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Effects of tetraconazole on antioxidant system in *Lemna minor*

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Abstract: Tetraconazole is a triazole fungicide widely used in agricultural fields and is potentially carcinogenic to humans. Previous studies have shown that this fungicide has toxic effects on plants and other non-target organisms. In this study, the impact of tetraconazole on the antioxidant system of duckweed (*Lemna minor*), a macrophyte plant, was evaluated. For this purpose, duckweed was exposed to tetraconazole at different doses (0.005, 0.01 and 0.02 ppm) for 7 days and the changes in photosynthetic pigments (chlorophyll a, chlorophyll b, carotenoids), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels were determined. In addition, changes in superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) enzyme activities and expression of genes encoding these enzymes were also measured. The results showed that tetraconazole exposure decreased photosynthetic pigment levels and increased MDA and H_2O_2 levels. In comparison to the control groups, the activities of SOD, CAT, POD and APX enzymes increased in a dose-dependent manner. Tetraconazole exposure also induced the mRNA expression levels of SOD, CAT and POD genes in *L. minor* in a dose-dependent manner. These results indicated that tetraconazole induced oxidative stress and activated the antioxidant system in duckweed.

Keywords: Antioxidant Enzyme; Gene expression; Hydrogen peroxide; *Lemna Minor*; Tetraconazole; Malondialdehyde

1 Introduction

Tetraconazole is an imidazole derivative with broad-spectrum antifungal properties and is an effective fungicide used against plant pathogens, especially in agriculture (Li et al. 2020). This fungicide is a steroid-type demethylation inhibitor. This compound, which both prevents the development of fungal spores and treats existing infections, is effective in all tissues of plants thanks to its systemic effect (Li et al. 2020). Tetraconazole inhibits ergosterol, the main component of the cell membrane, by inhibiting cytochrome P450 14-α sterol demethylase in the target organism (Amer et al. 2007). Tetraconazole plays an important role in combating diseases such as mold, rust and leaf spot, which are common in various plant species. In addition, it is used to control diseases such as septoria and rhynchosporium in sugar beet and cereals, apple ringspot on apples and powdery mildew on grapes (Abbassy et al. 2014; Castro-Sobrino et al. 2019). It aims to minimize environmental effects by showing high effectiveness at low usage doses. On the other hand, long-term use can lead to elevated levels of tetraconazole in the soil. Tetraconazole can migrate from soil to groundwater or be transported by rainwater to the aquatic environment, thus contaminating surface waters. In addition, triazole group fungicides such as tetraconazole can affect the photosynthesis rate, enzyme activities, hormone balance and yield of plants (Zhou et al. 1993; Zhou and Ye 1996). Fungicides in this group can trigger endocrine diseases in humans and animals due to their potential to affect steroid hormone biosynthesis (Li et al. 2012).

Lemna minor is a small monocotyledonous macrophyte with floating leaves and submerged roots that thrive in nutrient-rich stagnant or slow-flowing freshwater (Zhang et al., 2013). This aquatic macrophyte is fast growing, easy to culture and has a relatively simple structure, making it one of the model organisms used in ecotoxicology experiments (Song et al. 2012; Zuzelka et al. 2013). *L. minor* has an important ecological function as a primary producer. *L. minor*, which is abundant in freshwater ecosystems, is highly sensitive to organic and inorganic substances such as herbicides, pesticides and metals (Wang, 1990). Therefore, it is widely used to assess the effects of various pollutants on freshwater ecosystems.

This study aimed to investigate the physiological and molecular changes induced by tetraconazole in *L. minor* and to determine the effects of this compound on the antioxidant

system. In this context, *L. minor* was treated with tetraconazole at concentrations of 0.005, 0.01 and 0.02 ppm for 7 days. After seven days, changes in photosynthetic pigments, MDA and H_2O_2 levels, SOD, CAT, POD, APX activities, SOD, CAT and POD gene expressions were investigated.

2 Materials and Method

2.1 Plant Material and culture conditions

The duckweed (*Lemna minor* L.) used in this study was collected from the wetlands around Erzurum Airport. The collected plants were sterilized with 10% NaClO and 1% $HgCl_2$ for 2 minutes and washed several times with pure water. The plants were cultured in 1/10 Hoagland's solution for 3 months in the Plant Physiology Laboratory of the Department of Biology, Faculty of Science, Atatürk University. Toxicity testing was carried out according to the OECD guidelines. For the experiments, 600 healthy *L. minor* plants were selected. Approximately 150 (50X3) plants of the same size were used for each test group and the control. The experiments were carried out in 50 mL Petri dishes. Each Petri dish was filled with 50 mL of 10% Steinberg's solution and 50 *L. minor* plants. The experiments were carried out in the acclimatization room of Atatürk University, Department of Biology, at a temperature of 24±2 °C, 16/8 light/dark photoperiod and 60% humidity. Three different concentrations of tetraconazole (0.005, 0.01 and 0.02 ppm) were used in the experiments. The concentrations used in the experiments were determined based on preliminary experiments. While only 50 mL of 10% Steinberg solution was added to the control group, tetraconazole was added to the Steinberg solution in the other experimental groups. The experiments were carried out in 3 parallel experiments. Plants were harvested after 7 days and used for analysis.

2.1 Chlorophyll content

The procedure recommended by Witham et al. (1971) was used to determine chlorophyll a, chlorophyll b and carotenoid content. Fresh plant material (100 mg) was homogenized in 10 mL of 80% cold acetone for 24 h at 4°C in the dark. The extract was centrifuged at 3000 rpm for 5 min and the results were expressed as mg pigment per gram fresh weight.

2.3 Lipid peroxidation and H_2O_2 levels

To assess lipid peroxidation in *L. minor* plant treated with tetraconazole, the method proposed by Velikova et al. (2000) was used. Approximately 0.4 g of fresh leaves were ground and homogenized in 4 mL of 0.1% TCA. The homogenate was centrifuged at 13,800 rpm for 30 minutes. 1 ml of the supernatant was taken and 1 ml of 0.5% TBA solution was added. The reaction mixture was incubated in boiling water for 30 minutes and then the reaction was stopped by placing the tubes in an ice bath. The samples were centrifuged at 12,000 rpm for 5 minutes, the supernatant was removed and the absorbance value at 532 nm and the absorbance value for non-specific absorption at 600 nm were read. In order to determine the amount of hydrogen peroxide (H_2O_2), the determination was carried out by making some modifications to the method of Velikova et al. (2000).

2.4 Activities of antioxidant enzymes

0.5 g of fresh leaves were thoroughly extracted in liquid nitrogen in a porcelain mortar and the extract was homogenized in 5 ml of 0.1 M KH_2PO_4 (pH: 6.75), 1% PVP, 1 mM EDTA buffer. The homogenate was then centrifuged at 15,000 rpm for 15 min. The supernatant was carefully removed and used as enzyme source in the studies. Superoxide dismutase (SOD) activity was determined by the method of Agarwal and Pandey (2004) with minor modifications, catalase (CAT) activity was determined by observing the decomposition of H_2O_2 according to the method of Aebi (1984); peroxidase (POD) activity was measured by monitoring the oxidation of guaiacol in the presence of H_2O_2 according to the method of Yee et al. (2002), and ascorbate peroxidase (APX) activity was measured based on the decrease in absorbance at 290 nm according to the method of Nakano and Asada (1981).

2.5 Gene expression

To analyze the expression levels of SOD, CAT and POD genes related to the antioxidant system, total RNA was isolated from *L. minor* samples by following the manufacturer's protocol. The concentrations and purity of the obtained RNAs were determined by a NanoDrop spectrophotometer. 1 µg RNA and Quantitect Reverse Transcription kit (Qiagen) were used for cDNA synthesis. 2 µl cDNA and Quantifast SYBR Green RT-PCR kit (Qiagen) were used for real-time PCR. Actin was used as a reference gene in real-time PCR processes and each PCR process was performed in triplicate. The expression level of each gene was analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used in real-time PCR are presented in Table 1.

Table 1 The sequence of primers used for gene expression in *L. minor*.

Gene	Forward	Reverse
ACTIN	ATCCACTCTCACCGTGGTCT	CGGTGGTCTTCGAGTGTTGA
SOD	CCTGAAGCCTCCTCCTTACG	CCAGTGGAACTCCAGCGTC
CAT	ATGTTCCCTATCCCACCT	ATGAATCGTTCTTGCCTG
POD	AATGCCACGGAAGCCCTAA	CGATTGTATGCCCACCCGAG

2.6 Statistical analysis

The experiments were repeated three times and the data obtained are presented as mean ± standard deviation (SD). One-way ANOVA was used for statistical analysis of the data and multiple comparisons were performed with Dunnett's test. Significance limits were set as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. All statistical analyses were performed with GraphPad Prism 8.4 software.

3 Results and discussion

Changes in chlorophyll a, chlorophyll b and carotenoid amounts in *L. minor* plants treated with tetraconazole are

given in Figure 1. Compared to the control groups, 0.005 ppm tetraconazole applied to the plants did not significantly affect either chlorophyll a and b nor carotenoid amounts. On the other hand, the amount of chlorophyll a and carotenoid decreased significantly with increasing dose. Tetraconazole at a concentration of 0.02 ppm significantly decreased the amount of all three pigments compared to the control. The effect of tetraconazole on photosynthetic pigments in plants has not been investigated in the literature. However, another conazole fungicide, difenoconazole, was reported to reduce the amount of chlorophyll in wheat (Liu et al. 2021). Many abiotic factors can affect chlorophyll synthesis in plants (heavy metal stress, pesticides, etc.). The enzyme activity of δ-aminolevulinic acid dehydratase (ALAD), which has an important role in chlorophyll biosynthesis (Cenkci et al. 2010), may also be inhibited by the tetraconazole.

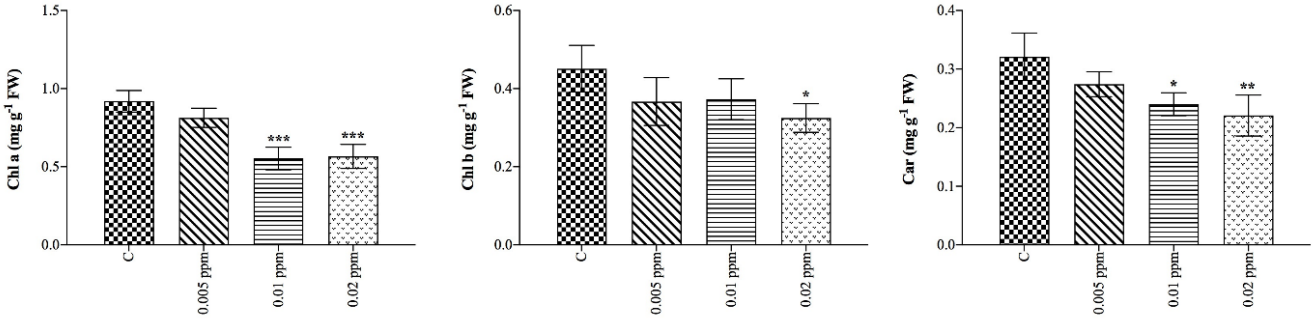

Fig. 1. Effect of tetraconazole on photosynthetic pigments in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001.

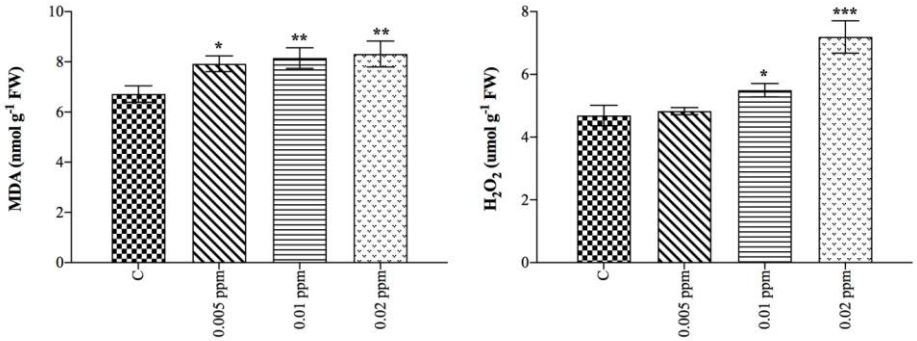

Fig. 2. Effect of tetraconazole on MDA and H_2O_2 levels in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001

Tetraconazole applied to *L. minor* significantly increased the amount of MDA in all treatment groups compared to the control, depending on the dose increase (Figure 2). In addition, the amount of H_2O_2 increased with the tetraconazole applied to the plants (Fig. 2). While this increase was statistically insignificant in the 0.005 ppm tetraconazole group compared to the control, it was found significant at $p < 0.05$ and $p < 0.001$ levels in the other groups. Especially tetraconazole applied to the plants at a concentration of 0.02 ppm increased H_2O_2 levels dramatically. Since the increase in MDA and H_2O_2 levels is known as a response to abiotic stress factors, they are considered an important indicator of stress.

In this study, MDA and H_2O_2 levels and SOD, CAT, POD and APX enzyme activities were measured to monitor the oxidative stress caused by tetraconazole treatment in *L.* minor. When compared with the control groups, tetraconazole applied to *L. minor* at a concentration of 0.005 ppm did not significantly affect the amount of antioxidant system enzymes SOD, CAT and POD.

The amount of APX increased significantly in the 0.005 ppm group compared to the control ($p < 0.05$) (Figure 3). Tetraconazole applied to the plants at a concentration of 0.01 ppm significantly ($p < 0.05$) increased SOD and CAT enzyme activity compared to the control, but did not change POD activity (Figure 3). In addition, APX activity increased significantly ($p < 0.01$) in the group treated with 0.01 ppm tetraconazole compared to the control. Tetraconazole applied to the plants at a concentration of 0.02 ppm increased SOD and POD activities at $p < 0.05$ and APX and CAT activities at $p < 0.01$ compared to the control groups (Figure 3).

Plants have enzymatic and non-enzymatic antioxidant defense systems to tolerate increased levels of ROS. SOD, CAT, POD and APX are members of the enzymatic antioxidant defense system. The enzyme SOD is involved in the dismutation of $O_2^{\bullet-}$ into O_2 and H_2O_2, while the enzymes CAT and POD scavenge H_2O_2. APX degrades H_2O_2 and uses ascorbate as substrate. The increase in antioxidant enzyme and mRNA expression levels observed in plants treated with high concentrations of tetraconazole indicates that the plant activates the antioxidant defense system against increased levels of ROS. However, the MDA increase observed in *L. minor* due to tetraconazole treatment indicates that lipid peroxidation in membranes increased

despite the antioxidant defense system. In parallel with our findings, Macar (2021) reported that tetraconazole increased MDA and induced the activities of antioxidant enzymes such as SOD and CAT in onion (*Allium cepa* L.) roots. It was also reported that diphenoconazole, one of the conazole fungicides, increased H_2O_2 and MDA levels in wheat and increased SOD, CAT, POD and APX enzyme activities (Liu et al. 2021).

Additionally, the transcript levels of SOD, CAT and POD genes, which are related to the antioxidant system, were up-regulated after tetraconazole exposure (Figure 4).

In conclusion, this study showed that tetraconazole not only altered photosynthetic pigments, antioxidant enzyme activities and MDA levels in *L. minor*, but also affected the expression of genes related to the antioxidant system at the transcript level.

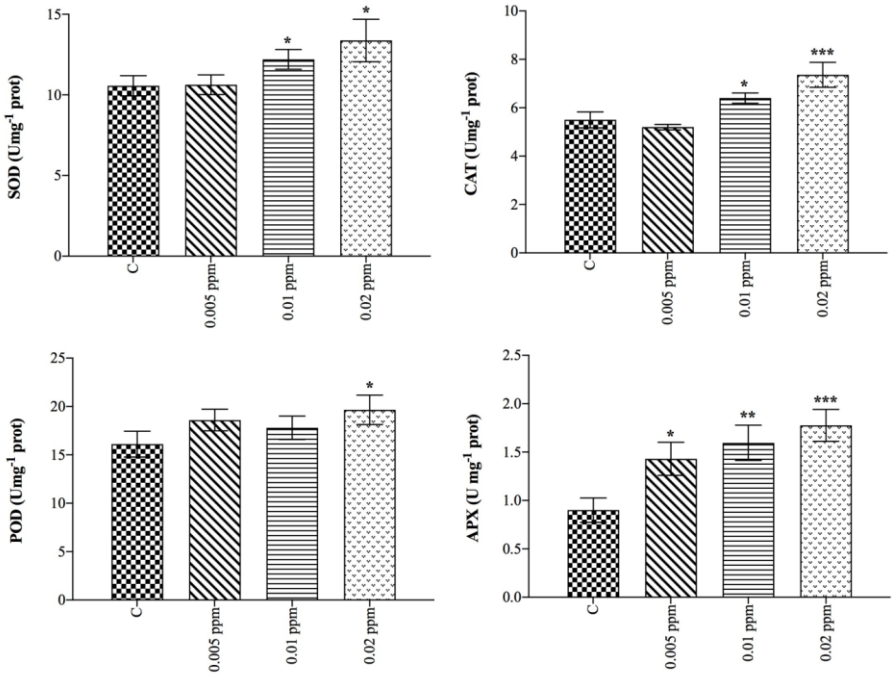

Fig. 3. Effect of tetraconazole on SOD, CAT, POD, and APX enzyme activities in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001.

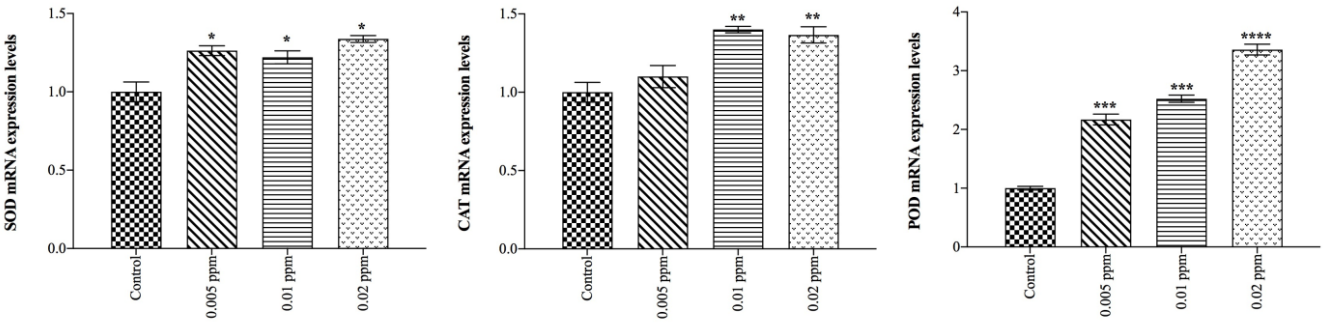

Fig. 4. Effect of tetraconazole on SOD, CAT, and POD mRNA expression levels in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

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Authors' contributions: OA: Design, data analysis, manuscript writing, laboratory experiments

Conflict of interest disclosure:

The author declares no conflict of interest.

References

Abbassy MA, Marzouk MA, Nasr HM, Mansy AS. (2014) Effect of imidacloprid and tetraconazole on various hematological and bio-chemical parameters in male albino rats (*Rattus norvegious*). Journal of Political Science and Public Affair 2(3):1-7.

Aebi H. (1984) Catalase in vitro. Methods Enzymol 105, 121–126.

Agarwal S, Pandey V. (2004) Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. Biol Plant 48:555-560.

Amer MM, Shehata MA, Lotfy HM, Monir HH. (2007). Determination of tetraconazole and diniconazole fungicide residues in tomatoes and green beans by capillary gas chromatography. Yakugaku Zasshi 127(6):993-999.

Castro-Sobrino L, Briz-Cid N, Figueiredo-González M, Sieiro-Sampedro T, González-Barreiro C, Cancho-Grande B, Rial-Otero R, Simal-Gándara J. (2019) Impact of fungicides mepanipyrim and tetraconazole on phenolic profile and colour of Mencía red wines. Food Control 98:412-423.

Cenkci S, Cigerci IH, Yildiz M, Ozay C, Bozdag A, Terzi H. (2010). Lead contamination reduces chlorophyll biosynthesis and genomic template stability in *Brassica rapa* L. Environ Experiment Bot 67:467-473.

Li Y, Nie J, Chang W, Xu G, Farooq S, Liu M, Zhang J. (2020) Enantioselective behavior analysis of chiral fungicide tetraconazole in apples with UPLC-MS/MS. Food Control 116: 107-305.

Macar O. (2021) Multiple toxic effects of tetraconazole in *Allium cepa* L. meristematic cells. Environ Sci Pollut Res 28:10092-10099.

Nakano Y, Asada K. (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880.

Song G, Gao Y, Wu H, Hou W, Zhang C, Ma H. (2012) Physiological effects of anatase TiO_2 nanoparticles on *Lemna minor*. Environ Toxicol Chem 31:2147-2152.

Velikova V, Yordanov I, Edreva A. (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Sci 151:59-66.

Wang W. (1990) Literature review on duckweed toxicity testing. Environ Res 52:7-22.

Yee Y, Tam NFY, Wong YS, Lu CY. (2002) Growth and physiological responses of two mangrove species (*Bruguira gymnorrhiza* and *Kandelia candel*) to waterlogging. Environ Experiment Bot 1:13.

Zezulka S, Kummerová M, Babula P, Vánová L. (2013) *Lemna minor* exposed to fluoranthene: Growth, biochemical, physiological and histochemical changes. Aquatic Toxicol 140-141:37-47.

Zhang B, Li X, Chen D, Wang J. (2013) Effects of 1-octyl-3-methylimidazolium bromide on the antioxidant system of *Lemna minor*. Protoplasma 250:103-110.

Zhou WJ, Ye QF. (1996) Physiological and yield effects of uniconazole on winter rape (*Brassica napus* L.). J Plant Growth Regul 15:69-73.

Zhou WF, Shen HC, Xi HF, Ye QF. (1993) Studies on the regulation mechanism of paclobutrazol to the growth of rape plant. Acta Agric Univ Zhejiang 19:316-320.

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Evaluation of the effects of immobilization on Spirulina *platensis* cultures

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Abstract: Different studies are carried out to reduce the production costs of *Spirulina platensis* biomass, which is produced in liquid cultures and has a high commercial value. These studies are in the direction of determining the optimum production method by changing the nutrient medium content and culture conditions. Immobilization is a method that changes the culture conditions. In our study, *Luffa cylindrica* (luffa), a natural fiber, was used for the immobilization of *S. platensis* cells, and the growth parameters and pigment production of the cultures were investigated. In two-factor experiments, week (first and second weeks) and culture type (free and immobilized cultures), statistically significant week*culture type interactions were found in optical density, dry weight, pH, and chlorophyll-a content. Immobilization did not increase the optical density and biomass production of the cultures. The highest optical densities and biomass productions were obtained in two-week-old free cultures, where the pH value was also found to be the highest. The highest values of chlorophyll-a and total carotene content were obtained from one-week immobilized cultures (30.06 µg/ml and 48.35 µg/ml, respectively). The fact that immobilization increased pigment production in one-week-old cultures indicates that when pigment production is targeted in *S. platensis* cultures, two-stage cultures that increase pigment yield via one-week immobilization after biomass production is completed can be used.

Keywords: *Spirulina platensis*; Immobilization; Luffa, Cell culture

1 Introduction

Spirulina plantesis is a genus of *Cyanobacteria* with high nutritional values. Its most striking feature is that it lives in waters with high carbonate and bicarbonate levels and pH values up to 11. It has a high fat and high nutritional value content such as provitamins, minerals, gamma-linolenic acid, carotene and xanthophyll pigments, and beneficial amino acids. Since centuries ago, Indigenous peoples living in Africa and Mexico have utilized *S. plantesis* as a food source (Venkataraman 1997; Miranda et al. 1998).

Luffa cylindrica (luffa) is a woody plant commonly known as gourd fiber and belongs to the family *Cucurbitaceae*, which is widely used for different purposes. L. cylindrica consists of 10% wood pulp (lignin), 30% semi-cellulose and 60% cellulose. It is widely distributed in North and East Australia, Central and South America, Asia, Africa, and the Mediterranean. It is a low-cost natural fiber that is harmless to human health and recyclable. *L. cylindrica* has a round or angular stem structure in terms of vegetative characteristics (Herklots 1972; Jeffrey 1990; Ghali et al. 2009). Due to these characteristics, it is an ideal natural material that can be used in immobilization studies to be placed inside the flasks.

Immobilization is a method that allows free cells in cell cultures to adhere to surfaces to obtain more production efficiency. This method, which is frequently used in plant cell cultures, is not required in *S. platensis* cultures due to the ability of algae to multiply without the need for attachment. Immobilization of cells on natural fibers in plant cell cultures has been found to produce different results in terms of both biomass production and secondary metabolite production (Nartop et al. 2013).

When *S. platensis* immobilization studies are examined, it is seen that the cells are trapped in semi-permeable membrane or gel structures and used in the cleaning of wastewater (Purev et al. 2023; Chen et al. 2023).

Our study aimed to investigate the growth parameters and contents of *S. platensis* cultures immobilized with luffa, a natural fiber. Therefore, the growth parameters of free and immobilized cell cultures were investigated comparatively

and their contents were determined by spectrophotometric methods.

2 Materials and Method

2.1. Materials

S. platensis starter culture was obtained from Çukurova University, Faculty of Fisheries (Adana, Turkey). The nutrient medium used was modified (Zarrouk's medium) and contained (g/L);); $NaHCO_3$ - 37.2 g/L; Na_2CO_3 - 16.12 g/L; K_2HPO_4 $3H_2O$ - 2.62 g/L; $NaNO_3$ – 4 g/L; NaCl - 4 g/L; $MgSO_4$ $7H_2O$ - 0.8 g/L; $CaCl_2 2H_2O$ - 0.04 g/L; $FeSO_4 7H_2O$ - 0.04 g/L; $EDTANa_2$ - 0.32 g/L; $ZnSO_4 7H_2O$ - 0.001 g/L; $MnSO_4H_2O$ - 0.0012 g/L; H_3BO_3 - 0.01 g/L; $Na_2MoO_4 2H_2O$ - 0.001 g/L; $CoCl_2 6H_2O$ - 0.0081 g/L; $CuSO_4 5H_2O$ - 0.00005 g/L; $FeSO_4 7H_2O$ - 0.7 g/L; $EDTANa_2$ - 0.8 g/L. This nutrient medium prepared with distilled water was sterilized in an autoclave at 121ºC for 15 minutes. All chemicals used in this study were purchased from Sigma and Acros Organics and were of analytical quality (97% purity).

Fig. 1 *Luffa cylindrica* (luffa) cut for use as immobilization material

Luffa cylindrica was used as immobilization material. Luffa was cut as shown in Fig 1, washed by rinsing with detergent water, rinsed three times with tap water, washed three times with distilled water, and placed at the bottom of the flasks. The mouth of the flasks was covered with cotton wool and aluminum foil and sterilized in an oven at 170ºC for 60 minutes (Nartop et al. 2013).

2.2. Methods

S. platensis cultures were subcultured continuously at 7-day intervals by half dilution before immobilization. The volume of the immobilized cultures formed by pouring half-diluted cultures into the flasks with luffa placed on the bottom was 500 ml and the working volume was determined as 400 ml. Cultivation was carried out at 23 µmol/m^2s light intensity, 3 L/min ventilation rate, and 26±1ºC. Cultures were terminated in the first and second weeks, filtered, fresh weights were determined, and dried in an oven at 40ºC, and dry weights were recorded (Nartop and Kuşku 2023).

Before the cultures were terminated in the first and second weeks, pH measurements were performed and optical densities of 3 ml samples were measured spectrophotometrically at 665 and 680 nm (Nartop and Kuşku 2023).

For chlorophyll-a and carotenoid analyses, 5 mg samples from each experiment were mixed with 5 ml of 70% ethanol, filtered after extraction in an ultrasonic bath for 60 min, and absorbance values at 665 and 450 nm were recorded. Chlorophyll-a (Dineshkumar et al., 2015) and carotenoid (Saefurahman et al., 2021) concentrations were calculated according to the following formulae; (A_{665}: Absorbance measured at 665 nm; A_{450}: Absorbance measured at 450 nm).

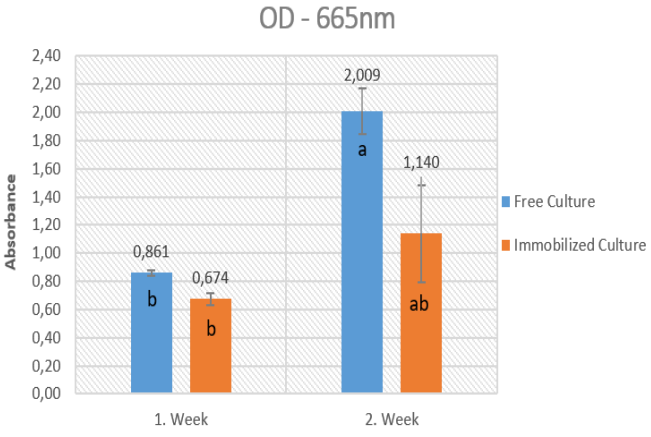
$$\text{Chlorophyll-a concentration } (\mu g/mL) = A_{665} \times 13.9 \quad (1)$$

$$\text{Carotenoid concentration } (\mu g/mL) = A_{450} \times 25.2 \quad (2)$$

In the study, each trial was carried out with three replications. Data analyses were performed by ANOVA and Tukey was used for post-hoc tests.

3 Results

In the spectrophotometric analysis at 665 nm, both weekly data and week*culture type interaction were statistically significant ($p<0.05$). The second week was in the first group with 1.574 and the first week was in the second group with 0.767 (Table 1). In the week*culture type interaction, two weeks of free cultures were in group A with 2.009, two weeks of immobilized cultures were in group AB with 1.140, and one week of free and immobilized cultures were in group B with 0.861 and 0.674, respectively (Fig 2).



(F_{week}= 17.68, p_{week}=0.014; $F_{interaction}$= 3.16, $p_{interaction}$=0.015)

Fig. 2 Optical densities of *S. platensis* free and immobilized cultures measured at 665 nm

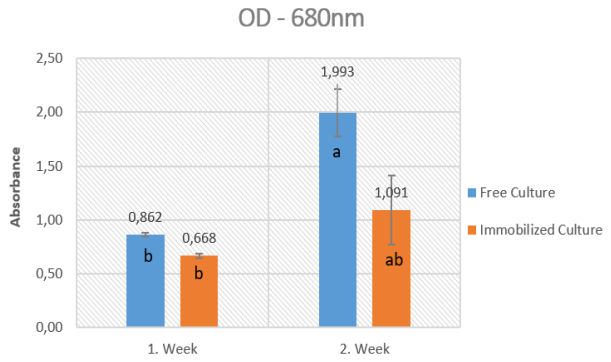
In the optical density measurement at 680 nm, both parameters and the interaction of the two parameters were statistically significant ($p<0.05$). Similar to the results obtained at 665 nm, the second week was in the first group with 1.542 and the first week was in the second group with 0.765 (Table 1). Free cultures were in group A with 1.427 and immobilized cultures were in group B with 0.879. In the week*culture type interaction, two-week free cultures were in group A with 1.992, two-week immobilized cultures were in group AB with 1.091, and one-week free and immobilized cultures were in group B with 0.862 and 0.668, respectively (Fig 3).

Table 1: Means and statistical groups of the growth parameters' results of *S. platensis* cultures depending on the duration and culture type.

Growth Parameter	Duration		Culture Type	
	1. Week	2. Week	Free Culture	Immobilized Culture
OD-665	0.767 ^{B}	1.574 ^{A}	1.435	0.907
OD-680	0.765 ^{B}	1.542 ^{A}	1.427 ^{A}	0.879 ^{B}
Fresh Weight (g)	0.659	0.873	1.025	0.507
Dry Weight (g)	0.060	0.073	0.096 ^{A}	0.035 ^{B}
pH	10.16 ^{B}	10.23 ^{A}	10.22	10.18
Chlorophyll-a (µg/ml)	28.17 ^{A}	14.30 ^{B}	21.95	20.52
Total Carotenoid (µg/ml)	47.88 ^{A}	33.57 ^{B}	44.52	36.90

1 Means marked with the same letter are statistically in the same statistical group ($p<0.05$).

The difference between the fresh weight values in the trials evaluated according to week and culture type was not statistically significant ($p>0.05$). The highest fresh weight was 1.329 g in two-week free cultures, 0.720 g in one-week free cultures, and 0.598 g and 0.416 g in immobilized cultures in the first and second weeks, respectively (Fig 4). The fresh weight results obtained were in parallel with the optical density measurements in free cultures, but the fresh weight in immobilized cultures was lower in the second week.



(F_{week}= 15.73, p_{week}= 0.017; $F_{culture\ type}$ = 7.83, $p_{culture\ type}$ = 0.049; $F_{interaction}$ = 3.26; $p_{interaction}$ = 0.015)

Fig. 3 Optical densities of *S. platensis* free and immobilized cultures measured at 680 nm

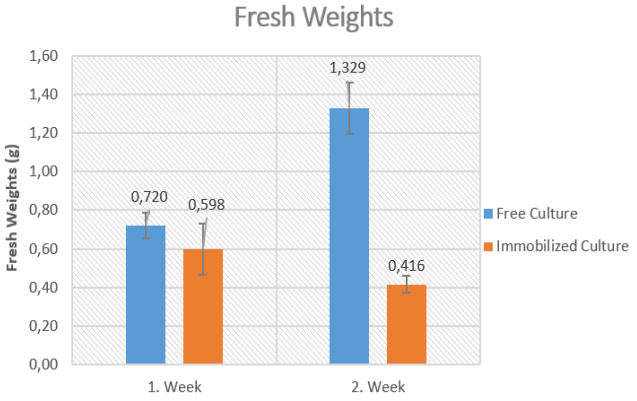
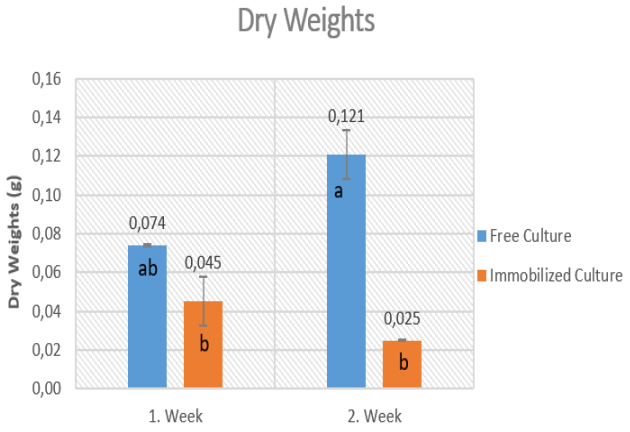


Fig. 4 Fresh weights of free and immobilized cultures of *S. platensis*

The results obtained in dry weights, culture type, and week*culture type interaction were statistically significant ($p<0.05$). Free cultures were in the first group with 0.096 g and immobilized cultures were in the second group with 0.035 g (Table 1). In the week*culture type interaction, two weeks of free cultures were in group A with 0.121 g, one week of free cultures were in group AB with 0.074 g, one and two weeks immobilized cultures were in group B with 0.045 g and 0.025 g, respectively (Fig 5).

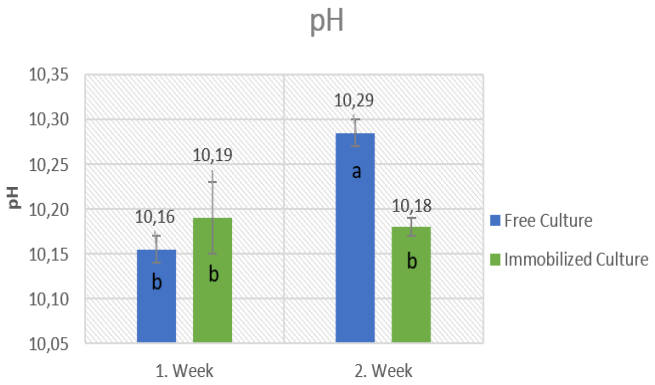
Fig 6 shows the pH values of S. *platensis* free and immobilized cultures. The week*culture type interaction was found to be statistically significant in the pH values obtained in the experiments ($p<0.05$). pH values were determined between 10.16 and 10.29 (Table 1). In free cultures, an increase was detected in the second week and the pH value of 10.29 was statistically in group A. The pH values of immobilized cultures in the first and second weeks were 10.19 and 10.18, respectively, and these values were in group B together with 10.16 obtained from free culture in the first week.



($F_{culture\ type}$= 49.99, $p_{culture\ type}$= 0,002; $F_{interaction}$ = 14.26, $p_{interaction}$ = 0.019)

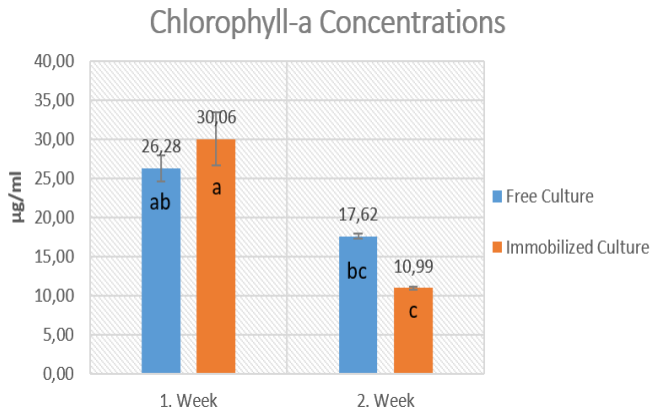
Fig. 5 Dry weights of free and immobilized cultures of *S. Platensis*

S. platensis chlorophyll-a and total carotene contents are given in Fig 7 and Fig 8. Chlorophyll-a contents were in the range of 10.99 - 30.03 µg/ml. In the results obtained in terms of chlorophyll-a concentrations, week and culture type*week interaction were found statistically significant ($p<0.05$). While the first week was in the first group with 28.17 µg/ml, the second week was in the second group with 14.30 µg/ml (Table 1). In the culture type*week interaction, one week of immobilized culture was in group A, one week of free culture was in group AB, two weeks of free culture was in group BC and two weeks of immobilized culture was in group C. One week of immobilization increased the chlorophyll-a content.



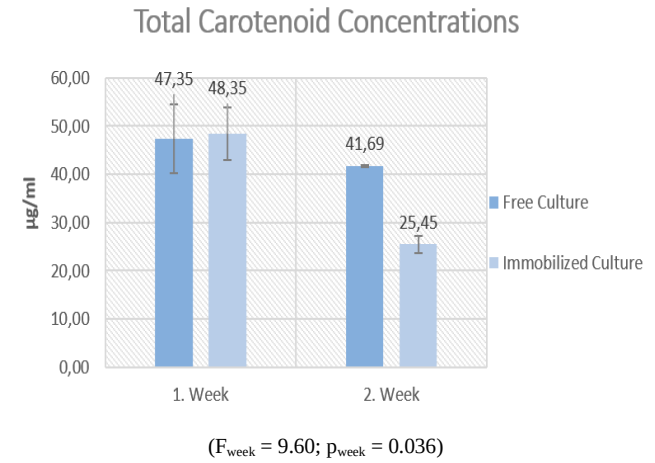
($F_{interaction}$ = 9.12; $p_{interaction}$ = 0.039)

Fig. 6 pH values of *S. platensis* free and immobilized cultures



(F_{week} = 52.83, p_{week} = 0.002; $F_{interaction}$ = 7.43, $p_{interaction}$ = 0.043)

Fig. 7 Chlorophyll-a concentrations of free and immobilised cultures of *S. platensis*



(F_{week} = 9.60; p_{week} = 0.036)

Fig. 8 Total carotene concentrations of free and immobilised cultures of *S. platensis*

Total carotene concentration values were determined in the range of 25.45-48.35 µg/ml. The difference between the data obtained according to the week was statistically significant ($p<0.05$) and the first week was in group A with 47.88 µg/ml, while the second week was in group B with 33.57 µg/ml (Table 1). The highest concentration was obtained in one week of immobilized cultures, but in the second week, the concentration decreased to a lower level than in free culture.

4 Discussion

Within the scope of our study, *S. platensis* cultures were immobilized with luffa, a natural fiber. The results of optical density measurements were parallel to each other at 665 nm and 680 nm. The highest optical density was found in two-week free cultures, while the week*culture type interaction was statistically significant in both measurements. Densities were higher in the second week as expected. Immobilized cultures were lower than free cultures. This indicates that free cells are retained in the luffa and therefore the optical density decreases. Fresh and dry weight data are in parallel with each other. Differences between fresh weights were not statistically significant, but culture type and culture type*week interaction were significant for dry weight differences. In terms of biomass accumulation, free cultures were more efficient than immobilized cultures, and the highest fresh and dry weight values were obtained in two-week free cultures. Biomass accumulation in two-week immobilized cultures was lower than in one-week immobilized cultures. This is in contrast to the situation detected in optical density measurements. This was considered as a sign that the number of cells increased over time, but the cells did not develop enough to increase the biomass.

Dry matter (biomass of liquid cultures) is an important parameter for *S. platensis* culture studies (Azgın et al., 2015). Biomass accumulation is a growth parameter that is often used in conjunction with optical density measurements for the development of cultures. Culture conditions such as temperature, light, and pH and the chemical composition of the nutrient medium (phytohormones, macro- and microelements, etc.) are known to affect the growth of *S. platensis* cultures (Abd El-Monem et al. 2018; Chen et al. 2010; Danesi et al. 2011; Romanenko et al. 2015; Gabr et al. 2020).

Immobilization is an application that changes the culture conditions and in our study, it was determined that immobilization did not increase the biomass. Lower biomass accumulation occurred in immobilized cultures compared to free cultures and biomass decreased in the second week. The pH value of the culture medium is one of the most important factors affecting the growth of S. *platensis* cultures. Thirumala (2012) reported that the optimum pH value was 10-11, but Fagiri et al. (2013) reported that the optimum pH value was 7-9. In our study, it was determined that the highest optical density and biomass accumulation were obtained at the highest pH value (10.29). The pH value was highest at two weeks of free cultures. In our study, fresh weights were determined between 0.416 - 1.329 g and dry weights between 0.025-0.121 g. In immobilized cultures, fresh weights were 0.598 g and 0.416 g and dry weights were 0.045 g and 0.025 g in the first and second weeks, respectively. Pandey et al. (2010) reported dry biomass in the range of 0.22 - 0.91 g. Ogbonda et al. (2007) determined it as 1.515 g at pH 10.

S. platensis pigment production is affected by light intensity (Chen et al. 2010; Danesi et al. 2011). However, other factors also affect pigment production. In our previous study, biosynthetic silver nanoparticles were found to affect pigment

production depending on the concentration (Nartop and Kuşku 2023). In this study, immobilization was found to affect pigment production. One-week immobilized cultures had the highest content in terms of both chlorophyll-a and total carotene. In the second week, pigment production decreased in both culture types, and the lowest value was determined in two-week immobilized cultures. In contrast to our findings, Thirumala (2012) reported that pigment accumulation increased as the culture period increased.

5 Conclusion

It is known that immobilization can cause biomass increase in plant cell cultures. In our study, it was observed that optical density and biomass did not increase with immobilization in *S. platensis* cultures, but the highest pigment production was obtained in immobilized cultures at the end of the first week. It can be recommended as a result of our study that when pigment production in *S. platensis* cultures is targeted, after obtaining high biomass, a short-term immobilization application for one week as a second step would be beneficial.

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Authors' contributions:

PN: Study conception, design, supervision, data analysis, tatistical analysis, literature review, manuscript writing, editing and laboratory experiments
EK: Laboratory experiments

Conflict of interest disclosure:

The authors declare that there are no real, potential, or perceived conflicts of interest for this article.

References

Abd El-Monem AM, Gharieb MM, Hussian AM, Doman KM (2018) Effect of pH on phytochemical and antibacterial activities of *Spirulina platensis.* Int J Appl Environ Sci 13(4):339–351

Azgın C, Işık O, Uslu L, Ak B (2015) A comparison the biomass of productivity, protein and lipid content of *Spirulina platensis* cultured in the pond and photobioreactor. J Biol Environ Sci 8(24):183–187.

Chen H-B, Wu J-Y, Wang C-F, Fu C-C, Shieh C-J, Chen C-I, Wang, C-Y, Liu Y-C (2010) Modeling on chlorophyll a and phycocyanin production by Spirulina platensis under various light-emitting diodes. Biochem Eng J 53:52–56. doi: 10.1016/j.bej.2010.09.004

Chen Z, Osman AI, Rooney DW, Oh W-D, Yap P-S (2023) Remediation of Heavy Metals in Polluted Water by Immobilized Algae: Current Applications and Future Perspectives. Sustainability 15:5128. doi: 10.3390/su15065128

Danesi EDG, Rangel-Yagui CO, Sato S, de Carvalho JCM (2011) Growth and content of Spirulina platensis biomass chlorophyll cultivated at different values of light intensity and temperature using different nitrogen sources. Braz Microbiol 42:362–373. doi: 10.1590/S1517-83822011000100046.

Dineshkumar R, Umamageswari P, Jayasingam P, Sampathkumar, P (2015) Enhance the growth of Spirulina platensis using molasses as organic additives. World J Pharm Res 4(6): 1057-1066.

Gabr GA, El-Sayed SM, Hikal MS (2020) Antioxidant activities of phycocyanin: a bioactive compound from Spirulina platensis. J Pharm Res Int 32(2): 73–85.

Ghali L, Msahli S, Zidi M, Sakli F (2009) Effect of pre-treatment of luffa fibres on the structural properties. Mater Lett 63(1): 61-63. doi: 10.9734/jpri/2020/v32i230407

Herklots GAC (1972) Vegetables in South-East Asia, Hafner Press, New York, pp. 326-333.

Jeffrey C (1990) An outline classification of the Cucurbitaceae. In: Bates, D.M., Robinson, R.W., Jeffrey, C. Biology and utilization of the Cucurbitaceae. Ithaca and London: Cornell University, pp. 449-463. doi: 10.7591/9781501745447-039

Miranda MS, Cintra RG, Barros SBM, Filho JM (1998) Antioxidant activity of the microalga Spirulina maxima. Braz J Med Biol Res 31(8):1075–1079. doi: 10.1590/s0100-879x1998000800007

Nartop P, Akay Ş, Gürel A (2013) Immobilization of Rubia tinctorum L. suspension cultures and its effects on alizarin and purpurin accumulation and biomass production. Plant Cell Tiss Org Cult 112(1): 123-128. doi: 10.1007/s11240-012-0212-z

Nartop P, Kuşku E (2023) Influence of bio-AgNP on growth and biochemical composition of *Spirulina platensis*, Biol Bull 50: 363-372

Ogbonda KH, Aminigo RE, Abu GO (2007) Influence of temperature and pH on biomass production and protein biosynthesis in a putative Spirulina sp. Biores Technol, 98(11): 2207–2211. doi: 10.1016/j.biortech.2006.08.028

Pandey JP, Pathak N, Tiwari A (2010) Standardization of pH and light intensity for the biomass production of Spirulina platensis. J Alg Biomass Util 1(2):93–102.

Purev O, Park C, Kim H, Myung E, Choi N, Cho K (2023) Spirulina platensis immobilized alginate beads for removal of Pb(II) from aqueous solutions. Int J Environ Res Public Health 20:1106. doi: 10.3390/ijerph20021106

Romanenko EA, Kosakovskaya IV, Romanenko PA (2015) Phytohormones of microalgae: Biological role and involvement in the regulation of physiological processes, Pt I: Auxins, abscisic acid, ethylene. Int J Alg 17(3): 275–289. doi: 10.1615/InterJAlgae.v17.i3.80

Saefurahman G, Rahman AA, Hidayatuloh S, Farobie O, Abidin Z. (2021) Continuous extraction of Spirulina platensis biopigments using different extraction sequences, IOP Conf Ser Earth and Environ Sci 749:012005. doi: 10.1088/1755-1315/749/1/012005

Thirumala M (2012) Optimization of growth of Spirulina platensis LN1 for production of carotenoid. Inter J Life Sci Biotechnol Pharm Res 1: 152–157.

Venkataraman L (1997) Spirulina platensis (Arthrospira): Physiology, Cell Biology and Biotechnology, ed. Avigad Vonshak, J Appl Phycol 9:295–296. ISBN: 0-203-48396-0

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Bulletin of Biotechnology

Determination of antibacterial activity of resveratrol on some pathogenic bacteria

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Abstract: This study aims to investigate the antibacterial effect of resveratrol, a natural polyphenol, on some pathogenic bacteria. Resveratrol is a compound found in plants such as red grapes and is known for its antioxidant, anti-inflammatory and antimicrobial properties. Due to these properties, it is considered as a potential agent in the prevention and treatment of bacterial infections. In this context, in this study, it was aimed to determine the antibacterial effects and effective dosage of resveratrol on *Salmonella typhimurium*, *Staphylococcus epidermidis*, *Pseudomanas aeruginosa*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, *Enterococcus faecalis*, *Enterobacter cloaceae*, *Escherichia coli*, and *Listeria monocytogenes*. Antibacterial activity studies were performed by agar well diffusion method. As a result of antibacterial activity studies, inhibition zones with a diameter of 14.0 ± 1.0 mm for *S. epidermidis*, 15.0 ± 0.5 mm for *E. faecalis*, 15.0 ± 0.5 mm for *E. cloaceae* and 16.0 ± 0.5 mm for *L. monocytogenes* were measured at 500 µg\mL dosage of resveratrol. However, resveratrol was found to have a bacteriostatic effect against *S. typhimurium*, *P. aeruginosa*, *Y. pseudotuberculosis*, *P. vulgaris* and *E. coli*. According to these results, it can be said that resveratrol may be effective against some pathogenic bacteria and may contribute to the development of new strategies for its use as an antibiotic adjuvant, although its use in terms of both food safety and public health is foreseen.

Keywords: Resveratrol, pathogenic bacteria, inhibition, antibacterial effect

1 Introduction

Bacterial infections pose a significant threat to both public health and food safety. The widespread use of antibiotics in the treatment of bacterial infections has particularly led to an increase in antibiotic resistance, making the development of alternative treatment strategies essential (Patel and Scott 2011). In this context, compounds derived from natural sources have emerged as a promising area for the discovery of new antibacterial agents (Das and Kapmaz 2019). Resveratrol, a natural polyphenol, is found naturally in plants and is most abundant in the skins of red grapes, blueberries, raspberries, mulberries, and peanuts (Das and Kapmaz 2019). Studies have reported that resveratrol has antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Additionally, it serves as a protective compound in plants against stress, infections, or ultraviolet (UV) radiation (Karameşe and Dicle 2022). Resveratrol attracts attention due to its numerous potential health benefits, particularly its protective effects against heart diseases, diabetes, cancer, and neurodegenerative diseases (Faydaoğlu and Sürücüoğlu 2013). Moreover, it has been shown to be effective against bacterial infections, making it important to consider as an alternative or adjuvant to antibiotics (Das and Kapmaz 2019). Although the literature indicates that resveratrol is effective against many pathogenic bacteria, the effects on various bacteria are not fully understood. However, studies have shown that resveratrol is effective against both Gram-positive and Gram-negative bacteria. The antibacterial activity of resveratrol is known to be mediated through mechanisms such as inhibiting bacterial cell wall synthesis, disrupting membrane permeability, or affecting bacterial metabolism (Karameşe and Dicle 2022).

Studies have observed that resveratrol possesses inhibitory properties against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Karameşe and Dicle 2022). Additionally, it has been revealed through studies that resveratrol can inhibit the biofilm-forming capabilities of bacteria, which makes them more resistant to antibiotics, and thus may be effective in the treatment of infections (Karameşe and Dicle 2022). In the face of the dramatic rise in antibiotic-resistant pathogens, the use of such molecules as direct or indirect antibiotic adjuvants is of great importance. Antibiotic adjuvants are known to inhibit

bacterial resistance mechanisms and make pathogens susceptible to the effects of antibiotics, thereby extending the lifespan and efficacy of existing antibiotic stocks and suggesting the development of new strategies (Vestergaard and Ingmer 2019). This study investigates the antibacterial efficacy of resveratrol against *Salmonella typhimurium*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Escherichia coli*, and *Listeria monocytogenes* bacteria and determines the effective dosage.

2.Materials and Methods

2.1 Resveratrol

In this study, the antibacterial efficacy of resveratrol (Resveratrol-(4-hydroxyphenyl-13C6, Merck), a natural polyphenol, was determined (Figure 1). A 30% aqueous solution of DMSO (Dimethyl sulfoxide - C2H6OS, Merck) was used as the solvent for resveratrol. The final concentration of resveratrol was prepared fresh before each use at 5 mg/mL.

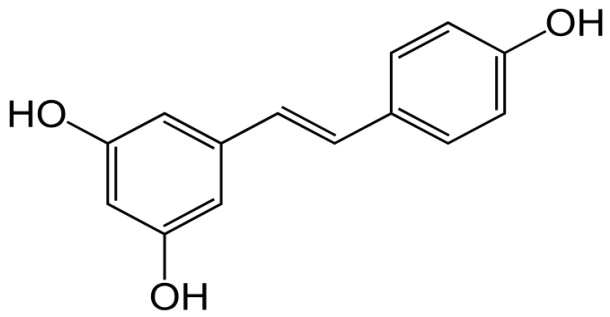

Figure 1. Chemical Structure of Resveratrol

2.2 Test Bacteria Used in the Study

A total of nine different bacteria were used as test bacteria in antibacterial activity studies (Table 1). The bacteria were grown in Nutrient Broth (NB, Merck) and Nutrient Agar (NA, Merck) for 16-18 hours at 37°C.

Table 1. Indicator Bacteria

Test Bacteria	Source
Salmonella typhimurium	ATCC 14028
Staphylococcus epidermidis	ATCC 12228
Pseudomanas aeruginosa	ATCC 27853
Yersinia pseudotuberculosis	ATCC 911
Proteus vulgaris	ATCC 13315
Enterococcus faecalis	ATCC 29212
Enterobacter cloaceae	ATCC 13047
Escherichia coli	ATCC 35218
Listeria monocytogenes	ATCC 7644

ATCC; American Type Culture Collection

2.3 Antibacterial Activity Test

The antimicrobial activities of resveratrol were determined using the agar well diffusion method (Aytar and Oryaşın 2019). The test bacteria included *Salmonella typhimurium*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Escherichia coli*, and *Listeria monocytogenes* the bacteria were inoculated into Nutrient Broth (NB) and incubated in a shaking water bath at 37°C for 16-18 hours. After incubation, the absorbance of the microbial cultures was measured at a wavelength of 600 nm using a spectrophotometer (Mapada, UV3100PC). The prepared cultures were diluted with sterilized dH2O to approximately 1 × 10^8 CFU/mL. A volume of 50 µL of the diluted bacterial cultures was added to 20 mL of soft Nutrient Agar (NA), spread onto the plate, and then allowed to gel. Wells were then created on the agar surface. A volume of 100 µL of the resveratrol (5 mg/mL) solution was added to the wells. Ciprofloxacin (30 µg/disk; Bioanalyse) was used as a positive control.

3. Results and Discussion

As a result of this study, inhibition zones were measured for resveratrol (500 µg/mL) with values of 14.0 ± 1.0 mm for *Staphylococcus epidermidis*, 15.0 ± 0.5 mm for *Enterococcus faecalis*, 15.0 ± 0.0 mm for *Enterobacter cloacae*, and 16.0 ± 0.5 mm for *Listeria monocytogenes*. When compared to the control group, it can be stated that the antibacterial activity was low. However, resveratrol was found to have a bacteriostatic effect against *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, and *Escherichia coli* (Table 2 and Figure 2).

Bacteriostatic effect is a type of effect that stops the growth and reproduction of bacteria. The bacteriostatic effect of resveratrol can occur through mechanisms such as disrupting the cell membrane, affecting gene expression, and influencing metabolic pathways (Altıok and Bayraktar 2009).

Table 2. Antibacterial Activity Properties of Resveratrol on Test Bacteria

	Inhibition Zone (mm)	
Test Bacteria	**Resveratrol**	**Control**
S. typhimurium	BS	36.0 ± 1.0
S. epidermidis	14.0 ± 1.0	20.0 ± 2.0
P. aeruginosa	BS	29.0 ± 1.6
Y. pseudotuberculosis	BS	26.0 ± 0.5
P. vulgaris	BS	36.0 ± 3.0
E. faecalis	15.0 ± 0.5	29.0 ± 1.2
E. cloaceae	15.0 ± 00	35.0± 1.0
E. coli	BS	35.0 ± 0.6
L. monocytogenes	16.0± 0.5	30.0 ± 1.5

BS: Bacteriostatic Effect, Control; Ciprofloxacin (30 µg/disk)

Figure 2. Inhibition Zones 1; *Salmonella typhimurium*. 2; *Staphylococcus epidermidis*. 3; *Pseudomonas aeruginosa*. 4; *Yersinia pseudotuberculosis*. 5; *Proteus vulgaris*. 6; *Enterococcus faecalis*. 7; *Enterobacter cloaceae*. 8; *Escherichia coli*. 9; *Listeria monocytogenes*.

Studies have shown that resveratrol has an inhibitory effect against various Gram (+) and Gram (-) bacteria. Some research has indicated that resveratrol exhibits antibacterial activity against *Staphylococcus aureus*, particularly antibiotic-resistant strains (MRSA), as well as *E. coli* and *Pseudomonas aeruginosa*. Resveratrol is being investigated for its direct antibacterial properties against antibiotic-resistant bacteria and as a potential adjunctive therapeutic agent (Paulo et al. 2010; Vestergaard et al. 2019).

Moreover, studies have demonstrated that resveratrol has a bacteriostatic effect against Gram (+) bacteria such as *Bacillus cereus* and *S. aureus*. However, the mechanisms underlying the antibacterial efficacy of resveratrol have not yet been fully elucidated (Paulo et al. 2010). Additionally, it has been reported that resveratrol can suppress biofilm formation in *S. aureus* (Cui et al. 2024). There are also studies indicating that resveratrol mitigates the pathogenic and inflammatory activities of *Porphyromonas gingivalis* and *Streptococcus mutans*. It is noted that resveratrol significantly reduces acid production and tolerance related to the virulence characteristics of *S. mutans*, inhibiting polysaccharide synthesis (Uysal et al. 2022).

Resveratrol has been shown to possess antibiofilm properties at low concentrations for some bacteria and, when combined with vancomycin, disrupts the expression of surface proteins, capsular polysaccharides, and genes associated with quorum sensing (QS).

it has been shown that resveratrol exhibits stronger activity against established biofilms (Cui and Wang 2024). Resveratrol is a naturally occurring polyphenolic compound belonging to the stilbene family, found in grapes, blackberries, blueberries, cranberries, blackcurrants, peanuts, Japanese knotweed, pine trees, legumes, and *Theobroma cacao* (cocoa). It is also present in related by-products such as red wine, dark chocolate, and fruit juices (Cui and Wang 2024).

Resveratrol has garnered attention not only as a potential natural antimicrobial agent but also for its possible functional and therapeutic applications. Therefore, its potential for use in both clinical applications and food preservation positions it as a polyphenol worthy of further research in the future. In this context, it suggests that at higher doses, resveratrol may exhibit bactericidal effects while also providing the opportunity for use as an antibiotic adjuvant due to its ability to inhibit bacterial growth.

4. Conclusion

In this study, the antibacterial effects of resveratrol against pathogenic bacteria such as *Salmonella typhimurium*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Escherichia coli*, and *Listeria monocytogenes* have been identified. Supporting this study, literature suggests that resveratrol could be a significant antibacterial agent against bacteria.

Although the antimicrobial effects of resveratrol are promising, there is a need for a comprehensive elucidation of this efficacy. In particular, it is essential to identify the antimicrobial activities of resveratrol against different microorganisms. Synergistic effect studies are important to investigate whether the combination of resveratrol with other antimicrobial agents enhances its antibacterial efficacy. Previous research has shown that resveratrol exhibits synergistic effects with some antibiotics, and it is necessary to optimize these effects with appropriate dosages. Such combination studies could potentially offer solutions to serious public health issues, such as antibiotic resistance, by enhancing the effectiveness of antibiotics.

Specifically, the combination of antibacterial agents and natural compounds has the potential to reduce toxicity and slow down the development of antimicrobial resistance, as they can be effective at lower doses. The low toxicity of resveratrol is anticipated to provide an advantage for its use in food safety or medical applications. However, it should be noted that further research is needed regarding the potential of resveratrol for use in combination with antibiotics and its synergistic effects.

5. References

Altiok D, Alitok E,Bayraktar O,Tihminoglu F (2009) Stability of trnas-resveratrol incorporated in chitosan microspheres,14th National Biomedical Engineering Meeting.

Aytar M, Oryaşın E, Başbülbül G, Bozdoğan B (2019) Standardization study of the agar well diffusion method Bartın University International Journal of Natural and Applied Sciences 2(2):138-145.

Cui W, Wang Y, Zhang L, Liu F, Duan G, Chen S, Yang H (2024) Recent developments in the use of resveratrol against *Staphylococcus aureus.* Infections Med Int. 4(6):1-9.

Daş T, Daş G, Kapmaz M (2019) Evaluation of the antibacterial and antifungal effects of resveratrol on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* In Vitro. Istanbul Gelisim University Journal of Health Sciences (8):739-750.

Faydoğlu E, Sürücüoğlu M (2013) Antimicrobial and antioxidant activities of medicinal and aromatic plants and their usage opportunities. Erzincan University Journal of Science and Technology 6(2): 233-265.

Karameşe M, Dicle Y (2022) The antibacterial and antibiofilm activities of resveratrol on Gram-positive and Gram-negative bacteria. Kafkas Journal of Medical Sciences 12(3): 201-206.

Kumar V, Yasmeen N, Pandey A, Ahmad Chaudhary A, Alawam A, Ahmad Rudayni H, Shaid M (2023) Antibiotic adjuvants: synergistic tool to combat multi-drug resistant pathogens frontiers in cellular and ınfection microbiology 13: 1293633.

Nakata R, Takahashi S, Inoue H (2012) Recent advances in the study on resveratrol. Biol Pharm Bull. 35(3):273-279.

Nøhr-Meldgaard K, Ovsepian A, Ingmer H, Vestergaard M (2018) Resveratrol enhances the efficacy of aminoglycosides against *Staphylococcus aureus.* Int J Antimicrob Agents 52(3): 390-396.

Otto M (2012) Molecular basis of Staphylococcus epidermidis infections In *Seminars in immunopathology* (Vol. 34, pp. 201-214). Springer-Verlag.

Patel K, Scott E, Brown V, Gescher A, Steward W, Brown K (2011) Clinical trials of resveratrol. Ann NY Acad Sci. 1215(1):161-169.

Paulo L, Ferrreira S, Gallardo E, Queiroz JA, Domingues F (2010) Antimicrobial activity and effects of resveratrol on human pathogenic bacteria. JMG 26:1533-1538

Kulkarni S, Cantó C (2015) The molecular targets of resveratrol. Biochim Biophys Acta. 1852(6):1114-1123.

Saran B, Karahan Z (2010) Overview of Antimicrobial Agents Turk Urol Sem 1,216-20.

Uysal M, Kaya B (2022) Resveratrol and its areas of effect Health Science-2022: Medical Research II, 155.

Vestergaad M, Ingmer H (2019)The Antibacterial and antifungal properties of resveratrol. Int J Antimicrob Agents 53 (6):716-723.

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Effects of deltamethrin on photosynthetic pigments and ascorbate-glutathione (ASA-GSH) cycle in *Lemna minor*

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Abstract: Deltamethrin is a synthetic pyrethroid insecticide that can cause adverse effects on non-target organisms. This study was designed to investigate the effects of different concentrations (0.001, 0.005 and 0.01 ppm) of deltamethrin on photosynthetic pigments and the ascorbate-glutathione (ASA-GSH) cycle in *Lemna minor*, a freshwater macrophyte. To assess the effect of deltamethrin on *L. minor*, photosynthetic pigments, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels, and the activities of some antioxidant enzymes (SOD, CAT and POD) and enzymatic and non-enzymatic antioxidants associated with the ASA-GSH cycle were measured. The results showed that exposure to deltamethrin decreased chl a, chl b and carotenoid levels and increased MDA and H_2O_2 levels. In addition, deltamethrin exposure significantly increased SOD, CAT and POD activities. The activities of ASA-GSH cycle enzymes (APX, GR, GPX, MDHAR and DHAR) decreased in *L. minor* exposed to 0.01 ppm deltamethrin, while GST activity increased. Exposure to low doses of deltamethrin increased ASA and GSH levels, while 0.01 ppm deltamethrin decreased the amounts of ASA and GSH compared to the control. Taken together, the present study revealed that different concentrations of deltamethrin inhibited photosynthetic activity, increased lipid peroxidation and caused oxidative stress and activated the antioxidant defense system of *L. minor* to eliminate the increased oxidative stress.

Keywords: Antioxidant enzyme; Ascorbate, Deltamethrin; Glutathione; *Lemna minor*; Malondialdehyde

1 Introduction

Deltamethrin is a synthetic pyrethroid insecticide and a powerful insecticidal chemical widely used in many fields such as agriculture, animal husbandry and public health. Obtained as a result of chemical modifications of natural pyrethrins, deltamethrin causes insects to die quickly, mainly due to its effects on the nervous system (Lu et al. 2019). By blocking sodium channels in nerve cells, it disrupts insects' nerve conduction, leading to paralysis resulting in death. Deltamethrin is also used to protect fish from ectoparasites (Abdelhalak et al. 2015). In India and some other developing countries, deltamethrin has been reported to be used to control mosquitoes carrying zika virus and dengue virus (WHO, 2010). Synthetic pyrethroids such as deltamethrin have high insecticidal activity and low toxicity to mammals and birds (Lu et al. 2019). Since the implementation of organophosphorus insecticide sales restrictions, the use of pyrethroid insecticides such as deltamethrin has increased significantly and pyrethroid insecticides have become the preferred choice in many agriculture-based countries over the past two decades (Kumar et al. 2016). However, due to their widespread use, pyrethroid insecticide contamination has become a major problem worldwide. Deltamethrin-contaminated waters have started to harm aquatic biota, and the consumption of food contaminated with this insecticide has become a danger to animal and human health (Barlow et al. 2001). While deltamethrin was initially thought to have low toxicity to mammals, several studies have reported toxic effects of this insecticide on non-target organisms (Lu et al. 2019). Deltamethrin has been reported to increase the amount of reactive oxygen species, cause oxidative stress, immunotoxicity and neurotoxicity in various organisms (Lu et al. 2019). Since the consumption of deltamethrin-treated foods may affect human health in the long term, further research on the toxicity of this pesticide is needed. In this study, the effect of deltamethrin on the antioxidant system of *L. minor*, a non-target aquatic organism, was evaluated. *L. minor* is a monocotyledonous plant of the Lemnaceae family

that grows in nutrient-rich freshwater. In addition to being food for fish and poultry, they have economic potential due to their small size and rapid reproduction and are used in research in biotechnology and ecology (Alp et al. 2023). Previous studies have shown that *L. minor* is sensitive to various environmental contaminants such as heavy metals and pesticides (Li et al. 2022). Therefore, *L. minor* has been used as a biomonitor to assess the ecotoxicity of various chemicals to the aquatic environment (OECD, 2006).

In this study, the effect of deltamethrin, which is widely used both in the world and in our country, on *L. minor*, a non-target organism, was evaluated. In the study, 3 different concentrations of deltamethrin were applied to *L. minor* and the changes in photosynthetic pigments, lipid peroxidation, hydrogen peroxide and enzymatic and non-enzymatic antioxidants related to the antioxidant system were analyzed.

2 Materials and Method

The duckweed (*Lemna minor* L.) used in this study was collected from the wetlands around Erzurum airport. The collected plants were stressed with 10% NaClO and 1% $HgCl_2$ for 2 minutes and then washed several times with pure water. The plants were cultured in 1/10 Hoagland's solution for 3 months in the Plant Physiology Laboratory of Atatürk University, Faculty of Science, Department of Biology. Toxicity testing was carried out according to the OECD guidelines. For the experiments, 600 healthy *L. minor* plants were selected. Approximately 150 (50X3) plants of the same size were used for each test group. The experiments were carried out in 50 ml Petri dishes. Each Petri dish was filled with 50 ml of 10% Steinberg's solution and 50 *L. minor* plants. The experiments were carried out in the acclimatization room of Atatürk University, Department of Biology, at a temperature of 24±2 °C, 16/8 light/dark photoperiod and 60% humidity. Three different concentrations of deltamethrin, 0.001, 0.005 and 0.01 ppm, were used in the experiments. The concentrations used in the experiments were determined as a result of preliminary experiments. While only 50 ml of 10% Steinberg solution was added to the control group, deltamethrin was added to the Steinberg solution in the other experimental groups. The experiments were carried out in 3 parallel experiments. Plants were harvested after 7 days and used for analysis.

The procedure recommended by Witham et al. (1971) was used to determine chlorophyll a, chlorophyll b and carotenoid content.

To determine lipid peroxidation in L. minor plant treated with tetraconazole, the method proposed by Velikova et al. (2000) was used. In order to determine the amount of hydrogen peroxide (H_2O_2), the determination was carried out by making some modifications to the method of Velikova et al. (2000).

Superoxide dismutase (SOD) activity was determined by the method of Agarwal and Pandey (2004) with minor modifications, catalase (CAT) activity was determined by observing the decomposition of H_2O_2 according to the method of Aebi (1984); peroxidase (POD) activity was measured by monitoring the oxidation of guaiacol in the presence of H_2O_2 according to the method of Yee et al. (2002), and ascorbate peroxidase (APX) activity was measured based on the decrease in absorbance at 290 nm according to the method of Nakano and Asada (1981). Glutathione reductase (GR) activity was analyzed by a modified method based on the protocol of Foyer and Halliwell (1976). Glutathione S-transferase (GST) activity was determined according to the method of Habig et al. (1974). Glutathione peroxidase (GPX) activity was determined et 470 nm based on the method described by Hasanuzzaman et al. (2012). Monodehydroascorbate reductase (MDHAR) activity was measured by the method of Miyake and Asada (1992) with minor modifications. Dehydroascorbate reductase (DHAR) activity was measured based on the method of Nakano and Asada (1981) with some modifications. Total ascorbate and GSH were measured using the methods proposed by Huang et al. (2005) and Yu et al. (2003), respectively, with some modifications. Briefly, 0.5 g tissue was grounded in 5% meta-phosphoric acid and 1mM EDTA, then centrifuged 12000 rpm for 10 min. Obtained supernatant was used for analysis ASA and GSH content.

The experiments were repeated three times and the results are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the data and Dunnett's multiple comparison tests were applied. Significance limits were set as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. All statistical analyses were performed with GraphPad Prism 8.4 software.

3 Results and Discussion

Chlorophyll a, chlorophyll b and carotenoid amounts showed a decreasing trend in parallel with the increase in deltamethrin concentration (Fig. 1). Compared to the control group, the chlorophyll a content of *L. minor* decreased by approximately 50% in the 0.01 ppm treated group. Chlorophyll b content decreased by approximately 36% in the 0.01 ppm treated group compared to the control group. Carotenoid content decreased by approximately 50% in the 0.01 ppm group compared to the control group. In parallel with these results, the inhibitory effect of deltamethrin on chlorophyll a, chlorophyll b and carotenoid pigments was also shown in maize, soybean and tomato plants (Bashir et al. 2007; Duran et. al. 2015; Touzout et al. 2021).

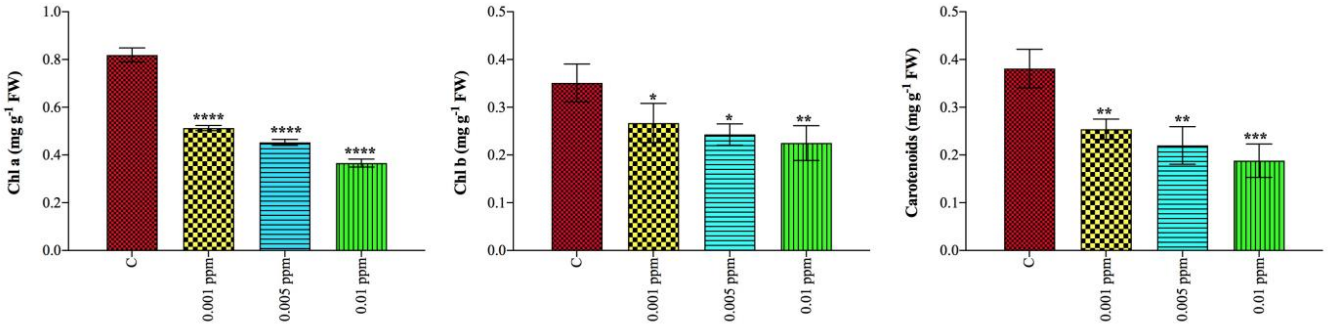

Fig. 1 Effect of deltamethrin on photosynthetic pigments in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

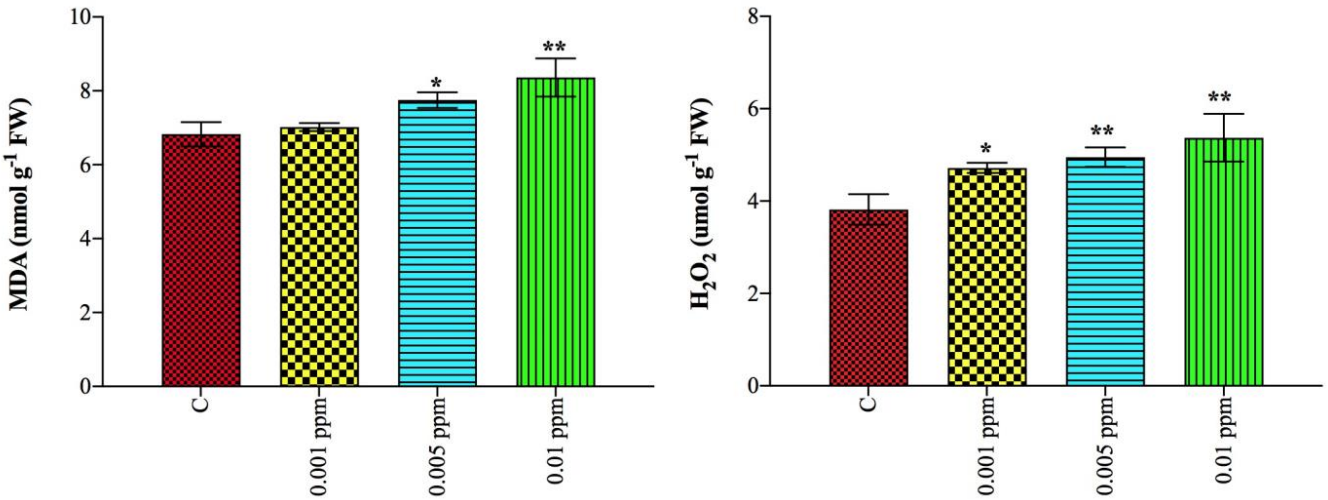

Fig. 2. Effect of deltamethrin on MDA and H_2O_2 levels in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Compared to the control group, deltamethrin at a concentration of 0.001 ppm did not change the MDA content in *L. minor* (Fig. 2). On the other hand, deltamethrin applied to *L. minor* at concentrations of 0.005 and 0.01 ppm significantly increased the MDA content compared to the control. In addition, deltamethrin applied to *L. minor* significantly increased the H_2O_2 content depending on the dose increase. This increase was approximately 40% in the 0.01 ppm group compared to the control (Fig. 2). These results showed that deltamethrin induced oxidative stress especially at high concentrations. In plants, reactive oxygen species (ROS) such as H_2O_2 are mainly produced in low amounts in different organelles. Under environmental stressors, ROS production affects the antioxidant system in plants and exceeds the antioxidant scavenging capacity, leading to oxidative stress. In parallel with our findings, it was reported that deltamethrin applied to tomato plants increased the amount of H_2O_2 (Touzout et al. 2021). As it is known, excessive production of ROS in plants demege cell membranes, disrupts membrane permeability and consequently prevents plant growth by negatively affecting the physiological activities of the plant. In our study, it was determined that especially high concentrations of deltamethrin increased the amount of MDA in *L. minor*. This was attributed to the H_2O_2 production induced by deltamethrin causing lipid peroxidation. Consistent with our results, deltamethrin was reported to increase MDA in maize and tomato (Duran et. al. 2015; Touzout et al. 2021).

Exposure to different concentrations of deltamethrin significantly affected the activities of SOD, CAT, POD, APX, GR, GPX, GST, MDHAR and DHAR enzymes in *L. minor*. SOD, CAT and GST activities increased significantly with the dose increase of deltamethrin (Fig. 3, Fig. 4), while APX, GPX and MDHAR activities decreased significantly in a dose-dependent manner (Fig. 4 and Fig. 5). While POD activity did not change with 0.001 ppm deltamethrin application compared to the control group, it increased with increasing dose (Fig. 3). GR activity decreased significantly in the 0.001 ppm deltamethrin treated group, while GR activity did not show a significant change in the other treatment groups compared to the control (Fig. 4). DHAR activity increased in the 0.005 ppm group and decreased in the 0.01 ppm group (Fig. 5).

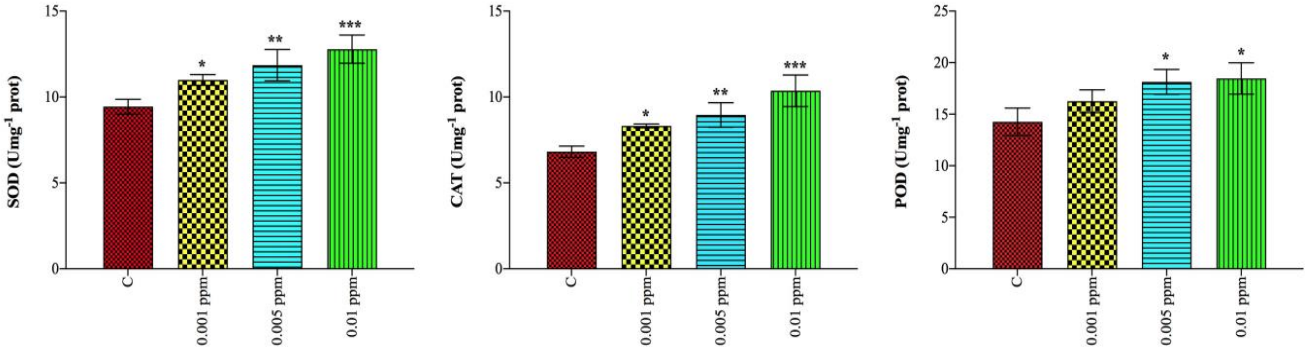

Fig. 3. Effect of deltamethrin on SOD, CAT, and POD enzyme activities in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

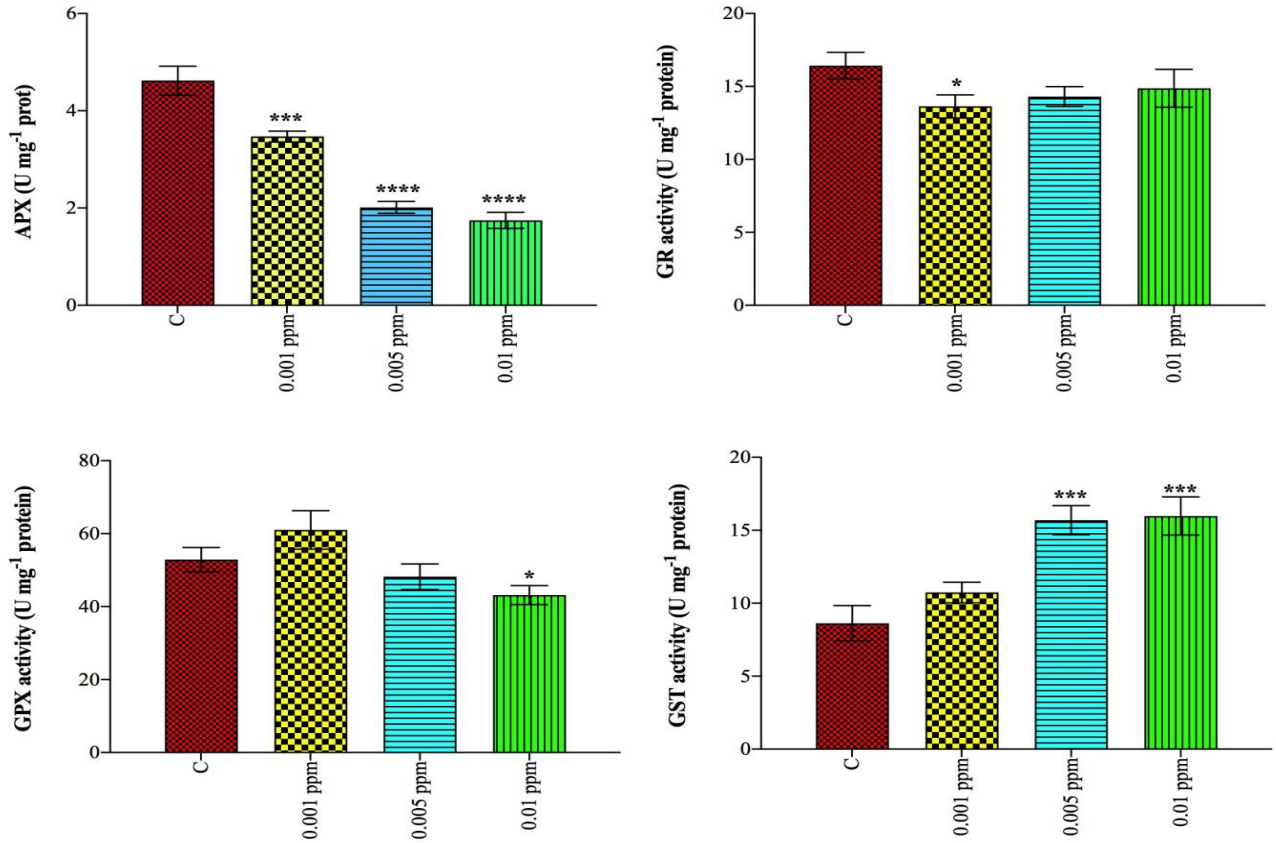

Fig. 4. Effect of deltamethrin on APX, GR, GPX, and GST enzyme activities in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

The increase of ROS in plants triggers a complex mechanism involving enzymatic (SOD, CAT, POD, APX, GR, GPX etc.) and non-enzymatic (ASA, GSH) systems to scavenge ROS. In plants, SOD functions as the first line of defense by catalyzing the conversion of $O_2^{\bullet-}$ to O_2 and H_2O_2. CAT, and POD are responsible for the scavenging of H_2O_2 and convert H_2O_2 to O_2 and H_2O. In our study, it was determined that the amount of SOD, CAT and POD increased parallel to deltamethrin administration (Fig. 3). Since these enzymes scavenge ROS, the increase in enzyme amounts with deltamethrin treatment is due to increased H_2O_2 accumulation. Similar to our results, deltamethrin was reported to increase SOD activity in soybean and CAT and POD activities in tomato (Bashir et al. 2007; Touzout et al. 2021). The amounts of ASA and GSH in deltamethrin-treated *L. minor* plants are given in Figure 5. Exposure to low doses of deltamethrin increased both ASA and GSH levels (Fig. 5). On the other hand, deltamethrin applied to *L. minor* at a concentration of 0.01 ppm decreased the amounts of ASA and GSH (Fig. 5). APX activity, one of the ASA-GSH cycle enzymes, decreased significantly with increasing deltamethrin dose. GR activity decreased in the 0.001 ppm treated group, but did not change in the 0.005 and 0.01 ppm treated groups compared to the control group. GPX, MDHAR and DHAR activities were increased by low doses of deltamethrin and significantly decreased in 0.01 ppm treated groups compared to the control. GST activity increased with increasing dose.

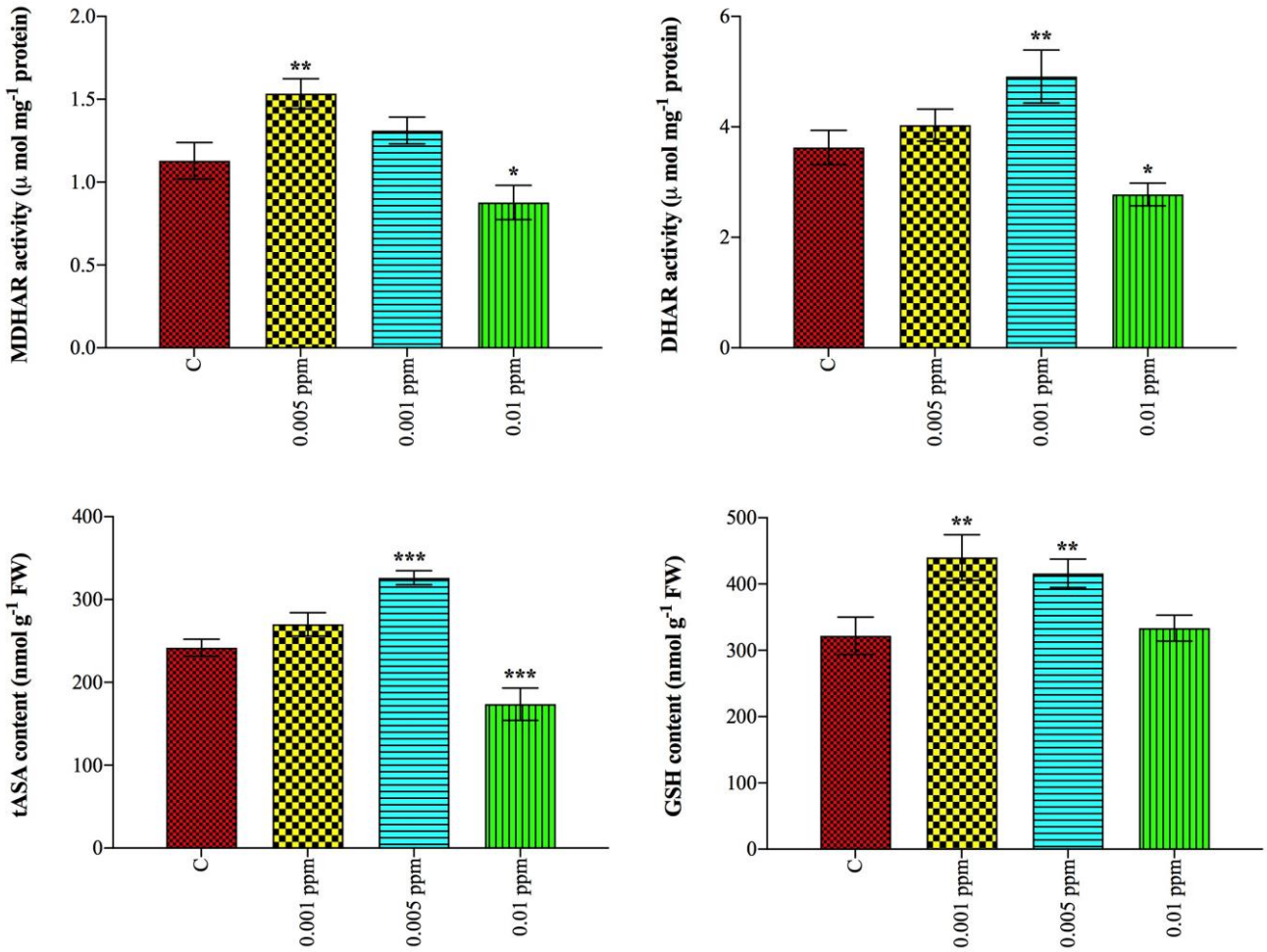

Fig. 5. Effect of deltamethrin on MDHAR and DHAR enzyme activities and ASA and GSH levels in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

ASA and GSH are the primary soluble antioxidants that protect plants from various environmental stressors. These antioxidants protect cells against ROS and maintain cellular redox balance. The amounts of these antioxidants increased in *L. minor* plants exposed to low dose (0.01 ppm) of deltamethrin. Similar to our results, low doses of deltamethrin increased the amounts of ASA and GSH in tomato and pea plants exposed to deltamethrin (Bashir et al. 2007; Touzout et al. 2021). The defense function of ASA against pesticide-induced oxidative stress in plants has been reported in many studies. It has also been reported that GSH eliminates reactive oxygen species in plants. In addition, GSH has been reported to protect cell membrane proteins and lipids against oxidation by inducing GST activity. In this context, in our study, it was also found that GST activity increased due to deltamethrin treatment. On the other hand, the ASA-GSH cycle is maintained by the activities of APX, GR, MDHAR and DHAR enzymes. Exposure to high doses of deltamethrin suppressed the activities of these enzymes, leading to a decrease in ASA and GSH levels and loss of redox status. In this case, it can be said that high doses (0.01 ppm) of deltamethrin applied to *L. minor* triggered oxidative stress and antioxidant enzymes and ASA-GSH cycle were ineffective in scavenging excess ROS.

Conclusions

As a result, the amount of photosynthetic pigments decreased and MDA and H_2O_2 levels increased in parallel with the increase in deltamethrin concentration. Deltamethrin exposure induced oxidative stress through ROS accumulation and led to disruptions in membrane structure by increasing lipid peroxidation. Increasing deltamethrin doses increased SOD, CAT and GST enzyme activities. This was interpreted as a defense mechanism to eliminate H_2O_2 accumulated in the plant. However, the activities of ASA-GSH cycle enzymes such as APX, GPX, GR, MDHAR and DHAR decreased at high doses, resulting in disruption of redox balance and increased oxidative stress. Low dose deltamethrin (0.001 ppm) treatment increased ASA and GSH amounts and supported the resistance of plants against oxidative stress. However, deltamethrin at 0.01 ppm concentration decreased the levels of these antioxidants and decreased ROS scavenging capacity. In conclusion, at low doses, deltamethrin activated some defense mechanisms to adapt to plant metabolism, but at high doses it increased oxidative stress and negatively affected the functionality of the antioxidant system. Our study showed that deltamethrin can cause loss of

photosynthetic pigment, lipid peroxidation and disruption of redox balance in plants. These findings are important for understanding the negative effects of pesticides on aquatic biota and promoting conscious use in the field of environmental toxicology.

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Authors' contributions: OA: Design, data analysis, manuscript writing, laboratory experiments

HU: Manuscript writing, EG: Laboratory experiments

Conflict of interest disclosure:

The authors declare no conflict of interest.

References

Abdelkhalek NKM, Ghazy EW, Abdel-Daim MM. (2015) Pharmacodynamic interaction of Spirulina platensis and deltamethrin in freshwater fish *Nile tilapia*, Oreochromis niloticus: impact on lipid peroxidation and oxidative stress. Environ Sci Pollut Res 22:3023–3031.

Agarwal S, Pandey V. (2004) Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. Biol Plant 48, 555-560.

Alp FN, Arikan B, Ozfidan-Knakcı C, Gelentürk C, Yildiztugay E, Turan M, Cavusoglu H. (2023) Hormetic activation of nano-sized rare earth element terbium on growth, PSII photochemistry, antioxidant status and phytohormone regulation in *Lemna minor*. Plant Physiol Biochem 194:361-373.

Barlow SM, Sullivan FM, Lines J. (2001) Risk assessment of the use of deltamethrin on bednets for the prevention of malaria. Food Chem Toxicol 39:407-422.

Bashir F, Siddiqi TO, Iqbal M. (2007) The antioxidative response system in *Glycine max* (L.) Merr. exposed to deltamethrin, a synthetic pyrethroid insecticide. Environ Pollut 147:94–100.

Duran RE, Kilic S, Coskun Y. (2015) Response of maize (*Zea mays* L. saccharata Sturt) to different concentration treatments of deltamethrin. Pestic Biochem Phys 124:15-20.

Foyer CH, Halliwell B. (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133:21-25.

Gong Y, Toivonen PMA, Lau OL, Wiersma PA. (2001) Antioxidant system level in '*Braeburn*' apple in related to its browing disorder. Bot Bul Acad Sin 42:259-264.

Habig WH, Pabst MJ, Jakoby WB. (1974) Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J Biol Chem 249(22):7130-7139.

Hasanuzzaman M, Nahar K, Alam MM, Fujita M. (2012) Exogenous nitric oxide alleviates high temperature induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by modulating the antioxidant defense and glyoxalase system. Aust J Crop Sci 6(8):1314-1323.

Huang C, He W, Guo J, Chang X, Su P, Zhang L. (2005) Increased sensitivity to salt stress in an ascorbate-deficient Arabidopsis mutant. J Experiment Bot 56:3041-3049.

Kumar A, Sasmal D, Bhaskar A, Mukhopadhyay K, Thakur A, Sharma N. (2016) Deltamethrin-induced oxidative stress and mitochondrial caspase-dependent sig- naling pathways in murine splenocytes. Environ Toxicol 31:808–819.

Li R, Luo C, Qiu J, Li Y, Zhang H, Tan H. (2022) Metabolomic and transcriptomic investigation of the mechanism involved in enantioselective toxicity of imazamox in *Lemna minor*. J Hazard Mater 425:127818.

Lu Q, Sun Y, Ares I, Anadón A, Martínez M, Martínez-Larrañaga M-R, Yuan Z, Wang X, Martínez M-A. (2019) Deltamethrin toxicity: A review of oxidative stress and metabolism. Environ Res 170:260-281.

Miyake C, Asada K. (1992) Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. Plant Cell Physiol 33:541-553.

Nakano Y, Asada K. (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880.

OECD. (2006) OECD guidelines for the testing of chemicals, revised proposal for a new guideline 221, Lemna sp. Growth inhibition test. OECD, Paris.

Touzout N, Mehallah H, Moralent R, Nemmiche S, Benkhelifa M. (2021) Co-contamination of deltamethrin and cadmium induce oxidative stress in tomato plants (*Solanum lycopersicum* L.). Acta Physiol Plant 43:91.

WHO (World Health Organization). (2010) Specifications and evaluations for public health pesticides- deltamethrin. World Health Organization, Geneva.

Yee Y, Tam NFY, Wong YS, Lu CY. (2002) Growth and physiological responses of two mangrove species (*Bruguira gymnorrhiza* and *Kandelia candel*) to waterlogging. Environ Experiment Bot 1:13.

Yu C-W, Murphy TM, Lin C-H. (2003) Hydrogen peroxide-induced chilling tolerance in mung beans mediated through ABA-independent glutathione accumulation. Funct Plant Biol 30: 955-963.

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Differential cytotoxicity of methanol and water extracts from *Bacopa monnieri* (L.) Wettst and *Ceratophyllum demersum* L. on HepG2 and THLE2 cells

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Abstract: This study evaluated the cytotoxic effects of methanol and water extracts from *Bacopa monnieri* (L.) Wettst and *Ceratophyllum demersum* L. on HepG2 (liver cancer) and THLE2 (normal liver) cell lines using XTT assay. The extracts were tested at concentration range of 12.5-400 µg/mL. In HepG2 cells, the water extract of *B. monnieri* at 400 µg/mL exhibited the highest cytotoxicity, reducing cell viability to 11.08%, which was statistically significant ($p < 0.05$) compared to other treatments. The methanol extract of *C. demersum* at 12.5 µg/mL had the least effect, maintaining 94.84% cell viability. For THLE2 cells, the water extract of *B. monnieri* (400 µg/mL) also showed the greatest reduction in cell viability (30.85%). The water extract of *C. demersum* at the same concentration resulted in similar viability (32.01%), with no significant statistical difference ($p > 0.05$). The lowest concentrations of methanol and water extracts of *C. demersum* showed minimal effects (97.55% and 97.74% viability, respectively). Median inhibitor concentration (IC_{50}) analysis revealed that *B. monnieri*'s water extract was most effective, with IC_{50} values of 68.45 µg/mL for HepG2 and 127.05 µg/mL for THLE2 cells. In contrast, *C. demersum*'s methanol extract had the highest IC_{50} values (173.35 µg/mL and 228.46 µg/mL, respectively), indicating lower cytotoxicity. Heatmap and cluster analyses highlighted the selective cytotoxicity of *C. demersum* on cancer cells with minimal effects on normal cells, showing its potential for targeted cancer therapy.

Keywords: anticancer activity; cell line comparison; extract potency; selective toxicity

1 Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer that begins in hepatocytes, the main cells of the liver. This disease is often a major cause of liver failure (García-Pras et al. 2021). The early stages of HCC are usually asymptomatic, but as it progresses, symptoms such as abdominal pain, loss of appetite, weight loss, jaundice, and abdominal swelling may occur. Factors such as hepatitis B and C virus infections, alcohol consumption, obesity, and diabetes increase the risk of HCC (Ruiz-Margáin et al. 2021). Blood tests, imaging techniques, and biopsy are used for diagnosis. Treatment options include surgery, radiotherapy, chemotherapy, ablation, and liver transplantation. The prognosis of HCC depends on the stage of the cancer and the success of treatment (Granata et al. 2021). Early diagnosis and treatment have a significant impact on the course of the disease. The risk of HCC can be reduced through hepatitis B and C vaccinations, a healthy lifestyle, and regular health check-ups (Flores et al. 2022).

The use of herbal products in the treatment of serious diseases such as cancer is an important topic that has been researched both historically and in modern times. While plants have been used to treat various ailments for thousands of years, modern medicine relies on evidence-based methods for the treatment of cancer (Dutta et al. 2019; Sharma et al. 2024). In this context, some herbal products can help reduce side effects such as nausea, vomiting, and fatigue caused by conventional treatments like chemotherapy or radiotherapy (Yazbeck et al. 2022; Bai et al. 2024). Certain plants may support the body's fight against cancer by strengthening the immune system. Additionally, some plants contain compounds that exhibit cytotoxic effects on cancer cells, inhibiting their growth or inducing cell death. This is one reason why plants have been used in traditional medicine for centuries to treat various diseases (Garcia-Oliveira et al. 2021; Esmeeta et al. 2022).

Bacopa monnieri (L.) Wettst has been used for many years in the treatment of cognitive problems such as memory and learning difficulties (Brimson et al. 2021). However, recent

studies have shown that *B. monnieri* may also have potential in cancer treatment. *B. monnieri* is a powerful antioxidant. Antioxidants prevent cell damage by neutralizing free radicals in the body, thus reducing the risk of cancer (Ghosh et al. 2021; Fatima et al. 2022). Chronic inflammation is associated with the development of many types of cancer, and the anti-inflammatory properties of *B. monnieri* may play a beneficial role in this process. Some studies suggest that *B. monnieri* may trigger programmed cell death (apoptosis) in cancer cells, potentially slowing or stopping tumor growth (Mishra et al. 2024).

Ceratophyllum demersum L., commonly known as the marsh flower, is a fully aquatic plant species (Engloner et al. 2023). This plant plays an important role in underwater ecosystems, but in recent years, it has attracted the attention of scientists due to its potential anticancer compounds. Some studies have shown that *C. demersum* extracts inhibit growth and promote cell death in various cancer cell lines (Masłyk et al. 2024). In particular, some research suggests that this plant is effective against colon, liver, and breast cancer cells (Saxena et al. 2021).

In cases where it is difficult to reproduce plants in natural environments, such as *B. monnieri* and *C. demersum*, tissue culture is a highly advantageous method. Since plants produced via tissue culture are genetically identical, the amount and type of bioactive compounds they contain are more homogeneous (Zuzarte et al. 2024). This provides a significant advantage in medical research and product development. Tissue culture offers great potential for the use of plants like *B. monnieri* and *C. demersum* for medical purposes. Through this method, it is possible to obtain standardized, disease-free, and large quantities of plant material (Haque et al. 2022; Jain et al. 2023).

HCC was likely chosen for this study due to its status as the most common type of liver cancer and a major cause of liver failure. This type of cancer has high mortality rates, particularly in later stages when treatment options become limited and less effective (Schlachterman et al. 2015). Additionally, HCC is often linked to hepatitis B and C infections, making it a type of cancer with known viral associations, especially in regions where these infections are prevalent (Min et al. 2023; Stroffolini and Stroffolini, 2023). By researching HCC specifically, scientists may find new ways to improve outcomes using alternative treatments, like herbal products from *B. monnieri* and *C. demersum*, which are thought to support cancer treatment by reducing side effects and possibly slowing cancer cell growth. In the present study, we examined the cytotoxic effects of different extracts obtained from *B. monnieri* and *C. demersum*, which were produced using tissue culture techniques, on human hepatocellular carcinoma (HepG2) cells. In addition, we tested side effect levels of the extracts on non-tumoral human liver (THLE2) cells.

2 Materials and Method

2.1 Plant tissue culture

In this study, sterile *C. demersum* and *B.monnieri* were obtained from the Biology Department of Karamanoğlu Mehmetbey University as plant material. Shoot tip explants from sterile and stock plants were isolated under sterile conditions and placed into the nutrient medium. Murashige and Skoog (MS) mineral salts and vitamins (Murashige and Skoog, 1968) were used as the nutrient medium in all experiments. As a plant growth regulator, 1 mg/L Benzyl Amino Purine (BAP) and 30 g/L sucrose were added in all trials. Experiments with *C. demersum* were conducted in a liquid medium without agar (Emsen and Dogan, 2018), while those with *B. monnieri* were performed in a medium containing 7 g/L agar (Dogan and Emsen, 2018; Dogan and Ugur, 2024). The pH of the nutrient medium was adjusted to between 5.6 and 5.8 using 1 N NaOH or KOH, or 1 N HCl, and then sterilized by autoclaving at 121°C and 1.2 atmospheres of pressure for 20 minutes. The cultures were placed in a plant growth chamber or room and incubated under a 16-hour light/8-hour dark cycle at a temperature of 24±1°C.

2.2 Preparation of the extracts

The plant samples (10 g) of *B. monnieri* and *C. demersum* were dried, ground into powder, and then extracted with methanol and water solvents (250 mL) using a Soxhlet extractor. The extracts were filtered, the solvent was removed, and the resulting dry powders were lyophilized (Emsen and Dogan, 2018).

2.3 Cell culture

Human liver cells, both cancerous (HepG2) and non-cancerous (THLE2), were grown in a Dulbecco's Modified Eagle Medium (DMEM) under specific conditions (with 10% heat-inactivated fetal bovine serum (FBS), 1% l-glutamine and 1% penicillin–streptomycin). Once the cells reached a certain density, they were used for experiments to test the toxicity of the samples being studied (Emsen et al. 2021).

2.4 Cytotoxic activity

Cells were plated at a density of 1×10^4 cells per well in 96-well flat-bottom microtiter plates and incubated at 37°C for 24 hours. After this incubation period, the medium was replaced with fresh medium containing various concentrations of plant extracts. The final extract concentrations in the wells were 12.5, 25, 50, 100, 200, and 400 µg/mL, with 0.5% dimethyl sulfoxide (DMSO) serving as the negative control. Following a 48-hour incubation, XTT reagent and activator (from Biological Industries, Beit Haemek, Israel) were added to each well as per the manufacturer's protocol. The plates were then incubated for an additional 4 hours at 37°C. Absorbance readings were taken at 450 nm using a spectrophotometer, with a blank serving as the background control. Cell viability was determined as a percentage using the formula: Viability = (Absorbance of extract / Absorbance of control) × 100 (Kok et al. 2023).

2.5 Statistical analyses

The extract activities were evaluated using one-way ANOVA followed by Duncan's test for post-hoc comparison. Probit regression analysis was employed to determine the median inhibitory concentration (IC_{50}) values. To explore the similarities and differences in cytotoxic activities, heatmap

analysis and hierarchical cluster analysis were performed using Ward's minimum variance method. All statistical analyses were conducted using SPSS software (version 21.0, IBM Corporation, Armonk, NY, USA).

3 Results

3.1 Cytotoxic activities of extracts

Cytotoxic effects of methanol and water extracts obtained from *B. monnieri* and *C. demersum* on HepG2 and THLE2 cells were tested by XTT analysis. The maximum concentrations (400 µg/mL) of the extracts were the applications that reduced the viability of both cells the most. Accordingly, the most effective application on HepG2 cells was 400 µg/mL concentration of water extract of *B. monnieri*. The mentioned application reduced cell viability to 11.08%. At the same time, this application had statistically ($p < 0.05$) different data than all other applications. The application with the least effect on HepG2 cell viability (94.84%) was the 12.5 µg/mL concentration of methanol extract of *C. demersum* (Fig. 1).

When we look at the studies on THLE2 cells, the application that decreased cell viability (30.85%) the most was the highest concentration (400 µg/mL) water extract of *B. monnieri*. The application that had statistically ($p > 0.05$) no different data (32.01%) from the mentioned application was the maximum concentration of the water extract of *C. demersum*. The applications that showed the lowest viability activity on THLE2 cells were the lowest concentration of methanol and water extracts of *C. demersum* (97.55 and 97.74%, respectively). In addition, it was determined that these data were statistically ($p > 0.05$) indistinguishable from each other (Fig. 2).

IC_{50} data were used to determine the effective concentration values of extract applications. Accordingly, the most effective application on HepG2 cells turned out to be the water extract of *B. monnieri*, which has the lowest IC_{50} value (68.45 µg/mL) (Table 1). Similarly, the most effective application (IC_{50}: 127.05 µg/mL) on THLE2 cells was the water extract of *B. monnieri* (Table 2). Considering the highest IC_{50} values (173.35 and 228.46 µg/mL, respectively) occurring on HepG2 and THLE2 cells, the methanol extract of *C. demersum* appeared to have the lowest effect (Table 1 and 2).

3.2 Heatmap and cluster analyses

Heatmap and cluster analyses were used to determine the relationship levels between different extract applications applied on HepG2 and THLE2 cells. According to the heatmap analysis calculated by considering the IC_{50} values on HepG2 and THLE2 cells, the different color gradients of the methanol and water extracts of *C. demersum* were remarkable. These results were particularly important in terms of the high cytotoxic potential of *C. demersum* on cancer cells, although it showed lower cytotoxic effects on normal cells (Fig. 3a).

When we look at the cluster analysis performed to support the heatmap analysis, the methanol extract of *C. demersum* formed a separate cluster on both cells. The other three extract applications were included in a separate group under Cluster 2. All these results showed that the methanol extract of *C. demersum* has a cytotoxic effect on cancerous liver cells with low side effects (Fig. 3b and c).

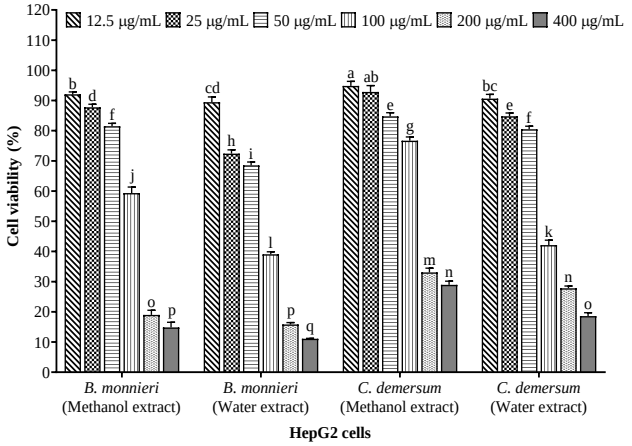

Fig. 1 Viability rates in HepG2 cells treated with different extracts from the plants (mean±standard deviation, $n = 3$) (Values indicated by different letters differ from each other at the level of $p < 0.05$.

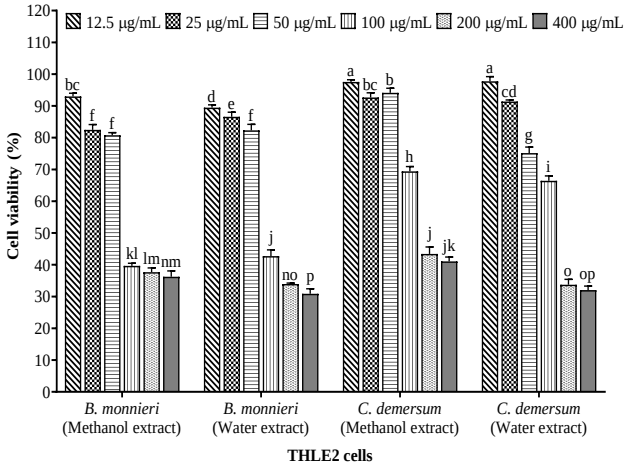

Fig. 2 Viability rates in THLE2 cells treated with different extracts from the plants (mean±standard deviation, $n = 3$) (Values indicated by different letters differ from each other at the level of $p < 0.05$.

Table 1 IC_{50} values (µg/mL) of extracts obtained from *B. monnieri* and *C. demersum* for cytotoxicity on HepG2 cells

Treatment	IC_{50} (Limits)	Slope ± Standard error (Limits)
B. monnieri (Methanol extract)	105.81 (97.29–115.39)	1.89 ± 0.07 (1.73–2.04)
B. monnieri (Water extract)	68.45 (62.61–74.82)	1.71 ± 0.07 (1.56–1.85)
C. demersum (Methanol extract)	173.35 (156.90–193.08)	1.71 ± 0.07 (1.55–1.86)
C. demersum (Water extract)	100.78 (91.90–110.83)	1.65 ± 0.07 (1.51–1.79)

Table 2 IC_{50} values (μg/mL) of extracts obtained from *B. monnieri* and *C. demersum* for cytotoxicity on THLE2 cells

Treatment	IC_{50} (Limits)	Slope ± Standard error (Limits)
B. monnieri (Methanol extract)	135.89 (120.83–154.26)	1.30 ± 0.06 (1.16–1.43)
B. monnieri (Water extract)	127.05 (113.85–142.79)	1.39 ± 0.06 (1.25–1.52)
C. demersum (Methanol extract)	228.46 (204.04–259.15)	1.63 ± 0.08 (1.47–1.79)
C. demersum (Water extract)	157.62 (142.54–175.62)	1.62 ± 0.07 (1.47–1.77)

4 Discussion

The results of this study provide significant insights into the cytotoxic effects of *B. monnieri* and *C. demersum* extracts on HepG2 cancer cells and THLE2 normal liver cells. The XTT analysis demonstrated that the water extract of *B. monnieri* exhibited the strongest cytotoxic activity on HepG2 cells, reducing cell viability to 11.08% at the maximum concentration of 400 μg/mL. This finding aligns with prior research, which highlights *B. monnieri*'s bioactive compounds, such as bacosides, that have been shown to induce apoptosis in cancer cells and inhibit tumor growth (Das et al. 2016; Smith et al. 2018; Aithal and Rajeswari, 2019). Moreover, the low IC_{50} value (68.45 μg/mL) further reinforces its potential as a promising therapeutic candidate for hepatocellular carcinoma treatment (Janani et al. 2010; Shefin et al. 2016).

Interestingly, the water extract of *B. monnieri* also demonstrated pronounced cytotoxicity on THLE2 cells, reducing viability to 30.85%, with a similarly low IC_{50} value (127.05 μg/mL). Although this suggests broad cytotoxic activity, it also raises concerns about its selectivity, as the effect on non-tumorous cells could limit its therapeutic window. This underscores the need for further investigation into *B. monnieri*'s selective toxicity to reduce potential side effects on healthy tissues (Ray et al. 2021; Malabadi et al. 2024). Recent findings have underscored *B. monnieri*'s pro-oxidant effects at high concentrations, which may contribute to its broad cytotoxicity, including potential effects on non-tumorous cells like THLE2 (Jyoti et al. 2007; Anand and Khanum, 2018). This broad activity raises questions regarding selectivity and highlights the need for further research into modifying *B. monnieri* extracts or dosing regimens to minimize harm to healthy cells.

In contrast, *C. demersum* exhibited a distinct cytotoxic profile, with its methanol extract showing the least impact on both HepG2 (IC_{50}: 173.35 μg/mL) and THLE2 cells (IC_{50}: 228.46 μg/mL). These findings are notable, as they suggest that *C. demersum* methanol extract may possess lower overall cytotoxicity, particularly on normal liver cells, making it a candidate for further investigation as a selective anticancer agent. Previous studies have identified bioactive compounds in *C. demersum*, such as flavonoids and phenolic acids, which may contribute to its selective anticancer properties (Awati et al. 2021; Masłyk et al. 2024). The heatmap and cluster analyses supported this distinction, with the methanol extract forming a separate cluster, emphasizing its potential for selective action against cancer cells.

This differential cytotoxicity, especially in the context of *C. demersum*, raises the possibility that specific compounds within this plant may target cancer cells more selectively. Compounds such as luteolin, apigenin, and phenolic acids have been reported in *C. demersum* extracts and are known for their anti-inflammatory, antioxidant, and pro-apoptotic activities (Eliašová et al. 2021; Nguyen et al. 2023). The relatively low cytotoxicity observed on THLE2 cells suggests that these compounds may have a targeted mechanism of action, potentially inducing apoptosis in cancer cells while sparing normal cells. Further studies are required to investigate the molecular pathways involved and to isolate the specific compounds responsible for this effect.

In terms of the broader clinical relevance, both *B. monnieri* and *C. demersum* exhibit mechanisms that may complement existing HCC therapies, such as chemotherapy, radiofrequency ablation, and immunotherapy. Current HCC treatments are often limited by resistance development, high recurrence rates, and adverse effects on liver function (Bruix et al. 2019; Chen et al. 2020). Combining conventional therapies with these plant-based extracts could potentially enhance treatment efficacy while reducing toxicity. For instance, *B. monnieri*'s ROS-inducing properties could sensitize cancer cells to chemotherapy agents that work through oxidative stress, while *C. demersum*'s compounds could provide an anti-inflammatory effect, possibly reducing adverse inflammatory responses often observed with HCC treatments (Mishra et al. 2019; Masłyk et al. 2024).

This study highlights the therapeutic potential of *B. monnieri* and *C. demersum* extracts in treating hepatocellular carcinoma. However, there are limitations that need to be addressed in future research. First, while XTT analysis provides valuable information on cytotoxicity, further studies, including mechanistic investigations of apoptosis, cell cycle arrest, and potential molecular targets, are necessary to fully understand the anticancer effects of these extracts (Cheung et al. 2023; Patra et al. 2023). Second, in vivo studies should be conducted to confirm the efficacy and safety of these extracts in more complex biological systems.

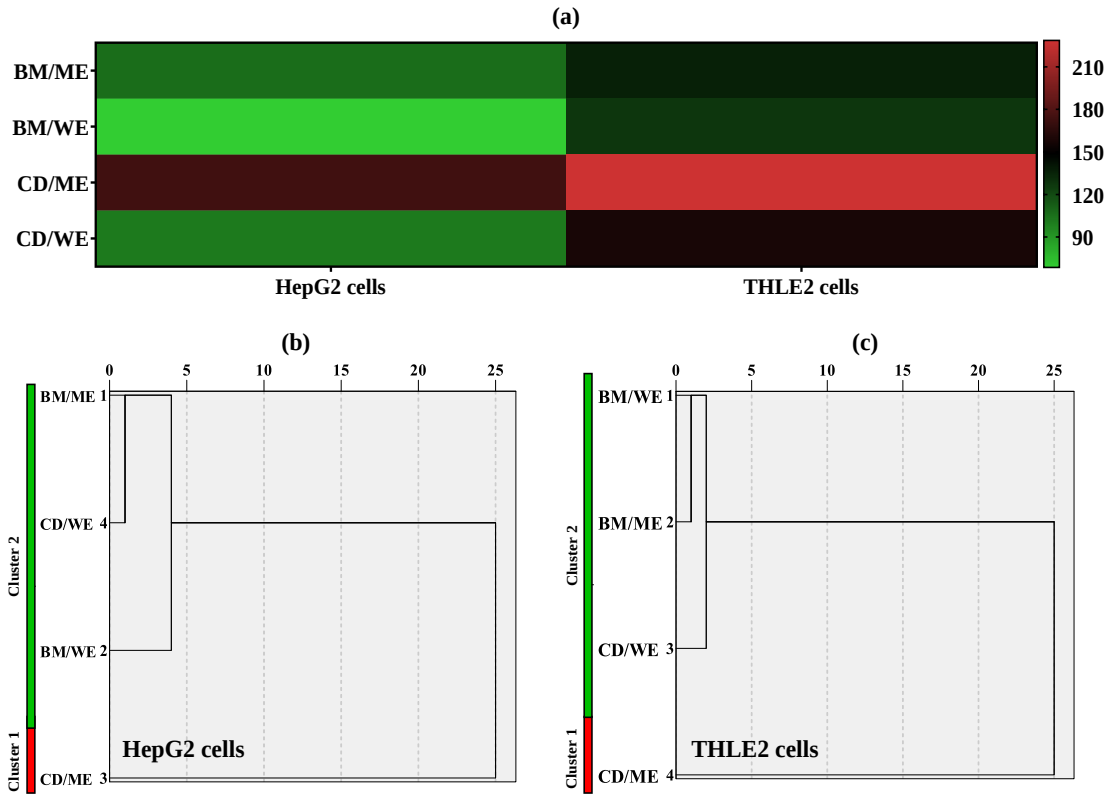

Fig. 3 (a) Heatmap based on IC_{50} values for cytotoxic activities of plant extracts and dendrogram (b) for HepG2 and (c) THLE2 cells (High and low activities were represented by red and green colour, respectively). BM/ME: Methanol extract of *B. monnieri*; BM/WE: Water extract of *B. monnieri*; CD/ME: Methanol extract of *C. demersum*; CD/WE: Water extract of *C. demersum*.

5 Conclusion

In conclusion, the study demonstrated that *B. monnieri* and *C. demersum* extracts exhibit significant cytotoxic effects on HepG2 liver cancer cells, with *B. monnieri* water extract being the most potent, reducing cell viability to 11.08%. Both plant extracts also showed varying degrees of cytotoxicity on non-tumorous THLE2 cells, with the water extract of *B. monnieri* having the most pronounced effect. However, the methanol extract of *C. demersum* displayed the lowest cytotoxicity on both cancerous and non-cancerous cells, suggesting its selective potential as a therapeutic agent with minimal side effects. Heatmap and cluster analyses further supported these findings, highlighting the distinct behavior of the *C. demersum* methanol extract, which formed a unique cluster, indicating its promising role in targeting cancer cells while sparing healthy ones.

Authors' contributions: B.E. designed the experiments. M.A. carried out the experiments. M.A., B.E., M.D. analysed the data and wrote the manuscript.

Conflict of interest disclosure:

The authors declare that they have no conflict of interest.

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References

Aithal MGS, Rajeswari N (2019). Bacoside a induced sub-G0 arrest and early apoptosis in human glioblastoma cell line U-87 MG through notch signaling pathway. Brain Tumor Res Treat 7(1):25–32.

Anand T, Khanum F (2018). Attenuation of cytotoxicity induced by tBHP in H9C2 cells by Bacopa monniera and bacoside A. Pathophysiology 25(2):143–149.

Awati SS, Singh SK, Wadkar KA (2021). In vitro antioxidant potential and anticancer activity of *Ceratophyllum demersum* Linn. extracts on HT-29 human colon cancer cell line. Res J Pharm Technol 14(1):28–36.

Bai L, Ni L, Lu J, Zhang YY, Yin Y, Zhang W, Duan X (2024). Relationship between nausea and vomiting and physical activity in patients with lung cancer undergoing first chemotherapy. Front Oncol 14:1396637.

Brimson JM, Brimson S, Prasanth MI, Thitilertdecha P, Malar DS, Tencomnao T (2021). The effectiveness of *Bacopa monnieri* (Linn.) Wettst. as a nootropic, neuroprotective, or antidepressant supplement: analysis of the available clinical data. Sci Rep 11(1):596.

Bruix J, da Fonseca LG, Reig M (2019). Insights into the success and failure of systemic therapy for hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol 16(10):617–630.

Chen Z, Xie H, Hu M, Huang T, Hu Y, Sang N, Zhao Y

(2020). Recent progress in treatment of hepatocellular carcinoma. Am J Cancer Res 10(9):2993.

Cheung AH, Hui CH, Wong KY, Liu X, Chen B, Kang W, To KF (2023). Out of the cycle: impact of cell cycle aberrations on cancer metabolism and metastasis. Int J Cancer 152(8):1510–1525.

Das DN, Naik PP, Nayak A, Panda PK, Mukhopadhyay S, Sinha N, Bhutia SK (2016). *Bacopa monnieri*-induced protective autophagy inhibits benzo[a]pyrenemediated apoptosis. Phyther Res 30(11):1794–1801.

Dogan M, Emsen B (2018). Anti-cytotoxic-genotoxic influences of in vitro propagated *Bacopa monnieri* L. Pennell in cultured human lymphocytes. Eurasian J Biol Chem Sci 1(2):48–53.

Dogan M, Ugur K (2024). Enhancing the phytoremediation efficiency of *Bacopa monnieri* (L.) Wettst. using LED lights: a sustainable approach for heavy metal pollution control. Environ Sci Pollut Res 31:53270–53290.

Dutta S, Mahalanobish S, Saha S, Ghosh S, Sil PC (2019). Natural products: An upcoming therapeutic approach to cancer. Food Chem Toxicol 128:240–255.

Eliašová A, Hrivnák R, Štefánová P, Svitok M, Kochjarová J, Oťaheľová H, Novikmec M, Pal'ove-Balang P (2021). Effects of ammonium levels on growth and accumulation of antioxidative flavones of the submerged macrophyte *Ceratophyllum demersum*. Aquat Bot 171:103376.

Emsen B, Dogan M (2018). Evaluation of antioxidant activity of in vitro propagated medicinal *Ceratophyllum demersum* L. extracts. Acta Sci Pol Cultus 17(1):23–33.

Emsen B, Sadi G, Bostanci A, Aslan A (2021). In vitro evaluation of cytotoxic, oxidative, genotoxic, and apoptotic activities of physodic acid from *Pseudevernia furfuracea* in HepG2 and THLE2 cells. Plant Biosyst 155(6):1111–1120.

Engloner AI, Németh K, Kós PB, Meglécz E, Bereczki J (2023). Genetic diversity of the submerged macrophyte *Ceratophyllum demersum* depends on habitat hydrology and habitat fragmentation. Front Plant Sci 14:1277916.

Esmeeta A, Adhikary S, Dharshnaa V, Swarnamughi P, Maqsummiya ZU, Banerjee A, Pathak S, Duttaroy AK (2022). Plant-derived bioactive compounds in colon cancer treatment: an updated review. Biomed Pharmacother 153:113384.

Fatima U, Roy S, Ahmad S, Ali S, Elkady WM, Khan I, Alsaffar RM, Adnan M, Islam A, Hassan MI (2022). Pharmacological attributes of *Bacopa monnieri* extract: current updates and clinical manifestation. Front Nutr 9:972379.

Flores JE, Thompson AJ, Ryan M, Howell J (2022). The global impact of hepatitis B vaccination on hepatocellular carcinoma. Vaccines 10(5):793.

Garcia-Oliveira P, Otero P, Pereira AG, Chamorro F, Carpena M, Echave J, Fraga-Corral M, Simal-Gandara J, Prieto MA (2021). Status and challenges of plant-anticancer compounds in cancer treatment. Pharmaceuticals 14(2):157.

García-Pras E, Fernández-Iglesias A, Gracia-Sancho J, Pérez-del-Pulgar S (2021). Cell death in hepatocellular carcinoma: pathogenesis and therapeutic opportunities. Cancers (Basel) 14(1):48.

Ghosh S, Khanam R, Acharya Chowdhury A (2021). The evolving roles of *Bacopa monnieri* as potential anti-cancer agent: a review. Nutr Cancer 73(11–12):2166–2176.

Granata V, Grassi R, Fusco R, Belli A, Cutolo C, Pradella S, Grazzini G, La Porta M, Brunese MC, De Muzio F (2021). Diagnostic evaluation and ablation treatments assessment in hepatocellular carcinoma. Infect Agent Cancer 16:1–22.

Haque MI, Singh PK, Ghuge S, Kumar A, Rai AC, Kumar A, Modi A (2022). A general introduction to and background of plant tissue culture: past, current, and future aspects. In: Advances in Plant Tissue Culture. Elsevier, pp 1–30

Jain S, Rathod M, Mishra G, Kumari V, Baksh H, Saxena S, Prasad L (2023). Plant tissue culture for medical therapy: unlocking the potential of medicinal plants. Curr J Appl Sci Technol 42(46):7–22.

Janani P, Sivakumari K, Geetha A, Ravisankar B, Parthasarathy C (2010). Chemopreventive effect of bacoside A on N-nitrosodiethylamine-induced hepatocarcinogenesis in rats. J Cancer Res Clin Oncol 136:759–770.

Jyoti A, Sethi P, Sharma D (2007). *Bacopa monniera* prevents from aluminium neurotoxicity in the cerebral cortex of rat brain. J Ethnopharmacol 111(1):56–62.

Kok O, Emsen B, Surmen B (2023). Screening of in vitro cytotoxicity and antioxidant potential of selected endemic plants in Turkey. J Taibah Univ Sci 17(1):2217369.

Malabadi RB, Sadiya MR, Kolkar KP, Mammadova SS, Chalannavar RK, Baijnath H (2024). Role of plant derived-medicine for controlling cancer. Int J Sci Res Arch 11(1):2502–2539.

Masłyk M, Lenard T, Olech M, Martyna A, Poniewozik M, Boguszewska-Czubara A, Kochanowicz E, Czubak P, Kubiński K (2024). *Ceratophyllum demersum* the submerged macrophyte from the mining subsidence reservoir Nadrybie Poland as a source of anticancer agents. Sci Rep 14(1):6661.

Min Y, Wei X, Xia X, Wei Z, Li R, Jin J, Liu Z, Hu X, Peng X (2023). Hepatitis B virus infection: an insight into the clinical connection and molecular interaction between hepatitis B virus and host extrahepatic cancer risk. Front Immunol 14:1141956.

Mishra S, Dwivedi S, Mallick S, Tripathi RD (2019). Redox homeostasis in plants under arsenic stress. In: Redox Homeostasis in Plants. Springer, pp 179–198

Mishra SR, Behera BP, Singh VK, Mahapatra KK, Mundkinajeddu D, Bhat D, Minz AM, Sethi G, Efferth T, Das S (2024). Anticancer activity of *Bacopa monnieri* through apoptosis induction and mitophagy-dependent NLRP3 inflammasome inhibition in oral squamous cell carcinoma. Phytomedicine 123:155157.

Murashige T, Skoog F (1968). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 50(1):151–159.

Nguyen DTC, Van Tran T, Nguyen TTT, Nguyen DH,

Alhassan M, Lee T (2023). New frontiers of invasive plants for biosynthesis of nanoparticles towards biomedical applications: a review. Sci Total Environ 857:159278.

Patra D, Bhavya K, Ramprasad P, Kalia M, Pal D (2023). Anti-cancer drug molecules targeting cancer cell cycle and proliferation. Adv Protein Chem Struct Biol 135:343–395.

Ray A, Gulati K, Rehman S, Rai N, Anand R (2021). Role of nutraceuticals as adaptogens. In: Nutraceuticals. Elsevier, pp 229–244

Ruiz-Margáin A, Román-Calleja BM, Moreno-Guillén P, González-Regueiro JA, Kúsulas-Delint D, Campos-Murguía A, Flores-García NC, Macías-Rodríguez RU (2021). Nutritional therapy for hepatocellular carcinoma. World J Gastrointest Oncol 13(10):1440.

Saxena MK, Singh N, Kumar S, Mp D, Datta S (2021). Potent pharmaceutical products from aquatic plants–review. Asian J Pharm Clin Res 14(1):48–63.

Schlachterman A, Craft Jr WW, Hilgenfeldt E, Mitra A, Cabrera R (2015). Current and future treatments for hepatocellular carcinoma. World J Gastroenterol 21(28):8478.

Sharma AN, Dewangan HK, Upadhyay PK (2024). Comprehensive review on herbal medicine: emphasis on current therapy and role of phytoconstituents for cancer treatment. Chem Biodivers 21(3):e202301468.

Shefin B, Sreekumar S, Biju CK (2016). Identification of lead molecules with anti-hepatitis B activity in *Bacopa monnieri* (L.) Wettst. and *Cassia fistula* L. through in silico method. J Pharm Biol Sci 11(5):16–21.

Smith E, Palethorpe HM, Tomita Y, Pei J V, Townsend AR, Price TJ, Young JP, Yool AJ, Hardingham JE (2018). The purified extract from the medicinal plant *Bacopa monnieri*, bacopaside II, inhibits growth of colon cancer cells in vitro by inducing cell cycle arrest and apoptosis. Cells 7(7):81.

Stroffolini T, Stroffolini G (2023). A historical overview on the role of hepatitis B and C viruses as aetiological factors for hepatocellular carcinoma. Cancers (Basel) 15(8):2388.

Yazbeck V, Alesi E, Myers J, Hackney MH, Cuttino L, Gewirtz DA (2022). An overview of chemotoxicity and radiation toxicity in cancer therapy. Adv Cancer Res 155:1–27.

Zuzarte M, Salgueiro L, Canhoto J (2024). Plant tissue culture: industrial relevance and future directions. In: Plants as Factories for Bioproduction: Recent Developments and Applications. Springer, pp 1–15

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Investigation of antiproliferative and apoptotic effects of *Rosmarinus officinalis* essential oil obtained by hydrodistillation on neuroblastoma cells

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Abstract: This study was designed to determine the potential antiproliferative and apoptosis inducing activities of *Rosmarinus officinalis* essential oil (RE) on neuroblastoma cancer cells. For this purpose, different concentrations of RE were applied to SH-SY5Y cells for 24 hours and cell viability was determined by MTT. In addition, the percentage of early, late and non-apoptotic cells was determined by AnnexinV/propodium iodide staining to determine the induction of apoptosis. In addition, the composition of RE was determined by GC-MS. In MTT assay, it was determined that the viability of SH-SY5Y cells decreased dose-dependently as a result of the application of different concentrations of RE. Moreover, 200 mg/ml RE treatment increased the percentage of cells in the late apoptotic phase. The main compounds of RE were determined as (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene, Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl, 1,8-Cineole, Camphor by GC-MS. In conclusion, RE is thought to be an important source of anti-proliferative and apoptosis inducing activity on neuroblastoma cells.

Keywords: Neuroblastoma; *Rosmarinus officinalis*; Apoptosis

1 Introduction

Neuroblastoma is a malignant tumour of neuroblasts, immature nerve cells found in various parts of the body. It usually affects children under 5 years of age. The tumour has the ability to grow rapidly and expand greatly, progressing to death (Quadir et al. 2024). NB is the most common extracranial solid tumour in children. It accounts for 8% of childhood cancers and 15% of childhood cancer deaths. NBs are neural crest developmental tumours arising from the sympathetic nervous system and most tumours occur in the adrenal medulla or along the paraspinal chain in the sympathetic ganglia. The clinical behaviour of NBs varies from spontaneous regression to relentless progression. These neuroblastic tumours show various histologies ranging from undifferentiated neuroblastoma to ganglioneuroblastoma or mature ganglioneuroma (Kamihara et al. 2024).

The most prominent features of malignant cell transformation and tumourisation are cell proliferation or irregularities in the apoptotic pathway. Apoptosis is programmed cell death and many physiological events are controlled by apoptosis (Letai 2017). Briefly explained, cells are eliminated by activating the apoptotic pathway as a result of mutations that occur in cells and cannot be repaired. However, as a result of irregularities in the apoptotic pathway, cells can escape from apoptosis and proliferate rapidly by entering the pathway of cancerisation. Moreover, it is thought that cells can be suppressed by activation of apoptosis in neuroblastoma cells (Fulda and Debatin 2003).

Previous studies have shown that plant materials play important roles in the activation of apoptosis and suppression of cell proliferation in different cancer cells. Especially essential oils have antiproliferative and apoptosis inducing activities due to important phytochemicals in their structure (Dhifi et al. 2016; Nieto 2017). Rosmarinus officinalis L. (Salvia rosmarinus Schleid), a plant belonging to the Lamiaceae family, is popularly known as Rosemary. It originates from the Mediterranean region, but can be found

all over the world. It is a shrubby perennial and aromatic herb with green leaves emitting a characteristic odour. R. officinalis can be used as a spice, as a natural preservative in the food industry and as an ornamental and medicinal plant. (González-Trujano et al. 2007; Pérez-Fons et al. 2010; Brewer 2011; Rašković et al. 2014). It exhibits important biological activities due to many phytochemicals in its structure (Hussain et al. 2010). It has been reported to have antioxidant (Hendel et al. 2024), anticancer (Wang et al. 2012), antibacterial (Bajalan et al. 2017), antifungal (Ozcan and Chalchat 2008) activities, especially in studies conducted with the essential oil of R. Officinalis. Although there are studies on the suppression of different cancer cells, studies showing the proliferative and apoptotic effects of this oil on neuroblastoma cells are very limited. Therefore, in this study, it was aimed to determine the antiproliferative and apoptosis inducing activities and chemical content of RE on neuroblastoma cells.

2 Materials and Method

2.1 Collection and extraction of *R.officinalis*

R.officinalis was collected from campus area of Gaziantep University. Then, washed with distilled water and was dried on blotting paper in the open air and in a room away from sunlight. Then, it was ground in a mortar before the extraction. 100 g of *R.officinalis* was placed in a Clavenger flask and extracted by hydrodistillation for 3 h. The *R.officinalis* essential oil (RE) was stored in the refrigerator (+4 C) until the experiments were started.

2.2 Maintenance and growth of SH-SY5Y cells

SH-SY5Y cells were grown with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic (Gibco, USA) supplemented in DMEM. Cells were maintained in an incubator with a 5% CO_2 supply at 37 °C. After cultivation, SH-SY5Y (6 x 10^4 cells/mL) cells were seeded onto 96 well plates and cultured for 24 h on DMEM medium with supplements. After 24 h incubation, the old medium was aspirated and replenished with a new medium containing different concentrations (50, 100, 150 and 200 µg/mL) of RE with serum-free medium and exposed for 24 h under specified conditions. RE was dissolved in DMSO (at concetration of 10% of dimethyl sulfoxide).

2.3 Determination of anti-proliferation activity

After preserving SH-SY5Y cells in DMEM for 24 hours, 96-well plates containing 70–80% confluent of lung cancer cell cultures were treated for 24 hours with various dilutions of RE (50, 100, 150 and 200 µg/mL). MTT was utilized to evaluate cell viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). Replaced the culture media with DMEM containing 1 mg/mL MTT (Sigma) and incubated at 37 °C for 15 minutes. The cells were then treated with MTT solution and dimethyl sulfoxide (DMSO, Sigma). The density of the cells will be measured at 550 nm with a colorimetric reader (BioTek instrument, USA).

2.4 Determination of apoptosis induction by AnnexineV and Propodium iodide (PI)

For the determination of apoptosis induction activity of RE, SH-SY5Y cells were seeded with a $1x10^6$/mL density to 6-well plates and lowest (50µg/mL) and highest (200 µg/mL) concentrations of RE were applied for a period of 24 hours. Annexin V/PI apoptosis detection kit was used to measure the apoptosis according to manufacturer' recommended protocol. Results were measured in Becton-Dickinson flow cytometer.

2.5 Statistically analysis

Statistical analysis was carried out using GraphPad Prism 8.0.2. program. Dunnett's test was used for statistical evaluation for antiproliferation, apoptosis and the mRNA levels. In evaluation of statistical, the terms "*", "**" and "***" were meant $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

3 Results

3.1 Determination of antiproliferative activity

It was observed that the viability of SH-SY5Y cells decreased as a result of RE treatment for 24 hours (Figure 1). Figure 1 shows that 50 µg/ml RE concentration had no effect on cell viability, but 100 ($p<0.05$), 150 ($p<0.01$) and 200 ($p<0.01$) µg/ml RE concentrations inhibited cell proliferation in a concentration-dependent manner. After the application of 100, 150 and 200 µg/ml concentrations, 71.31%, 61.53% and 25.58% of SH-SY5Y cells remained viable, respectively.

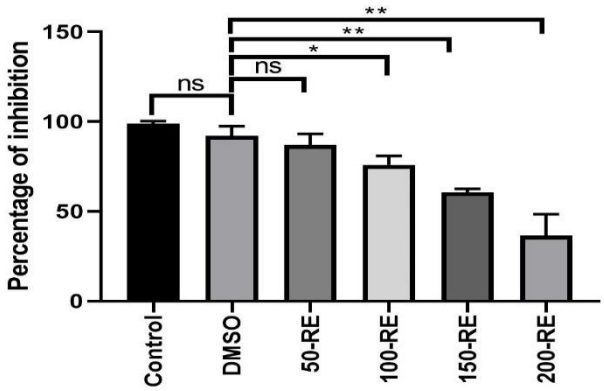

Fig. 1 The antiproliferative activity of RE on SH-SY5Y cells

3.2 Induction of apoptosis

In this experiment, cells were exposed to the lowest and highest dose of RE for 24 hours and then stained with AnnexinV/PI and early apoptotic, late apoptotic and non-apoptotic cell percentages were determined by flow cytometry. When Figure 2 was analysed, it was determined that 50 µg/ml RE concentration did not affect cell percentages in non-apoptotic early apoptosis and late apoptosis phases compared to control. On the other hand, after the application of 150 µg/ml RE concentration, the amount of viable cells decreased, the percentage of cells in the non apoptotic phase increased from 0.3% to 7.0%, the percentage of cells in the early apoptotic phase increased from 0.1% to 1.5% and the percentage of cells in the late apoptotic phase increased from 2.2% to 16.5%.

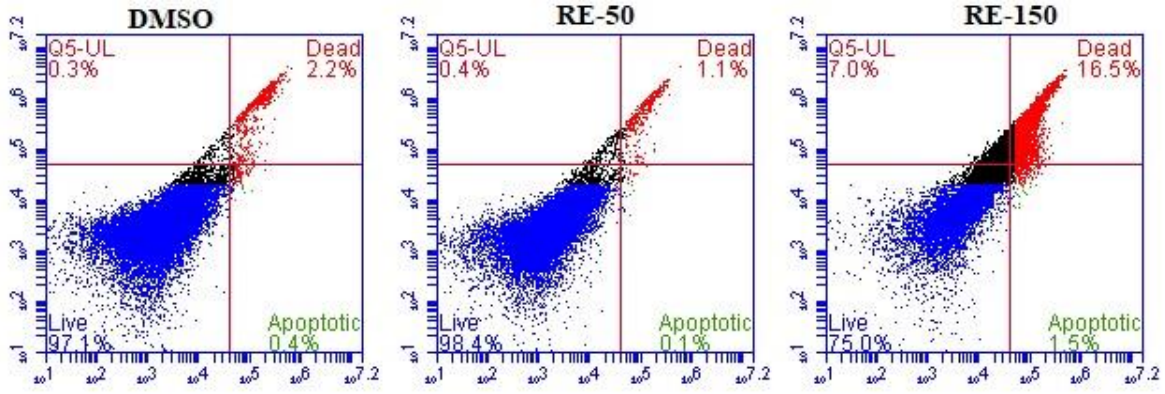

Fig. 2 Determination of apoptosis induction after RE application

3.3 Screening of RE content by GC-MS

As a result of screening the content of RE by GC-MS, 57 compounds were determined (Table 1). The identified compounds constitute 100 % of the total oil. However, the main compounds of RE were (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (24.66%), Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl (13.48%), 1,8-Cineole (11.21%), Camphor (6.71%).

Table 1 Content of RE by GC-MS

No	R.Time	Name	%
1	1.901	1,3-Pentadiene, 2-methyl-, (E)-	0.09
2	3.494	Tricyclene	0.14
3	3.719	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	24.66
4	4.109	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1R)-	0.06
5	4.215	Camphene	3.27
6	4.765	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	1.64
7	4.955	Sabinene	0.05
8	5.006	Verbenene	0.44
9	5.060	2-Pentanone, 4-hydroxy-4-methyl- (CAS)	0.02
10	5.586	.beta.-Myrcene	4.58
11	5.801	.alpha.-Terpinene	0.87
12	6.088	D-Limonene	4.17
13	6.221	1,8-Cineole	11.21
14	6.553	Furan, 2-pentyl-	0.03
15	6.611	trans-.beta.-Ocimene	0.18
16	6.771	.gamma.-Terpinene	1.85
17	7.122	Benzene, 1-methyl-4-(1-methylethyl)- (CAS)	1.27
18	7.324	.Alpha.-Terpinolene	1.38
19	8.947	.Alpha.-Fenchone	0.04
20	9.510	Benzene, Methyl(1-Methylethenyl)-	0.06
21	9.558	Filifolone	0.07
22	9.846	Trans-Sabinene Hydrate	0.13
23	9.895	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans-	0.22
24	10.246	.Alpha.-Campholene Aldehyde	0.09
25	10.492	Chrysanthenone	0.21
26	10.629	Camphor (CAS)	6.71
27	10.834	trans-3(10)-Caren-2-ol	0.34
28	10.954	1,6-Octadien-3-ol, 3,7-dimethyl-	2.85
29	11.025	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-, (1.alpha.,2.alpha.,5.alpha.)-	0.89
30	11.189	p-menth-2-en-1 –ol	0.08
31	11.321	Pinocarvone	0.24
32	11.517	Bornyl acetate	3.64
33	11.647	Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl-	0.10
34	11.722	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	2.36
35	11.801	Caryophyllene	0.23
36	11.906	Spiro [bicyclo[3.3.0] octan-6-one-3 - cyclopropane]	0.17
37	12.027	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	0.05
38	12.344	Cyclohexanone, 5-methyl-2-(1 - methylethylidene)-	0.71
39	12.416	(S)-cis-Verbenol	0.20
40	12.622	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, (S)- (CAS)	0.24
41	12.726	(S)-cis-Verbenol	0.71
42	12.885	Cyclopentane, 1 -acetoxymethyl-3-isopropenyl-2-methyl-	0.09
43	12.980	.Alpha. Terpineol	2.89
44	13.079	endo-Borneol	1.17
45	13.208	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	13.48
46	13.395	p-Mentha-1,5-dien-8-ol (CAS)	0.10

47	13.448	Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl- (CAS)	0.22
48	13.606	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)- (CAS)	0.20
49	14.140	.alpha.-Campholenal	0.19
50	14.439	2-Heptene, 5-ethyl-2,4-dimethyl- (CAS)	0.27
51	14.579	Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl- (CAS)	1.38
52	15.342	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	0.18
53	15.416	cis-Myrtanol	0.40
54	15.577	trans-Geraniol	2.69
55	17.142	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)- (CAS)	0.14
56	17.611	2-Cyclopenten-1-one, 3-methyl-2-(2-pentenyl)-, (Z)-	0.25
57	19.209	Benzene, 1,2-dimethoxy-4-(2-propenyl)- (CAS)	0.10
		Total (%)	100.00

4 Discussion

Millions of people pass away from cancer every year. Medicinal plants are used in cancer research by many researchers due to the different chemicals they contain and the low possible side effects of these chemicals. Rosmarinus officinalis is one of the most well-known plants among medicinal plants and its oil and other extracts have been tested for the suppression of cancer types. Significant cytotoxic effects have been reported in many anticancer studies with essential oil and extracts of R. officinalis. Santos et al. (2016) reported that RE had no cytotoxic activity on Hela cells. However, Jardak et al. 2017 reported that 0.01 and 0.253 µl/ml doses exhibited potent cytotoxic activity on cervix (HeLa) and breast (MCF7) cancer cells, respectively. In a different study, Hussain et al. (2010) reported that RE suppressed the proliferation of MCF7 cells. In addition, Dolghi et al. 2022 determined that the viability and proliferation of HCT-116 colorectal cancer cells decreased significantly, especially at doses of 100-500 µg/ml. In a different study, the proliferation of HepG2 and ECV304 cells was determined at doses of 508.7 µg/ml and 525.7 µg/ml, respectively (Becer et al. 2023). In our study, possible antiproliferative effects of RE on neuroblastoma cells, one of the childhood tumours, were tested. In the light of the data obtained, it was determined that especially 100 and 200 mg/ml doses suppressed neuroblastoma cells. In this context, it can be said that RE has agents that can reduce the proliferation and viability of different cancer cells. In addition, in previous studies, the number of studies showing the cytotoxic activity of RE on neuroblastoma cells is very limited and therefore the results obtained in our study are important in terms of cytotoxicity.

It was stated that neuroblastoma cells would be suppressed by inducing apoptosis. With the activation of caspases involved in both mitocontrial and extrinsic apoptotic pathways, the DNA of neuroblastoma cells can be fragmented and the cells are removed (Fulda and Debatin 2003). However, it has also been shown that neuroblastoma cells are suppressed by non-apoptotic cell death pathways such as necrosis (Fulda and Debatin 2003). In our study, it was determined that early and late apoptotic and non-apoptotic neuroblastoma cell percentages increased at 200 µg/ml concentration. It can be concluded that RE induces apoptotic cell death and non-apoptotic cell death.

It has been shown in previous studies that RE collected from different geographies contain different types and amounts of chemicals (Santos et al. 2016). They identified a total of 19 compounds in RE and 1,8-cineole (52.2%), Camphor (15.2%) and alpha-pinene (12.4%) were the main compounds of the oil. In another study, the main components of RE were 1,8-cineole (23.56%), camphene (12.78%), camphor (12.55%) and β-pinene (12.3%). Dolghi et al (2022). They determined 23 compounds in RE. However, the main components of the oil were eucalyptol (33.592%), α-pinene (12.239%), L-camphor (12.2%), β-Thujene (9.709%), β-pinene (9.435%), camphene (5.723%). Husaain et al. 2010 determined the main compounds of RE as 1,8-cineol (38.5%), camphor (17.1%), -pinene (12.3%), limonene (6.23%), camphene (6.00%), and linalool (5.70%). However, Becer et al. 2023 found the main compounds of RE as camphor (15.1%), verbenone (14.3%), α-pinene (13.6%), 1,8-cineole (11.8%), and borneol (7.9%). In addition, the main compounds of RE in our study were (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (24.66%), bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl (13.48%), 1,8-Cineole (11.21%), Camphor (6.71%). It is thought that the different effects on different cancer cells may be due to the type, amount and interaction of these chemicals with each other.

5 Conclusion

In conclusion, antiproliferative and apoptotic effects of RE on neuroblastoma cells SH-SY5Y were determined in this study. However, it is recommended to determine the levels of caspases, BCL2, caspase inhibitors and necrotic factors such as RIPK and MLK1 for non-apoptotic effects in order to understand the apoptotic effects more clearly.

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Authors' contributions:

PY: Methodology, Data curation, writing draft. MP: Methodology, Material collection, Data curation. MK: Writing, editing draft. DK: Methodology, Data curation. ÖY: Supervision, Data curation, Writing, editing draft.

Conflict of interest disclosure:

The authors declare that there is no conflict of interest.

References

Bajalan I, Rouzbahani R, Pirbalouti AG, Maggi F (2017) Antioxidant and antibacterial activities of the essential oils obtained from seven Iranian populations of Rosmarinus officinalis. Ind Crops Prod 107: 305-311.

Brewer MS (2011) Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Compr Rev Food Sci Food Saf 10:221–47.

Dhifi W, Bellili S, Jazi S, Bahloul N, Mnif W (2016) Essential oils' chemical characterization and investigation of some biological activities: A critical review. Medicines 3(4): 25.

Dolghi A, Coricovac D, Dinu S, Pinzaru I, Dehelean CA, Grosu C, Chioran D, Merghes PE, Sarau CA (2022) Chemical and Antimicrobial Characterization of *Mentha piperita* L. and Rosmarinus officinalis L. Essential Oils and In Vitro Potential Cytotoxic Effect in Human Colorectal Carcinoma Cells. Molecules (Basel, Switzerland) 27(18):6106.

Fulda S, Debatin K M (2003) Apoptosis pathways in neuroblastoma therapy. Cancer Lett 197(1-2): 131-135.

González-Trujano ME, Peña EI, Martínez AL, Moreno J, Guevara-Fefer P, Déciga-Campos M López-Muñoz FJ (2007) Evaluation of the antinociceptive effect of Rosmarinus officinalis L. using three different experimental models in rodents. J Ethnopharmacol 111(3): 476–482.

Hussain AI, Anwar F, Chatha SAS, Jabbar A, Mahboob S, Nigam PS (2010) Rosmarinus officinalis essential oil: antiproliferative, antioxidant and antibacterial activities. Braz J Microbiol 41: 1070-1078.

Jardak M, Elloumi-Mseddi J, Aifa S, Mnif S (2017) Chemical composition, anti-biofilm activity and potential cytotoxic effect on cancer cells of Rosmarinus officinalis L. essential oil from Tunisia. Lipids Health Dis 16:1-10.

Kamihara J, Diller LR, Foulkes WD, Michaeli O, Nakano Y, Pajtler KW, Perrino M, Scollon SR, Stewart DR, Voss S, Weksberg R, Hansford JR, Brodeur GM (2024) Neuroblastoma Predisposition and Surveillance-An Update from the 2023 AACR Childhood Cancer Predisposition Workshop. Clin Cancer Res 30(15):3137–3143.

Letai A (2017) Apoptosis and cancer. Annu Rev Cancer Biol 1(1): 275-294.

Nieto G (2017) Biological activities of three essential oils of the Lamiaceae family. Medicines 4(3): 63.

Özcan MM, Chalchat JC (2008) Chemical composition and antifungal activity of rosemary (Rosmarinus officinalis L.) oil from Turkey. Int J Food Sci Nutr 59(7-8): 691-698.

Pérez-Fons L, Garzón MT, Micol V (2010) Relationship between the antioxidant capacity and effect of rosemary (Rosmarinus officinalis L.) polyphenols on membrane phospholipid order. J Agric Food Chem 58(1): 161–171. https://doi.org/10.1021/jf9026487.

Qadir MI, Ahmed B, Noreen S (2024) Advances in the Management of Neuroblastoma. Crit Rev Eukaryot Gene Expr 34(2):1–13.

Rašković A, Milanović I, Pavlović N, Ćebović T, Vukmirović S, Mikov M (2014) Antioxidant activity of rosemary (Rosmarinus officinalis L.) essential oil and its hepatoprotective potential. BMC Complement Altern Med 14:225.

Santos PASR, Avanço GB, Nerilo SB, Marcelino RIA, Janeiro V, Valadares MC, Machinski M (2016) Assessment of cytotoxic activity of rosemary (Rosmarinus officinalis L.), turmeric (Curcuma longa L.), and ginger (Zingiber officinale R.) essential oils in cervical cancer cells (HeLa). Sci World J 2016(1): 9273078.

Wang W, Li N, Luo M, Zu Y, Efferth T (2012) Antibacterial activity and anticancer activity of Rosmarinus officinalis L. essential oil compared to that of its main components. Molecules 17(3): 2704-2713.