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RESEARCH PAPER

Callus and suspension culture techniques optimized for use in carrot breeding studies (*Daucus carota* ssp. *sativus* var. *atrorubens* alef and *D. carota*)

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Abstract

In this study, studies were conducted to optimize callus and suspension culture methods for in vitro mutation breeding in purple and orange carrots. Following this, the developed mutant lines were subjected to in vitro salt stress selection. The study determined the optimal agar dose. The first germination, 50% germination, root-cotyledon formation and genuine leaf formation in carrot seedlings were analyzed daily in the study, which was continued with the determined 7g/L agar dose. The cotyledon and hypocotyl explants from the seedlings were cultivated for callus production in mixtures of MS-1, MS-2 and MS-3 nutritional medium. In the second and fourth weeks following the second subculture, callus production percentages and weights were measured. The MS-3 (1 mg/L 2,4-D + 0.5 mg/L kinetin) nutritional medium and hypocotyl explant were found to be particularly effective at producing callus. The second subculture's data on the number of plants that had successfully regenerated per callus, showed that the MS nutritional medium with 0.2 mg/L Thidiazuron (TDZ) was the best medium for plant regeneration. The dispersed calli were grown in a nutritional medium designed for suspension culture in a nutrient medium mixture comprising MS+0.1 mg/L kinetin. The results obtained with the optimization steps were used in the ongoing study.

Introduction

Plant breeding research is labor-intensive and time-consuming, and as a result, the cost is expensive. Because research using traditional methods takes a long time, breeding studies are now being planned to be merged with plant tissue culture techniques. The tissue culture technique makes it possible to offer all of the conditions required by the plant in the most efficient manner, minimizing the time of breeding studies and saving labor and total cost. From the 1920s to the present, totipotency has been exploited in plant tissue culture techniques like cell and callus culture. On the one hand, plant reproduction, disease-free material production, and techniques to shorten the breeding period were used, on the other hand, cell-level studies were developed (Babaoğlu, 2001). Callus and cell suspension cultures are a couple of these. It will be used for genetic research at the molecular level, the creation of secondary metabolites with high medicinal value, the development of new cell lines resistant to/tolerant of environmental and non-environmental stress factors such as salinity, drought, heat, and various diseases, as well as applications that are reduced to the cell level in plant regeneration technique through cell suspension

culture materials that can be obtained in a lot less time than traditional methods (Neumann et al., 2009).

In the technological development stage we are in, callus and cell suspension culture techniques are used in tolerant plant breeding studies, selecting resistant/tolerant plants to biotic and abiotic stress factors, and tolerant plants are grown from them (Taner, 2002, Taner et al., 2004).

Carrot is a cool climate vegetable in the Umbelliferae (Apiaceae) family that belongs to the genus Daucus. Carrots grown from seed are a biennial, open-pollinated, diploid vegetable. There is a genetic basis for distinct species within the genus Daucus. There are currently 22 Daucus species described, the majority of which are diploid with chromosomal counts ranging from n=x=9 to n=x=11. Cultivated carrots in these species are divided into two groups: Western Europe and East Asia. Western origins have orange, yellow, red, or white roots, less hairy leaves, and are less prone to blossoming even when not exposed to cold temperatures for an extended period of time. East Asian carrots, on the other hand have anthocyanin-rich reddish purple or yellow roots, are prone to early flowering, and have hairy leaves (Rubatzky et al., 1999).

The majority of purple carrots grown in our country are grown by local inhabitants. Local population non-uniformity in fruit shape, size, color, and yield is the greatest difficulty for farmers. The high rate of a purple hue in the plants is regarded as a selection factor for the production of seeds for the following generation (Montilla et al., 2011). Because the carrot is a foreign pollinated, two-year plant, its selection efficiency is limited, its duration is prolonged, and the breeding process is likewise delayed under these conditions (Erişdi, 2015). Despite the fact that carrots are a crop with significant agricultural value, our nation has very few breeding studies for the creation of new varieties. There aren't many studies in the literature on carrots that focus on developing lines with high tolerance to abiotic stressors. Although salt tolerance and herbicide resistance are topics that are widely discussed in international sources, research on these topics and the application of biotechnological techniques in our country are fairly restricted.

All genotypic differentiations that take place *in vitro* are referred to as somaclonal variation, and they are considered a new solution in situations where natural variety eventually declines throughout breeding experiments or when it is challenging to introduce variation. In long-term cultures or short-term conditions with high concentrations of plant growth regulators, plant cells in culture that can generate callus or totipotent new plants may lose this ability. Genetic or chromosomal problems may result in phenotypic and genotypic changes in plants grown from cell cultures. The most significant benefit of somaclonal variation is that it makes cell-level selection for many features easier (Babaoğlu, 2001).

The importance of using mutation-inducing applications to increase genetic diversity is rising. Mutation breeding has proven successful in creating plants with high tolerance to abiotic environmental stress conditions, including salt and drought, in a variety of plant species. The use of both chemical and physical mutagens, such as EMS (ethyl methane sulfonate) and gamma radiation, increases the frequency of mutation in plant cells and has several uses in fostering the production of genetic variety. Important agricultural crops like rice, wheat, potatoes, soybeans, peppers, and peanuts have seen the development of hundreds of novel cultivars with salinity, drought tolerance, yield enhancement, and early maturity (Bado et al., 2015; IAEA, 2022).

Shortening the time needed for breeding research by utilizing mutagen-induced somaclonal variation, which would enable the selection of carrots that can withstand salt stress at the cellular level. In earlier years, certain plant species have used mutation-inducing applications to enhance the variety between cells through the use of physical mutagen applications, which may be used as an auxiliary technique (Kantoğlu et al., 2009).

Selection of tolerant/resistant new cell lines because of filtrate applications of *in vitro* mutation and stress-causing disease agents is a method with successful examples (Rus et al., 2000, El Hadrami et al., 2005, Arıcı, 2006, Bükün et al., 2009). Cell lines and cultivars developed with the same method have been tested against abiotic stress factors. Cell lines were selected and new cultivar candidates were determined in many different species orange (Ben-Hayyim & Kochba, 1983), tobacco (Watad et al., 1983), rice (Winicov,1996), tomato (Rus et al., 2000), potato (Queiros et al., 2007), strawberry (Torun et al., 2007), and fig (Emek & Erda, 2008) using salt stress applied *in vitro.*

Materials and Methods

The research was carried out at Recep Tayyip Erdogan University's Faculty of Agriculture Tissue Culture and Physiology Laboratories between 2017-2021.

Commercial orange (*Daucus carota* L.) (Nantes - Arzuman Tohum Company) and purple (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) Hatay local carrot cultivars' seeds were used.

Sowing Seeds and Agar Dose Trial

During the study's initial phase, surface sterilization was applied to the seeds of the orange and purple carrot varieties. The seeds were submerged for one minute in a 70% ethyl alcohol solution after being packed in tiny bags, rinsed under running water and surface sterilized. The seeds were then maintained for 20 minutes in a solution containing 20% commercial sodium hypochlorite and 1-2 drops of Tween-20. After being rinsed three times for five min. with sterile

distilled water and placed on sterile drying paper, the seeds were brought ready for sowing. Seeds of orange and purple carrot varieties were sown on Murashige and Skoog (MS) (Murashige & Skoog, 1962) basic nutrient medium containing 30 g/L sucrose and solidified with 6 g/L, 7 g/L, 8 g/L, and 15 g/L agar. The amount of agar used to determine the germination status of the seeds sown as 10 seeds in each petri dish and jar, and after which the plant growth was the best. After sowing the seeds, the petri dishes were kept in the room temperature climate chamber for 2 weeks, and the development of the plants was monitored daily (Ipek, 2002).

In carrot seedlings whose plant growth was observed for two weeks, the initial germination, 50% germination, root formation, cotyledon formation, and genuine leaf formation were all evaluated and reported on a continuous basis. Additionally, the percentage of seeds that germinated on the seventh and fourteenth days in the nutritional media was recorded, and the statistical difference between the averages was analyzed.

Callus Obtaining Studies

The optimal agar dosage determined in MS-1, MS-2 and MS-3 nutritional medium combinations was used to cultivate the cotyledon and hypocotyl explants collected from the seedlings acquired two weeks after sowing both orange and purple carrot seeds (Table 1).

Table 1. Plant growth regulator contents of the nutrient medium prepared for callus development

Nutrient mediums	Plant growth regulator contents
MS-1	1 mg/L 2,4-D
MS-2	1 mg/L 2,4-D + 0.1 mg/L kinetin (Ipek, 2002)
MS-3	1 mg/L 2,4-D + 0.5 mg/L kinetin (Herdem, 1998)

Hypocotyl and cotyledon explants subcultured every two weeks in nutritional media MS-1, MS-2, and MS-3 with different combinations of plant growth regulators showed callus development. In order to determine which nutrient medium and explant were most effective at producing callus, each application used 10 explants that were cultured in 10 petri dishes. Data on the percentage of callus formation were collected twice, in the second and fourth weeks. The callus weights were determined in the fourth week.

Plant regeneration medium from the callus

For plant regeneration, callus tissues were obtained from purple and orange carrot explants and cultured in combinations of MS-4, MS-5 and MS-6. 10 purple and 10 orange carrot calli in ten petri dishes were cultured in three different nutrient media for each application. The best medium combination for plant regeneration was discovered after the second subculture, when the number of plants that had grown from each callus had been counted from the calli subcultured every three weeks (Table 2).

Table 2. Nutrient media plant growth regulator ingredients prepared for plant regeneration from callus

Nutrient mediums	Plant growth regulator contents
MS-1	MS
MS-2	MS+0.1 mg/L kinetin
MS-3	MS+ 0.2 mg/L TDZ

Plant regeneration studies in suspension culture

Purple and orange carrot calluses were grown in a liquid nutritional medium with MS-2 prepared for plant regeneration from suspension cultures. Twenty 250 mL flasks containing 50 mL of nutritive media were filled, their mouths were sealed with aluminum foil, and they were autoclave sterilized. In sterile liquid nutritional medium, purple and orange carrot calluses weighing 1 g were cultivated. The prepared cultures were incubated for three weeks at 25°C and 110 rpm in an orbital shaker incubator. In suspension cultures that were subcultured twice every two weeks, embryo development and plant regeneration took place. After filtering the cultures, their final weights were calculated.

Statistic Evaluation

The JMP 13 package software was used to establish the ideal nutrient medium concentration for callus development, the ideal nutrient medium quantity for carrot genotype seed germination, and the ideal nutrient medium for plant regeneration from callus. The analyzes were carried out according to the factorial trial design in random plots. To identify significant differences in pairwise and triple comparisons, the LSMeans Student's t technique for LSD was used. Prior to statistical analysis, % parameter values underwent an arcsin transformation.

Results & Discussion

Sowing Seeds and Agar Dose Trial

On MS nutritional media with different concentrations of solidifying agar, similar results were achieved when comparing the daily growth data of purple and orange carrot genotypes from carrot seeds (Table 3).

While germination was shown to begin on the third day, it was found that 50% of germination and root production began on the fourth day, in contrast to the MS nutritional media that was solidified with 6 g, 8 g and 15 g agar. In nutritional media containing 7 g/L agar, cotyledon development began on the sixth day, but genuine leaf creation wasn't seen until the thirteenth day. When the development period was monitored, it was found that the purple carrot genotype responded faster to the nutritional medium consolidated with 7 g/L agar in terms of germination and days, even if there was no variation in plant growth.

Table 3. Various aspects affect the daily variation of plants developed from purple and orange carrot seeds

Carrot varieties	Agar doses (g/L)	First germination	50% germination	Root formation	Cotyledon formation	Persistent leaf formation
Purple	6	4	5	6	7	15
	7	4	6	7	8	19
	8	3	5	5	7	16
	15	4	6	4	6	19
Orange	6	4	5	6	8	16
	7	4	5	5	7	15
	8	4	6	6	9	19
	15	5	7	7	10	21

When the orange carrot cultivar's daily growth on MS nutrient media containing 7 g/L agar was investigated, root formation began on the fourth day, followed by cotyledon formation on the seventh day. It was discovered that the earliest nutritional medium for the orange carrot cultivar was solidified with 7 g/L agar when real leaf production began on the 15th day. The germination rate of purple carrot seeds on the seventh and 14th days and their response to the medium at different agar concentrations were statistically evaluated.

The differences between the averages for all parameters were found to be statistically significant at the level of 0.05 when the statistical evaluation of the germination percentage rates of native purple carrot seeds on the seventh and 14th days in MS basic nutrient media solidified with different amounts of agar was examined (Table 4).

Table 4. The germination rate of purple carrot seeds on the seventh and 14th days and their response to different agar concentrations

Agar Doses (g/L)	Time (days)	Percentage of germination (agar x time)	Average germination percentage (agar X time)
6	7	54.26 ^{cd}	59.07 ^{a}
	14	63.88 ^{ab}	
7	7	55.31 ^{c}	62.51 ^{a}
	14	69.71 ^{a}	
8	7	49.35 ^{cd}	56.98 ^{ab}
	14	64.61 ^{ab}	
15	7	46.33 ^{d}	51.65 ^{b}
	14	56.98 ^{bc}	
LSD		*8.14*	*5.76*
Cv %		15.86	15.86
		*P<0.05	*P<0.05

The seed germination percentage on the 14th day was determined to be the greatest in the nutritional media solidified with 7 g agar, with an average of 69.71 based on the findings of the interaction between the agar dose and the germination period. With a germination rate of 59.07% in the nutritional medium containing 6g/L agar and 62.51% in the nutrient medium containing 7 g/L agar, the average germination rate according to the nutrient medium was statistically in the same group.

Statistics were used to assess the orange carrot seeds' germination rate on the seventh and 14th days as well as how they responded to the medium at various agar concentrations (Table 5).

Table 5. Orange carrot seed germination rates on various agar combinations of nutritional media

Agar Doses (g/L)	Time (days)	Percentage of germination (agar x time)	Averagegerminati on percentage (agar X time)
6	7	50.35	57.15 ^{ab}
	14	63.94	
7	7	55.80	61.25 ^{a}
	14	66.69	
8	7	49.09	56.85 ^{ab}
	14	64.61	
15	7	45.64	53.35 ^{b}
	14	61.05	
LSD		*N.S*	*4.34*
Cv%		12.05	12.05
		P>0.05	*P<0.05

In MS basic nutritional medium, the variations between the seed germination % and averages on the seventh and fourteenth days in terms of the agar content and time parameters were not determined to be statistically significant. However, the maximum germination rate was 66.69% on nutritional media containing 7 g/L agar on day 14. Although the combined influence of nutrient media and time parameters on seed germination averages was determined to be statistically significant at the 0.05 level, the seed germination percentage on the 14th day was the greatest with an average of 61.25 in the nutrient medium solidified with 7 g/L agar. In nutrient media containing 7 g/L agar, root, cotyledon, and true leaf formations take 5, 7, and 15 days, respectively, to form in the case of orange carrot genotypes. Purple carrot genotypes, on the other hand, showed significantly faster plant growth on a daily basis than the other agar concentrations, taking place in 4, 6, and 13 days. In light of all the findings, the nutrient media in the subsequent investigation were solidified using 7 g/L agar.

The effects of various concentrations of activated carbon and jasmonic acid on seed germination, shoot, cotyledon, and first leaf development of carrot plants were examined under in vitro nutritional conditions by Ozsan et al., (2020) in their study on orange and purple carrots. As a result, it was discovered that both species' in vitro-grown seeds began to enlarge on the second day in the control medium. In contrast to orange carrots, where the formation of the epicotyl was discovered on the eighth day and real leaves were seen on the 10th day, the first cotyledon leaves appeared on the fourth day and the cotyledons on the sixth day. At the earliest, true leaf development took place after 10 days. The greatest germination rates were found to be 86.7% in orange carrots and 83.2% in purple carrots in the same study's analysis of seed germination rates.

Callus Obtaining Studies

Cotyledon and hypocotyl explants of purple and orange carrots were cultivated in 3 different concentrations of MS nutritional medium containing different ratios of kinetin for the measurement and optimization of the callus growth medium (Table 6).

Table 6. Purple carrot callus formation percentages at various nutritional medium concentrations

Auxin/Cytokinin dose	Explant	Time (Week)	Callus percentage
1 mg/L 2,4-D	Hypocotyl	2	39.783 de
		4	47.307 b-e
	Cotyledon	2	33.750 e
		4	36.217 e
1 mg/L 2,4-D+0.1 Kinetin	Hypocotyl	2	43.269 b-e
		4	62.918 a
	Cotyledon	2	33.625 e
		4	49.690 bc
1 mg/L 2,4-D+0.5 Kinetin	Hypocotyl	2	54.041 ab
		4	65.209 a
	Cotyledon	2	39.674 de
		4	53.883 ab
LSD	*(dose x explant x time)*		*12.34***
Cv%		23	**P <0.01

By using statistical analysis of variance, it was possible to compare the percentages of callus development of the hypocotyl and cotyledon explants of the cultured purple carrot genotype at various concentrations and times. The interaction of nutritional medium, explant, and time was shown to be significant at the 0.01 level when the callus formation in the purple carrot explants put in the callus growth media was studied. The best callus development rate, 65.19%, was found in hypocotyl explants at the end of the fourth week (second subculture) in MS media with 1 mg/L 2,4-D + 0.5 mg/L kinetin. In the same group, 62.918% of calluses formed from hypocotyl explants cultivated in MS media containing 1 mg/L 2,4-D + 0.1 mg/L kinetin. An average of 54.041% callus was seen in the second week hypocotyl explants in MS nutritional medium with 1 mg/L 2,4-D + 0.5 mg/L kinetin when the results were evaluated. The average callus density measured from the fourth week cotyledon explants was 53.883%, and variance analysis revealed that these two values belonged to the same group. The outcome was in a separate group even though 49.690 ratios of cotyledon explants were obtained in MS nutritional medium with 1 mg/L 2,4-D + 0.1 mg/L kinetin. The lowest values in the table are for the cotyledon in MS nutrient medium containing 1 mg/L 2,4-D + 0.1 mg/L kinetin, with average rates of 36.217% in the fourth week and 33.750% in the second week obtained from cotyledon explants in MS nutrient medium containing 1 mg/L 2,4-D callus formation, with an average rate of 33.625% obtained from the explants in the second week.

The interaction of the average % of callus formed in different weeks and in the nutrient medium containing different auxin/cytokinin doses in orange carrots was found to be significant, and the differences between them were statistically compared and analyzed for variance (Table 7).

Table 7. Orange carrot callus formation percentages at various nutritional medium concentrations

Auxin/Cytokinin dose	Time (Week)	Callus percentage
1 mg/L 2,4-D	2	42.366 [d]
	4	60.127 [b]
1 mg/L 2,4-D + 0.1 kinetin	2	38.484 [e]
	4	58.498 [b]
1 mg/L 2,4-D+0.5 kinetin	2	49.464 [c]
	4	74.507 [a]
LSD	*(dose x time)*	*3.75*
Cv%	8.42	**P <0.01

It was determined that the difference between the findings was statistically significant at the 0.01 level when the percentage of callus produced in orange carrots according to the second and fourth week data at different nutritional medium concentrations were evaluated. The rate of 74.507 was found to produce the greatest results in MS nutritional medium, which contained 1 mg/L 2,4 D+0.5 mg/L kinetin at the fourth week.

The average callus percentages of 60.127% and 58.498% obtained at the end of the fourth week in MS nutritional media containing 1 mg/L 2,4 D and 1 mg/L 2,4 D + 0.1 mg/L kinetin were the next highest values, while the analysis of variance was the next highest. The results showed that in the second week of MS nutritional medium with 1 mg/L 2,4 D + 0.5 mg/L kinetin, the average per callus formation was 49.464%. The callus formation rate was 42.366% in the second week of the MS nutritional medium containing 1 mg/L 2,4 D, and it was 42.366 percent in the second week of the MS nutrient medium containing 1 mg/L 2,4 D + 0.1 mg/L kinetin. However, in the second week of MS nutritional medium with 1 mg/L 2,4 D + 0.1 mg/L kinetin, an average of 38.484% callus values was observed, and the findings were statistically different in different groups.

The average callus percentages were compared in range carrots in order to determine the importance of the interactions between various nutritional media and explants (Table 8).

Table 8. Average callus percentages of orange carrots at the end of the fourth week consisting of different explants in different nutrient media

Auxin/Cytokinin dose	Explant	Callus percentage
1 mg/L 2,4 D	Hypocotyl	55.957 [b]
	Cotyledon	46.536 [d]
1 mg/L 2,4 D + 0.1 kinetin	Hypocotyl	54.409 [b]
	Cotyledon	42.573 [e]
1 mg/L 2,4 D + 0.5 kinetin	Hypocotyl	72.986 [a]
	Cotyledon	50.409 [c]
LSD	*(Dose x explant)*	*3.75*
		**P <0.01

The statistical comparison of the change in the callus formation percentage for various nutrient media and explants revealed that the differences between the averages were significant at the 0.01 level. The callus formation rate obtained from hypocotyl explants in MS nutrient medium containing 1 mg/L 2,4-D + 0.5 mg/L kinetin had the highest value, which was 72.986%. According to the analysis's findings, while the rate of callus formation from hypocotyl explants in MS nutrient medium containing 1 mg/L 2,4-D was 55.957%, 54.409% of callus formed in the same group in MS nutrient medium containing 1 mg/L 2,4-D + 0.1 mg/L kinetin. The following group had an average of 50,409%, which was derived from cotyledon explants in MS nutrient medium that contained 1 mg/L 2,4-D + 0.5 kinetin. The lowest callus formation rates in the table were found to be 46.536% for the MS medium containing 1 mg/L 2,4-D and 42.573% for MS medium containing 1 mg/L 2,4-D + 0.1 mg/L kinetin. These values were statistically in different groups.

The average callus weights of the petri dishes were calculated from callus tissues consisting of 10 orange and 10 purple carrot hypocotyl explants cultured in 10 petri dishes and in MS nutrient media with 3 different plant nutrient regulator concentrations. Variance analysis was used to determine the effect of callus weight on various carrot cultivars and nutrient media concentrations, and it was discovered that the differences between the averages were significant at the 0.05 level (Table 9).

It was determined that the best nutrient medium for callus formation was MS nutrient medium containing 1 mg/L 2,4-D + 0.5 mg/L kinetin after taking into account the results of hormone dose and variety interaction.

When the amount of callus formation in this nutrient medium was compared between genotypes, purple carrots averaged 931.28 g callus weight, while orange carrots averaged 933.16 g callus weight, and the values were statistically in the same group. Purple carrot and orange carrot genotypes in MS nutrient medium with 1 mg/L 2,4-D had the lowest ratio, with average callus weights of 496 g and 558.92 g, respectively.

Table 9. Average weights of the calluses on purple and orange carrots in various nutrient media

Auxin/Cytokinin dose	Variety	Average Weight of Callus (g)
1 mg/L 2,4-D	Purple	496.00 [e]
	Orange	558.92[d]
1 mg/L 2,4-D + 0.1 mg/L kinetin	Purple	698.26 [c]
	Orange	761.58 [b]
1 mg/L 2,4-D + 0.5 mg/L kinetin	Purple	931.28 [a]
	Orange	933.16 [a]
LSD	(*Dose x variety*)	*27.34*
Cv%	4.02	*P<0.05

These findings showed that callus formation is stimulated by the addition of auxin (2,4-D) and cytokinin (kinetin) to the nutrient medium for callus development.

The local purple carrot cultivar produced an average of 708.51 g and the Nantes orange carrot cultivar produced an average of 751.22 g callus when the averages of the different carrot cultivars were compared. The differences between the averages were statistically significant.

With these findings, the effect of genotype on regeneration capacity was demonstrated once more. Murashige and Skoog medium worked well as a nutrient medium to help carrot explants develop callus when 2,4-D was added to the medium.

The success of these two factors in promoting callus formation in carrots has been demonstrated in the past (Dodds & Roberts, 1982). There are those who use 2,4-D at doses as high as 2 mg/L (Bradley et al., 1984), even though 1.0 mg/L is the usual dosage (Torres, 1989; George & Sherrington, 1984).

Although there was no discernible difference in callus weights between the use of 0.5 mg/L and 1.0 mg/L kinetin, the callus formed at 0.5 mg/L was initially preferred because it appeared to be more easily dispersed and better suited for suspension culture.

Hormone-free medium was used to ensure plant growth from calluses. Plant growth was hampered by the addition of 2,4-D or kinetin to the nutritional medium, even at low concentrations. A modest rate of shoot differentiation (21.0%) was seen in MS medium supplemented with only 0.2 mg/L kinetin, but these forms could not produce a healthy and full plant.

While 2,4-D continues to promote callus development, kinetin enhanced the callus' greenish hue but did not significantly improve callus development. Kinetin and Benzil Adenin (BA) have been shown to impede development in callus culture (Fujimura & Komamine, 1975).

Plant regeneration medium from the callus

For plant regeneration from purple and orange carrot calli, which were developed in nutrient medium with 1 mg/L 2,4-D and 0.5 mg/L kinetin, subcultures were made in MS nutrient medium with 0.1 mg/L kinetin or 0.2 mg/L TDZ. From the 10th day of culture, purple and orange carrot calluses showed differentiation. The plantlets that had grown from the calli at the end of the fourth week were counted, and the ideal medium combination for plant regeneration was identified (Figure 1.).

Figure 1. Plantlets regenerated from orange and purple callus tissues.

The amount and duration of external auxin application, as well as the nitrogen compositions in the nutrient medium, were discovered to be the most important chemical factors in the study for stimulating somatic embryogenesis. It is also reported that the presence of auxin, which stimulates the formation of embryogenic cells, must decrease to very low levels after this stage, and that the decrease in the amount of nitrogen stimulates the embryogenesis event (Reinert, 1973). In fact, taking cells into auxin-free environments had a positive impact on embryonic development in our study.

When purple and orange carrot genotypes were cultured in MS nutrient media containing various plant growth regulators and without plant growth regulators for plant regeneration, the average number of plants that were regenerated from callus tissues was evaluated statistically. The differences between the averages were found to be significant at the 0.05 level (Table 10).

Table 10. Number of the plants regenerated from calli of carrots

Variety	**Purple**		
Nutrient mediums	MS	MS+0.1 mg/L kinetin	MS+0.2 mg/L TDZ
Average	11.16 ^{b}	14.76 ^{a}	16.68 ^{a}
LSD	2.38		
Cv%	11.52		
	*P<0.05		
Variety	**Orange**		
Average	6.96 ^{b}	7.76 ^{ab}	8.76 ^{a}
LSD	*1.37**		
Cv%	12.09		
	*P <0.05		

According to statistics, the MS nutrient medium with 0.2 mg/L TDZ had the highest average growth, with 16.68 plants that grow on average from the purple carrot genotype's callus tissues. In MS nutrient medium containing 0.1 mg/L kinetin, an average of 14.76 plants were able to regenerate, but statistically, this value was in the same group as MS nutrient medium containing 0.2 mg/L TDZ. In MS medium without a plant growth regulator, an average of 11.16 plants recovered, but the value defined a statistically distinct group.

When plant regeneration from orange carrot callus tissues was evaluated for the number of plants developed, MS nutrient medium containing 0.2 mg/L TDZ emerged as the dominant nutrient medium, producing an average of 8.76 plants. The average number of regenerated plants obtained from MS basic nutrient medium was 7.76, followed by MS nutrient medium containing 0.1 mg/L kinetin, which produced the fewest average plants at 6.96.

Plant regeneration studies in suspension culture

Explants taken from three-week-old purple and orange carrot seedlings' hypocotyls and cotyledons were cultured for the formation of calluses in nutrient media containing various concentrations of 2,4-D and kinetin.

The dispersed calli were placed in nutritional media prepared for suspension culture in a nutrient medium combination comprising MS + 0.1 mg/L kinetin. The purple and orange carrot calli that had formed in the chosen nutrient medium combination containing 1 mg/L 2,4-D were brought into culture.

10 purple and 10 orange carrot calluses were made into suspension cultures and placed in a sterile nutritional medium. The shaker's purple carrot calluses began to differentiate at the end of the second week, and the third week saw the regrowth of the plants. The dispersed calli were subcultured in MS broth medium without a plant growth regulator after three weeks. Herdem, (1998) demonstrated somatic embryogenesis in hormone-free nutritional media solidified with 1% agar following culturing in liquid medium twice, once with 1 mg/L 2,4-D and once without auxin, in reference experiments. Similarly, it has been observed that cells must first recover from the effects of auxin in hormone-free liquid medium before being moved to agar media in order to achieve somatic embryogenesis (Torres, 1989, Karataş, 2013).

In our lab settings, with the optimization study carried out in the light of studies on carrot varieties, the ideal composition of the nutrient medium and the process to use were identified. Orange carrots of the Nantes variety and local purple carrots were used to create new plants in *vitro* using callus and cell suspension culture techniques.

Conclusion

In this publication, which is the optimization phase of a doctoral thesis, the best media and growth combinations in callus and suspension cultures were determined, taking into account the genotypic differences of orange and purple carrots. Purple carrots were used to produce plant regeneration from tissues exposed to various gamma rays by callus mutagenesis, and by calculating the effective radiation dosage, it was feasible to identify salt stress-tolerant mutants in this type of carrot. After that, the technique was prepared for use in breeding studies.

The next stage will include collecting seeds from salt-tolerant carrot plants to investigate if there is a segregation in the progeny with regard to this trait. The calli samples from this investigation are also still being replicated. By regenerating the plant and adding various biotic or abiotic stress factors at this stage, mutations at the cellular level can be revealed. This approach is believed to be a strategy that may be tested in the breeding of resistance to different abiotic environments, diseases, pests, and stress situations. It is well known that mycoplasma illnesses in carrots have recently started to spread throughout our nation. There is currently no known resistance to these illnesses. A fairly efficient method for producing genetic variety for this kind of resistance is mutation breeding. As a result, our nation has optimized a method that is crucial for utilizing the advantages of biotechnological advances in the carrot industry.

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BIOTECH STUDIES

REVIEWPAPER

Obtaining haploid plants by irradiated pollen culture in oil seed crops

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Abstract

Haploid plant production is of great importance to shorten the breeding period in plant breeding programs. Obtaining pure lines in plant growing programs require an intensive work with huge labor and time. Obtaining one hundred percent homozygous pure lines is a key point for the improvement and development of new cultivars. Haploid plants with a single set of homozygous chromosomes have become a valuable tool in plant breeding. Dihaploid plants that are homozygous at all loci with doubling of their chromosomes can be propagated by seed and reach full homozygosity in a single generation. Traditional methods take seven years to reach homozygosity. Dihaploidization methods provide significant advantages in terms of gaining homozygosity in a short period of one year and bringing pure lines into agriculture. Anther culture and irradiated pollen technique are among the most widely used techniques in this respect; where physical or chemical agents are used to induce mutated pollen grains and anthers that are subsequently employed to develop dihaploids through *in vitro* cultures. These techniques are a good source to facilitate gene mapping, cytogenetic research, and evolutionary studies. Irradiated pollen culture techniques have been applied to many oilseed crops to obtain pure lines. This study highlights some salient features of producing dihaploids using irradiated pollen grains and their maintenance.

Introduction

There is a deficit in production and demand of vegetable oil, therefore, Türkiye spends a large amount of budget to import vegetable oil to meet the demand for increased vegetable oil and products (Arıoğlu et al., 2020). In addition to increasing the current cultivation areas, improving cultural techniques, and preferring high-yielding varieties can be good solutions to increase yield (Şanver & Göksoy, 2019).

It is essential to breed varieties suitable for changing climatic conditions especially to cold, heat, and drought stresses, which are the main constraints that limit the yield of many crops. Haploid plant studies were reported first by Blakeslee et al. (1922) and have since become an important topic for researchers, especially in plant breeding. Haploid plants can be obtained by *in vitro* culture of anthers or isolated microspores (androgenesis), ovules (gynogenesis) or *in vitro* rescue of parthenogenetic embryos induced by *in situ* pollination with irradiated pollen (parthenogenesis) (Gonzalo et al., 2011). It can occur spontaneously in nature and can also be induced in different ways. Irradiation of pollen grains and anthers is one method among them.

Initial research aimed to evaluate the effects of radiation on pollen germination and pollen tube growth. Sestili and Ficcadenti (1996) has reported that radiation could also be used to render pollen generatively ineffective without affecting the ability to stimulate egg cells, thus allowing formation of parthenogenic embryos.

The leading plants from which vegetable oil is obtained include soybean, sunflower, cottonseed (cotton), rapeseed, peanut, sesame, safflower, castor oil, poppy, flax, hemp, jojoba, corn (from corn germ), olive, date palm, and coconut. Most of them except jojoba,

date palm, and coconut can be successfully cultivated in Türkiye with their high adaptation capabilities. The development of fast and effective methods and their adaptation to the agronomic process will make a great economic contribution to Turkish vegetable seed oil industry (Arıoğlu et al., 2010).

Haploid methods used in oilseed plants

Haploid and double haploid methods is desired by plant breeders in order to shorten the process of obtaining and developing new varieties. Double haploid plants are homozygous at each locus. They can be used as parents to grow F1 hybrids. Many studies have successfully obtained haploid plants in different plant species. The induction of maternal haploid embryos by pollination with irradiated pollen has been successfully used in many species (Grouh et al., 2017; Kurtar et al., 2020; Shahhosseini, 2022; Zhao et al., 2022).

Due to the successful use of haploid and doubled haploid technology in maize, the technique has also been investigated and developed for various oilseed crops with varying success rates. There has been limited success in soybeans. Several successful *in vitro* anther culture experiments have been conducted to produce haploid plants in soybean but are not commercially sufficient and viable (Lulsdorf et al., 2011).

Two methods, 'maternal haploid' and 'paternal haploid', are used to obtain haploid plants by *in vivo* technique. The method of using the reducing line as the pollen donor, i.e., the father, is called paternal haploid, and the method of using the reducing line as the pollen receiver, i.e., the mother, is called maternal haploid (Yorgancılar et al., 2019). Maternal induction is the most widely used and preferred method in modern maize breeding programs due to its higher efficiency and more reliable source of stable inducer lines than paternal induction. In a program to develop soybean doubled haploid lines using *in vivo* haploid induction, maternal induction would be the preferred method due to the possibility of using male sterile lines (Friederich, 2020).

Aktaş (2018) aimed to develop a haploidization procedure in his study with sunflower (*Helianthus annuus* L.). Therefore, the researcher used irradiated pollen technique as an alternative to the previously studied anther and microspore cultures. 16 dissimilar sunflower breeding lines were used as experimental material. Results showed that the effectiveness of the method is highly dependent on the genotype of the recipient lines.

Studies have shown that it is possible to use irradiated pollen to produce double haploids through parthenogenesis (Aktaş et al., 2018; Bidney & Scelonge, 1997; Kaya, 2004; Todorova et al., 1997).

Sesame (*Sesamum indicum* L.) is one of the most important oil crops. Gamma irradiation is a reasonable tool to induce variability in sesame and is advantageous in increasing pollen viability (Audu et al., 2021; Ryu, Doo & Kim, 1992).

Haploid studies for the variety development in cotton breeding studies are helpful to shorten breeding period and providing economic gains (Bajaj & Gill, 1997; Korkunç et al., 2017).

Brassica napus L. is one of the most important oil crops in the world. It is more prone to tissue culture studies than other Brassicaceae. Mutants with high oil content and high oleic acid, low linolenic and erucic acid contents were obtained through mutations and microspore culture (Ahmad, Macdonald & Ingram, 1991; Fletcher et al., 1998; Kučera et al., 2002; Seyis et al., 2014).

Obtaining haploid with irradiated pollen

Obtaining a sufficient number of haploids in breeding programs has been among the objectives of plant breeders. Interspecific crosses, delayed pollination, temperature shocks, use of irradiated pollen, use of chemicals, and plant growth regulators have been tried (Dwivedi et al., 2015; Puolimatka & Pauk 2000; Zheng et al., 2001).

Errors that occur during DNA replication cause mutation and then natural radiation in the environment triggers this mutation that causes a change in the hereditary material. The individuals modified as a result of this natural mutation is known as a spontaneous mutant. With the discovery of ionizing (X and γ-rays) and non-ionizing (UV) radiation, the mutations can also be realized artificially (Spencer-Lopes et al., 2018).

Various types of radiations are available for plant mutation. Gamma rays, which induce disruption of the unstable nucleus of an atom, are generally preferred and widely used. They have a shorter wavelength and therefore have more energy compared to x-rays. They can also be placed in a climate chamber, greenhouse, or field with the advantage of using them at dissimilar stages of plant development (Spencer-Lopes et al., 2018). Cobalt-60 (^{60}Co) and Cesium-137 (^{137}Cs) isotopes are the main sources of gamma rays (Lerouge & Simmons, 2012).

Pollen irradiation can give plants new positive or negative traits by making some hereditary changes in the structure and number of chromosomes or the physical and chemical structures of their genes (Yaman, 2014). The success of the irradiated pollen technique depends on the genotype of the plant, the physiological stage of the female plant, culture conditions, ambient compositions, and radiation dose (German, 2011).

The stages of obtaining haploid plants with irradiated pollen are as follows;

i. Cultivation of the donor plant: The oil crops are grown in fields or greenhouses.
ii. Emasculations: Removal of anthers before anthesis to prevent self-pollination. Then they are covered.
iii. Pollen collection and irradiation: Pollen at the appropriate stage is collected and irradiated at different doses.

iv. Emasculated irradiated pollen given to the plant: Pollens irradiated at different doses are given to the emasculated plant and covered.
v. Embryo rescue: 12-20 days after pollination, the trays are collected and brought to the laboratory environment and the immature embryos are regenerated to obtain plants.
vi. Haploid plant regeneration and ploidy: Haploid plants regenerated from embryos are grown under climatic chamber conditions.
vii. Ploidy level determination: Ploidy analyzer or flow cytometry devices are used to determine ploidy levels.
viii. Colchicine treatment: Different doses of colchicine are applied to plantlets that have completed root formation.
ix. Doubled haploid plants: Colchicine applied plantlets are evaluated morphologically and cytologically. Necessary maintenance procedures are carried out in the greenhouse for those which are found to be double haploid.

Gamma radiation in plant breeding

The induction of mutations through physical agents allows the acquisition of genetic variations of agricultural importance that are not found in plants existing in nature (Pérez-Jiménez et al., 2020). The formation of free radicals, induced by the exchange promotes structural and metabolic changes in the plant. For example, 50 Gy gamma radiation produced a sensitizing effect on chloroplasts. Moreover, high Gy doses affect protein synthesis, hormonal balance, enzymatic activity, gas, and water exchange (Riviello-Flores et al., 2022). Nowadays, new variations are needed to develop more nutritious, resistant, and productive varieties (Amri-Tiliouine et al., 2018; Shuryak et al., 2019). Obtaining these variations in a controlled manner in a short time has enabled the rapid development of breeding studies.

Gamma-ray is the most efficient source for irradiation. Its simple application, good penetration, reproducibility, high mutation frequency, and low lethality problems are of great advantage (Chahal & Gosal, 2002). The most widely used gamma-ray sources are Cobalt-60 (^{60}Co) and Cesium137 (^{137}Cs). Gamma-irradiated pollen can germinate on the stigma, grow, and reach the embryo sac. Although it cannot fertilize the egg cell and polar nuclei, it stimulates the development of haploid embryos (Blasco et al., 2016). This method requires immature embryo rescue under *in vitro* conditions.

Radiation dose and source

Radiation causes changes in the generative nucleus of sperm cells that induce parthenogenetic development of the egg to form haploid embryos (Dal et al., 2016). Beneficial mutations that can be induced by physical mutagenic agents such as ionizing radiation (X-rays and gamma rays), non-ionizing radiation (ultraviolet) and corpuscular radiation (protons, neutrons, alpha and beta particles) are the changes in genotypic structure that increase the variability of species and facilitate their adaptation to various selection pressures. These sources have a 94% effect on the generation of mutant types (Al-Safadi & Simon, 1990; Ludovici et al., 2020; Spencer-Lopes et al., 2018).

Although seeds or whole plants can be irradiated, pollen irradiation has advantages such as the rare formation of chimeras and homozygous plants. The disadvantages are the difficulty of obtaining material and the duration of viability. Efficient radiation doses depend on pollen type. In many practical cases, the absorbed dose is not measured directly but is calculated from the measured number of ions produced in the air by ionizing radiation (Spencer-Lopes et al., 2018).

Plants are affected by physical and chemical mutagens to varying degrees. One of the most important factors for successful mutation breeding and achieving the goal of the study is the determination of the optimum mutagen dose. The dose required for a given experiment depends on the desired return, but mutagenic treatment can also have undesirable consequences, such as death and infertility. The genotype of the selected material can also alter the susceptibility to mutagenic treatments (Kundu et al., 2014, Kundu et al., 2016). Lower doses are generally preferred, as dose increases can lead to undesirable severe mutations. Therefore, preliminary analyses are required to determine the appropriate doses for each plant material.

Pollen irradiation

Artificial mutation of plant pollen using ionizing radiation (protons, neutrons, alpha, beta, gamma, and x-rays) causes chemical changes in the plant. It acts together with molecules and atoms on the production of free radicals in the cell. These radicals cause changes in the physiology, biochemistry, anatomy, and morphology of plants depending on the radiation levels. The amount of moisture in the material is also important for the effect of radiation on DNA. Radiation causes random ionization and excitation events in the environment it passes through.

In practice, male flowers are collected one day before flowering in plants grown under controlled conditions in the greenhouse or the open field, hermaphrodite flowers (emasculated) are castrated and closed to prevent uncontrolled pollination. Afterward, the petals of the male flower are removed, the pollen grains are collected and irradiated with gamma-ray doses (250, 350, 450, and 550 Gy) determined according to the plant using a radiation source (e.g.,^{60}Co) and then stored at room temperature overnight and the next day the female flowers are pollinated using gamma-irradiated pollen.

Irradiated pollen surviving test

Sensitivity tests must be performed to determine the mutagen dose. Determining the most effective and

efficient mutagen dose (RD50, EMD) is a prerequisite for success. Irradiation dose, irradiation duration, pollen age, and genotypes can affect pollen viability. The viability, which indicates the quality characteristics of irradiated pollen, varies according to genotype, and is highly affected by the gamma-ray dose applied (Hayati et al., 2022).

The viability of pollen kept at room temperature and 50% humidity decreases rapidly (Giovannini et al., 2017). Therefore, irradiated pollen should be stored under cold conditions. In order to measure the response of irradiated pollen to irradiation and to determine the effect of different doses, it is important to determine the appropriate dose by pollen viability tests (Kurtar et al., 2020). Different pollen viability tests are performed to determine viability percentages.

2,3,5, triphenyl tetrazolium chloride (TTC) (1%) solution is preferred to determine pollen viability. A viability test should be performed before aceto-orcein staining, since this may stain all pollen. Pollen viability is separated according to pollen character (viable, semi-viable, non-viable). After dropping 1 drop of the prepared solutions on the slide, the pollens are sprinkled on this drop covered with a coverslip, holding it at room temperature for 4-6 hours or more to before observing them under a light microscope. In the preparations examined under the microscope, the dark-stained, light and non-stained pollens on slides could be identified as living, semi-living and non-living in the same order (Özer, 2016; Stanley & Linskens, 1974).

In the iodized potassium iodide (IKI) method, 1 g of potassium iodide and 0.5 g of iodine are dissolved in 100 mL of distilled water for the IKI solution. Pollen viability counts are made five minutes after pollen is placed in a solution of IKI. Pollen grains with dark spots (dark red or brown color) are considered alive (Sulusoglu & Cavusoglu, 2014).

In vivo pollination

Pollen and floral biology of the plants to be used in the studies should be known for inducing better and improved conditions for their use in breeding programs. Emasculated flowers are covered with a cloth bag to eliminate the risk of contamination before pollination with irradiated pollen. Emasculation is carried out one day before the separation of anthers. After irradiation, pollen kept at +4℃ is released early the next morning to ensure pollination in field conditions. The female flowers are then isolated again with cloth bags to prevent unwanted pollen contamination.

To promote fruit or seed development after pollination with irradiated pollen, it has been proposed to apply growth regulators to the calyx of pollinated flowers; however, this may induce parthenocarpy rather than haploidy (Sestili & Ficcadenti, 1996). *In vitro* culture is necessary to rescue haploid plants in many cases. Embryos formed after pollination are isolated and regenerated under *in vitro* conditions on dissimilar nutrient media pre-optimized according to the genotypes.

Embryo rescue and chromosome doublin

Gamma-irradiated pollen can germinate on the stigma, grow along the style, and reach the embryo sac. Although it cannot fertilize the egg cell and polar nuclei, it stimulates the development of haploid embryos (Musial & Pzrywara, 1998). This method requires immature embryo rescue under *in vitro* conditions (Blasco et al., 2016).

To preserve the immature embryos formed in the flowers of plants pollinated with irradiated pollen, field controls must be carried out. The application of this method starts with the castration of the donor plant. Pollen is collected from the donor plant and exposed to the appropriate dose of irradiation. Embryo maturation time varies according to species (Shu et al., 2011). This timing occurs 10-15 days after pollination depending on the climate.

During this process, the embryos are removed, placed in the most suitable nutrient medium for regeneration under *in vitro* conditions and allowed to develop. The most important issue in plant development and rooting is determining the ploidy level. Some plants may exhibit spontaneous folding.

Knowing the ploidy level of living plants is very important for the effective use of genetic resources. Ploidy level in a plant greatly affects the performance of the plant (Sakiroglu & Kaya, 2012). Two methods are generally used to determine the ploidy level in plants. The first of these methods is to determine the number of chromosomes with the help of a microscope and the other is the flow cytometry method. However, it is a significant disadvantage that the processes are long and laborious. Moreover, it is known that it is not a very efficient method as it will increase the possibility of error for plants with small chromosomes (Wanner et.al., 1991). Flow cytometry (FCM) has become a convenient and useful tool for determining ploidy levels in plant breeding. It is a device and technique for quick and reliable measurement of cells or other biological particles for physical or chemical properties in a liquid stream. By using fluorescent dyes that bind to the structure of DNA in this technique, usable information is created by transmitting different wavelengths of the device to the system by laser radiation. Propidium iodide, ethidium bromide and acridine orange are fluorescent dyes commonly used in flow cytometry (Bohanec, 2003; Demirel, 1995; Demirel et.al. 2019).

Aktaş (2018) used the irradiated pollen technique in this study and obtained haploid plants with embryo recovery. He reported spontaneously formed six haploids in his study.

In order for haploid plants to attach seeds and continue their generation, chromosome numbers must be multiplied with the help of chemicals such as hexachlorocyclohexane, acenaphthene, chloral hydrate, ethyl mercuricloride, nitrogen perotoxide, colchicine, caffeine, sulfinilamide. After the chromosomes of the plants are folded, they are transferred to the greenhouses for acclimatization.

Conclusion

Developing tolerance to increased stress in plants is crucial for the development of the future agricultural sector and to reduce the risk. As in other plants, a number of physiological, biochemical, and molecular mechanisms are involved in the development of tolerance to various stresses in oil crops.

With the use of effective scientific and analytical approaches in agriculture, the number of faster and more effective breeding studies will increase in the future. Climate change, pollution, changing natural areas, population growth, and intensive agricultural practices increase the importance of agricultural production and biodiversity. The decrease in biodiversity brings along many environmental and economic problems. With the development of biotechnology, efforts to increase our agricultural resources and quality should continue rapidly. Biotechnological approaches that can be used to increase the effectiveness of breeding programs increase the effectiveness of the studies and ensure the growth and sustainability of the agricultural economy.

To meet the increasing population demand, mutation breeding can be considered a good option to modify existing superior varieties.

Gamma irradiation, an irradiation method used in combination with haploid techniques as a tool for plant breeding, offers several opportunities in agricultural and food applications. If irradiation technology is used in conjunction with targeted biotechnological methods, it has a potential to play an important role in the accelerated breeding.

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BIOTECH STUDIES

RESEARCH PAPER

Re-visiting lactate dehydrogenase from a different dimension: a model bioinformatics study for wrestling

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Abstract

Sports bioinformatics is of great importance in the understanding of sports performance from different perspectives. Accumulated bio-sequences in databases provide considerable contributions to compare proteins in different organisms. In Kingdom of Animalia, some animals have experienced evolution for excellent athletic performances in nature. The present paper exhibits a model *in silico* approach for the evaluation of sports performance by comparing lactate dehydrogenases (LDH) in humans (*Homo sapiens)* and saltwater crocodiles (*Crocodylus porosus)*. The results show that a high sequence similarity is observed between the LDHs from *H. sapiens* and *C. porosus* with minor modifications. The stability and grand averages of hydrophobicity index values for studied LDHs were found as 24.79–25.18 and -0.006 –0.020 in *H. sapiens* and *C. porosus*, respectively. In conclusion, the identification of amino acid modifications in important enzymes of specific animals that are related to sports physiology are lessons we learn from from nature, which can open a new gate for the development of sports performance and talent selection.

Introduction

Lactate dehydrogenase (LDH, EC:1.1.1.27) is an important housekeeping enzyme in human metabolism. The main role of the enzyme is to catalyse the oxidation-reduction reaction between pyruvate and lactate. In anaerobic conditions, glycolysis should be continued in performance requiring activities. However, depleted nicotinamide adenine dinucleotide (NAD^+) levels must be replenished in muscle cells. LDH catalyses the conversion of pyruvate into lactate. In this reaction, while pyruvate is reduced into lactate, $NADH+H^+$ is oxidised into NAD^+. The formed NAD^+ provides glycolysis to continue in human metabolism under anaerobic conditions (Voet & Voet, 2004). After the completion of the genome project in 2000, biosequence based data has increased in various data banks such as Uniprot and PDB (Berman et al., 2000; The Uniprot Consortium, 2021). Comparison of the data from different organisms may provide important information in different areas. As an example, the sport "wrestling" resembles many natural events in nature. The crocodiles should grab their prey very fast and then they should show an excellent performance within minutes which is based on rapid rotation about the longitudinal axis of the body (Fish et al., 2007). Similarly, in wrestling, the athletes should also exhibit enormous performance in a limited time such as 5 min (Yard & Comstock, 2008). The leg lace technique is an important technical move found in all positions of freestyle wrestling. For wrestlers, this technique is important for their performance in competitions (Yard & Comstock, 2008). This technique is very similar to the hunting move of the crocodiles since they have to grab their prey and then spin very fast. These events are partly anaerobic and lactate dehydrogenase is of great

importance (Baldwin et al., 1995; Bennett et al., 1985; Owerkowich & Baudinette, 2008). This is the aim why LDH is selected to be investigated in this model study.

In order to compare these events in *Homo sapiens* and saltwater crocodile *Crocodylus porosus* (hereafter *C. porosus*) at molecular levels, LDH was selected to be investigated. The sequence-based properties from crocodile and human-originated LDHs were compared by using bioinformatics tools in the present study. This model paper is the first scientific study on the use of sports bioinformatics in wrestling.

Materials and Methods

FASTA formats of the human (*H. sapiens*) and crocodile (*C. porosus*) LDHs were retrieved from uniprot.org (The Uniprot Consortium, 2021). The accession numbers of the human and crocodile LDHs in uniprot.org are P00338 and A0A7M4G2G2, respectively. The protein parameters such as amino acid composition both number and percentage, pI values, the total number of negatively charged residues (Asp + Glu) and the total number of positively charged residues (Arg + Lys), estimated half-life, Instability index, aliphatic index, grand average of hydropathicity values were computed by using protparam tool developed by Gasteiger et al (2005). Multiple sequence analysis was carried out by Clustal Omega (1.2.4 version) (Sievers et al., 2011). 3D models of the LDHs were studied by using Swiss-Model (Bertoni et al., 2017; Bienert et al., 2017; Studer et al., 2020; Studer et al., 2021; Waterhouse et al., 2018). The outline of the study is shown in Figure 1. In bioinformatics, FASTA formats are obtained after isolation and sequencing experiments. 3-D structures of the sequences can be modelled by various tools such as SwissModel. The superposition of the sequences exhibits similarities and also differences of the proteins compared. The superposition of the sequences exhibits similarities and also differences of the models.

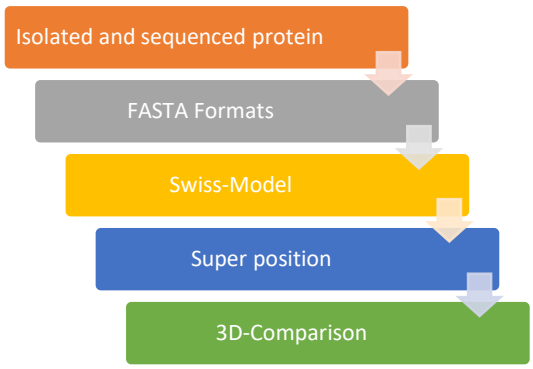

Figure 1. The architecture of the study.

Results and Discussion

Amino acid numbers and percentages of human and crocodile LDHs obtained from the protparam tool are depicted in Table 1. Leu and Val have been found as amino acids in both species in terms of max number and percentage. This could be explained by the high hydrophobic nature of the enzyme, especially in the inner sides of the enzymes. From the results, no irregular amino acids were detected in both human and crocodile LDHs. The percentages of Cys and Trp in human and crocodile LDHs were found to be 1.5% and 1.7%, respectively.

Table 1. Amino Acid Numbers and Percentages in the Lactate Dehydrogenases from Homo sapiens and Crocodylus porosus

Amino acids	*H. sapiens*		*C. porosus*	
	#	%	#	%
A	18	5.4	20	5.5
B	0	0.0	0	0.0
C	5	1.5	7	1.9
D	18	5.4	20	5.5
E	18	5.4	21	5.8
F	7	2.1	9	2.5
G	26	7.8	27	7.4
H	7	2.1	19	5.2
I	23	6.9	23	6.3
K	28	8.4	29	8.0
L	38	11.4	37	10.2
M	9	2.7	11	3.0
N	15	4.5	11	3.0
O	0	0.0	0	0.0
P	11	3.3	10	2.8
Q	12	3.6	9	2.5
R	11	3.3	11	3.0
S	24	7.2	27	7.4
T	14	4.2	15	4.1
U	0	0.0	0	0.0
V	34	10.2	42	11.6
W	6	1.8	6	1.7
X	0	0.0	0	0.0
Y	8	2.4	9	2.5
Z	0	0.0	0	0.0

These results show that disulphide bridges are not common compared to other proteinic structures. Even if Cys is not at the minimum level in crocodile LDH, the percentage is very close to the min value (1,9%). The total number of negatively charged residues (Asp + Glu), the total number of positively charged residues (Arg + Lys), estimated half-life, instability index, aliphatic index, grand average of hydropathicity values in *H. sapiens* and *C. porosus* LDHs are given in Table 2. There are three amino acids difference between the total number of negatively charged residues (Asp + Glu) and the total number of positively charged residues (Arg + Lys) in *H. sapiens* LDH, the difference between these amino acids is only one in *C. porosus* LDH.

Regarding enzyme stability of LDHs from *H. sapiens* and *C. porosus*, it is almost the same since they have the same values. Instability indexes of the studied LDHs were found as 24.79 and 25.18 in *H. sapiens* and *C. porosus*, respectively.

Table 2. Protein parameters of the lactate dehydrogenases from *H. sapiens* and *C. porosus* (*mammalian reticulocytes, *in vitro*)

Protein Parameters	*H. sapiens*	*C. porosus*
Extinction coefficient (M^{-1} cm^{-1})	45170	46785
Estimated half-life* (hours)	30	30
Instability index	24.79	25.18
Aliphatic Index	106.78	103.53
Grand average of hydropathicity	-0.006	0.020

These results exhibit that both enzymes are very stable, and they are resistant to being degraded easily. Grand averages of hydrophobicity index values in human and crocodile LDHs were found to be very different. They are -0.006 and 0.020, respectively. Grand averages of hydrophobicity index values in bioinformatics are used to estimate the hydrophobicity value of a polypeptide sequence (Gasteiger et al., 2005). Positive and negative values are explained with the adjectives "hydrophobic" and "hydrophilic" peptides. Based on this definition it is said that although human LDH is a hydrophilic polypeptide, crocodile LDH is a hydrophobic polypeptide. This could have been due to the amino acid sequence difference of both species. A pairwise sequence comparison of human and crocodile LDHs were depicted in Figure 2.

From the result, it could be said that the amino acid sequences of human and crocodile LDHs are very similar. However, there are some differences among the sequences as follows. First, the symbols are important to understand in Figure 2.

Figure 2. Pairwise sequence comparison of human and crocodile LDHs via Clustal omega (1.2.4).

The symbols "*", ":" and "." show the same amino acids, amino acids with very similar physicochemical properties, and similar physicochemical properties, respectively. Glu and Asp can be given as examples of amino acids with very similar physicochemical properties, Ile and Val are also examples of amino acids with similar physicochemical properties since both are hydrophobic. However, their structures (Ile and Val) are not very same compared to amino acids such as Glu and Asp. If there is no symbol in the pairwise sequence comparison, it could be explained with the deletion or insertion. The binding site for NAD^+ in human LDH are at the 99th, the positions between 29-57th and 138th positions. The positions at the 106th, 138th, 169th, and 248th are reported for the substrate binding site. His at the position of 193 is located at the active site and it plays as role for proton acceptor. When we compare this pattern with that of crocodile LDH, it is the same. The nucleotide-binding domain of the LDH in humans is very important (Read et al., 2001). The pairwise sequence alignment in our study clearly shows that this residue in crocodile LDH is also conserved. Two mutageneses are reported for human LDH and they are at the position of 56th and 99th. Asp at the position of 56th is reported for the wild-type human LDH. If it is substituted with Ala, this change abolishes interaction with MP31 which is a micropeptide and it limits lactate-pyruvate conversion in mitochondria (Huang et al., 2021). Since lactate-pyruvate conversion is of paramount importance in both anaerobic and aerobic exercises, this substitution should be noted for further investigations. Similarly, if the amino acid at the position of 99 (Arg) is substituted with Ala, this case also resulted in decreased interaction with MP31 (Huang et al., 2021). When these regions (Asp and Arg) are checked in crocodile LDH, it is seen that these regions are also conserved. Amino acid modifications are also reported in the uniprot.org records for P00338 (human LDH). The positions with the modifications are 2 (N-acetylalanine), 5 (N6-acetyllysine), 5 (N6-succinyllysine), 10 (Phosphotyrosine), 14 (N6-acetyllysine), 18 (Phosphothreonine), 57 (N6-acetyllysine), 57 (Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in SUMO2)), 81 (N6-acetyllysine), 118 (N6-acetyllysine), 118 (N6-succinyllysine), 126 (N6-acetyllysine), 224 (N6-acetyllysine), 232 (N6-acetyllysine), 239 (Phosphotyrosine), 243 (N6-acetyllysine), 309 (Phosphothreonine), 310 (Phosphoserine), 318 (N6-acetyllysine), 318 (N6-succinyllysine) and 322 (Phosphothreonine). In light of these modifications, we wanted to compare these regions in crocodile LDH. When we revisit the pairwise sequence alignment, N-terminal regions are different in both LDHs. The position-18 is Thr in human LDH and the relevant modification is phosphorylation. On the other hand, this residue is substituted with His in crocodile LDH and it is not possible to observe phosphorylation in this residue. The high similarity starts at the position of 20 of the human LDH compared to the sequence of crocodile LDH. The second amino acid in human LDH is Ala and it is reported with acetylation (Bienvenut et al., 2012; Gauci et al., 2009). Since this amino acid does not exist in crocodile LDH, this modification may be of importance. As Bienvenut et al (2012) mentioned that the modifications at N-terminal peptides are of great importance for the crucial processes of the proteins such as activity, stability, and subcellular locations. Inasmuch as there is a clear difference between the initial sequences of the human LDH compared to the

sequence of crocodile LDH, the initial regions may have importance in the enzymatic activity. However, there is a great need for more structural investigation to reveal the importance of the initial sequences. It is very interesting to note that even if the initial sequences are different, the amino acid position-5 is conserved in both species and the succinylation and acetylation of lysine are also possible in crocodile LDH (Choudhary et al., 2009). The position of 10 in human LDH is very also important to be compared with crocodile LDH due to amino acid differences in this region (Mayya et al., 2009). It is Tyr in human and it is His in crocodile LDH. The hydroxyl residue of Tyr is generally important in enzymatic activities through phosphorylation. Since there is no phosphorylation residue in the His of crocodile LDH, this region should also be noted for the enzymatic activity of LDH. Although initial sequences are seen differently in both enzymes, Lys residue is conserved in both species and this region (position 14 in human LDH) is mentioned with acetylation in Uniprot.org (Choudhary et al., 2009). The position of 57 in a human LDH is Lys and it is mentioned that it is modified with acetylation (Choudhary et al., 2009). This region is conserved in crocodile LDH and similar modification is most likely to be observed in crocodile LDH. Glycyllysine isopeptide (Lys-Gly) interchain with G-Cter in SUMO2 was reported by Hendriks et al (2017). The position of 81 in both species is the same and it is Lys. Acetylation is reported in this residue (Henriks et al., 2017). The latter explanation is also valid for the position of 118, 126, 224, 232, 243, and 318 (Choudhary et al., 2009). The position of 239 in both species is the same and it is Tyr. Phosphorylation is mentioned in Uniprot.org for this position (Bian et al., 2014; Huang et al., 2021; Zhou et al., 2013). The last modification residue is positioned at 322. When amino acids are compared for this position, Thr is found for both enzymes. Modelling of the lactate dehydrogenase from *C. porosus* was carried out through the Swiss Model (Waterhouse et al., 2018). The Swiss Model template 5nqb.1.A (Rabbit Muscle L-lactate dehydrogenase in complex with malonate) was selected for modelling (Alam et al., 2017) and the structure (ribbon model) of the lactate dehydrogenase is shown in Figure 3.

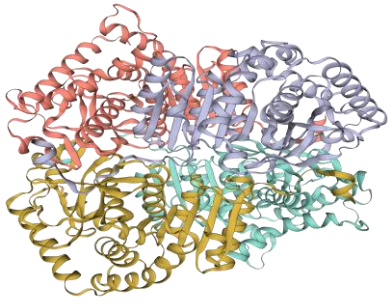

Figure 3. Modelling of the lactate dehydrogenase from *C. porosus* via Swiss Model (Waterhouse et al., 2018).

The sequence identity percentage was found as 88.48%. The plot related to the local quality estimation versus residue number was drawn in Figure 4.

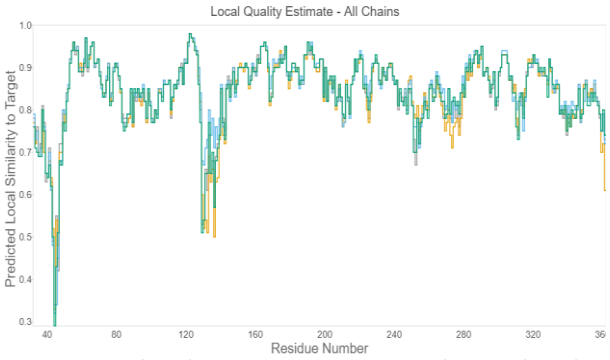

Figure 4. Local quality estimation versus residue number plot.

Qmean Z-Scores as QMean, CβQMEANDisco Global values were found as 0.86 and 0.84, respectively (Studer et al., 2021; Waterhouse et al., 2018). Normalised QMEAN4 Score, which is composed of four statistical potential terms and shows the quality of the model, versus the residue number plot was shown in Figure 5.

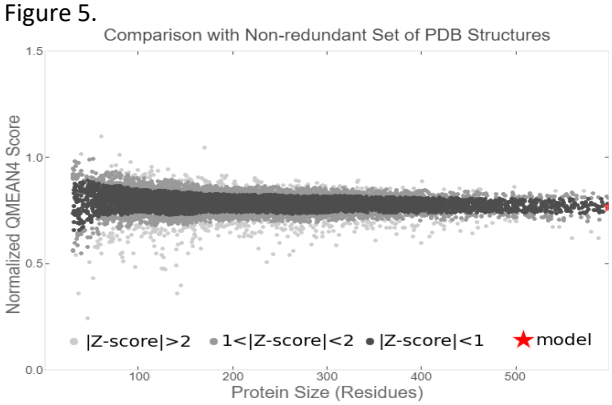

Figure 5. Normalised QMEAN4 Score versus residue number plot.

Since the values in Figure 5 are considered to be high, the model is acceptable. The template 5nqb.1.A was selected since it does not contain any ligand and also it has a homo-tetramer structure. Moreover, the method for the modelling was X-ray and the resolution is 1.58 A. When scientific literature was examined, generally lactate dehydrogenase is used in sports science to evaluate athletic performance. Here we review some of the lactate dehydrogenase-based papers. Hoff et al (2016) investigated the brains of the hooded seal (*Cystophora cristata*), the ferret (*Mustela putorius furo*), and the mouse (*Mus musculus*) to provide evidence of whether these animals have enhanced cerebral capacity for anaerobic energy production. The study revealed significant differences in the mRNA, protein expression of lactate dehydrogenase (LDHA and LDHB), and the LDH activity in the ferret brain compared to the other two animals. The researchers did not observe significant differences in the LDHA and LDHB sequences. The results also show that the high hypoxia tolerance of seals for

anaerobic energy production cannot be explained by the seal brain's enriched capacity. Inaddition to the above, the study addressed that the hooded seal's cerebral tolerance to hypoxia may be partially affected by the different LDH isoenzymes. The study conducted by Barranco et al (2017) investigated some enzymes (creatine kinase (CK), LDH, and aspartate aminotransferase (AST) results in saliva to see the impact of intensive sports training (Futsal) on eleven young males. After Futsal training, while dramatic increases are found in CK, LDH, and AST in serum samples, significant increases are determined for CK and LDH in saliva. There was no change in saliva AST after the intensive training. The study highlighted that changes in CK and LDH in saliva can be used as a potential indicator to determine muscle injuries and stress. In a study comparing the CK and LDH concentrations of 20 men while doing resistance training, it was reported that serious muscle damage could be caused if one minute of rest intervals was applied (Rodrigues et al., 2010). Rumley et al. (1985) focused on the CK and the LDH isoenzymes in serum. The study consisted of 35-50 years aged men who did marathon training for 30 weeks. It was determined that marathon training did not have a significant effect on muscle CK and LDH release. However, it has been mentioned that isoenzyme distribution changes occur in muscles during endurance training. Similar scientific reports can be found in sports science-based literature. However, the enzymatic activity of LDH or its concentrations are measured to estimate lactate levels or muscle injuries in the athletes in these investigations. As can be seen from this paper, there are plenty of amino acid modifications and also variants that could affect enzyme activities. Observation of significantly elevated activities in the athletes could be associated with individual differences in the LDHs. From this point, it is highly suggested to isolate the LDH from the elite athletes. The results within this paper can be used to compare with the sequence of the isolated enzymes. Swiss-Model clearly provides a big contribution to the understanding the 3-Dimensional structures of the enzymes studied.

As can be seen from Figure 6, not only 3-dimensional structures but also different characteristics such as polarity, amino acid sequence similarities, sizes, and charges can also be shown on the 3-D structures. Any modification on the enzyme structure can also be interpreted from these images (Figure 6).

Observation of different modifications in the amino acid sequences of elite athletes may open a new route of scientific investigations in the sports sciences. Obtaining important amino acid modifications in elite athletes (Olympic and World Champions) may be used as important biomarkers in talent selection. The results mentioned in this paper can be used to compare the amino acid sequences of Olympic and World Champions. A sample figure is also drawn to explain the latter (Figure 7).

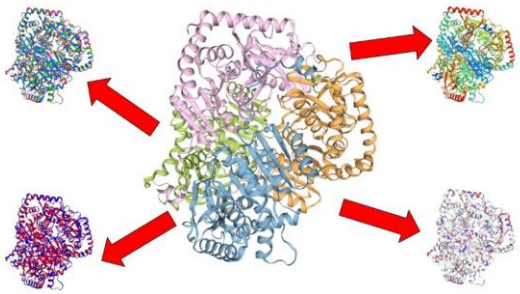

Figure 6. Four different drawings of the lactate dehydrogenase in Swiss-Model based on the different characteristics. Left-upper: Clustal, Left-down: size, Right-upper: rainbow, Right-down: charged amino acids.

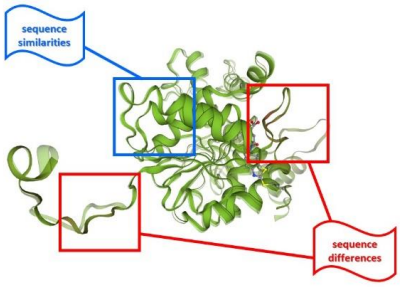

Figure 7. Superposition of two different lactate dehydrogenase structures. Sequence differences and similarities are shown within the figure.

Two sequences can be compared by superposition in Swiss-Model and this could give important ideas to other sports scientists about the enzymes in elite athletes. Moreover, the methodology mentioned in this paper could also be extended to other sports disciplines.

Conclusion

Comparison of LDHs in humans and crocodiles by using the *in silico* tools show that bioinformatics may have a potential application area in sports science. Possible modifications and/or mutations in side chains of amino acids may alter the enzymatic activity. In this route, bioinformatics may provide a great contribution to sports biochemistry and physiology by analysing sequence of the enzymes which are important in athletic performance. The animals such as crocodiles have-long years-experienced evolution for better physical performance for their survival in nature. Therefore, the sequence similarities, differences, and also important modifications of the selected animals could be used in talent selection. Moreover, understanding the modifications at amino acid sequences in elite athletes may also contribute to the latter. The lessons learned from nature may open a new gate in sports science. To get the full picture, more enzymes and also genes from different animals with different adaptations may be used in bioinformatics analysis in sports. In conclusion, sports bioinformatics is waiting to be explored: Let's start for the ideas from other sports disciplines.

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BIOTECH STUDIES

RESEARCH PAPER

Neuroprotective role of chrysin against bupivacaine induced apoptosis and oxidative stress in SH-SY5Y cell line

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Abstract

Chrysin, a natural flavonoid, has a strong neuroprotective effect in many neurodegenerative diseases. Therefore, we aimed to investigate the neuroprotective effect of chrysin against bupivacaine-induced neurotoxicity in SH-SY5Y cells. According to the results of XTT analysis, the non-toxic concentration of chrysin was determined and the cells were treated with bupivacaine alone and together with this determined chrysin dose. According to the results of RT-qPCR analysis, the level of caspases increased in the group treated with only bupivacaine compared to the control group, while the expression of antioxidant enzymes decreased. When compared with the group treated with bupivacaine alone, it was determined that while the expression of caspases decreased in the group in which bupivacaine and chrysin were treated together, the expression of antioxidant enzymes increased. According to the ELISA results, SOD and CAT activities were decreased in the group treated with bupivacaine alone compared to the control group. SOD and CAT activities increased in the presence of chrysin treated with bupivacaine compared to the group treated with bupivacaine alone. The obtained data showed that chrysin may play a neuroprotective role by inducing the expression of antioxidant enzymes while inhibiting apoptosis against bupivacaine-induced neurotoxicity in SH-SY5Y cells.

Introduction

Regional anesthesia is the temporary elimination of nerve conduction and pain sensation in certain parts of the body without causing loss of consciousness (Ardon et al., 2019). Local anesthetics are used in blocking the nerves going to the area to be operated and in the management of post-operative pain (Yu et al., 2017). Bupivacaine is the most toxic local anesthetic widely used in clinical practice for epidural anesthesia, nerve blockade, and postoperative analgesia (Zhao & Wang, 2020; Kendall et al., 2018). In studies conducted so far, it has been determined that long-term and high-dose treatment with local anesthetics is highly effective as an analgesic, but it also causes neurotoxic damage and serious neurological complications (Wang et al., 2021; Niu et al., 2014). Bupivacaine treatment triggers a series of signaling pathway cascades that cause neuronal apoptosis-mediated neurotoxicity (Li et al., 2013). Increased reactive oxygen species, decreased antioxidant response systems, caspase activation, and mitochondrial dysfunction are among the markers of apoptosis induced by local anesthetics (Bouderba et al., 2012).

Oxidative stress occurs when redox homeostasis associated with cell survival is disrupted. When the level of ROS (reactive oxygen species) produced as by-products of oxygen metabolism in the body exceeds the antioxidant capacity, it causes oxidative stress called cellular redox imbalance and this induces cellular biomolecular damage (Su et al., 2013; Nirmaladevi et al., 2014). Cellular antioxidant systems directly remove free

radicals and maintain the intracellular redox state by converting ROS into more stable molecules such as H_2O and O_2 through antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Sukprasansap et al., 2020; Reddy, 2008). Oxidative stress caused by the cumulative increase of ROS also plays an important role in many physiological processes including cellular apoptosis and in the pathologies of neurodegenerative disorders (Wang et al., 2018). Although the mechanisms underlying bupivacaine-induced neurotoxicity are not fully understood, it is widely believed that activation of the apoptotic pathway can induce nerve damage and neurotoxicity (Werdehausen et al., 2009; Ji et al., 2015). Considering that the use of anesthetics in surgical procedures is inevitable, it becomes very important to develop new neuroprotective strategies against bupivacaine-induced neurotoxicity (Zhao & Wang, 2020).

Chrysin (5,7-Dihydroxyflavone) is a natural phytochemical flavonoid abundant in honey, propolis, and blue passion flower, which have great economic and medicinal value, as well as various mushrooms, and plants (Mani & Natesan, 2018). In vivo, in vitro, and clinical studies carried out so far chrysin has been shown to have many pharmacological activities such as antioxidant, anticancer, antibacterial, anti-inflammatory, antidiabetic, and antidepressant (Karthikeyan et al., 2013; Xiao et al., 2014; Ahad et al., 2014; Filho et al., 2015; Song et al., 2016). It has also been determined that chrysin has a neuroprotective effect in many neurodegenerative disorders due to neuron damage (Sathiavelu et al., 2009; Zhang et al., 2015; Souza et al., 2015). In this study, we aimed to investigate the anti-apoptotic and antioxidant response-mediated neuroprotective potential of chrysin against bupivacaine-induced neurotoxicity in SH-SY5Y neuroblastoma cells.

Materials and Methods

Cell culture and treatment

SH-SY5Y (Human Neuroblastoma cells) purchased from ATCC were cultured in DMEM-F12 medium (Sigma-Aldrich, USA) supplemented with 10 % fetal bovine serum (FBS) (Capricorn, Germany) and 100 U/ml penicillin-streptomycin (10 mg/mL) at 37°C in a humidified atmosphere containing 5 % CO_2. Chrysin was obtained from Sigma-Aldrich (D7321 Merck; Germany) and dissolved at a final concentration of 0.1 % DMSO to prepare a stock solution. The stock solution was stored at -20°C until used in the experiments and diluted with a culture medium to prepare different concentrations.

Cell viability assay

The cytotoxic effects of bupivacaine and chrysin on SH-SY5Y cell viability were evaluated by XTT cell proliferation assay (Biological Industries, 20-300-1000). SH-SY5Y cells were seeded into 96-well plates at a density of 2x104 cells/well. After 24 hours (h) of incubation, cells were treated with different concentrations of chrysin (5-10-15-20-40-50-75-100 µM) and bupivacaine (200-400-500-750-1000 µM) for 24 h. XTT assay was carried out to determined IC50 value of bupivacaine and chrysin. To assess the therapeutic efficacy of chrysin against bupivacaine toxicity, cells were pretreated with different concentrations of chrysin (5-10-15-20-40-50-75-100 µM) for 6 h and then incubated with the bupivacaine of effective IC50 dose (500 µM) for 24 h. According to the results of XTT analysis, the non-toxic concentration of chrysin was detected 10 µM at 24th hours. XTT solution was added to each well and incubated for 4 h at 37 °C. After incubation, the OD (optical density) absorbance values of the wells were measured at a wavelength of 450 nm and 630 nm (reference absorbance) on an ELISA microplate reader (BioTek, Epoch). In subsequent analyses, cells were co-treated with 10 µM chrysin, 500 µM bupivacaine as a combination dose. The detail of the cell viability assay has been described in our previous study (Çınar Ayan et al., 2022).

RT-qPCR

To evaluate the apoptosis pathway and antioxidant enzymes mediated protective effect of chrysin against bupivacaine-induced neurotoxicity in SH-SY5Y cells, real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed. First, SH-SY5Y cells were seeded in 6-well plates at a density of 2,5x104 cells/well and incubated for 24 h at 37 °C. After dose treatment to the cells for 24 h, RNA isolation was performed with RiboEx reagent (GeneAll, 301–001) from the sample wells of the dose and control group. The concentrations and quality of the isolated RNA samples were determined by reading the absorbance at 260 and 280 nm with a nanodrop instrument, a UV spectrophotometer. DNase I enzyme (Thermo Scientific, USA) was used to remove possible DNA contamination from RNA samples. Purified RNAs were reversed to cDNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad, 170–8891) according to the manufacturer's instructions. The primer sequences of CASP3, CASP7, CASP8, CASP9, SOD1, SOD2, SOD3, CAT genes associated with the apoptosis pathway, and antioxidant response enzymes were designed with IDT PrimerQuest (https://eu.idtdna.com/Primerquest/Home/Index). RT-qPCR was performed using SYBR on an Applied Biosystems thermocycler. The RT-qPCR conditions were as follows: 95°C for 4 min, 40 cycles of amplification (95°C for 10 sec, 60°C for 60 sec, and 72°C for 4 min). The detail of the RT-qPCR assay has been declared in our previous study (Güçlü et al., 2022).

Superoxide dismutase (SOD) activity assay

The SOD enzyme activity was measured by using a Superoxide dismutase (SOD) activity assay kit (BioVision-K335-100) (ELISA based) according to the manufacturer's protocol. For this analysis, cells were seeded into a 6-wells plate at a density of 5x105

cells/well and incubated overnight. Afterwards, cells were treated with dose groups (Bupivacaine and chrysin alone and together) for 24 h. Cells were lysed in ice-cold 0.1 M Tris/HCl, pH 7.4 containing 0.5 % Triton X-100, 5 mM β-ME, 0.1 mg/ml PMSF. The obtained cell lysate was centrifuged at 14000 x g for 5 min at 4 °C. The collected supernatant contains total SOD activity from cytosolic and mitochondria. Sample, blank 1, blank 2, and blank 3 wells were determined in a 96-well plate. While 20 µl of Sample Solution was added to the sample and blank 2 well, 20 µl H_2O was added to Blank 1 and Blank 3 well. Afterwards, 200 µl of WST Working Solution was added to all wells. 20 µl of dilution buffer was added to blank 2 and blank 3 wells, and 20 µl enzyme working solution was added to the sample and blank 1 wells and mixed well. After the plate was incubated for 20 min at 37 °C, the absorbance of each well was measured at 450 nm using a microplate reader.

Catalase (CAT) activity assay

Catalase is an antioxidant enzyme that catalyzes the conversion of hydrogen peroxide, an undesirable by product of aerobic respiration, into water and oxygen. The catalase enzyme activity was measured by using a CAT activity assay kit (BioVision-K335-100) (ELISA based) according to the manufacturer's protocol. Afterwards, cells were treated with dose groups (Bupivacaine and chrysin alone and together) for 24 h. Cells were homogenized in 0.2 ml cold assay buffer and centrifuged at 10000 x g for 15 minutes at +4 °C, then the phase of supernatant was collected for assay. The total volume in each well was made up to 78 µl, and 50 µl of sample supernatant and 28 µl of assay buffer were added to the wells. In separate wells, 50 µl of sample supernatant and 28 µl of assay buffer were added to prepare sample high control (HC), making the total volume to 78 µl. To inhibit the catalase activity in the samples, 10 µl of stop solution was added to the sample HC and incubated at 25 °C for 5 min. For the H_2O_2 standard curve, stock 0.88 M H_2O_2 was diluted with distilled water and a 1mM H_2O_2 solution was prepared. Afterwards, an H_2O_2 standard was created at different concentrations of 0-2-4-6-8-10 nmol/well in 96 plate from a 1 mM H_2O_2 solution. To initiate the catalase reaction, 12 µl of 1 mM H_2O_2 was added to each well and incubated at 25°C for 30 min. After incubation, 50 µl developer buffer containing 46 µl of Catalase assay buffer, 2 µl of OxiRed Probe, and 2 µl of HRP solution were added to each well, mixed, and incubated at 25°C for 10 min. The absorbance (OD) of each well was measured in a microplate reader at a wavelength of 570 nm.

Statistical analysis

Data were repeated in triplicate. All results were presented as mean ± SD (standard deviation). Cell viability analysis, comparison of control versus treatment groups, and comparison between groups were performed with GraphPad Prism version 8.0.2 using Student's t-test and one-way ANOVA test. P<0.05 values were considered statistically significant.

Results & Discussion

SH-SY5Y human neuroblastoma cells are widely used to investigate the neurotoxicity of local anesthetics because they can mimic the biological properties of neuron cells. Therefore, SH-SY5Y cells were used as an in vitro neuronal damage model. In this study, we aimed to investigate the protective role of chrysin mediated by apoptosis and oxidative stress against bupivacaine-induced neuronal damage in SH-SY5Y cells.

Firstly according to the XTT cell viability test, it was observed that bupivacaine inhibited SH-SY5Y cell proliferation in a dose-dependent manner. And the IC50 dose, which was half maximal inhibitory concentration (killed about half of the cells), was determined as 500 µM at 24 h (Figure 1). Therefore the dose for bupivacaine was used as 500 µM in subsequent experiments. In previous studies in the literature, the neurotoxic IC50 dose (about 50% cell growth inhibition) of bupivacaine in SH-SY5Y cells was found to be in the range of 500-1000 µM (Wen et al., 2013; Wang et al., 2019; Zhao & Wang, 2020). With our cell viability test results, we have demonstrated that bupivacaine can cause neuronal cell death by inducing neurotoxicity, in agreement with previous studies (Dhanalakshmi et al., 2015; Zhang et al., 2019).

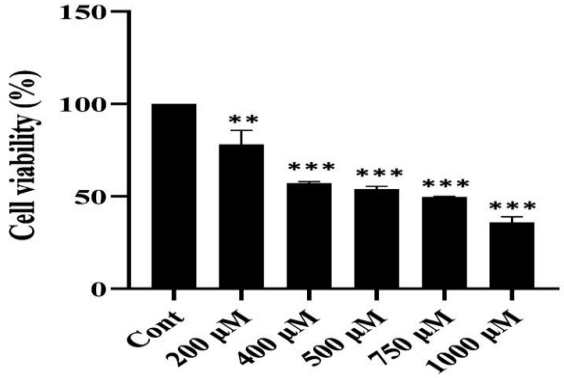

Figure 1. Cytotoxic effects of bupivacaine on SH-SY5Y cells. Different concentrations of bupivacaine were treated in SH-SY5Y cells for 24 h dose dependent manner. Each group was subjected to least three independent experiments. Cell viability assay determined by XTT cell proliferation assay (*P <0.05, **P <0.01, and ***P<0.001).

It was also determined that cell viability decreased in a dose-dependent manner when cells were treated with chrysin alone (Figure 2). SH-SY5Y cells were treated for 24 h in combination with bupivacaine (500 µM) after 6 hours of pretreatment with different concentrations of chrysin. Chrysin significantly inhibited bupivacaine-induced cell death, resulting in an increase of 45.1078, 41.6888, and 44.0577 % in cell viability at 5 µM, 10 µM, and 15 µM concentrations, respectively. When the cytotoxic effects of both chrysin alone and in combination with bupivacaine on cell viability were

evaluated together, the non-toxic chrysin dose was determined as 10 µM for 24 h (Figure 3).

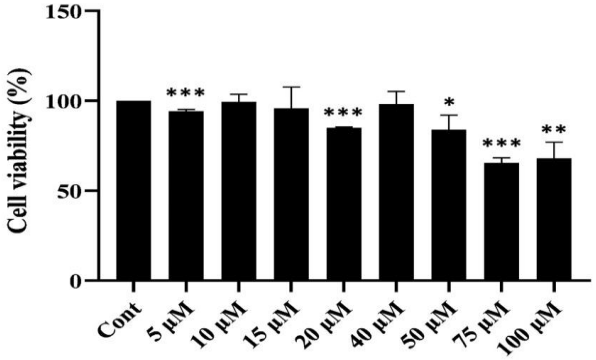

Figure 2. Antiproliferative effects of chrysin on SH-SY5Y cells in a dose-dependent manner. Cells were treated with different concentrations of chrysin for 24 h. Each group was subjected to least three independent experiments. Cell viability was determined by XTT assay and presented as a percentage relative to untreated control cells (100%) (*P <0.05, **P <0.01, and ***P<0.001).

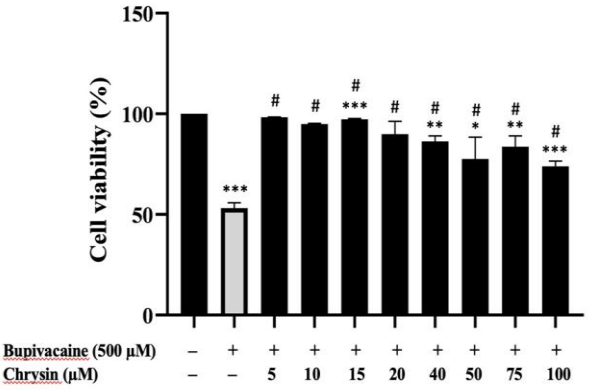

Figure 3. Effects of different concentration of chrysin against bupivacaine-induced oxidative damage in SH-SY5Y cells using XTT assay. Each group was subjected to least three independent experiments. After pretreatment with various concentrations of chrysin (5, 10, 15, 20, 40, 50, 75 and10 µM) for 6 h, cells were treated with 500 µM bupivacaine for 24 h (*P<0.05, **P<0.01, and ***P<0.001 versus control group; #p <0.05 versus bupivacaine group).

It is known that foods rich in antioxidants have protective effects against cancer, cardiovascular diseases, and neurological degeneration (Wollgast & Anklam, 2000). Medicinal plant-based natural phenolic compounds induce apoptosis of many cancer cells by targeting multiple pathways involved in cell death. Flavonoids are phenolic compounds commonly found in natural plant structures (Lim et al., 2018). Chrysin is a naturally occurring flavonoid, especially in honey and propolis. In a study, it was determined that chrysin decreased cell proliferation by inducing apoptotic cell death in the PC-3 human prostate cancer cell line (Samarghandian et al., 2011). In another study, it was found that chrysin inhibited cell proliferation by inducing apoptosis in malignant glioma U87-MG and U-251 cells in a dose-dependent manner (Parajuli et al., 2019).

Against the triggered ROS production, the expression level of Nrf2, an important transcription factor to initiate antioxidant mechanisms, is increased. After Nrf2 activation, it separates from the Nrf2-Keap1complex and translocates to the nucleus. Activation of the Nrf2/ARE signaling pathway increases the expression of antioxidant enzymes (SOD, CAT, GSH, and GST), thus preventing cellular damage caused by oxidative stress (Zhang et al., 2021). Oxidative stress results from the disruption of the redox balance between ROS production and antioxidant mechanisms that remove ROS. Thus, the amount of ROS in the cell increases cumulatively (Lin & Beal, 2006). Increased ROS in the cell causes neuronal damage by inducing apoptosis (Moldogazieva et al., 2018). Previous studies have shown that chrysin exerts a neuroprotective effect against neuronal damage induced by different agents by decreasing the level of ROS in the cell and increasing the level of antioxidant enzymes (El-sisi et al., 2017; Belli et al., 2019; Khezri et al., 2020). It has been determined that chrysin can affect oxidative stress by increasing the intracellular expression of antioxidant enzymes such as SOD, CAT, and GPx (Vedagiri & Thangarajan, 2016). In addition, it has been demonstrated that chrysin has neuroprotective effects through different pathways such as antioxidant, anti-inflammatory and antiapoptotic mechanisms (Mishra et al., 2021).

In this study, SOD and CAT activity in control and treatment groups were evaluated at the protein level by ELISA assay. According to the results obtained, it was determined that CAT activity decreased significantly in the group treated with bupivacaine alone (4.81 mU/mL) compared to the control group (8.74 mU/mL), and increased in the group treated with bupivacaine in the presence of chrysin (8.04 mU/mL) compared to the group treated with bupivacaine alone (4.81 mU/mL) (Figure 4B). Similar to the results of CAT activity, it was identified that SOD activity decreased significantly in the group treated with bupivacaine alone (66.57%) compared to the control group (103.92%), and increased in the group treated with bupivacaine in the presence of chrysin (80.88%) compared to the group treated with bupivacaine alone (66.57 %) (Figure 4A).

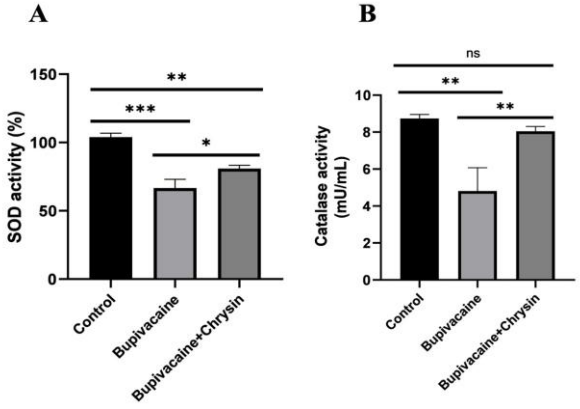

Figure 4. Antioxidative effects of chrysin against bupivacaine-induced oxidative stress in SH-SY5Y cells. The activity of SOD and CAT were measured using ELISA (Colorimetric based)

assay. Each group was subjected to least three independent experiments. Bupivacaine (500 µM) treatment significantly decreased the levels of SOD (A) and CAT (B) activity as compared to control cells, while chrysin (10 µM) pretreatment significantly increased the levels of SOD (A) and CAT (B) as compared to bupivacaine alone treated cells (*P<0.05, **P<0.01, and ***P<0.001).

According to the RT-qPCR results obtained, it was determined that CASP8 and CASP9 gene expression associated with apoptosis increased 8.3 and 6.24 times in the group treated with only bupivacaine, respectively, while the expression of SOD2, SOD3 and CAT associated with oxidative stress decreased 1.79, 4.7, 2.95 times, respectively. It was determined that SOD2, SOD3 and CAT gene expression in the group treated with bupivacaine in the presence of chrysin increased by 6.81, 3.67 and 4.34 times, respectively, compared to the group treated with bupivacaine alone, while the expression of CASP8 and CASP9 genes decreased by 12.23 and 9.14 times, respectively (Figure 5).

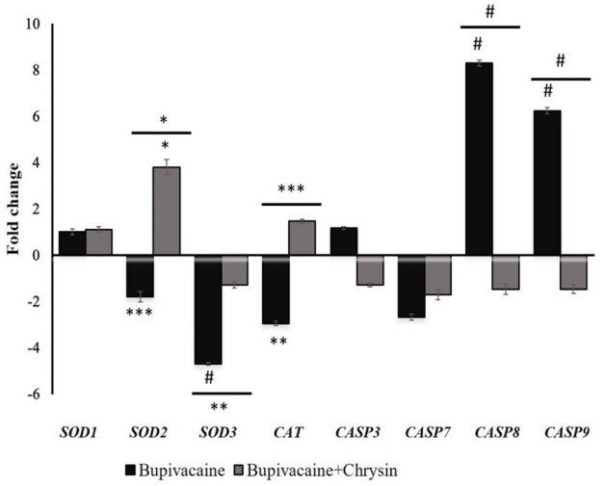

Figure 5. Neuroprotective effect of chrysin against bupivacaine-induced SH-SY5Y neuronal cell death on level of genes expression associated with apoptosis and antioxidant enzymes (*P<0.05, **P<0.01, ***P<0.001 and #P<0.0001). Each group was subjected to least three independent experiments.

Conclusions

Our results revealed that the pretreatment of SH-SY5Y cells with chrysin exerted a protective effect on bupivacaine-induced neurotoxicity. In this study, it was determined that chrysin suppressed oxidative stress by inducing antioxidant enzyme expressions and could resist neuronal cell death mediated by the bupivacaine-induced apoptosis pathway. Therefore, it shows that the mechanism underlying the neuroprotective effects of chrysin is due to the inhibition of oxidative stress and apoptosis pathway. Our findings from this study suggest that chrysin may be a potential neuroprotective agent candidate against bupivacane-induced neurotoxicity if it can be performed in neuron cells in vivo.

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RESEARCH PAPER

Comparison of biochemical and antioxidant activities of ultrasonic-assisted extraction with different solvents in olive leaf

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Abstract

Olive leaves are considered to have great potential as natural sources of antioxidants and phenolic compounds. In this study, dried olive leaves were extracted using four different solvents (water, methanol, ethanol, and 80:20(v/v) methanol-water) with ultrasonic-assisted extraction. The biochemical (total phenolics and flavonoids, total protein, free amino acids, total soluble, and reducing sugars) and antioxidant activities (CUPRAC, DPPH, FRAP, and FIC) of these extracts were evaluated. Total phenolics content was significantly affected by the different solvents and the highest total phenolics content was obtained in methanol-water (234 mg g^{-1}) extraction. The highest total flavonoid (47 mg g^{-1}) and total protein (5,1 mg g^{-1}) content were obtained in methanol extraction. Yield of the free amino acids was lowest in ethanol (1,5 mg g^{-1}), while it was highest in water (2,3 mg g^{-1}) and methanol-water (2,2 mg g^{-1}) extractions. The highest total soluble sugars were obtained from methanol-water (70,4 mg g^{-1}) and ethanol (65,4 mg g^{-1}) extractions, while the highest total reducing sugar contents were obtained from methanol (112,2 mg g^{-1}) and methanol-water (111,6 mg g^{-1}). While methanol-water extraction showed the highest antioxidant capacity with 0,63 mmol TR g^{-1} CUPRAC value, it also showed the strongest radical scavenging activity with 1,09 mmol TR g^{-1} DPPH radicals value and 0,065 mmol TR g^{-1} FRAP potential value. FIC capacity was higher in water than in other solvent extraction methods. Methanol and methanol-water solvents were the most effective solvents for measuring phenolic and antioxidant activities in olive leaves.

Introduction

Olive (*Olea europaea* L.), belonging to the Oleaceae family, is an evergreen tree with hard branches, grayish bark, and high-value oil, used in the food, medicine, and cosmetic industries. Since olive leaves are cheap, edible and considered a source of various biologically active compounds (Şahin & Şamlı, 2013), they can be used as a by-product after harvesting, and oil extraction of olive fruits. Leaves are lanceolate, positioned opposite to each other with short-stalked mucronate, green top, and hoary bottom surfaces (Castejón et al., 2020). Olive leaves have been used in traditional medicine for the treatment of illnesses. The dried leaf decoction is used to treat diabetes (Alarcon-Aguilara et al., 1998), and the leaf extracts are used to treat stomach and intestinal conditions as well as to clean the mouth (Bellakhdar et al., 1991). They also have been used to treat hypertension and induce diuresis from fresh leaf extracts (Pieroni et al., 1996; Ribeiro et al., 1986). Due to the phenolic and flavonoid compounds they contain, olive leaves have demonstrated that they exhibit antimicrobial activity, including antiviral, antifungal,

and antibacterial effects against various pathogenic microorganisms, as well as containing a number of biologically active compounds that support health-promoting effects (Sánchez-Gutiérrez et al., 2021; Topuz & Bayram, 2022).

Metabolites in the leaves have crucial roles in fulfilling the vital functions of the olive tree (Papoti & Tsimidou, 2009), and olive leaves contain many pharmacologically active compounds, especially antioxidants. Olive leaves have gained increasing attention from the scientific and industrial community due to their rich oleuropein content. Secoiridoids and flavonoids other than oleuropein may contribute to antioxidant activity in leaf extracts (Goulas et al., 2010). Antioxidants typically have the ability to delay or prevent the initiation and spread of oxidative chain reactions. Phenolic and flavonoid compounds from plants are an antioxidant class that acts with free radical scavenging ability because the formation of free radical derivatives during the oxidation process and the propagation chain of stable derivatives during this reaction are inhibited by antioxidant activities (Castejón et al., 2020). Additionally, these compounds function as metal chelators to inhibit the production of hydroxyl radicals (Gouvinhas et al., 2017).

The yields of many beneficial biological components in plant samples are largely dependent on the extraction method and period. The effectiveness of extraction is affected by changes in the extraction parameters (type of extraction solvent, solvent concentration, extraction time, and extraction temperature) (Xu et al., 2017). The biochemical content and antioxidant capacity of plant extracts can be used with maximum efficiency when the extraction parameters are optimized (Chew et al., 2021). Plant extracts consist of different active biological component structures, physicochemical properties, and polarities, and these properties affect extraction efficiency. Therefore, biochemical contents and antioxidant capacities cannot be determined by using a single universal method. Many methods are used for the extraction of biochemical and antioxidant components from leaves, but solvent extraction is the most widely method (Wissam et al., 2016). Several solvents have been used to extract the polyphenols from the samples effectively. It has been reported in many studies that the most efficient extraction of polyphenols is usually obtained from polar solvents rather than non-polar solvents (Liu et al., 2007; Wissam et al., 2016). Therefore, water and organic solvents (methanol and ethanol) are commonly used in the extraction of samples. Consequently, the specification of optimum extraction parameters for each sample is required for the correct determination and evaluation of biochemical contents and antioxidant capacities. While determining the extraction yield, the cost, safety, and environmental effects of the process should also be considered. The use of conventional extraction methods has begun to decline because of the following reasons: i) time-consuming extraction protocols of plant samples; ii) the loss of some phenolic and flavonoid compounds due to oxidation caused a decrease in antioxidant capacity and ionization (Şahin & Şamlı, 2013). The ultrasonic-assisted extraction technique can be used as an alternative, which has proven to be more effective than other techniques in recent years (Chew et al., 2021).

Although studies on the antioxidant capacities of olive leaves and fruits are plentiful (Dobrinčić et al., 2020; Goulas et al., 2010; Topuz & Bayram, 2022), and have received increasing attention, there are few studies on the effect of ultrasonic-assisted extraction with different solvents on the biochemical content and antioxidant capacity (Ahmad-Qasem et al., 2013; Şahin & Şamlı, 2013). Therefore, the aim of the study was to investigate to what extent the biochemical content and antioxidant capacity are affected by ultrasonic-assisted extraction with different solvents of dried olive leaves, which are sources of bioactive components and can be used for commercial purposes throughout the year.

Materials and Methods

Plant material

The leaves of Memecik variety were used in the study. Olive leaves were collected from an orchard in Karatepe village (37° 55´ N, 28° 05´ E), Köşk, Aydın in March 2021. The olive trees in the orchard were irrigated with flood irrigation in summer. Leaf samples were collected between 9 and 10 AM and branches containing mature leaves of 1-year old shoots were selected from the entire perimeter of the trees to minimize environmental and orientation variability in the samples. After sampling, the leaves were immediately cleaned of dust and then dried at room temperature (RT) for 14 days. Finally, the dried samples were placed in airtight opaque glass jars and stored in a dry, dark, and cool place until analysis.

Chemicals

Methanol (99%), ethanol (99%), and glycerol (99%) were purchased from ISOLAB Laborgeräte GmbH (Isolab, Germany). (±)-Catechin hydrate, phenol, sulfuric acid (%99), sodium borate, 2-cyanoacetamide, copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), neocuproine (Nc-$C_{14}H_{12}N_2$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron(II) chloride and ferrozine were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium carbonate, sodium nitrate, aluminum chloride, sodium hydroxide, ninhydrin, sodium citrate, *L*-valine, potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), gallic acid, trichloroacetic acid (TCA) were purchased from Merck (Merck Company, Darmstadt, Germany). All other chemicals used were analytical grade and obtained from either

Sigma-Aldrich or Merck.

Preparation of leaf extracts

Ultrasonic-assisted extraction of olive leaves with four different solvents (water, methanol, ethanol, and methanol-water (80:20, v/v) was carried out in accordance with a method described by Zhang et al. (2009) with minor modifications. Prior to extraction of the dried leaves, the samples were powdered with an electric grinder (SCM 2934, Sinbo, Türkiye) and the obtained powder was immediately used for extraction. The samples (1 g) were extracted in an ultrasonic bath system (LAB.ULT.4045, interior dimensions: 300 mm × 150 mm × 100 mm) using 40 mL of solvent. General extraction parameters were: temperature: 70 °C; time: 10 min; solid/solvent ratio: 1:40 (w/v), maximal nominal output power: 150 W, and ultrasonic frequency: 40 kHz. The extracts were then filtered through a 0,45 µm PTFE filter (Isolab, Germany) and filtered aliquots were stored in the dark at -20 °C until further analyses.

Determination of total phenolic and flavonoid contents

The total phenolics of the samples, extracted with different solvents, were determined using the modified Folin-Ciocalteau method (1927). The extract solution (0,1 mL) was mixed with 2,5 mL of deionized water and 0,1 mL of Folin-Ciocalteu reagent (Merck Company, Darmstadt, Germany), and the reaction was terminated using 0,5 mL of 20% sodium carbonate. The reaction mixture was incubated at RT for 30 min in the dark and the absorbance was measured at 760 nm with a UV–vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The standard curve was prepared using different concentrations of gallic acid (GA).

Total flavonoids were measured by the aluminum chloride reaction (Sakanaka et al., 2005). The extract solution (0,25 mL) was mixed with 1,25 mL of deionized water, and 75 µL of 5% sodium nitrate. After 6 min, 0,15 mL of 10% aluminum chloride was added; after 5 min, 0,5 mL of 1 M sodium hydroxide was added. The absorbance of all the sample solutions against a blank was measured at 510 nm and (±)-catechin concentrations were used to construct the standard curve.

Determination of protein and free amino acids contents

The protein content in the different solvent extracts was measured by the Bradford method (Bradford, 1976). The extract solution (0,1 mL) was mixed with 3 mL of Bradford reagent and gently vortexed. The reaction mixture was incubated for 5 min at RT for the color formation and the absorbance was measured at 595 nm with a UV–vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The standard curve was prepared using bovine serum albumin (BSA).

Total free amino acids were measured by the ninhydrin method (Lee & Takahashi, 1966). The extract solution (0,1 mL) was mixed with 1,9 mL of reaction solution (0,5 mL 1% ninhydrin + 1,2 mL glycerol + 0,2 mL 0,5 M sodium citrate). The reaction mixture was boiled in a water bath for 12 min, and cooled in an ice bath and absorbance was measured at 570 nm. Total free amino acids content was calculated using a standard curve prepared with *L*-valine.

Determination of total soluble and reducing sugar contents

Total soluble sugar was measured by the phenol-sulphuric method (Dubois et al., 1956). The extract solution (1 mL) was mixed with 0,5 mL of 5% phenol and 2,5 mL of concentrated sulfuric acid. The reaction mixture was incubated for 10 min at RT for the color formation and the absorbance of all the sample solutions against a blank was measured at 490 nm. Total soluble sugar content was calculated using a standard curve prepared with glucose.

Total reducing sugar was measured using the Somogy-Nelson method (Somogyi, 1952). An aliquot of each extract (1 mL) was mixed with 2,5 mL of sodium borate (100 mM, pH 9.0) and 0,5 mL of 1% 2-cyanoacetamide and vortexed well. The reaction mixture was boiled in a water bath for 12 min, and cooled in an ice bath and absorbance was measured at 280 nm. A standard curve prepared with glucose.

Cupric Reducing Antioxidant Capacity (CUPRAC) method

The CUPRAC test was performed according to the method of Apak et al. (2006). CUPRAC reactions were set up as follows: 1 mL of 0,01 M copper(II) chloride, 1 mL of 0,0075 M neocuproine solution, and 1,0 mL of 1 M ammonium acetate buffer solution (pH 7.0) were added successively into a glass tube. Subsequently, X mL of extract solution and "1,1 – X" mL deionized water were added to obtain a total volume of 4,1 mL and mixed well. Absorbance against a reagent solution without a sample was measured at 450 nm after 30 min. The antioxidant activity was calculated as Trolox equivalents per g of dry weight (mmol TR g^{-1} DW), through a calibration curve with Trolox standard.

$$CUPRAC\ (mmol\ TR\ g^{-1}) = \frac{A}{\varepsilon_{TR}} x\ \frac{V_m}{V_s}\ x\ D_f\ x\ \frac{V_E}{m}$$

Where; *A*: Sample absorbance measured at 450 nm; ε_{TR}: molar absorption coefficient of TR compound in the CUPRAC method (1,67 × 10^4 L mol^{-1} cm^{-1}); *Vm*: Total volume of CUPRAC method measuring solution; *Vs*: Sample volume (mL); *Df*: Dilution factor (if needed); V_E: Volume of the prepared extract (mL); *m*: The amount of sample taken in the extraction process (g).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging method

DPPH radical-scavenging activity was measured according to Bener et al. (2022). DPPH method was applied as follows: X mL of extract solution, "2 – X" mL

99% ethanol, and 2 mL of 0,2 mM of DPPH• solution were added to a glass tube and mixed well. The reaction mixture was incubated at RT in the dark for 30 min. The absorbance of the samples was measured at 515 nm against ethanol with a UV-vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The free radical-scavenging activity was expressed as Trolox equivalents per g of dry weight (mmol TR g^{-1} DW) and calculated according to the following equation:

$$DPPH\ (mmol\ TR\ g^{-1}) = \frac{\Delta_A}{\varepsilon_{TR}} x\ \frac{V_m}{V_s}\ x\ D_f\ x\ \frac{V_E}{m}$$

where ε_{TR}: molar absorption coefficient of TR compound in the DPPH method (2,16 × 10^4 L mol^{-1} cm^{-1}), V_s: sample volume, V_m: total volume of method, *Df*: dilution factor (if needed), V_E: extract volume and, *m* is the mass of dry extract.

Ferric reducing antioxidant power (FRAP) method

The ferric reducing antioxidant power was measured according to the method of Berker et al. (2007). The following reactions were set up to measure FRAP activity: X mL of extract solution, "1 – X" mL 96% ethanol, 2,5 mL of 0,2 M phosphate buffer (pH 6.6), and 2,5 mL of 1% potassium ferricyanide solution were added to a glass tube and incubated for 20 min in a water bath at 50 °C. After incubation, 2,5 mL of 10% trichloroacetic acid (TCA) was added, and thoroughly mixed. An aliquot of 2,5 mL was mixed with 2,5 mL of distilled water and 0,5 mL of 0,1% ferric chloride solution; then the absorbance of the resulting Prussian blue solution was measured at 700 nm after 2 min against a reagent blank. Ferric reducing antioxidant power activity was expressed as Trolox equivalents per g of dry weight (mmol TR g^{-1} DW) and calculated according to the following equation:

$$FRAP\ (mmol\ TR\ g^{-1}) = \frac{A}{\varepsilon_{TR}} x\ \frac{V_m}{V_s}\ x\ D_f\ x\ \frac{V_E}{m}$$

Where; *A*: Sample absorbance measured at 700 nm; ε_{TR}: molar absorption coefficient of TR compound in the FRAP method (1,77 × 10^4 L mol^{-1} cm^{-1}); *Vm*: Total volume of FRAP method measuring solution; *Vs*: Sample volume (mL); *Df*: Dilution factor (if needed); V_E: Volume of the prepared extract (mL); *m*: The amount of sample taken in the extraction process (g).

Ferrous ion-chelating (FIC) method

The ferrous ion-chelating ability was determined in accordance with a method described by Decker & Welch (1990) with minor modifications. FIC reactions contained 1 mL of extract solution, 3,7 mL of distilled water, and 100 µL of 2 mM iron(II) chloride. The reaction was initiated by the addition of 200 µL of 5 mM ferrozine. The reaction was well mixed and incubated for 20 min at RT. After incubation, absorbance was determined at 562 nm against a blank. Distilled water (1 mL) was used as a blank instead of the ferrozine solution, which helped with error correction due to the uneven color of the sample solutions. FIC was calculated as follows:

$$FIC\ (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 was the absorbance without samples and A_1 was the absorbance of the sample.

Statistical analysis

All analyzes were performed with three replicates. Results were subjected to analysis of variance (ANOVA) using SPSS Statistics 22.0 software (IBM, Armonk, NY, USA). Duncan's multiple range test (Duncan, $P \leq 0.05$) was used to separate significant differences between the means. To show the relationship between the biochemical contents and antioxidant capacities, Pearson's linear correlation analysis (Heatmap correlation) was calculated using OriginPro software (version 2021, OriginLab, Northampton, MA). All values were expressed as mean ± standard deviation and the results were based on dry weights (DW) of samples.

Results and Discussion

The mechanical activity of ultrasound accelerates the distribution of the solvent toward the tissues, and thus the bioactive substances in the plant tissue are easily transferred to the solvent. There is no single or standard extraction method for bioactive compounds in plants. Methods and solvents differ according to the active substance desired to be obtained (Ignat et al., 2011). The solubility of compounds found in plants is affected by the polarity of the solvent in which they are dissolved. It is therefore very difficult to have a single procedure for all plant components (Garcia-Salas et al., 2010). In the ultrasonic-assisted extraction method, the biochemical and antioxidant capacities of the samples can be extracted most effectively by using hot or cold solvents. In the present study, the components of the olive leaf dried by ultrasonic-assisted extraction using four solvents of different polarities were investigated in detail.

Organic solvents (such as ethanol, methanol, and acetone), distilled water, and aqueous mixtures are commonly used for solvent extraction. The results of the extractions from olive leaves with different solvents obtained through the ultrasonic-assisted extraction are listed in Figure 1. In this study, total phenolic and total flavonoid contents in the ultrasonic-assisted extraction of olive leaves with water, methanol, ethanol, and methanol-water solvents were determined. The highest total phenolic content was obtained in the order of methanol-water > methanol > ethanol > water (Figure 1a) and the highest total flavonoid content in the order of methanol > ethanol > methanol-water > water (Figure 1b). The highest total phenolic content was obtained from methanol-water (234 mg g^{-1}) and methanol (192 mg g^{-1}) solvent extracts, and the highest total flavonoid content was obtained from methanol (47 mg g^{-1}) and ethanol (46 mg g^{-1}) solvent extracts. Olive leaves are rich in antioxidants, such as phenolics

and flavonoids (Ahmad-Qasem et al., 2013; Dobrinčić et al., 2020; Hannachi et al., 2019; Talhaoui et al., 2014), and values in the current study were higher than the total polyphenol content (61,09 to 92,49 mg g^{-1}) in olive leaves in ultrasonic-assisted extraction with ethanol solvent (50%, v/v) reported by Dobrinčić et al. (2020). Phenolic and flavonoid compounds are potent chain-breaking antioxidants, and recent studies have focused specifically on determining total phenolic and total flavonoid contents (Chew et al., 2021; Dobrinčić et al., 2020; Guo et al., 2011). Flavonoids and polyphenols are located in the cell wall, while free phenolic compounds are found in the cell vacuoles and phenolic and flavonoid compounds can be extracted with suitable solvents (Chew et al., 2021). The total phenolic content of leaves of different olive cultivars was between 52,1-60,4 mg g^{-1} in the extracts obtained from methanol-water solvent (80:20) (Talhaoui et al., 2014), and the total phenolic content was 66 mg g^{-1} in the ultrasonic-assisted extraction of olive leaves with ethanol-water solvent (80:20) by Ahmad-Qasem et al. (2013). Kenaf (*Hibiscus cannabinus*) leaves extracted with 95% ethanol yielded significantly higher total flavonoid content (48,195 mg CHE g^{-1}), while water extract yielded the lowest total flavonoid content (3,843 mg CHE g^{-1}), which is consistent with the results of the current study (Chew et al., 2021). Regarding the use of water in ultrasonic-assisted extraction, it was observed that ultrasounds were effective in extracting phenolic compounds even when applied for a short time (Ahmad-Qasem et al., 2013). As a result of different solid/liquid ratios and different times at ultrasonic-assisted extraction of olive leaves, the total polyphenol content ranged from 0,897 to 5,309 g GAE (gallic acid equivalent) 100 g^{-1}, and the total flavonoid content ranged from 0,042 to 0,239 g RE (rutin equivalent) 100 g^{-1} (Hannachi et al., 2019). Therefore, the solubility of various phenolic and flavonoid components in olive leaves was significantly affected by differences in solvent polarities (Lafka et al., 2013).

Flavonoids can interact with and bind proteins, affecting the antioxidant capacity of sample extracts (Arts et al., 2002; Packer et al., 1999). Therefore, the effect of ultrasonic-assisted extraction with different solvents on protein content was also investigated. While the total protein content in olive leaves was not significantly affected by extraction with methanol and ethanol solvents, the total protein content was significantly reduced in methanol-water, and water extracts (Figure 1c). Total protein content was found to be 5,1 mg g^{-1}, 4,9 mg g^{-1}, 3,5 mg g^{-1}, and 1,9 mg g^{-1} in the leaf extracts obtained with methanol, ethanol, methanol-water, and water solvents, respectively. The total protein content of creosote bush (*Larrea tridentata*) leaves in different organic solvents (methanol, ethanol, or acetone, in a concentration of 90%, 70%, 50%, or 30% v/v) and water extracts changed between 5,79-131,84 mg g^{-1} (Martins et al., 2012). The lowest protein content in the extracts of creosote bush was observed in water extracts, while the highest protein content was observed in methanol (90%, v/v) extracts. The results support the opinion that flavonoids in samples extracted with different solvents interact by binding proteins in the extracts, which may affect their antioxidant capacity.

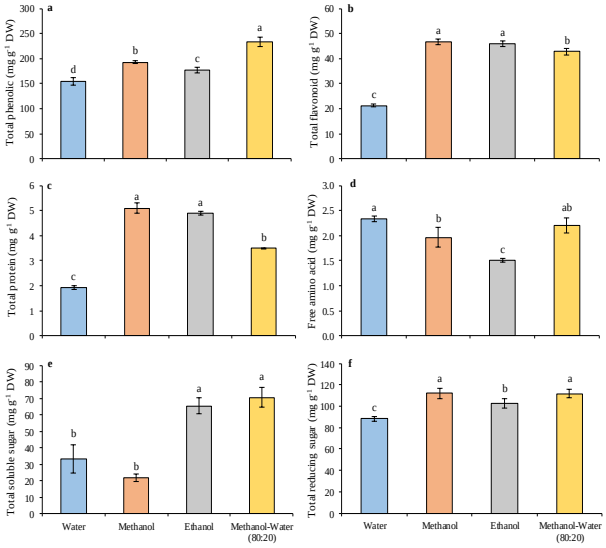

Figure 1. Total phenolic (a), total flavonoid (b), total protein (c), free amino acid (d), total soluble sugars (e) and total reducing sugars (f) in olive leaf extractions with four different solvents. Results represent means ± standard deviation. Different letters indicate significant differences at $P \leq 0.05$ level.

Free amino acids, an important group of polar nitrogen metabolites, are the precursors of various cell components, such as proteins, nucleotides, phenylpropanoids, and alkaloids (Zhang et al., 2017). The total free amino acids content was highest in extracts obtained from water (2,3 mg g^{-1}) and methanol-water (2,2 mg g^{-1}), but the yields were not statistically different ($P \leq 0.05$) (Fig 1d). However, free amino acids content was significantly lower in olive leaves extracted with methanol (2,0 mg g^{-1}) and ethanol (1,5 mg g^{-1}). The total free amino acid content in the ultrasonic-assisted extraction of olive leaves with methanol solvent was reported to be 0,09% (Luo et al., 2019). The free amino acid content of tea flowers (*Camellia sinensis*) extracted with distilled water at 90 °C was 8089 µg g^{-1} (Wang et al., 2010).

Total soluble sugars are metabolic substrates that play a fundamental role in structure and metabolism at the cellular and whole plant levels (Afzal et al., 2021). Ultrasonic extraction with different solvents of olive leaves changed the total soluble sugar content (Figure 1e). The soluble sugar content was highest in methanol-water (70 mg g^{-1}) and ethanol (65 mg g^{-1}) extractions. However, extractions with water (33 mg g^{-1}) and methanol (22 mg g^{-1}) yielded significantly lower soluble sugar contents compared to the methanol-water and ethanol extractions. This probably changed depending on the polarity of each solvent and the solubility of soluble sugars in them. The total soluble

sugar content in the ultrasonic-assisted extraction of olive leaves with methanol solvent was reported to be 14,14% (Luo et al., 2019). The total soluble sugar content of olive leaves extracted with 80:20 (v/v) ethanol-water at 85 °C was 18,20-17,71 mg g^{-1} (Eris et al., 2007).

Reducing sugars play an important role in metabolic pathways and assist in the production of secondary metabolites (Khatri & Chhetri, 2020). The values obtained from different extraction solvents for the reducing sugar content of olive leaves are given in Figure 1f. The highest reducing sugars contents were obtained from methanol (112,2 mg g^{-1}) and methanol-water (111,6 mg g^{-1}) extracts, and the lowest from ethanol (102,8 mg g^{-1}) and water (88,2 mg g^{-1}) extracts. The reducing sugar content in olive leaves varied considerably with respect to the solvent used, possibly depending on the polarity of each solvent and the solubility of the reducing sugars in them.

Four different techniques (CUPRAC, DPPH, FRAP, and FIC) were used to determine the antioxidant capacities of the extracts which are highly sensitive methods with reproducible results. It is recommended that at least two methods should be included to evaluate antioxidant capacity in extracts (Boeing et al., 2014). The CUPRAC assay uses copper(II)–neocuproine reagent as the oxidizing agent and is a very fast way to evaluate the antioxidant activity of extracts in a short time. In this method, the increase in absorbance was measured at 450 nm based on the reduction of copper(II)-neocuproin to the highly colored copper(I)-neocuproin chelate as a result of the color change from light blue to orange-yellow. Figure 2a shows that the copper(II) ion-reducing ability changes significantly depending on the extraction with different solvents ($P \leq 0.05$). The CUPRAC activity in methanol-water and methanol extracts was 0,63 and 0,50 mmol TR g^{-1}, respectively, while the activity in ethanol and water extracts was 0,43 and 0,25 mmol TR g^{-1}, respectively. CUPRAC activity in 12 different solvent extractions (water, ethanol (50, 80, and 100 %), methanol (50, 80, and 100 %), acetone (50, 80, and 100 %), methanol-DMSO, and ethanol-DMSO of yuzu (*Citrus junos*) ranged from 702.4 to 2195,2 mg TE 100 g^{-1} in peels, from 348,7 to 1067,1 mg TE 100 g^{-1} in pulp, from 338,9 to 785,1 mg TE 100 g^{-1} in seeds (Assefa & Keum, 2017). In addition, aqueous forms of organic solvents gave better results for CUPRAC assay in yuzu. The CUPRAC activity in leaves of Chondrolia Halkidiki, Kalamon, and Koroneiki olive cultivars extracted with 70:30 (v/v) methanol-water at 70 °C changed between 271-398 mM TE g^{-1} (Yancheva et al., 2016). The alteration in CUPRAC capacity when olive leaves are extracted with different solvents might be the difference in the polarity of the solvents, changing the extraction efficiency of certain antioxidant compound groups and affecting the antioxidant properties of the samples.

The DPPH assay measures the radical scavenging ability of extracts. DPPH radical scavenging activity was higher than CUPRAC and FRAP capacities, and DPPH radical scavenging activity was found to be in the order of methanol-water (1,09 mmol TR g^{-1}) > methanol (1,01 mmol TR g^{-1}) > ethanol (0,99 mmol TR g^{-1}) > water (0,46 mmol TR g^{-1}) (Figure 2b). Regarding solvent extracts, the DPPH radical scavenging activity in the ethanolic extract of Chinese truffle (*Tuber indicum*) was 1,61 mg mL^{-1} for the EC_{50} value (Guo et al., 2011). Hon-shimeji (*Hypizigus marmoreus*) scavenged DPPH radical by 59,7% and 34,0% at 5 mg mL^{-1} ethanolic and cold-water extracts (Lee et al., 2007), whereas scavenging abilities in the ethanol extract of bracket fungus (*Laetiporus sulphureus*) was 14%, 26%, 55% and 86% inhibition at 100, 200, 400 and at 800 µg mL^{-1} concentrations, respectively (Turkoglu et al., 2007). As a result of the extraction of olive leaves with methanol-water solvent (80:20), DPPH radical scavenging activity Arbequina leaves had the highest scavenging activity with 7,2 µg mL^{-1} for EC_{50}, followed by Sikitita and Picual cultivars with 11,3 µg mL^{-1} for EC_{50} (Talhaoui et al., 2014). This indicates that cultivars differ in their antioxidant capacity.

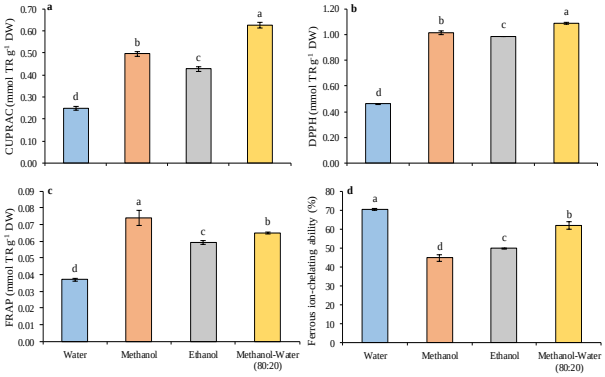

Figure 2. Results of CUPRAC (a), DPPH (b), FRAP (c) and FIC (d) assays from olive leaf extracts with four different solvents. Results represent means ± standard deviation. Different letters indicate significant differences at $P \leq 0.05$ level.

FRAP test was used to determine the reducing ability of antioxidant compounds in olive leaf extracts. FRAP functioned as a reducing agent with the ability to donate a single electron or hydrogen atom for the reduction of antioxidants (Rabeta & Nur Faraniza, 2013). The lowest FRAP capacity was observed in water extract with 0,037 mmol TR g^{-1} and was determined as 0,059, 0,065 and 0,074 mmol TR g^{-1} in ethanol, methanol-water and methanol extracts, respectively (Figure 2c). The FRAP capability of ultrasonic extraction of kenaf leaves with ethanol solvent was 18,39 mg TEAC g^{-1} (Chew et al., 2021). Ahmad-Qasem et al. (2013) reported that the FRAP capacity was between 41,1-89,2 mg TR g^{-1} in olive leaves extracted with ultrasonic-assisted extraction with ethanol-water (80:20) solvent. The FRAP capacity in olive leaves was due to the presence of antioxidant compounds involved in electron transfer. These antioxidant compounds have the ability to neutralize free radicals,

transform them into stable compounds and terminate the reactions initiated by free radicals (Muddathir et al., 2017).

Excess iron in the body causes the production of hydroxyl radicals (OH•) by stimulating lipid peroxidation (Guo et al., 2011). Moreover, the OH• is very toxic even at low concentrations and damages macromolecules (e.g. DNA, lipids, and proteins), causing their structural disorders (Özyürek et al., 2008). Metal ions chelation may be important to prevent the production of hydroxyl radicals that can damage biomolecules. In addition, natural metal chelating compounds, including phenolics and flavonoids, are desirable instead of synthetic chelating agents, which are associated with the problem of toxicity (Gulcin & Alwasel, 2022). Ferrous ions are also considered the most effective pro-oxidants widely used in the food industry. Ferrozine generates complexes with the Fe^{2+} ion, but the chelating agents in the reaction disrupt the complex formation, causing a decrease in the color intensity in the reaction. Therefore, the decrease in color intensity provides an estimate of the ion-chelating activity for the chelator in the reaction. FIC activity was expressed as percent inhibition of ferrozine-Fe^{2+} complex formation in olive leaves with different solvents. As shown in Figure 2d, all solvent extractions interfered with the formation of the ferrous and ferrozine complex and thus olive leaves have ferrous ion-chelating ability. The highest ferrous ion-chelating ability in olive leaves was observed in water (70%) and methanol-water (62%) extractions, while the lowest was observed in ethanol (50%) and methanol (45%) extractions. The ethanolic extracts of Chinese truffle had ferrous ion-chelating abilities of 73,4% at 6 mg mL^{-1} and 77,4% at 12 mg mL^{-1}, and the FIC ability of the samples increased as the concentration increased (Guo et al., 2011). Lee (2007) observed that the FIC abilities of Hon-shimeji were 3,30% and 62,9% in the 0,5 mg mL^{-1} ethanolic and cold-water extracts and 79,2% and 94,1% in the 5 mg mL^{-1} ethanolic and cold-water extracts, respectively. The metal chelating activity was 14,24%, 3,71%, and 18,53% in eggplant (*Solanum melongena*) extracts with 70% methanol, 70% ethanol, and 70% acetone, respectively (Boulekbache-Makhlouf et al., 2013).

Overall, the hydromethanol mixture (methanol-water (80:20)) was the most effective solvent for the ultrasonic-assisted extraction of biochemical content and antioxidant capacity from olive leaves. Other studies have shown that hydroalcoholic mixtures (aqueous mixtures of methanol and ethanol) were more effective than pure solvents in biochemical and antioxidant capacity analyzes (Wang et al., 2009; Waterman & Mole, 1994). The effects of CUPRAC, DPPH radical-scavenging, and FRAP had different antioxidant and radical scavenging activities in solvent extracts, and the lowest antioxidant activity was observed in water extracts regardless of the assay used. The olive leaves extracted with methanol and methanol-water solvents showed higher antioxidant activity than those extracted with the other solvents. In addition, total phenolic content is associated with antioxidant capacities, because the structural properties of phenolic compounds are responsible for antioxidant activity (Katalinic et al., 2004). In the study, antioxidant activities calculated against reactive oxygen species were higher in methanol and methanol-water solvents, consistent with the total phenolic content observed in different solvent extracts. The differences between the findings of the current study and the other studies may come from varietal differences, cultivation practices, environmental and geographical factors, and different plant parts used in the studies.

Based on the results of ultrasonic-assisted extraction with four different solvents, heat-mapped correlation analysis was conducted to examine the relationship between biochemical contents and antioxidant capacity. The result of the correlation analysis based on the extraction of olive leaves with different solvents is provided in Figure 3. Of the 45 coefficients, five were significant at the $P \leq 0.05$ level. Of these five significant correlations, four were positively correlated and one was negatively correlated with each other. The significant positive correlation (r = 0,97) between total phenolic content and CUPRAC may indicate that phenolic compounds mainly contribute to CUPRAC capacity. The significant positive correlation (r = 0,96) between total flavonoid content and DPPH may indicate that flavonoids mainly contribute to DPPH capacity. In addition, the positive correlation of total reducing sugars with DPPH (r = 0,95) and FRAP (r = 0,97) indicates that they are significantly correlated. The significant negative correlation (r = -0,98) between total protein content and FIC assay indicates that proteins have an effect on ferrous ion-chelating abilities. Prolonged ultrasonic-assisted extraction of kenaf leaves with ethanol revealed a weak positive correlation between total flavonoid content and DPPH capacity, as it could cause more losses of flavonoid compounds (Chew et al., 2021). The observation of a linear correlation (r = 0,90) between total protein content and total flavonoid in the extraction of creosote bush leaves with different organic solvents (methanol, ethanol, or acetone, in a concentration of 90%, 70%, 50%, or 30% v/v) and distilled water (Martins et al., 2012) was consistent with the linear correlation (r = 0,93) between total protein and total flavonoid in current results.

We have evaluated the biochemical content and antioxidant capacity of the extracts based on the ultrasonic-assisted extraction of olive leaves with water, methanol, ethanol, and methanol-water solvents. Methanol and ethanol were the best solvents for extracting total flavonoids and total protein; methanol-water extraction gave better results for total phenolics, soluble sugars, CUPRAC and DPPH capacity. The total free amino acids were better extracted with water and methanol-water, and the reducible sugars

with methanol and methanol-water solvents. FRAP assay was better when samples were extracted with methanol and FIC assay with water. Therefore, no single solvent can simultaneously extract all the molecules and antioxidant capacity of a sample.

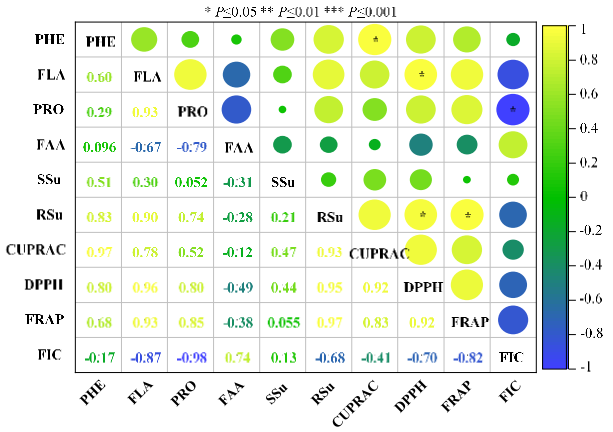

Figure 3. Relationships and correlations between examined parameters generated by a heat map using mean values. The color scale indicates the intensity of the normalized mean values of the different parameters [PHE, total phenolic; FLA, total flavonoid; PRO, total protein; FAA, free amino acid; SSu, total soluble sugars; RSu, total reducing sugars; CUPRAC, cupric reducing antioxidant power; DPPH, DPPH radical-scavenging; FRAP, ferric reducing antioxidant power; FIC, Ferrous ion-chelating].

Conclusion

In this work, the effect of ultrasonic-assisted extraction with different solvents of dried olive leaves on biochemical content and antioxidant capacity was investigated. The results showed that methanol and methanol-water solvents were better extraction solvents for measuring the biochemical content and antioxidant capacity of olive leaves. The extracts obtained from the ultrasonic-assisted extraction of olive leaves with methanol or methanol-water are also a valuable natural product sources with antioxidant capacity and can be used in industrial applications, such as medicine and food. However, due to the toxicity of methanol, the next step in research efforts should focus on finding lesser or non-toxic solvents that can provide high extraction results, such as methanol.

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