ALAD). Inhibition of ALAD leads to an increase in the concentration of substrate D-aminolevulinic acid (ALA) in blood and urine, thus producing hydrogen peroxide and superoxide radical [25, 28]. The progression of the mechanisms may cause the cell to be vulnerable to oxidative stress and even to death [9].

4. OXIDATIVE STRESS

The deterioration of the balance between the antioxidant defense system and the production of free radicals, that is, the ratio of prooxidant and antioxidant, is defined as oxidative stress [20, 29]. The intracellular ROS concentration may vary in some cases due to the continuous production and consumption of ROS. At levels where the ROS concentration is stable, the amount of ROS produced is equal to the amount of ROS consumed. However, the concentration of ROS may be responsible for the alteration of the oxidative stress state and thus the damage to cells, tissues, and organs. Under normal conditions, the balance between ROS production and consumption is achieved by keeping the ROS ratio constant, while the occurrence of oxidative damage causes the ROS level to increase. In such a case, the increase in ROS level occurs as a temporary, that is acute oxidative, thanks to its antioxidant potential. If the antioxidant potential is not in balance, chronic oxidative stress is seen because of faster production of ROS [27].

Factors such as smoking, heavy metals, radiation, infection cause an increase in oxidative stress. Oxidative stress also damages other macromolecules, especially lipids. As a result, it causes tissue damage, chronic diseases and even death [30]. Oxidative stress plays an important role in the formation of many chronic and degenerative diseases (cancer, autoimmune diseases, cardiovascular diseases, etc.) [12, 22]. Especially since the brain is the organ most affected by oxidative damage, free radicals are the cause of many pathological cases [29].

Oxidative stress forms the basis of especially toxicological studies [20, 29]. For this reason, it is important to investigate the formation mechanisms of free radicals and the responses to these radicals in terms of evaluating oxidative stress [31].

5. RELATIONSHIP OF LEAD WITH ROS AND OXIDATIVE STRESS

Lead increases ROS production and causes oxidative stress. ROS induced by environmental lead damage lipids, proteins, DNA, antioxidant defense systems and cellular structures [32, 33, 34].

When the Pb concentration increases, the balance between reactive oxygen species (ROS) and antioxidants changes. The increase in ROS ($O_2^{\cdot}$, H_2O_2, $NO^{\cdot}$, $ONOO^{\cdot}$, $OH^{\cdot}$) leads to a decrease in antioxidant defense power. Thus, oxidative stress and lead poisoning occur. Lead also affects antioxidant enzyme (Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx)) activity (Figure 1) [35, 36]. Oxidative stress begins with the effect of lead (Pb) due to two different ways. First, ROS such as hydroperoxides, singlet oxygen and hydrogen peroxide (H_2O_2) are produced, and secondly, antioxidant reserves are depleted [9].

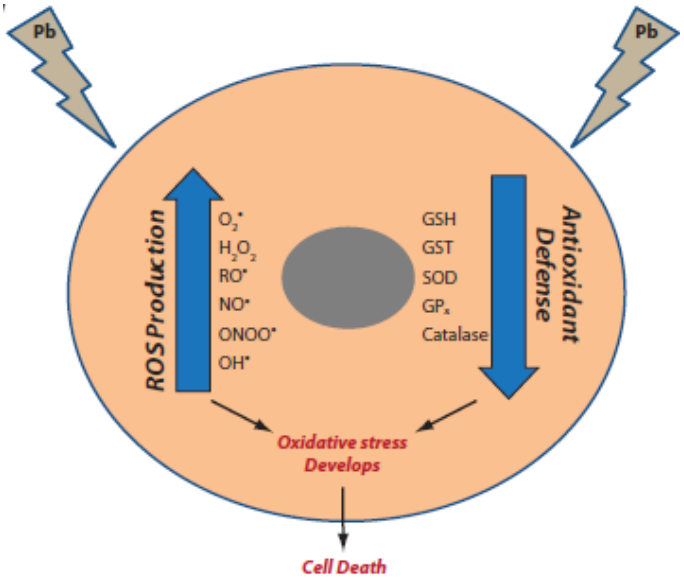

Figure 1. Development of oxidative stress in a lead-exposed cell [9].

Studies on lead exposure show that there are effects such as malondialdehyde (MDA) increase, lipid peroxidation, weakening of antioxidant defense mechanisms [37, 38, 39, 40]. In vivo and in vitro studies of lead exposure reveal increased production of ROS. The resulting oxidative stress causes changes in the structure and composition of fatty acids in the cell membrane [32, 41]. The increase in lipid peroxidation can affect the activities of membrane enzymes, endocytosis and exocytosis, solute transport, and signal transmission [42].

In addition, the enzyme Delta-aminolevulinic acid dehydratase (ALAD) is adversely affected by lead [41, 43]. As a result of the lead effect, D-aminolevulinic acid (ALA) accumulation is observed and this accumulation increases the amount of ROS and thus causes the formation of oxidative stress. It is stated that 4,5-dioxovaleric acid, the oxidation product of ALA, is an effective alkylating agent for DNA [32].

6. RELATIONSHIP OF LEAD WITH ANTIOXIDANT ENZYME ACTIVITY

Increased oxidative stress is one of the main causes of health problems such as cancer, diabetes, asthma, neurodegeneration, inflammation, developmental and reproductive disorders. An imbalance in cellular redox homeostasis is one of the consequences of heavy metal exposure [44]. One of the systems affected by oxidative stress caused by lead exposure is the antioxidant defense system [32].

ROS production is a common phenomenon in normal metabolic activities, but this is tightly regulated by the antioxidant system. Some enzymatic [Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx)] and non-enzymatic (glutathione) antioxidants stand out to control ROS formation. SOD catalyzes the formation of oxygen (O_2) and hydrogen peroxide (H_2O_2) from superoxide radicals by dismutation reaction. CAT and GPx enzymes act to decompose H_2O_2 into water (H_2O) and O_2. The imbalance between the antioxidant system and ROS causes an increase in oxidative stress and thus damage to cellular events [44, 45, 46, 47].

It has been revealed that there are changes in the activities of enzymes such as superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), glutathione peroxidase (GPx) because of lead exposure [48, 49, 50].

Glutathione (GSH) has sulfhydryl groups in its structure and is an important antioxidant. It exists in both reduced (GSH) and oxidized (GSSG) forms. GSH stabilizes reactive oxygen species by donating electrons. After donating electrons, it combines with another glutathione under the action of glutathione peroxidase and forms glutathione disulfide (GSSG). GSH is produced again by glutathione reductase from the formed GSSG. Under oxidative stress conditions, the GSSG concentration is higher than the GSH concentration. Lead neutralizes glutathione by binding to the sulfhydryl groups in the structure of glutathione [9]. In addition, GSH levels decrease when enzymes such as δ-amino levulinic acid dehydratase (ALAD), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione-S-transferase are inhibited by the effect of lead [5].

As mentioned before, lead has an effect on lipid peroxidation. However, since lead does not participate in oxidation-reduction reactions, this effect occurs indirectly. Lead causes lipid peroxidation by acting on enzymes working against free radicals and GSH levels. As a result of the lead effect, GSH is oxidized to GSSG. The GSH/GSSG ratio is a reliable parameter of oxidative stress. Glutathione, which contains high levels of sulfur, is affected by Pb. Lead binds strongly to -SH groups in the structure of GSH. Thus, the GSH level decreases. In addition, inhibition of glutathione reductase (GR) and glutathione-S-transferase enzymes by lead also negatively affects the maintenance of GSH level [35, 51,52].

SOD and CAT concentrations decrease because of exposure to lead. This situation also disrupts the superoxide radical removal mechanism. Lead can also cause enzyme inhibition by displacing the zinc ion required for the activity of antioxidant enzymes [9].

It has been reported that SOD, CAT and GPx enzymes work irregularly in the renal cortex, renal medulla and thoracic aorta in rats given 100 ppm lead acetate in drinking water. It has been stated that the dysregulation of these enzymes leads to hypertension [53]. It has been observed that blood lead levels of people who are engaged in painting profession are ≤400 μg/L and in this case, SOD and CAT activities decrease. At the same time, an increase in lipid peroxidation was observed [54]. In a study conducted with factory workers, it was stated that lead changed the antioxidant defense by inhibiting the sulfhydryl groups of antioxidant enzymes such as SOD, CAT and GPx [50]. It has been reported that rats exposed to lead acetate have significant decreases in hepatic and erythrocyte GPx, GST, CAT, SOD and GSH contents. At the same time, it was stated that MDA and H_2O_2 concentrations increased significantly. It was observed that antioxidant levels did not improve after removal of lead acetate [55].

7. PREVENTION OF LEAD TOXICITY

Considering the toxic effects of lead, it is almost impossible to remove lead from the body or reverse its harmful effects. Therefore, it is generally preferred to take preventive measures [9]. A three-step preventive approach model is recommended for lead toxicity, which includes an individual intervention, a preventive medicine strategy, and a public health strategy. If lead is detected in the blood, medical intervention is performed to control the poisoning results and prevent lead accumulation. The public health strategy operates at the population level and has a wider impact. The public health strategy aims to reduce lead exposure in living spaces. Public health proposes strategies such as banning the establishment of industrial establishments near the living area and banning the use of lead where another suitable substance can be used [9]. It is reported that nutrition plays an important role in the prevention of toxicity caused by lead. It is stated that nutrients such as vitamins, minerals and flavonoids may be beneficial in protecting the body from lead. These nutrients are effective in eliminating the effects of oxidative stress [32]. It is reported that antioxidant nutrients such as vitamin C, carotenoids, selenium, vitamin E and similar have protective effects [56].

Vitamin C is an important antioxidant that prevents lipid peroxidation and reduces the amount of ROS [56]. It is stated that because of lead exposure, lipid peroxidation with the effect of vitamin C is significantly inhibited in rat liver and brain tissues, and CAT level increases in kidney tissue [57]. It has

been reported that lead-induced ROS production is increased in rat sperm cells. It was observed that the increase in ROS decreased by 40% when water supplementation with vitamin C was given [54]. In animals exposed to lead, vitamin C supplementation exerts a protective effect by significantly affecting lead levels and lead-related biochemical changes in blood, liver, and kidneys [56].

Carotenoids are among the most common fat-soluble phytonutrients. It is stated that carotenoids show antioxidant properties by defending cell membranes and lipoproteins against ROS [59]. By consuming nutrients with rich carotenoid content, tissues and cells are protected from oxidative damage. Carotenoids play a role in scavenging singlet oxygen and peroxyl radicals [60].

β-carotene is found in yellow-orange vegetables, fruits, and green vegetables. β-carotene is found in yellow-orange vegetables, fruits and green vegetables. It shows an antioxidant effect with its activities such as scavenging singlet oxygen, scavenging free radicals and protecting lipids from oxidative degeneration [41].

Lycopene, which is found in red-colored fruits and vegetables such as tomatoes and watermelons, is known to have a strong antioxidant property [59, 61]. In clinical studies, lycopene is characterized as an important micronutrient that plays a role in oxidative stress and cancer-related disorders. Lycopene can reduce oxidative stress by scavenging oxygen-derived radicals and prevent the formation of ROS [62]. Inclusion of lycopene-rich foods in the diet can provide protection against oxidation of lipids, proteins, and DNA [61, 62].

Vitamin E, which is of plant origin, has an important role in the circulatory system, nervous system and reproductive systems [41]. Vitamin E has tocopherol (α, β, ɣ, δ) and tocotrienol (α, β, ɣ, δ) forms. The most bioactive form of vitamin E is α-tocopherol [63]. ROS, which are released because of cell metabolism, affect the polyunsaturated fatty acids of phospholipids in the membranes of cells and organelles. Thus, these fatty acids are converted to hydroperoxides by peroxidation. The number of free radicals increases with the decomposition of hydroperoxides [14]. In this case, vitamin E terminates the lipid peroxidation chain reactions by giving hydrogen to the polyunsaturated fatty acids in the structure of the phospholipids in the membranes [14, 59, 63]. In this respect, vitamin E does not prevent radical formation in an environment with high lipid content. However, it minimizes the formation of secondary radicals [17].

The combined use of vitamin E and other antioxidants is highly effective in preventing or reducing lead toxicity [32]. It is stated that chelation of vitamin E alone or with $CaNa_2EDTA$ (a drug used in lead poisoning) reduces lipid peroxidation in brain and liver tissues of rats [57]. It was determined that aspartate aminotronsferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities, cholesterol, triglyceride, LDL levels increased and GSH levels decreased in rats exposed to lead acetate through drinking water. It has been reported that with the concomitant use of vitamins E and C, lipid hydroperoxide levels decrease and GSH levels return to normal. The use of combined doses of vitamins E and C increases the protective effect [64].

In the treatment of oxidative stress caused by lead poisoning, the method of increasing the antioxidant capacity of cells can be used. Antioxidants (pyridoxine, methionine, S-adenosylmethionine, N-acetylcysteine, alpha-lipoic acid, captopril, taurine, homocysteine) which have thiol group (compounds containing -SH group) in their structures bind to compounds that lead tends to bind to, and lead removal is ensured. Thus, oxidative damage is prevented [33].

8. CONCLUSION

As a result of the understanding that lead has industrially important qualities, health problems such as lead poisoning have also emerged. It is known that lead has no biological function and lead exposure

causes serious health problems. The development and effective implementation of individual intervention, preventive medicine strategy and public health strategies are the first measures to be taken against lead toxicity.

Oxidative stress caused by lead is quite severe. ROS production is increased, thus causing damage to DNA, enzymes, proteins and membrane lipids. Increased ROS production causes depletion of antioxidant defense system elements in cells. The use of naturally occurring antioxidants such as some vitamins, carotenoids and herbal antioxidants separately or in combination for the prevention of lead-induced oxidative stress and lead toxicity has been reported in some studies. In addition, the widespread use of sulfur-containing antioxidants as protective and therapeutic is an effective method in preventing oxidative damage. In order to prevent lead toxicity, it is important to carry out and develop experimental studies both in terms of nutrition and the use of sulfur compounds after lead exposure.

CONFLICT OF INTEREST

The author stated that there are no conflicts of interest regarding the publication of this article.

REFERENCES

[1] Ünsal V. Biochemical approach to heavy metals. Editors: Köse O, Kirik E. New Approaches in Scientific Research-1, Ankara, Türkiye; Berikan Matbaacılık, 2018. pp. 107-123.

[2] Dixit R, Malaviya D, Pandiyan K, Singh UB, Sahu A, Shukla R, Singh BP, Rai JP, Sharma PK, Lade H. Bioremediation of heavy metals from soil and aquatic environment: An overview of principles and criteria of fundamental processes. Sustainability 2015; 7, 2189–2212.

[3] Méndez-Armenta M, Nava-Ruiz C, Fernández-Valverde F, Sánchez-García A, Rios C. Histochemical changes in muscle of rats exposed subchronically to low doses of heavy metals. Environ Toxicol Phar 2011; 32(1), 107-112.

[4] Assi MA, Hezmee MNM, Haron AW, Sabri MY, Rajion MA. The detrimental effects of lead on human and animal health, Vet World 2016; 9(6), 660-671.

[5] Ahamed M, Siddiqui MKJ. Environmental lead toxicity and nutritional factors. Clin Nutr 2007; 26(4), 400-408.

[6] Mutlu N, Ersan Y, Nur G, Koç E. Protective effect of caffeic acid phenethyl ester against lead acetate-induced hepatotoxicity in mice. Kafkas Univ Vet Fak Derg 17(Suppl A) 2011; 1-5.

[7] Spivey, A. The weight of lead: Effects add up in adults. Environ Health Persp 2007; 115, 30-36.

[8] Özbolat G, Tuli A. Effects of heavy metal toxicity on human health. Archives Medical Review Journal 2016; 25 (4), 502- 521.

[9] Flora G, Gupta D, Tiwari A. Toxicity of lead: a review with recent updates. Interdiscip Toxico 2012; 5(2), 47.

[10] Abadin H, Taylor, J, Buser M.C, Scinicariello, F, Przybyla, J, Klotzbach, J.M, Diamond G.L, Chappell L.L, McIlroy L.A. Toxicological profile for lead: Draft for public comment, Agency for Toxic Substances and Disease Registry, USA, 2019.

[11] Erişir M. The effect of Naringenin on oxidative stress in some tissues (heart, lung, brain, spleen, muscle) of lead-treated rats. Atatürk Üniversitesi Vet Bil Derg 2018; 13(1), 34-41.

[12] Kabel AM. Free radicals and antioxidants: Role of enzymes and nutrition. World Journal of Nutrition and Health 2014; 2(3), 35-38.

[13] Toyokuni S. Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 1999; 49(2), 91-102.

[14] Akkuş, I. Serbest Radikaller ve Fizyopatolojik Etkileri, first ed., Konya, Türkiye: Mimoza Yayınları, 1995.

[15] Üner N, Oruç E, Sevgiler Y. Oxidative stress-related and Atpase effects of etoxazole in different tissues of Oreochromis niloticus. Environ Toxicol Phar 2005; 20(1), 99-106.

[16] Miller JK, Brzezinska-Slebodzinska E, Madsen, FC. Oxidative stress, antioxidants, and animal function. J Dairy Sci 1993; 76(9), 2812-2823.

[17] Young IS, Woodside JV. Antioxidants in health and disease. J Clin Pathol 2001; 54(3), 176-186.

[18] Akpoyraz M, Durak I. Serbest radikallerin biyolojik etkileri. The Journal of the Faculty of Medicine 1995; 48:253-262.

[19] Bahorun T, Soobrattee MA, Luximon-Ramma V, Aruoma OI. Free radicals and antioxidants in cardiovascular health and disease. Internet Journal of Medical Update 2006; 1(2), 25-41.

[20] Kopáni M, Celec P, Danišovič L, Michalka P, Biró C. Oxidative stress and electron spin resonance. Clinica chimica açta 2006; 364(1), 61-66.

[21] Kurtdede E, Pekcan M, Karagül H. Free radicals, reactive oxygen species and relationship with oxidative stress. Atatürk Üniversitesi Vet Bil Derg 2018; 13(3), 373-379.

[22] Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci 2008; 4(2), 89.

[23] Derviş E. Oral antioksidanlar. Dermatoz 2011; 2(1), 263-267.

[24] Kulaksız R. In vitro evaluation of saanen buck semen frozen with extenders added different antioxidants. PhD, Ankara University, Ankara, Turkiye, 2009.

[25] Charkiewicz AE, Backstrand JR. Lead toxicity and pollution in Poland. Int J Environ Res Public Health 2020; 17(12), 4385.

[26] Foyer CH, Noctor G. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxidants & Redox Signaling 2009; 11(4), 861-905.

[27] Akbulut C, Kaymak G, Esmer HE, Yön ND, Kayhan FE. Oxidative stress mechanisms induced by heavy metals and pesticides in fish. Ege J Fish Aqua Sci 2014; 31(3): 155-160.

[28] Patrick L. Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. Alternative Medicine Review 2006; 11(2).

[29] Mercan U. Importance of free radicals in toxicology. Journal of Yüzüncü Yıl University Faculty of Veterinary Medicine 2004; 15 (1-2), 91-96.

[30] Sezer K, Keskin M. Role of the free oxygen radicals on the pathogenesis of the diseases. Fırat University Veterinary Journal of Health Sciences 2014; 28(1), 49-56.

[31] Yazıcı C, Köse K. Melatonin: The antioxidant power of darkness. Erciyes University Journal of Health Sciences 2004; 13(2), 56-65.

[32] Hsu PC, Guo YL. Antioxidant nutrients and lead toxicity. Toxicology 2002; 180(1), 33-44.

[33] Çaylak E. Lead toxication and oxidative stress in children and antioxidant effects of thiol compounds. J Child 2010; 10(1), 13-23.

[34] Ebrahimi M, Khalili N, Razi S, Keshavarz-Fathi M, Khalili N, Rezaei N. Effects of lead and cadmium on the immune system and cancer progression. J Environ Health Sci 2020; 18(1), 335-343.

[35] Lee JW, Choi H, Hwang UK, Kang JC, Kang YJ, Kim KI, Kim JH. Toxic effects of lead exposure on bioaccumulation, oxidative stress, neurotoxicity, and immune responses in fish: A review. Environ Toxicol Phar 2019; 68, 101-108.

[36] Collin MS et al. Bioaccumulation of lead (Pb) and its effects on human: A review. Journal of Hazardous Materials Advances 2022; 7, 100094.

[37] Sandhir R, Gill KD. Effect of lead on lipid peroxidation in liver of rats. Biol Trace Elem Res 1995; 48(1):91-7.

[38] Yiin SJ, Lin TH. Lead-catalyzed peroxidation of essential unsaturated fatty acid. Biol Trace Elem Res 1995; 50(2):16772.

[39] Gürer H, Ercal N. Can antioxidants be beneficial in the treatment of lead poisoning? Free Radic Bio Med 2000; 29(10):927-45.

[40] Çaylak E, Aytekin M, Halifeoglu I. Antioxidant effects of methionine, alpha-lipoic acid, N-acetylcysteine and homocysteine on lead-induced oxidative stress to erythrocytes in rats. Exp Toxicol Path 2008; 60(4-5):289-94.

[41] İriş C, Çınar M. Antioxidant vitamins and its effects on heavy metal toxicity. Bulletin of Veterinary Pharmacology and Toxicology Association 2019; 10(3), 135-151.

[42] Adonaylo VN, Oteiza PI. Pb^{2+} promotes lipid oxidation and alterations in membrane physical properties. Toxicology 1999; 132(1), 19-32.

[43] Süzen HS. Delta-aminolevulinic acid dehydratase (ALAD) genetic polymorphism in Turkish population. J Fac Pharm 2001; 30(1), 27-38.

[44] Paithankar JG, Saini S, Dwivedi S, Sharma A, Chowdhuri DK. Heavy metal associated health hazards: An interplay of oxidative stress and signal transduction. Chemosphere 2021; 262, 128350.

[45] Canli Ö et al. Myeloid cell-derived reactive oxygen species induce epithelial mutagenesis. Cancer Cell 2017; 32, 869–883.

[46] Habtemariam S. Modulation of reactive oxygen species in health and disease. Antioxidants 2019; 8(11), 513.

[47] Elzagallaai AA, Sultan EA, Bend JR, Abuzgaia AM, Loubani E, Rieder MJ. Role of oxidative stress in hypersensitivity reactions to sulfonamides. The Journal of Clinical Pharmacology 2020; 60(3), 409-421.

[48] Ito Y, Niiya Y, Kurita H, Shima S, Sarai S. Serum lipid peroxide level and blood superoxide dismutase activity in workers with occupational exposure to lead. Int Arch Occ Env Hea 1985; 56(2), 119-127.

[49] McGowan C, Donaldson WE. Changes in organ nonprotein sulfhydryl and glutathione concentrations during acute and chronic administration of inorganic lead to chicks. Biol Trace Elem Res 1986; 10(1), 37-46.

[50] Chiba M, Shinohara A, Matsushita K, Watanabe H, Inaba Y. Indices of lead-exposure in blood and urine of lead-exposed workers and concentrations of major and trace elements and activities of SOD, GSH-Px and catalase in their blood. The Tohoku Journal of Experimental Medicine 1996; 178(1), 49-62.

[51] Blaylock R.L. Neurodegeneration and aging of the central nervous system: Prevention and treatment by phytochemicals and metabolic nutrients. Integrative Med 1998; 1(3), 117-133

[52] Matović V, Buha A., Đukić-Ćosić D & Bulat Z. Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. Food Chem Toxicol 2015; *78*, 130-140.

[53] Farmand F, Ehdaie A, Roberts CK, Sindhu RK. Lead-induced dysregulation of superoxide dismutases, catalase, glutathione peroxidase, and guanylate cyclase. Environ Res 2005; 98(1), 33-39.

[54] Mohammad IK, Mahdi AA, Raviraja A, Najmul I, Iqbal A, Thuppil V. Oxidative stress in painters exposed to low lead levels. Arhiv za higijenu rada i toksikologiju 2008; 59(3), 161.

[55] Omobowale TO, Oyagbemi AA, Akinrinde AS, Saba AB, Daramola OT, Ogunpolu BS, Olopade JO. Failure of recovery from lead induced hepatoxicity and disruption of erythrocyte antioxidant defence system in Wistar rats. Environ Toxicol Phar 2014; 37(3), 1202-1211.

[56] Patra RC, Rautray AK, Swarup D. Oxidative stress in lead and cadmium toxicity and its amelioration. Vet Med-Us 2011; 2011, 9.

[57] Patra RC, Swarup D, Dwivedi SK. Antioxidant effects of α tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. Toxicology 2001; 162(2), 81-88.

[58] Hsu PC, Hsu CC, Liu MY, Chen LY, Guo YL. Lead-induced changes in spermatozoa function and metabolism. J. Toxicol. Environ. Health 1998; 55, 45-64.

[59] Nimse SB, Pal D. Free radicals, natural antioxidants, and their reaction mechanisms. RSC Advances 2015; 5(35), 27986-28006.

[60] Stahl W, Sies H. Antioxidant activity of carotenoids. Mol Aspects Med 2003; 24(6), 345-351.

[61] Hedayati N, Naeini MB, Nezami A, Hosseinzadeh H, Wallace Hayes A, Hosseini S, Imenshahidi M, Karimi G. Protective effect of lycopene against chemical and natural toxins: a review. Biofactors 2018; 45, 5–23.

[62] Bacanli M, Başaran N, Başaran AA. Lycopene: is it beneficial to human health as an antioxidant?. Turkish Journal of Pharmaceutical Sciences 2017; 14(3), 311.

[63] Altıner A, Atalay H, Bilal T. Vitamin E as an antioxidant. Balıkesir Health Sciences Journal 2017; 6(3), 149-157.

[64] Bashandy SA. Beneficial effect of combined administration of vitamin C and vitamin E in amelioration of chronic lead hepatotoxicity. The Egyptian Journal of Hospital Medicine 2006; 23(1), 371-384.