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Research Article

In vitro production of tropane alkaloids from *Brugmansia suaveolens*

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Abstract: For thousands of years, secondary metabolites have been utilized as medications, flavors, pesticides, and dyes. For the generation of valuable secondary metabolites, *in vitro* plant culture techniques have been viewed as beneficial alternatives to whole plants. *Brugmansia suaveolens* is an ornamental plant including anticholinergic agents which are employed in medicine. Callus induction was performed from leaf and internode explants cultured on Murashige and Skoog's medium supplemented with different concentrations and combinations of plant growth regulators (PGRs) with 6 treatments. The highest callus induction response was obtained from the leaf-originated explants (73%) on the medium supplemented with 0.4 mg/L KIN and 0.2 mg/L NAA which produced friable callus in 4 weeks. The cell suspension culture of *B. suaveolens* was established in shake flasks using friable calli. The extraction protocol of tropane alkaloids was optimized, atropine and scopolamine were obtained efficiently. The data could provide technical support for the large-scale production of valuable alkaloids of *B. suaveolens in vitro* systems with improved strategies.

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Scopolamine,
Suspension culture.

1. INTRODUCTION

Plants have a diverse group of well-recognized phytochemicals which are named as secondary metabolites. Secondary metabolites have been used as drugs, flavors, insecticides, and dyes for thousands of years. The applications of *in vitro* plant culture techniques have been seen as beneficial alternatives to whole plants for the production of valuable secondary metabolites (Baque *et al*., 2012). The secondary metabolites are being extracted efficiently from plants as their chemical synthesis is complex and requires expensive instrument use. However, large amounts of plant materials are needed for the extraction of secondary metabolites. Unfortunately, the collection of plants from their natural habitats threatens the existence of different types of living organisms and environments (Kumar and Gupta, 2008). Production of the secondary metabolites by plant tissue culture system could be accomplished efficiently using callus, cell suspension, and organ (embryo, root, and shoot) cultures. The following are

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some of the benefits of *in vitro* plant culture systems for the generation of secondary metabolites:

1) Whole plant and organs can be produced under controlled conditions independently of external factors (eg., climate, soil content); 2) Controlled plant cell and tissue cultures can yield a source of defined standard phytochemicals in large volumes with improved quality; 3) Cultured plant cells would be free of microbes.

Several *in vitro* plant cell culture applications have been performed for large-scale production of secondary metabolites to produce higher amounts than in intact plants (Ikeuchi *et al.,* 2013; Chandran *et al.,* 2020). Many reports have described that some approaches have been applied to increase their productivity. Shoot cultures of *Bacopa monnieri* were established for the production of bacoside A by Praveen *et al.,* (2009) and when compared to field-grown plants, the regenerated shoots had a 3-fold increase in bacoside A content. Similarly, *in vitro* regenerated shoots of *Nothapodytes nimmoniana* yielded higher amounts of camptothecin when compared to their mother plants (Dandin & Murthy, 2012).

The accumulation of secondary metabolites is improved by some strategies such as transformation, elicitor treatment, mutagenic chemical, and bioreactor use. The scaling up of the tropane alkaloid anisodamine in hairy root cultures of two ecotypes of *Brugmansia candida* plants by *rolC* gene expression was achieved (Cardillo *et al.,* 2013). The release of scopolamine and hyoscyamine into the media after elicitor treatment of B. candida roots was previously documented in a study (Pitta-Alvarez *et al.,* 2000). In addition, special bioreactor systems have been devised for the large-scale cultivation of plant cells for the production of bioactive compounds with efficient applications.

Alkaloid group is currently used in medicine and this group includes the analgesics morphine and codeine, the anticancer agent vinblastine, the gout suppressant colchicine, and the sedative scopolamine. Tropane alkaloid group is a typical secondary metabolite of certain *Solanaceous* genera including *Atropa, Hyoscyamus, Duboisia, Scopolia,* and *Mandragora.* Tropane alkaloids are a unique group of compounds that are commonly employed as parasympathetic nervous system blockers. They are known to prevent the binding of acetylcholine to its receptor and as a result have effects on heart rate, respiration, and functions in the central nervous system (anticholinergic poisoning). Atropine and scopolamine are the major alkaloids in *Solanaceae* family plants. It is reported that tropane alkaloid content is different in the tissues and development stages of plants (Ghorbanpour *et al.,* 2013). Atropine is a well-known tropane alkaloid and is used for treating organophosphate poisoning and exposure to some chemical weapons. Scopolamine (sometimes called hyoscine) is a pharmacological drug that is used to treat nausea, vomiting, motion sickness, and smooth muscle spasms. Scopolamine has a tenfold higher commercial demand than atropine. Scopolamine was also suggested as a protective metabolite of *B. suaveolens* against insects (Alves *et al.,* 2007; Sarin, 2005).

Many efforts have been undertaken to improve practical tropane alkaloids production methods using plant tissue culture techniques (Dehghan *et al.,* 2012). A variety of *Solanaceous* plants have been examined for the production of medically important alkaloids through callus, suspension, and hairy root cultures (Pitta-Alvarez *et al.,* 2000; Cardillo *et al.,* 2010; Chandran *et al.,* 2020).

B. suaveolens is an ornamental plant known as angel trumpet. It is a member of *Solanaceae* family and is considered toxic with some medical properties. *B. suaveolens* produces atropine, scopolamine, and hyoscyamine alkaloids as defense molecules which are organic esters exhibiting hallucinogenic, antispasmodic, diaphoretic, and diuretic activities (Pitta-Alvarez *et al.,* 2000; Anthony *et al.,* 2009). *In vitro* tropane alkaloid production of *Brugmansia* species is carried out in a few scientific works and they are mostly performed with hairy root cultures.

The main objective of this study was to establish optimal *in vitro* culture conditions by using different combinations of PGRs. Also, an efficient extraction protocol of tropane alkaloids was optimized, and the presence of scopolamine and atropine was determined qualitatively.

2. MATERIAL and METHODS

2.1. Plant Material and Seed Germination

Mature seeds of *B. suaveolens* were collected in September of 2019 in Gebze, Kocaeli, Turkey and a voucher specimen was deposited. The seeds were immersed in water for one hour and the seed coat was removed gently. The seed surface sterilization was performed in 70% (v/v) ethanol for 2 min and in 50% (v/v) commercial bleaching (5.25% w/v solution of sodium hypochlorite) including Tween 20 (two drops for 100 ml solution) for 10 min. Then the seeds were rinsed four times in sterile distilled water. The disinfected seeds were blotted with sterile filter paper and cultured on PGR free Murashige Skoog (MS) (1962) basal medium containing 3 % sucrose (w/v) and 0.6 % agar (w/v) with pH 5.8. The seeds were incubated at 25±2 °C with a relative humidity of 55-60 % under dark for two weeks. Then, the cultures were transferred to 16 h light /8 h dark photoperiod conditions by cool white fluorescent lamps with an intensity of 3000 lux.

2.2. Establishment of Callus and Cell Suspension Cultures

Calli were induced from leaf and internode (epicotyl and hypocotyl pieces) explants of 8 weeks old *in vitro* germinated seedlings of *B. suaveolens*. The leaf pieces with 0.5 m^2 and the internodes with 1 cm length were excised from the seedlings and were placed on full-strength semisolid MS basal media. The media were supplemented with a synthetic auxin and different combinations of auxins and cytokinins including; 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), benzyl aminopurine (BAP), indole-3-acetic acid (IAA), thidiazuron (TDZ) and kinetin (KIN).

The explants were placed on 90 mm petri dishes for callus development. Normally, plants are subjected to variable stresses *in vitro* culture systems. Considering the stress effects of plant tissue culture systems, concentrations of PGRs were used in minimal amounts in the culture media of *B. suaveolens.* The PGR free MS basal medium was used as a control treatment. In addition, one set of the treatments of the cultures were incubated under dark. The frequency of callus induction on semisolid MS medium was calculated according to the following equation:

$$\text{Callus induction frequency (\%)} = \frac{\text{The number of calli formation}}{\text{Total number of explant used}} \text{x}100$$

Following the callus formation on the edges and at the tips of the explants, the induced calli were dissected from the explants and transferred onto the same ingredients containing fresh medium for the subsequent 4 weeks. The calli were harvested from the second passage of cultures and co-cultivated on semisolid MS media for the fourth passage where the assessment of the 6 treatments was performed to find out the effective experimental design for callus growth of *B. suaveolens*. Inoculum weight of callus for fourth subculturing was 1.0± 0.05 g as initial fresh weight ($FW_{initial}$).

Cell suspension cultures of *B. suaveolens* were initiated by transferring fourth-round subcultures of leaf-derived, friable calli into liquid MS3 medium (containing 0.4 mg/L KIN+0.2 mg/L NAA). The best cell growth performance of the suspension cultures was observed with the MS3 medium. Two sizes of flasks (100 and 250 ml) were used with different volumes (1/8 and 1/5) of the growth media. Erlenmeyer flasks of 100 ml and 250 ml sizes were filled with 1/5 and 1/8volume of liquid MS3 growth medium. The flasks with 100 ml size were filled with 12.5 m and 20 ml and the culture flasks with 250 ml size were filled 31.25 ml and 50 ml of the growth medium. Actively growing friable calli clumps were selected for suspension culture

starting material. The callus clumps were slightly chopped with a scalpel and cell suspension culture was inoculated with 1.0±0.05 g of calli biomass ($FW_{initial}$) to initiate suspension cultures.

The inoculated flasks of the suspension cultures were placed on a rotary orbital shaker at 100 rotations per minute and incubated under the same photoperiod, temperature, and humidity conditions as the callus cultures. The culture medium was refreshed with a new liquid medium at the end of 2 weeks, and the suspension cultures were maintained for 4 weeks.

2.3. Assessment of the Culture Growth

After 30 days of fourth cultivation in liquid MS medium, the calli were morphologically assessed and final fresh weight (FW) was obtained. Cell growth of the cultures was expressed as FW, dry weight (DW), and growth index (GI). Rating of callus was also evaluated as callus score by size between 1-7 mm.

The calli were washed with distilled water and were blotted on tissue paper at the end of cell suspension culture period of 4 weeks. The FW of the proliferated calli was obtained by weighing. The callus and the cell suspension culture biomass were oven-dried at 40°C for 24 h and DW was obtained. The growth of the cultures was represented as GI by using the equation given below.

$$\mathrm{GI} = \frac{(\text{Final fresh weight of biomass} - \text{Initial fresh weight of inoculum})}{\text{Initial fresh weight of inoculum}}$$

2.4. Tropane Alkaloid Extraction from Plant Material

Tropane alkaloid extraction was carried out from 4th subcultured callus cultures and leaf samples. The leaf material was collected from the natural habitat in flowering time. The dried samples (5 g DW) were ground to fine powder by using a grinder and alkaloid extraction was performed as described by Kamada *et al.,* (1986) with some modifications. Briefly, an appropriate volume of extraction buffer $CHCI_3$/MeOH/NH_4OH (I5/5/1) was added onto the powdered materials, and the mixture was sonicated in an ultrasonic water bath for 10 min. The slurry was macerated for 24 hours at room temperature. The crude extract was filtered through filter paper, washed twice with chloroform (CHCl3), and evaporated. The residue was dissolved in 2 ml of sulfuric acid (98% v/v) and 5 ml of CHCI3 to separate CHCI3 phase. The aqueous phase was adjusted to pH 10 with 25% ammonium hydroxide (NH4OH) solution in the ice bucket. Alkaloid residue was extracted twice with $CHCI_3$ and filtered by adding anhydrous Na_2SO_4 under vacuum at 40°C.

2.5. Qualitative Analyses of Tropane Alkaloids

Qualitative estimation of the alkaloid content of *B. su*aveolens leaf extract was carried out using thin-layer chromatography (TLC) on silica gel alumina TLC plates (20X20 cm Silica gel 60 F_{254} plates). The alkaloid leaf extract of *B. suaveolens* and alkaloid standards of atropine and scopolamine were spotted onto the silica plate. The separation of the spots was performed on the plate using solvent systems of chloroform/acetone/ammonia: methanol (3/17) with a combination of 5/4/1 as mobile phase. Following the separation, the spots were visualized under short and long-wavelength ultraviolet lights (254 and 365 nm) and immediately the plate was sprayed with Dragendorff's Reagent to clarify the spots of tropane alkaloids.

The alkaloid extracts of the callus and the leaf samples and atropine standard (Sigma-Aldrich) were analyzed by High-Performance Liquid Chromatography (HPLC) system (Shimadzu) equipped with a Zorbax Extend C18 column (100x4,6 mm, 3,5 µm particle size) and a UV-VIS detector. The data series of standard atropine dilutions over a range of 150 – 6555 µg/ml were used to construct a calibration graph by plotting the peak area versus the corresponding concentration.

The samples were filtered through a 0.45 µm membrane prior to the HPLC assay. The mobile phase was optimized with potassium acetate/acetonitrile (82/18, v/v, pH 3.5) with a flow rate of 1 $ml.min^{-1}$ at 40 °C and total analysis time was 10 min. The injection volume of the samples was set at 10 µl and elution was monitored at 210 nm (Koetz *et al.,* 2017).

2.6. Statistical Analyses

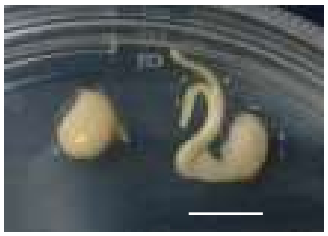
Experimental designs of the callus and the cell suspension cultures were repeated four times with a sample size of 6 replications. The influence of the experimental designs was analysed by one-way analysis of variance (ANOVA) to detect significant differences between the means of the data. All the treatments were conducted in a randomized design.

3. RESULTS

3.1. Plant Material and Seed Germination

The uncoated seeds of *B. suaveolens* were germinated on PGR free semisolid MS basal medium with 80% germination rate (Figure 1). The seed germination of B. suaveolens was evaluated using different chemical treatments by Montanucci et al. (2012). They found that the seed germination was reduced to various extents by physical and chemical treatments.

Figure 1. *In vitro* germination of *B. suaveolens* seeds on MS basal medium after 14 days (Scale bar: 1 cm).



3.2. Establishment of Callus and Cell Suspension Cultures

In the present work, *in vitro* culture system leading to the induction of callus was initiated from the internode and the leaf explants of 8 weeks old *in vitro* raised seedlings of *B. suaveolens*. Different PGRs were used either singly or in combination (Table 1) to evaluate their influences on callus induction.

Table 1. Effects of different PGR treatments on callus induction from internode and leaf explants of *B. suaveolens* after 6 weeks of cultivation.

		Internode derived		Leaf derived explant	
Treatment	PGR (mg/L)	Callus morphology**	Callus score*	Callus morphology**	Callus score*
MS0	PGR free MS	DG, B, c	+	G, DG, B, c	+
MS1	2,4-D (0.5)	L, f, r	++	WG, f, r	++
MS2	2,4-D+Kin (0.2+0.5)	W, L, f	++	G, LG, f, g	++
MS3	Kin+NAA (0.4+0.2)	LG, f, g	+++	LG, f, g	+++
MS4	BAP+NAA (0.5+0.5)	L, g	+	WG, g	+
MS5	BAP+2,4-D+Kin (0.5+0.2+0.5)	L, G, c	++	WG, G, co	++
MS6	TDZ+IAA (0.1+0.1)	L, WG, c	++	G, WG, c	++

*Callus formation: (+) weak (1 mm diameter), (++) moderate (up to 4 mm diameter), (+++) good (up to 7 mm diameter).

**Colour; Whitish (W), Light green (L), Yellowish (Y), Green (G), Lush green (LG), Dark green (DG), Brownish (B) and form; Friable (f), Globular (g), Compact (c), Root formation (r), Cotton like (co).

Callogenic response of the explants was observed in the second week through the swelling of the explants on 6 different MS media. Subsequently, the formation of friable and compact calli forms was observed at the cut tips of internode sticks and on the edges of the leaves. Callogenesis was observed on both types of explants with different morphology and growth rate. The best response of callus formation was observed with friable and green calli of the leaf explants. The leaf explants exhibited the highest frequency of callus induction (73%) which was greater than that obtained in the internode explants (59%). Callus induction of the explants has also been performed under the dark. The highest callus induction frequency was obtained (55%) with the leaf explants compared to the induction frequency of internode explants (45%) under dark. Generally, the induced calli were observed as light yellow and the color turned brownish after two weeks under dark.

It is obvious that the callogenesis process of *B. suaveolens* was positively affected under photoperiod conditions. The light was beneficial for the production of callus and cell suspension cultures compared to dark. The effects of different PGR on callus induction were assessed based on callus morphology and callus score after 6 weeks of culturing of the explants (Table 1). The appearance of the calli was observed as friable, globular, compact, root forming, and cotton-like. The color of the calli was various on different growth media. The callus score was also evaluated based on the diameter of the propagated callus.

3.3. Assessment of the Culture Growth

Assessment of the 6 treatments was performed for callus propagation using the fourth subculture as callus score, FW, DW, and GI as shown in Table 2.

Table 2. Effects of different PGR treatments on callus growth of 3^{rd} cultivation of leaf-derived explants of *B. suaveolens* after 4 weeks.

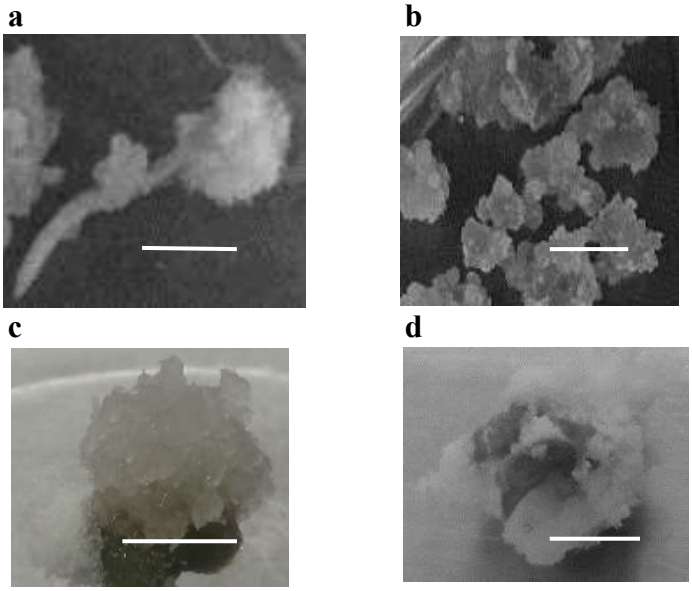
Treatment	PGR (mg/L)	Callus morphology	Callus score*	FW** (gr/culture)	DW (g)	Growth Index (GI)
MS0	PGRs free MS	Dark green, compact	+	1.42±0.19	0.12±0.01	0.42
MS1	2,4-D (0.5)	Whitish, root form	++	2.62±0.30	0.19±0.01	1.62
MS2	2,4-D+Kin (0.2+0.5)	Green, semi friable	++	3.38±0.27	0.32±0.01	2.38
MS3	Kin+NAA (0.4+0.2)	Lush green, friable, globular	+++	3.92±0.29	0.37±0.00	2.92
MS4	BAP+NAA (0.5+0.5)	Whitish, loose	+	1.61±0.41	0.09±0.01	0.61
MS5	BAP+2,4-D+Kin (0.5+0.2+0.5)	Whitish, cottony, leaf	++	1.15±0.21	0.06±0.01	0.15
MS6	TDZ+IAA (0.1+0.1)	Greenish, compact	++	2.04±0.32	0.16±0.01	1.04

*(+) weak (1 mm diameter), (++) moderate (up to 4 mm diameter), (+++) good (up to 7 mm diameter)
**FW: Fresh weight final. Callus inoculum: 1 g/culture. Data represent mean values (± *SE*) of three repeats each with six replicates. Level of significance $p < .05$.

Compact, dark green, and small (around 1mm diameter) callus forms were observed on the PGR free MS medium (MS0) after 2 weeks of cultivation, and the cultures became brownish after 4 weeks of cultivation. Interestingly, a transition state was observed where some tiny leaf structures formed on some explants after 5 weeks on PGR free MS medium. 2,4-D is the most often used synthetic auxin for callus induction with fast-growing calli. While the explants of *B.*

suaveolens were cultured on MS1 medium supplemented with 0.5 mg/L 2,4-D, light green, friable calli formation were observed under photoperiod conditions. In addition, distinctly different morphology root formation was observed on some callus aggregates after the fourth subculturing on MS1 media (Figure 2a). In addition, the calli became necrotic after 4 weeks when subcultured on the medium containing 2,4-D. Combinations of auxins and cytokinins produced more callus than auxin alone, according to our findings. It is reported that 2,4-D amounts ranging from 1.0 mg/L to 3.0 mg/L in the culture medium resulted in a high degree of browning of callus after 3 weeks (Dong *et al.,* 2015). Liu et al. (2018) performed a study with miniature rose that 3.0 to 5.0 mg/L 2,4-D concentration caused high degree browning of callus and abnormal embryo appearances. In order to reduce the inhibitory effect of 2,4-D, it is advised not to use high concentrations in the callus induction process. Importantly, browning is causing cell death by affecting cell growth in plant tissue culture (Dong *et al.,* 2015; Sarin, 2005). The browning of callus was observed after extended periods of the culture.

Figure 2. The morphology of leaf-derived calli of *B. suaveolens* on different semisolid media after 4 weeks of cultivation, **(a)** Root forming callus on MS1 medium, **(b)** Callus on MS3 medium, (**c)** Callus on MS4 medium, **(d)** Callus on MS5 medium. (Scale bars: 1 cm.)



The highest values of FW (3.92±0.29 g), DW (0.37±0.00 g), and GI (2.92) data were obtained in a combination of the semisolid MS3 medium supplemented with 0.4 mg/L KIN + 0.2 mg/L NAA. The MS3 treatment produced friable and lush green calli (Figure 2b). Based on the mean of FW (3.38±0.27 g) and GI (2.389) data, the MS2 treatment (0.2 mg/L 2,4-D +0.5 mg/L KIN) provided an efficient callogenesis after the MS3 treatment. Montanucci et al. (2012) performed callus induction and plant regeneration studies of *B. suaveolens* with different combinations of 2,4-D and KIN and they obtained 66 % callus induction with 0.5 mg/L 2,4- D +0.5 mg/L KIN combination on MS medium.

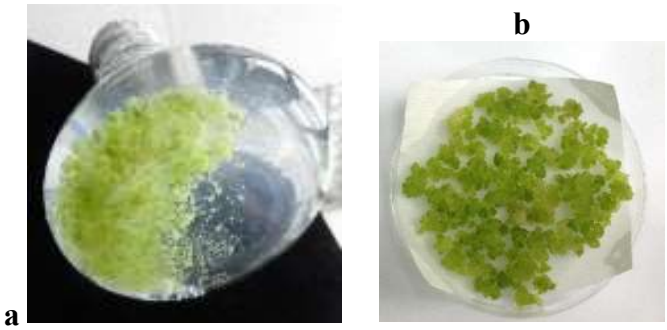
MS4 treatment has a combination of 0.5 mg/L BAP+ 0.5 mg/L NAA resulting in formation of whitish, loose callus forms (Figure 2c) and 1.61±0.41g FW was recorded in 4 weeks of cultivation. However, previously a study was performed by our group using BAP and NAA supplemented media for callus induction of *Hyoscyamus niger*. The media containing 0.25 mg/L BAP+0.25 mg/L NAA and 0.5 mg/L BAP+0.5 mg/L NAA performed friable, green callus formation with leaf and stem explants of *H. niger* which is a member of the *Solanaceae* family.

The effect of MS6 medium supplemented with 0.1 mg/LTDZ+0.1 mg/LIAA promoted light green, compact callus formation. Cultivation of the leaf-derived calli on MS6 medium resulted in 2.04±0.32g FW and 1.04 GI at the end of 4 weeks. The calli became brown on MS6 medium when the cultivation prolonged more than 4 weeks.

The lowest callus FW (1.15±0.21 g) was obtained on the treatment of MS5 medium (0.5 mg/LBAP+0.2 mg/L2,4-D+0.5 mg/LKIN). MS5 medium was poor at propagating standard callus forms like other calli-forming media. In MS5 medium is caused to produce cotton-like cotton-like white tissue forms (Figure 2d) with some tiny leaves in some callus aggregates. Because of the cotton-like calli appearance and leaf formation in some callus aggregates, FW and GI data of this treatment were not included in the statistical analyses.

The cell inoculum size was used 1.0± 0.05 g/culture for callus and cell suspension cultures of *B. suaveolens.* Inoculum size is an important parameter on cell growth and has a positive effect on the metabolite yield of cell suspension cultures. A suitable inoculum size can provide higher biomass production and accumulation of secondary metabolites. Lee and Shuler (2000) studied the effect of cell inoculum density on ajmalicine production of *Catharanthus roseus* cells. The study's findings revealed that increasing inoculum density resulted in higher ajmalicine concentrations. However, high inoculum size could be growth limiting *in vitro* cultures because of the accumulation of cell metabolites, toxic products, dead cells, and oxygen depletion during the stationary phase. It is also reported that a higher inoculum size does not produce high cell biomass. The suspension culture media were refreshed at the 15th day and the cultures were terminated after 30 days of culturing under photoperiod conditions. Cell aggregates of suspension cultures were composed of greenish, irregular, friable aggregates between 0.1 and 0.8 mm in diameter (Figure 3).

Figure 3. Suspension cell cultures of *B. suaveolens* after 4 weeks of inoculation, **(a)** In the 250 ml size flasks bottom view, (**b)** Filtered cell aggregates on filter paper.



Biomass data (FW and DW) of the cell suspension cultures of *B. suaveolens* were maintained with different container sizes and medium amounts. The maximum mean biomass of FW (4.85±0.46 g/culture) and DW (0.4185±0.56 g/culture) were obtained in the 250 ml size flask containing 1/8 volume (31.25 ml) of growth medium (Table 3).

The flasks with 250 ml size and with 1/5 volume (50 ml) of growth medium had the higher FW (4.60±0.33 g/culture) and DW (0.34 g/ culture) values of cells than the cell data of 100 ml size flasks. The 12.5 ml medium containing 100 ml flasks produced a bit higher FW (3.63±0.58 g) having cells than the high amount medium (20 ml) containing flasks of the same size (FW: 3.44±0.24 g). The same situation was observed with 250 ml flask cultures. It is concluded that a low amount (1/8) of growth medium is more effective than a high amount (1/5) of growth medium in biomass production of cell suspension cultures. This biomass growth could be explained by the positive effect of high aeration in big culture containers on the shaker. The

assessment of the results showed that the highest cell growth on callus and cell suspension cultures were obtained on the medium MS3 which was the best medium for callogenesis of *B. suaveolens*.

Table 3. Effects of container size and medium amount on growth parameters of *B. suaveolens* suspension cultures.

Erlen Size (ml)	Medium (ml)	Callus Morphology	FW (g/culture)	DW (g)	GI
100	12.5	Greenish, friable	3.62±0.58	0.25±0.01	2.62
100	20	Greenish, friable	3.44±0.24	0.28±0.00	2.44
250	31.25	Greenish, friable	4.85±0.46	0.41±0.02	3.85
250	50	Greenish, friable	4.60±0.33	0.34±0.00	3.60
100*	12.5	Yellowish, compact	2.98±0.25	0.27±0.11	1.98
100*	20	Yellowish, compact	2.76±0.25	0.34±0.51	1.76
250*	31.25	Yellowish, compact	3.62±0.24	0.29±0.01	2.62
250*	50	Yellowish, compact	3.40±0.45	0.30±0.03	2.40

Medium: MS3. Callus inoculum: 1 g/culture. *PGRs free MS medium. Data are means (±*SE*) of three repeats each with six replicates. Level of significance $p < .05$.

Comparing the biomass propagations of the semisolid and liquid cultures of *B. suaveolens*, the cell suspension culture system seems more promising. This is the first report on the establishment of the cell suspension cultures of *B. suaveolens* for tropane alkaloid production. To our knowledge, there is no previous report related to the cell suspension cultures of *B. suaveolens.* When compared to the overall plant system, cell suspension culture studies for the generation of secondary metabolites under controlled conditions are preferable. Plant growth regulators are important for the growth, development, and synthesis of secondary metabolites. Media composition, explant type, media strength, and presence of light have significant effects on *in vitro* plant development. These factors are critical for variation in biomass weight and production of secondary metabolites. Media strength was also assessed on callus induction of *B. suaveolens* that whole MS media promoted better callus growth than half-strength MS media in the presence of PGRs.

3.4. Tropane Alkaloid Extraction from Plant Material

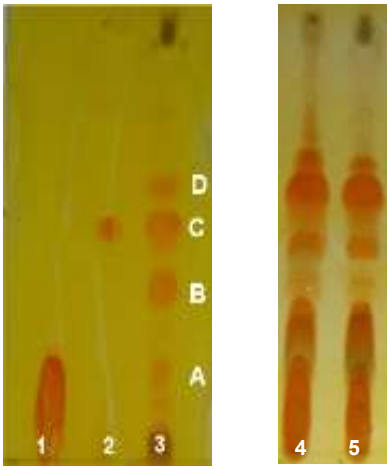
The alkaloid extraction of the plant materials of *B. suaveolens* was performed efficiently as described in the material and methods section. After the chloroform washing step, a dark brown alkaloid extract was obtained and it was kept at 4°C until further use. Chloroform extracts of the samples were applied onto the TLC plate and purity of scopolamine and atropine extracts were observed.

3.5. Qualitative Analyses of Tropane Alkaloids

The chloroform extract of leaf and alkaloid standards of atropine and scopolamine were spotted onto the silica TLC plate. The chromatography was performed in the related solvent system and the plate was sprayed with Dragendorff's reagent for the visualization of the spots. After spraying with the reagent, the plate was exposed to daylight and orange color spots of tropane alkaloids were appeared (Figure 4). Atropine (Figure 4A) and scopolamine (Figure 4B) spots of the leaf sample were observed clearly on the plate compared with the standard spots of atropine and scopolamine. This demonstrates that the modified alkaloid extraction protocol was effective for target alkaloid extraction of *B.* suaveolens. TLC technique is used in qualitative

analyses for the initial screening of plant extracts for routine alkaloid analysis before more sophisticated instrumental chromatography analyses.

Figure 4. TLC chromatogram of alkaloid standards and extraction of *B. suaveolens* leaf sample: **1.** Atropin standard, **2.** scopolamine standard, **3.** *Brugmansia* leaf extract. Atropine band (**A**), scopolamine band **(C**), and other tropane alkaloid molecules (**B, D**). Crude extracts of *Brugmansia* leaf extract (**4. and 5**). The derivatizing agent is Dragendorff's reagent.



Qualitative estimation of the callus the leaf extracts of *B. suaveolens* was performed by HPLC (Figure 5). The atropine peak at the retention time of approximately 2.5 minutes was observed clearly. It was compared with literature data and concluded that the peaks between 1.25 and 2.0 min could be other alkaloids, most probably including scopolamine peak.

A linear calibration curve was obtained with a correlation coefficient (r^2) of 0.9977. Based on the atropine standard curve, the percentages of atropine in total extracts of the leaf and callus were 66.52% and 55.78%, respectively. The amounts of atropine obtained from the leaf and the callus were 18.87 ± 0.19 mg/g dry weight and 6.94±0.19 mg/g dry weight, respectively. The callus has the capacity to biosynthesize atropine as the leaf. The results confirmed that atropine was produced in the callus of *B. suaveolens* as the major tropane alkaloid which resulted in 55.78% of total alkaloids. Several studies were performed to quantify tropane alkaloids in the *Solanacea*e family's plants. *Atropa belladonna* is the most known tropane alkaloid producer and the atropine amount was quantified in the leaves as 112.86 µg/ml (Koetz *et al.,* 2017). Statistically, the results of the ANOVA showed that there were no significant differences among the treatments for FW and GI of the callus and cell suspension cultures (Table 2). The result is significant at $p < .05$.

4. DISCUSSION and CONCLUSION

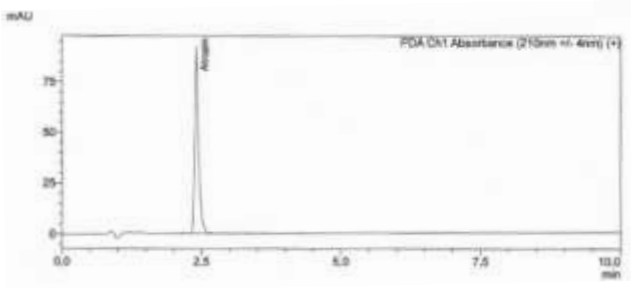
The goal of this study was the establishment of *in vitro* growth systems of *B. suaveolens* and to improve the tropane alkaloid extraction technique from the leaf and the callus materials. The induction of callus cultures and establishment of cell suspension cultures of *B. suaveolens* were performed efficiently using different combinations of PGRs and the best growth medium was determined. *B. suaveolens* is not a widely known tropane alkaloid having species and not involved much in scientific studies. In addition, the extraction protocol of tropane alkaloids was modified efficiently, atropine and scopolamine were determined qualitatively and quantitatively using chromatography methods of TLC and HPLC.

According to the data obtained in this study, we can conclude that *in vitro* growth of *B. suaveolens* cells is promising for the production of main tropane alkaloids. *In vitro* culture systems with different strategies could be considered as an alternative source for the production of valuable phytochemicals. Further studies could focus on investigating atropine and

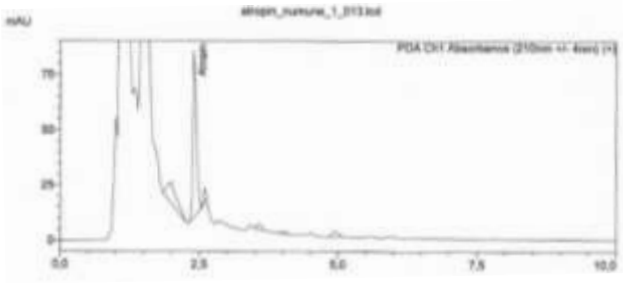
scopolamine productivity of the cultures under scale-up conditions using elicitor and bioreactor. The results could serve as a background for the large-scale production of valuable alkaloids of *B. suaveolens in vitro* plant systems with improved strategie

Figure 5. HPLC chromatograms of (a) atropine as a standard alkaloid, (b) alkaloid extraction of the leaf sample, (c) alkaloid extraction of callus sample of *B. suaveolens* by UV-VIS detector at 210 nm at a with a flow rate of 1 ml/min.

a



b



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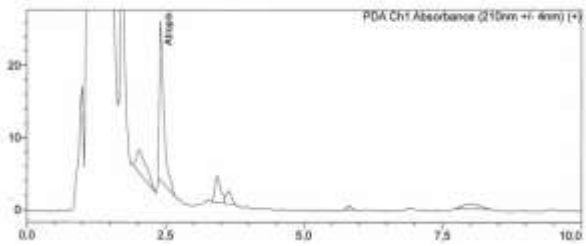

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). **Ethics Committee Number**: The relevant publication was approved at the TUBITAK MAM GMBE's Ethics Committee Editorial Board meeting of 08.09.2020.

Authorship contribution statement

Tijen Talas Ogras: Investigation, Plant resource, Plant based cultures, and Writing the manuscript. **Elif Tahtasakal:** Chemical extraction and purification methodology, and Formal Analysis. **Selma Ozturk:** Investigation, and Funding.

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Research Article

Persea americana Mill.: As a potent quorum sensing inhibitor of *Pseudomonas aeruginosa* PAO1 virulence

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Abstract: The emergence of bacteria resistant to conventional antibiotics and the inability of these antibiotics to treat bacterial biofilm-induced infections cause millions of deaths every year.

This situation has prompted scientists to develop alternative strategies to combat infectious diseases. Among these, researches on phytochemicals to reduce bacterial virulence in *Pseudomonas aeruginosa* have gained momentum in recent years. The main reasons behind this are the production of virulence factors and biofilm formation, all of which are under the control of quorum sensing (QS) system. Hence, inhibition of the QS pathways is an eligible strategy for the control of microbial pathogenesis.

For the first time in the present study, the methanolic seed extract of avocado was evaluated for its anti-QS activity against *P. aeruginosa* PAO1. The results of the experiments carried out proved that the extract has inhibitory activity on the regulation of virulence and biofilm formation. Phytochemical analysis resulted in the identification of epicatechin, catechin, chlorogenic acid, caffeic acid, quercetin, kaempferol, vanillin, ferulic acid in the extract. Then, the mechanism of action for the extract was investigated through molecular docking. Docking outcomes demonstrated that the major components, catechin, epicatechin, chlorogenic acid, could bind to the receptors of QS competitively. Hence, the mode of action for the extract might be through the inhibition of the QS. Considering the computational analysis results and the literature, it is thought that the anti-QS activity of the extract prepared from avocado seeds may be related to the synergistic effect of the phytochemicals it contains.

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1. INTRODUCTION

Antimicrobial resistance is defined as the capacity of microorganisms to develop various mechanisms that inactivate antimicrobial agents. As one of the most serious threats to global health, it causes millions of deaths and results in huge financial losses each year (Bery *et al*., 2013). Since the discovery of new conventional antibiotics targeting bacterial killing or

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inhibition to overcome the threat of resistance is not a permanent solution, this situation has prompted scientists to develop alternative strategies to combat infectious diseases. Among these, down-regulation of QS system associated with bacterial virulence is the most remarkable strategy in the last few decades (Jiang *et al*., 2019).

QSis a survival mechanism of bacteria and provides resistance against antimicrobial chemotherapeutics by controlling a variety of pathophysiological processes related to bacterial functions through cell-to-cell signaling (Rutherford & Bassler, 2012). This population density-dependent intercellular communication network is formed by signal molecules called autoinducers (Smith & Iglewski, 2003). The autoinducer concentration that reaches the critical threshold causes changes in the gene expression of the bacteria as a result of interaction with the QS receptors and triggers the regulation of a range of biochemical processes. In this fashion, bacteria gain the ability to adapt to environmental changes important for growth, adhesion, antibiotic resistance, and virulence (Parsek & Greenberg, 1999). QS-associated biofilm formation and production of virulence factors reduce sensitivity to antibacterial therapy. In this regard, inhibition of the QS system is vital in combating life-threatening bacterial infections (Vysakh *et al*., 2018).

The opportunistic Gram-negative bacterium *Pseudomonas aeruginosa* is responsible for a broad spectrum of infectious diseases and is classified as a major cause of nosocomial infections (Lyczak *et al*., 2000). It has been listed by World Health Organization (WHO) as one of the 12 major pathogens of critical priority that are considered the greatest threat to human health (Tacconelli *et al*., 2018). Pseudomonal infections are correlated with high morbidity and mortality and are really difficult to treat due to the high resistance of bacteria to multiple classes of disinfecting agents. The main reasons behind this antimicrobial resistance are the production of virulence factors (like pyocyanin, rhamnolipids, exotoxins, proteases, elastases) and biofilm formation, all of which are under the control of the QS system (Pompilio *et al*., 2015). Hence, inhibition of the QS pathways is an eligible strategy for the control of microbial pathogenesis.

Medicinal plants that have inspired the discovery of new medicines have attracted great attention throughout the ages (Rather *et al*., 2021). Recently, there has been increased interest in studying the phytochemicals responsible for QS inhibition in the treatment of infections caused by resistant microorganisms (Mohabi *et al*., 2017).

Persea americana Mill. is one of two species belonging to the genus *Persea* (the other is *P. schiedeana*) and is the most studied member of this genus. It is an evergreen tree classified into the family Lauraceae and native to tropical America. Today, it is widely cultivated commercially for its edible fruit known as "avocado" in tropical and subtropical regions (Hurtado-Fernández *et al*., 2018). Avocado consumption has increased tremendously with increased awareness of its health benefits. It is considered as one of the healthiest fruits due to its rich nutritional composition, which includes vitamins, minerals, proteins and monounsaturated fatty acids (Dreher & Davenport, 2013). Its unique phytochemical content has paved the way for this fruit to be researched for medicinal applications, and so far different parts of the fruit have been studied for their antioxidant, anticancer, anti-inflammatory, antimicrobial, antidiabetic, hypolipidemic, hepatoprotective, antihemolytic and wound healing activities (Nayak *et al*.,2008; Rodriguez-Carpena *et al*., 2011; Pahua-Ramos *et al*., 2012; Nabavi *et al*., 2013; Alkhalaf *et al*., 2019; Umoh *et al*., 2019). However, there is no literature data currently exists on the anti-QS activity of any parts of avocado.

In the present study described here, we aimed to evaluate the QS inhibitory activity of the methanolic seed extract of avocado against *P. aeruginosa* PAO1. In addition, the mechanism of action for QS inhibition detected was explored through computational analysis.

2. MATERIAL and METHODS

2.1. Plant Material and Extract Preparation

Samples of matured avocado fruits were collected from the Manavgat region of Turkey. The herbarium sample was identified as *P. americana* Mill. by Asst. Prof. Gülsen Kendir and has been deposited with voucher number AEF 30121 in Herbarium of Ankara University Faculty of Pharmacy.

The seeds were removed from the succulent parts of the fruits by knife and washed with distilled water. After that, they were sliced and dried in an oven at 36 °C to a constant weight. They were then ground into powder using a grinding machine (Waring 8011 EB). Eight grams of seed powder was subjected to ultrasonic extraction with 80 mL for 45 min. The methanolic seed extract was filtered and the filtrate was evaporated to dryness at 36 °C using a rotary evaporator (Heidolph Hei-Vap Rotary Evaporator). At the end of the process, the crude extract remaining in the flask was weighed and the amount recorded, then dissolved with dimethylsulfoxide (DMSO) and transferred to a vial.

2.2. Phytochemical Screening

The phytochemical analysis of methanolic seed extract was carried out High-Performance Liquid Chromatography (HPLC) technique. HPLC conditions were presented in Table 1.

Table 1. Chromatographic conditions.

Chromatographic conditions	Time (min.)	A (%)	B (%)
Detector:	0	93	7
Photo Diode Array Detector (λ max.: 278 nm)	20	72	28
Autosampler:	28	75	25
SIL–10AD vp	35	70	30
System controller:	50	70	30
SCL-10A vp	60	67	33
Pump:	62	58	42
LC-10AD vp	70	50	50
Degasser:	73	30	70
DGU-14a	75	20	80
Column heater:	80	0	100
CTO-10 A vp			
Column:			
Agilent Eclipse XDB C-18			
(250 mm × 4.6 mm), 5 µm			
Column temperature:			
30 °C	81	93	7
Mobile phases:			
A: acetic acid-water (3:97 v/v), B: methanol			
Flow rate:			
0.8 mL/min.			
Injection volume:			
20 µL			

2.3. Screening Seed Extract for QS Inhibitory Activity

2.3.1. *Antibacterial activity*

QS inhibitors should reduce virulence rather than bacterial growth, in contrast to standard antimicrobials. Therefore, firstly, the agar well method was used to determine the concentration with no antibacterial effect on PAO1 (Holder & Boyce, 1994). Overnight cultures of bacteria were prepared by adjusting to 0.5 McFarland turbidity. Five mL soft agar (0.5% agar) with

bacterial cultures, added on the Muller-Hinton Agar (MHA) medium and 6 mm diameter wells were opened on the media. 100 µL of the extract was added to the well. Antibacterial activity was determined by measuring the zone diameters after 24 hours of incubation at 35 °C. The test was carried out in triplicate.

2.3.2. *Biofilm formation assay*

Biofilm is a virulence trait associated with QS known to protect pathogens from host defense as well as antibiotics by acting as a diffusion barrier (Xu *et al*., 2000). Centers for Disease Control and Prevention (CDC) reported that approximately 65-80% of infections are caused by biofilm and this reveals the need for new treatment options to be developed in this regard (Qu *et al*., 2016).

The anti-biofilm activity of the seed extract was investigated on *P. aeruginosa* PAO1 strain using the crystal violet method (O'Toole 2011; Önem *et al*., 2018). 10 µL of an overnight culture of PAO1 (OD at 600 nm=0,05) was added to a 96-well microplate containing 160 µL of freshly prepared Luria–Bertani Broth (LBB) medium and 20 µL of the seed extract. Microplate incubated at 37°C for 48 h. After the incubation, the culture on the plates was drained and washed 3 times with sterile water. By adding 125 µL aqueous solution of crystal violet (0.1%) to the wells, the biofilm layer was dyed for 30 min, then the paint was poured and the excess was washed with distilled water. Two hundred µL of 95% ethanol was added and the reaction mixture was read spectrophotometrically at 570 nm. PAO1 culture and LBB were used as positive and negative controls, respectively. All experiments were repeated three times unless otherwise mentioned. The inhibition that occurred in biofilm formation was calculated according to the following formula:

*OD: Optic Density

$$\text{Inhibition rate (\%)} = [(OD_{\text{in control}} - OD_{\text{in treatment}}) \times 100]/OD_{\text{in control}}$$

2.3.3. *Elastolytic assay*

Elastase B also named LasB is an extracellular virulence factor of *P. aeruginosa* and this metalloprotease is involved in the invasiveness of this pathogen in the host tissues due to its ability to hydrolysis of immunologically important molecules such as antibodies (Bever & Iglewski, 1988; Galdino *et al*., 2019).

The elastolytic activity of the seed extract was determined with Elastin Congo Red (ECR) test Ohman *et al*., 1988). This test helps to measure the elastase activity in the supernatant of PAO1 culture using ECR as substrate. Elastase B degrades elastin and this causes the congo red dye to be released into the supernatant. Elastolytic activity is determined by spectrophotometric quantification.

During the procedure, 100 µL of the seed extract was mixed with 10 mL LBB containing OD 0.05 at 600 nm PAO1 culture and left to incubate at 37°C by shaking for 16-18 h. Afterward, 100 µL of the supernatant part of this culture was transferred to a tube and 900 µL ECR buffer was added. This mixture was incubated at 37°C for 3 h with shaking at 200 rpm. After the incubation, the sample was centrifuged at 4500 rpm for 5 min. The supernatant of the sample was transferred to a cuvette and its optical absorption at 495 nm wavelength was measured spectrophotometrically (BioTek -Epoch 2 Microplate Spectrophotometer). The reference PAO1 strain was used as a positive control in this experiment. The negative control was sterile LBB.

2.3.4. *Pyocyanin inhibition assay*

Pyocyanin is a QS-controlled secondary metabolite exclusively produced by *P. aeruginosa.* Therefore, this redox-active toxic compound plays a role as an important biomarker in the identification of this pathogen (Reyes *et al*., 1988). As one of the virulence factors, it contributes

to the persistence of pseudomonal infections. Reactive oxygen species associated with pyocyanin have been found to increase the survival ability of this opportunistic pathogen by helping to escape host defense, competing with other pathogens, and causing damage to the host tissue (Lau *et al*., 2004).

Pyocyanin inhibition assay was conducted as described by Essar *et al*., 1990. 10mL of LBB medium together with 100 µL of plant extract left for incubation at 37ºC for 16-18 h with shaking. After the incubation period, 5 mL of chloroform was added to the medium and vortexed for 30 sec. The sub-phase formed in the medium and separated from chloroform was transferred to tubes as 2 mL. One mL HCl-water mixture (0.2 mol/L HCl) was added to it and vortexed for 30 sec again. The absorbance of the pink phase formed on the upper part of the tubes was measured at 520 nm. Untreated PAO1 was served as a positive control.

2.4. Statistical Analysis

The experiments were carried out in triplicate according to the randomized plot design and the data obtained were subjected to variance analysis using the JMP 8 packet statistics program. Statistical differences were marked by the LSD multiple comparison test.

2.5. Molecular Docking

The crystal structure of LasR was obtained from PDB (protein data bank). The structure utilized in the molecular docking (PDB ID: 6MWL) has a resolution of 1.50 Å (Paczkowski *et al*., 2019). GRID box was specified in a manner that included the bound ligand inside the protein structure. The protein structures were prepared by deleting water molecules, adding polar hydrogens, and assigning Gasteiger charges. The structures of the ligands analyzed were obtained from PubChem (Kim *et al*., 2021). Similarly, the ligands were prepared for docking by adding polar hydrogens and assigning Gasteiger charges. Then, AutoDock Vina was run after the parameters were assigned properly (Trott & Olson, 2010). The results were visualized and analyzed with Biovia Discovery Studio 3.5 (2020). The docking process was validated by performing redocking with the bound ligand in the structures utilized.

3. RESULTS

3.1. Confirmation of the anti-QS activity

Before the anti-quorum sensing experiments, an antibacterial activity test was performed to determine the concentration where the plant extract did not have antibacterial activity and it was observed that there was no activity up to 238 mg.

The results of the antibiofilm formation assay are given in Figure 1. The extract inhibited biofilm formation of PAO1 by 38% (2.38 mg/mL concentration).

Figure 1. Biofilm formation inhibition of plant seed extract.
**The difference between averages with different letters is important, $p<0.01$ ($SD\pm$)

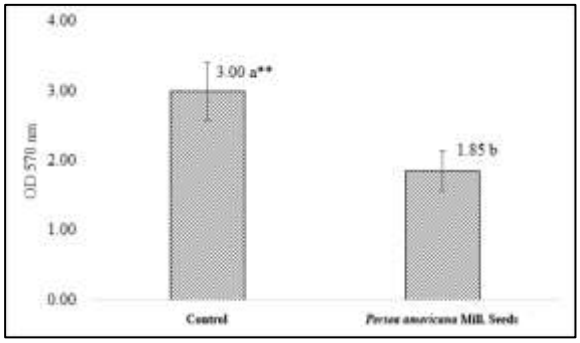
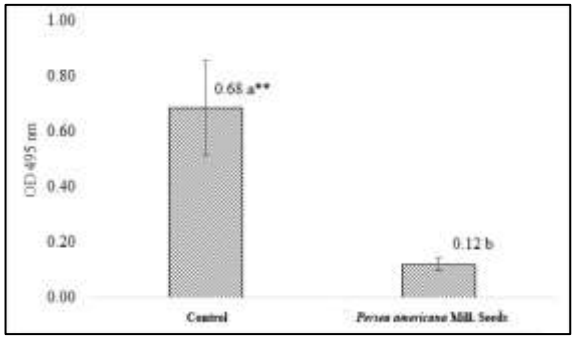
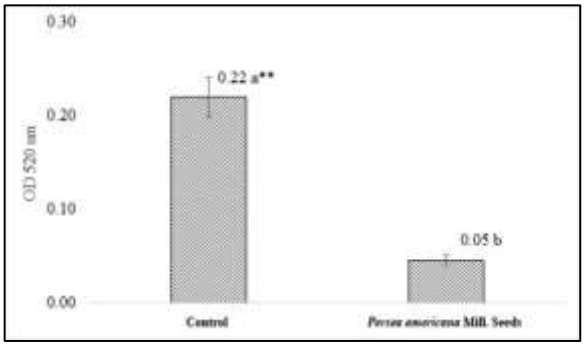


Figure 2. Elastase inhibition activity of plant seed extract.
**The difference between averages with different letters is important, $p<0.01$ ($SD\pm$)



Results of the elastolytic assay are shown in Figure 2. Elastase inhibition rate of the extract was found as 83%. Figure 3 presents the results of the pyocyanin inhibition assay. The percentage of pyocyanin inhibition of the extract was calculated as 79%.

Figure 3. Pyocyanin inhibiton activity of plant seed extract.
**The difference between averages with different letters is important, $p<0.01$ ($SD\pm$)



3.2. Results of HPLC Analysis

HPLC chromatogram of the methanolic extract of *P. americana* seeds shows the presence of gallic, *p*-hydroxybenzoic, chlorogenic, caffeic, ferulic acids and, catechin hydrate, epicatechin, vanillin, quercetin dihydrate, kaempferol. Figures 4a & 4b show a standards chromatogram and a sample chromatogram, respectively. Epicatechin had the highest concentration (222.15 µg/mL) followed by catechin with a concentration of 209.95 µg/mL, then chlorogenic acid with a concentration of 97.65 µg/mL, with the presence of *p*-hydroxybenzoic acid, caffeic acid, quercetin, kaempferol, vanillin, ferulic acid and gallic acid with concentrations of 82.2, 33.45, 21, 8.75, 6.25, 4.45 and 3.35 µg/mL, respectively (Table 2). Chemical structures of determined compounds in the methanolic extract of avocado seeds are shown in Figure 5.

Figure 4. A) HPLC chromatogram for standards, B) HPLC chromatogram for the main phenolic compounds identified in the methanolic extract of *P. americana* Mill. seeds. 1:gallic acid; 2:protocatechic acid; 3:catechin; 4:p-hydroxybenzoic acid; 5:chlorogenic acid; 6:caffeic acid; 7:epicatechin; 8:syringic acid ; 9:vanillin; 10:p-coumaric acid; 11:ferulic acid; 12:sinapinic acid; 13:benzoic acid; 14:o-coumaric acid; 15:rutin; 16:hesperidin; 17:rosmarinic acid; 18:eriodictiol; 19:cinnamic acid; 20:quercetin; 21:luteolin; 22: kaempferol; 23:apigenin

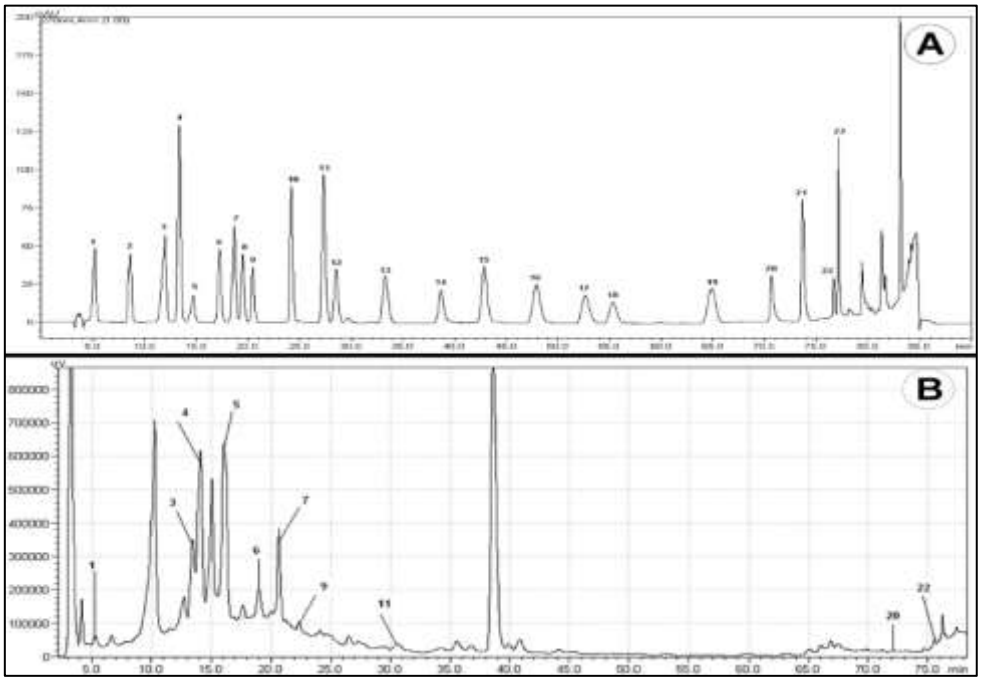
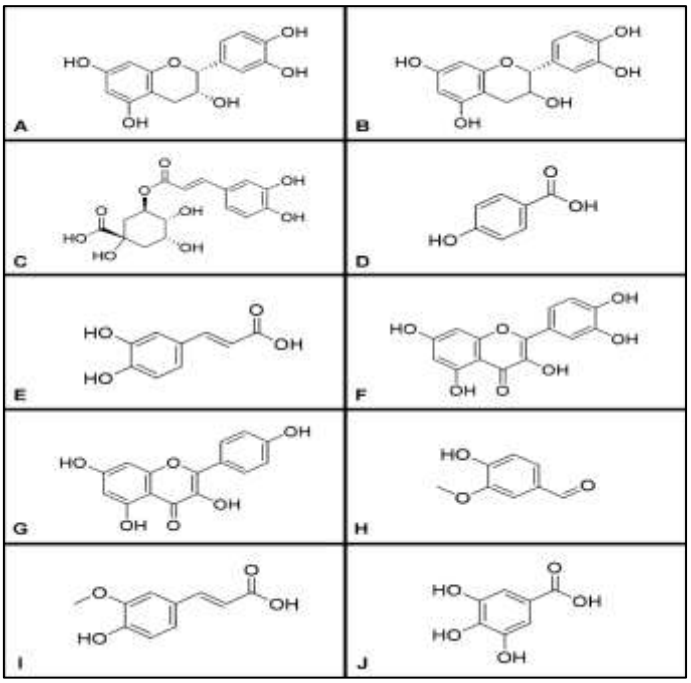


Table 2. Concentrations of the main phenolic compounds identified in the methanolic extract of *P. americana* Mill. seeds.

Phytochemicals	Concentrations (µg/mL)	Retention time (min)
Gallic acid	3.35	5.41
Catechin	209.95	13.50
p-Hydroxybenzoic acid	82.2	14.60
Chlorogenic acid	97.65	16.17
Caffeic acid	33.45	18.84
Epicatechin	222.15	20.57
Vanillin	6.25	22.23
Ferulic acid	4.45	30.23
Quercetin	21	72.94
Kaempferol	8.75	77.13

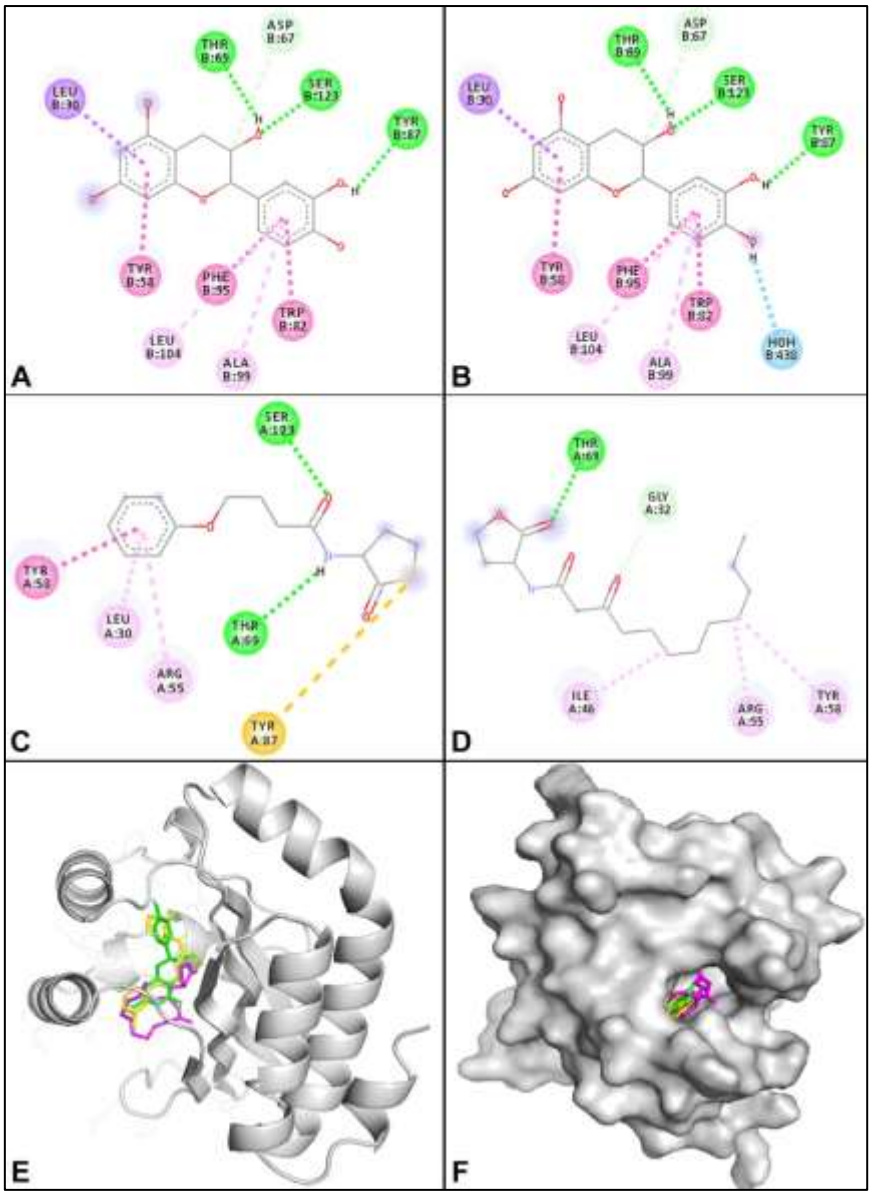
Figure 5. Chemical structures of determined compounds in the methanolic extract of *P. americana* Mill. seeds. A) Epicatechin, B) Catechin, C) Chlorogenic acid, D) *p*-hydroxybenzoic acid, E) Caffeic acid, F) Quercetin, G) Kaempferol, H) Vanillin, I) Ferulic acid, J) Gallic acid.



3.3. Results of Molecular Docking Studies

Molecular docking outcomes showed that catechin and its isomer epicatechin had relatively good interaction with LasR, a crucial receptor involved in the QS system (Figure 6). Hence, the ligands analyzed could bind to LasR very well. The binding energy of catechin, bound ligand and OdDHL (N-3-Oxo-Dodecanoyl-L-Homoserine Lactone) were recorded as -11.9 kcal/mol, -10.5 kcal/mol and -9.0 kcal/mol respectively. Furthermore, the interactions of catechin and epicatechin in the presence of water in the structure of the protein were investigated. In the presence of water molecules, the detected interactions were the same as the interactions without water. The only difference was the interaction with W438 (Figure 6 A&B) (Lie *et al*., 2011).

Figure 6. Binding mode of A) catechin B) catechin in hydrated protein structure C) bound ligand D) OdDHL with LasR. E) superimposition of the ligands inside the structure, F) ligands inside the binding pocket of LasR (green-epicatechin, yellow-bound ligand, magenta-OdDHL)



4. DISCUSSION and CONCLUSION

The emergence of bacteria resistant to conventional antibiotics and the inability of these antibiotics to treat infections caused by bacterial biofilms prove the need for new strategies in the treatment of bacterial infections (Saleem *et al*., 2010). Recently, researches on phytochemicals to reduce bacterial virulence in *P. aeruginosa* has gained momentum.

For the first time in the present study, the seed extract of avocado was evaluated for its anti-QS activity against *P. aeruginosa* PAO1. Since methanol can extract a variety of bioactive phytochemicals better than the others, it was used as a solvent for the extraction of secondary metabolites. The results of the experiments carried out proved that the methanolic seed extract has inhibitory activity on the regulation of virulence and biofilm formation. Phytochemical analysis performed on the extract resulted in the identification of epicatechin, catechin, chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, quercetin, kaempferol, vanillin, ferulic acid, and gallic acid.

There are some data in the literature regarding the anti-QS activity of detected bioactive phytochemicals and the promising anti-QS and anti-biofilm activity of the extract has been associated with the synergistic effect of these phenolic compounds in its composition. Phenolic plant secondary metabolites are among the most investigated naturally occurring phytochemicals due to their health-promoting benefits (Bhuyan and Basu, 2017). These phytocompounds have attracted scientific interest in terms of their various biological activities, especially their antioxidant properties (Lattanzio *et al*., 2018). Additionally, some studies in recent years have provided evidence of the anti-QS and anti-biofilm activities of the phenolic phytoconstituents (Ugurlu *et al*., 2016). Flavonoidal compounds have been documented to interfere with the regulation of QS-associated pathways in PAO1. In the study by Vandeputte and associates, it was found that catechin has inhibitory activity on elastase and pyocyanin production and biofilm formation by downregulating QS gene expression in PAO1. On the other hand, it was determined that epicatechin also had an inhibitory effect on pyocyanin production (Vandeputte *et al*., 2010). Lahiri and colleagues have indicated that the catechin from *Azadirachta indica* leaf extract is extremely active in preventing dental biofilm and this compound can be used in the treatment of biofilm-related chronic infections (Lahiri *et al*., 2021). Quercetin, a flavonoid commonly found in the plant kingdom, has gained importance as a QS system inhibitor. Quyang et al. reported the inhibitory activity of this compound on virulence factors production and biofilm formation in PAO1 (Ouyang *et al*., 2021). The anti-QS property of kaempferol, another flavonoidal compound, has been proven in a study investigating the effectiveness of phytochemicals obtained from *Camellia nitidissima* Chi flowers on PAO1 (Yang *et al*., 2018). Different studies showed the inhibitory potential of cinnamic acid derivatives against QS-controlled behaviours in PAO1. Wang and coworkers proved that chlorogenic acid regulated QS system and reduces the pathogenicity of *P. aeruginosa* by weakening virulence factors (Wang *et al*., 2019). In a study that investigated the effects of some phenolic secondary metabolites on QS-related virulence factor production of PAO1, caffeic and ferulic acids have been found to be active. The action mechanisms of these phenolic acids have been shown to be the reduction of pyocyanin production and blockage of biofilm formation (Ugurlu *et al*., 2016). Various studies have suggested that several benzoic acid derivatives present anti-QS activity against PAO1. In a study conducted by Plyuta et al., it was determined that gallic acid at a concentration of 200 μg/mL inhibits the formation of PAO1 biofilms by 30%. In the same study, *p*-hydroxybenzoic acid and vanillin have been found to inhibit bacterial biofilm formation by reducing the swarming motility of the PAO1 strain in the concentration range of 400–800 μg/mL (Plyuta *et al*., 2013).

Phytochemical analysis of avocado extract revealed that catechin, epicatechin and chlorogenic acid are the first three most abundant components. Catechin and epicatechin were found to be the major components of the extract. In this study, the postulation was the QS inhibition effect detected might result from the inhibition of the QS receptors by these components synergistically. This premise was explored through molecular docking. Molecular docking outcomes demonstrated that catechin and epicatechin could inhibit the QS system by inhibiting LasR competitively. They bound to the ligand-binding domain of LasR with hydrogen bonding (Thr69, Tyr87, Ser123) and eight more hydrophobic interactions. The binding was realised at relatively low binding energy. In addition, the major components and the natural ligand (OdDHL) had common interaction points at Thr69 and Tyr58. According to the computational analysis here, catechin and epicatechin are expected to have stronger interaction with the LasR than the natural ligand (Figure 1). This in turn gives them the opportunity to inhibit the QS system by interfering with the binding of agonists to the receptor (Bottomley *et al*., 2007). Furthermore, previous experimental studies, which were supported by computational analysis, reported that chlorogenic acid had QS inhibition effect (Wang *et al*., 2019; Onem *et al*., 2021).

To sum up, catechin, epicatechin and chlorogenic acid have the potential of inhibiting the QS system. Hence, the avocado extract, which consists of these compounds as major components, might inhibit this system and thus decrease bacterial virulence.

In conclusion, considering the computational analysis outcomes and literature data it is thought that the anti-QS activity of the methanolic extract prepared from avocado seeds may be due to the synergistic effect of phenolic phytochemicals in its content. Further studies are planned to undertaken to determine the anti-QS activity of different doses of isolated compounds thought to be responsible for the activity.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Ebru Onem: Investigation, Supervision; **Fatma Tugce Guragac Dereli:** Writing-original draft; **Ayse Gul Ozaydin:** Methodology; **Evren Arin:** Resources, Methodology; **Muhammed Tilahun Muhammed:** Formal Analysis

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Inhibitory effect on acetylcholinesterase and toxicity analysis of some medicinal plants

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Abstract: This study aimed to analyse the inhibition of different extracts of *Rosmarinus officinalis, Pistacia terebinthus* and *Sideritis dichotoma* on acetylcholinesterase enzyme of *Drosophila melanogaster*. Additionally, the biological features including antioxidant activity, phenolic contents, antibacterial effects and *in vivo* toxicities were identified using radical scavenging, Folin-Ciocalteu, disc diffusion methods, and larval (eclosion) assay using *Drosophila*, respectively. Also, GC-MS was used to determine of the terpene-derivative compositions of the plants. IC_{50} values on acetylcholinesterase were determined between 0.57±0.02-2.54±0.11µg μL^{-1} for ethanol, 0.86±0.05-2.19±0.15µg μL^{-1} for methanol and 1.98±0.13-4.76±0.24µg μL^{-1} for water extracts. Inhibition types of *Rosmarinus, Pistacia* and *Sideritis* were uncompetitive, competitive and competitive, respectively. The antioxidant activities of the extracts were between 77.87±1.72-96.94±1.84% against DPPH and 90.57±2.18-98.18±2.36% against $ABTS^+$ radicals. GC/MS results showed that carvacrol and thymol were the major monoterpenes of *Pistacia* and *Sideritis*, while limonene and borneol were the main monoterpenes of Rosmarinus. The strongest antibacterial activities were observed with *Rosmarinus* and *Sideritis* against *Staphylococcus aureus* and *Escherichia coli*, respectively with an inhibition zone larger than 15 mm. According to the *in vivo* toxicity study, all extracts were found non-toxic to *Drosophila*, and they ameliorated H_2O_2 induced decrease of puparation, survival rate and eclosion values.

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1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that shows memory loss as a primary symptom and increased incidences are observed in industrialized countries having elderly populations. Although the pathogenesis of AD could not be fully elucidated, the most clarified hypothesis is the lack of the acetylcholine (ACh) molecule, known as the cholinergic hypothesis(Cavdar *et al.*, 2019). ACh molecule acts as a neurotransmitter in the synaptic gap and provides information flowing among neurons, so the cholinergic hypothesis is explained by the deficiency of acetylcholine and the loss of the cholinergic system (Adewusi *et al.*, 2011). The predominant marker of cholinergic system deficiency can be an increased activity of

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acetylcholinesterase (AChE) (EC 3.1.1.7), which degrades ACh, and/or the inhibition of cholineacetyltransferase, which is involved in the synthesis of acetylcholine (Fu *et al.,* 2004). The recovery of ACh can be carried out by inhibition of AChE with utilized inhibitors. Therefore, many AChE inhibition studies have been done to solve this problem. Many synthetic drugs are available on the market such as tacrine, donepezil, rivastigmine and galanthamine as AChE inhibitors (Yang *etal.,* 2015; Cavdar *et al.,* 2019;Dave *et al.,* 2000). In fact, AChE inhibitors for AD treatment are the only group of drugs in which a certain success ratio is achieved, but their use have been limited due to their detrimental side effects (Colovic, *et al*., 2013).

Another reason for the progression of AD is oxidativestress that leads to neurotoxicity through the generation and spread of reactive oxygen species (ROS)(Zhao& Zhao, 2013).Therefore, AD prevention or treatment with natural antioxidants should be considered as an alternative approach. Some medicinal plants are used as natural components of AChE inhibitors instead of synthetic drugs because of their prosperous antioxidant capacities. For example, huperzin A is a promising drug for treating AD symptoms with a very strong and reversible inhibitory effect on AChE and it is isolated from a plant, *Lycopodium serratum* (Thunb.) Trev. (Syn. *Huperzia serrata* Thunb.) (Ozarowski *et al*., 2017; Wang *et al*., 2006). In addition, there are more interesting results in the literature about the inhibitory effects of some other plant extracts like *Salvia miltiorrhiza radix* extracts which have stronger inhibitory capacities than huperzin A (Ozarowski *et al.,* 2017). Some *Salvia* species were also reported as memory enhancers because of their monoterpene compositions that lead to strong and reversible anti-acetylcholinesterase activities both *in vitro* and *in vivo* (Bahadori *et a*l., 2016; Perry *et al*., 2000). Another examples showing the advantage of strong antioxidant activities to deal with neurodegenerative diseases are *Gingko biloba* and *Panax ginseng* plants (Bastianetto *et al.,* 2000; Chang *et al.,* 2016).

In this study, *Rosmarinus officinalis* L (*RO*), *Pistacia terebinthus* L (*PT*) and *Sideritis dichotoma* Huter (*SD*) plant samples with known chemical profiles were analyzed in detail for the AChE inhibition capacities. The results were compared with the previous findings in which some of the extracts in different concentrations were found as ineffective. In addition, the antioxidant properties were revealed by DPPH and ABTS radical scavenging methods, the phenolic contents were identified by Folin-Ciocalteu method and terpenes in these plant extracts were analyzed using gas chromatography coupled to mass spectrometry (GC-MS). The antimicrobial effects of the extracts against pathogenic bacteria were also analyzed in this study by disc diffusion method because it is known that the dysbiosis of microbes, which can occur because of the pathogenic bacteria invading the intestine, may lead to brain dysfunctions and AD may be associated with that (Angelucci *et al*., 2019). Considering the potential use of these plants for therapeutic purposes, it is also necessary to better understand the toxicities in living organisms. Therefore, *in vivo* toxicities of *RO, PT* and *SD* were analyzed in this study using *Drosophila melanogaster* as a model organism because of the many developmental mechanisms they share with mammals (Macedo *et al*., 2017).

2. MATERIAL and METHODS

2.1. Materials

Rosmarinus officinalis (*RO), Pistacia terebinthus (PT)* and *Sideritis dichotoma (SD)* were collected and identified by Prof. Dr. Serap DOĞAN at their ripening period in Balikesir, Turkey. The body, leaf, flower parts and fruits of the collected plants were powdered with a grinder mill after drying at room temperature in the dark. All chemicals were purchased from Sigma-Aldrich.

2.2. Methods

2.2.1. *Preparation of Plant Extracts*

The powdered *RO, SD* and *PT* samples were prepared with MeOH, EtOH and water solvents. A 0.5 g portion of powdered samples were dissolved in5 mL solvent. It was kept in a fridge (+4°C) overnight. Then, it was centrifugated for 10 min at 4000 rpm, and supernatant was removed. After the centrifugation, the pellets were rewashed with 5 mL and 2 mL of solvents. Then, the supernatants were combined. Solvents were removed by evaporation process. The residuals were stored at -20 °C until analysis. Stock solutions of the extracts were prepared to use as 25 mg mL^{-1} for all analyzes.

2.2.2. *Preparation of Enzyme Extract*

100 mg of *D. melanogaster* larvae were homogenized by tissue homogenisator in 1mL of 50 mM phosphate buffer (pH 8.0) containing 300 mM sucrose. The homogenate was centrifuged at 4000 g for 4 min at 4 ºC. Supernatant was separated and used for experimental purposes (Assis *et al.,* 2012).

2.2.3. *Enzyme Activity and Inhibition*

AChE enzyme reaction was measured spectrophotometrically by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of thiocholines with DTNB. AChE activity and inhibition assays were performed according to the methods decribed by Ellman et al. (Ellman et al., 1961) and Senol et al. (Şenol *et al.,* 2010).

In order to determine the enzyme activity,150 µL of 0.1mM phosphate buffer (pH 8.0), 20 µL of 10 mM DTNB and 20 µL of AChE solutions were combined in a 96-well microplate with a multi-channel automatic pipette, and then incubated at 37 °C for 15 minutes. After the incubation, the reaction was started with the addition of 10 µL of 10 mM acetylcholine iodide and monitored by a microplate reader at 412 nm(Şenol *et al.,* 2010). The experiments were assayed in triplicate.

For inhibition assay, test mixtures (200 µL total volume) were prepared with 0.1 mM phosphate buffer (pH 8.0, 120-155 µL of 0.1mM), substrate solutions (ACh and DTNB) at various concentrations prepared in buffer (2.5 -22.5 µL of 10mM), the inhibitor solution (25µg μL^{-1}) at fixed concentrations and 20 µL enzymatic extract solutions. Blank (reference) sample contained all of the components except the enzyme extract with a final volume of 190 µL. The reaction was initiated by adding the substrate to the assay medium. The IC_{50} values were determined for all extracts in this way. The types of inhibition were determined using an extract of each plant sample with the best IC_{50} value. The inhibition kinetic analysis of *D. melanogaster* AChE was determined in the absence and in the presence of *RO*-EtOH, *SD*-EtOH, and *PT*-MeOH at two different concentrations. Inhibition constants (Ki and Ki') were concluded from the Lineweaver–Burk plots (Doğan*et al*., 2011).

2.2.4. *Determination of antioxidant capacities*

2.2.4.1. DPPH radical scavenging activity. The antioxidant capacities of *RO, SD* and *PT* were determined using 1,1–diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity (Blois, 1958). A 0.024 g portion of DPPH was dissolved in 100 mL MeOH. Then, 0.05mL of plant extract, 2.5 mL of DPPH solution and 2.5 mL of MeOH were added into a test tube and were kept in the dark for 1 h. For the control, MeOH was used instead of a sample. Spectrophotometric measurements were done at 517 nm. The radical scavenging activity of the samples were calculated using the following formula;

Antioxidant Activity (%) = [1- (absorbance of sample / absorbance of control)] x 100

2.2.4.2. ABTS radical scavenging activity. ABTS radical scavenging activity of the samples were performed by the method of Re et al. (Re *et al.,* 1999). $ABTS^+$ radical solution was prepared using equal volumesof 7 mM ABTS salt and 2.4 mM ammonium persulphate and kept in dark overnight. After then, the solution was diluted with MeOH until an absorbance of 1.50±0.01 at 734 nm was obtained. This absorbance was recorded as a control. For the sample analysis, 2.95 mL of the $ABTS^+$ solution and 0.05 mL of sample (extract) were added in a 3 mL cuvette. Measurements were done at 734 nm by a UV-Visible spectrophotometer (Perkin Elmer lambda-35 UV-Visible spectrophotometer). The measurements were performed in triplicate for each extract. ABTS radical scavenging activity (%) of the extracts were calculated with the following formula;

Antioxidant Activity (%) = [1- (absorbance of sample / absorbance of control)] x 100

2.2.5. *Determination of total phenolic content*

The phenolic contents of *RO, SD* and *PT* were analyzed by the Folin-Ciocalteu method (Dogan *et al*., 2010). A 3.5 mL portion of distilled water, 0.25 mL Folin reagent, and 0.25 mL of extract were combined in a test tube and incubated in the dark for 3 min at room temperature. $NaCO_3$ was added to the test tube (1 mL of 20%) and incubated for 40 min at 40 °C. For the control sample, MeOH was used instead of the plant extract. After the 40 min, absorbance values of all samples were measured at 685 nmby UV-Visible spectrophotometer. Total phenolic compounds were identified using the gallic acid calibration curve, and the results were calculated as *µg gallic acid/g*.

2.2.6. *GC-MS analysis for the composition of terpene derivatives*

The composition of the plant extracts' terpene derivatives were performed by capillary GC/MS using Shimadzu 6890N Network GC-2010 plus system combined with Shimadzu GC/MS-QP2010 ultra mass spectrometerdetector.

In order to perform GC analysis 30m x 0.25 mm x 0.25 µm HP Innowax Capillary column was used. The oven program was adjusted to keep the column'sinitial temperature at 60 ºC for 10 min after injection, rise to 220 °C with 4 ºC/min heating ramp for 10 min and increase to 240 °C with 1 ºC/min heating ramp. The injector temperature was adjusted to 250 °C, carrier gas was helium, in let pressure was 20.96 psi, linear gas velocity was 28 cm/s, column flow was 1.2 mL/min, the split ratio was 40:1 and injection volume was 1.0 µL.

MS conditions were adjusted as follows; ionization energy:70 eV; ionsource temperature: 280 °C; integral temperature: 250 ºC; and mass range: 34–450 atomic mass units. Identification of the terpenes in the *RO, PT* and *SD* were determined by comparison of their mass spectra and retention times with the GC/MS Wiley and Nist Mass Spectral Searchlibrary. The proportion of the compounds were calculated from the GC peak areas by the normalization method.

2.2.7. *Disc diffusion method for antibacterial activity*

The bacteria were maintained on Muller-Hinton agar (MHA). Two bacteria strains were selected, including the Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and the Gram-negative bacteria *Escherichia coli* (ATCC 8739). Antibacterial activities of the samples were evaluated using the paper disc agar diffusion method defined by National Committee for Clinical Laboratory Standard. The paper discs (6mm diameter) were incubated with the extracts overnight. After, the impregnated discs were placed on petri dishes inoculated with bacteria strains (10^5 CFU/mL). The petri dishes were incubated at 37 °C for 24 h. Finally, the diameters of the inhibition zones forming around the discs were measured to evaluate antibacterial activities of the extracts.

2.2.8. *In vivo toxicological analyses*

2.2.8.1. Fly rearing conditions of *Drosophila melanogaster* strains. The standard growth medium was prepared by dissolving sugar (43 g), agar (9 gr), semolina (90 gr), yeast (25 gr), antifungal drug (200 µl, Mikostatin-Deva Holding-228/97) and propionic acid (5 ml) in 500 ml of water (Chung *et al*., 2009; Yakovleva *et al*., 2016). 25 g of media was then proportioned into sterile glass cultures vials and the flies were kept in glass bottles at 22 °C.

2.2.8.2. The larval (eclosion) assay. The assay was performed according to Liu et al. with minor modifications (Liu *et al*., 2015). All of the *D. melanogaster* flies used in this study were Oregon R wild-type strains. 25 adult male and female *D. melanogaster* flies were placed into cultures bottles. After 48 ± 4 hours of incubation, 1st instar larvae were collected and rinsed with distilled water. Plant extracts (25 mg/L) and H_2O_2 (6.5 µg/mL) were directly applied to the growth media. The negative control was prepared without any treatment and the positive control was prepared by adding H_2O_2 (6.5 µg/mL). Equal numbers of 1st instar larvae were added into the experimental bottles and then incubated at 22 °C until they became adults. The pupae and eclosed adult fly numbers were counted (Liu *et al*., 2015). The puparation %, survival rate % and eclosion % were calculated according to the previous studies *(Depetris-Chauvin et al*., 2017; Liu *et al*., 2015; Macedo *et al*., 2017; Rand *et al*., 2014; Riaz *et al*., 2018) using the following formulas;

$$Puparation\ \% = \frac{Numberofpupae}{Numberoflarvae}\ x\ 100$$

$$Survival\ rate\ (\%) = \frac{Numberofadultflies}{Numberoflarvae}\ x\ 100$$

$$Eclosion\ \% = \frac{Numberofadultflies}{Numberofpupae}\ x\ 100$$

2.2.9. *Statistical analysis*

The standard error (SE) was calculated using three biological repeats, paired student *t* test was used and $p<0.05$ was determined as statistically significant for *in vivo* toxicological analyses. Other findings were presented as mean ± standard deviation ($\bar{X} \pm s$) of three biological repeats by Anova Test. All of the calculations and statistics of this study were performed by Microsoft Office Excel.

3. RESULTS

3.1. Enzyme Activity and Inhibition Results

The kinetic constants of the AChE enzyme obtained from *D. melanogaster* were presented in Table 1. They were calculated from Lineweaver-Burk equation using the acetylcholine substrate. The Michaelis constant (Km) and maximum reaction velocity (Vmax) values were calculated from the Lineweaver–Burk double reciprocal plots and values for the acetylcholine substrate were calculated as 1.94 mM and 17.95 EU/mL min, respectively.

Table 1. Kinetic values of AChE of *Drosophila melanogaster.*

Substrate	Km (mM)	Vmax (EU/mL min)	Vmax/Km (EU/mL min mM)
Acetycholine iodide	1.94	17.95	9.26

It is well known that most of the medicinal plants possess antioxidant activities. This property makes them very effective protectors against various diseases and memory deficits, in addition to their capacity of reducing the toxicities of toxic agents or other drugs (Karimi *et al*.,

2015). *RO*, *PT* and *SD* are the plants used to treat many diseases by local people. However, there was not enough data in the literature about their inhibition capacities on AChE that would make them natural alternatives of synthetic drugs without detrimental side effects leading to serious disorders in human metabolism (Colovic *et al.,* 2013). Therefore, this study aimed to analyse the AChE inhibition types and capacities of *RO, PT* and *SD* extracts prepared with EtOH, MeOH and water. The enzyme inhibition assay results were given in Table 2, Table 3 and at Figure 1.

Table 2. IC_{50} values of the plant samples on AChE of *Drosophila melanogaster.*

Samples	IC_{50} (µg/µL)		
	MeOH extract	EtOH extract	Aqueous extract
PT	0.86±0.05	2.54±0.11	4.76±0.24
SD	2.19±0.15	2.01±0.08	2.54±0.14
RO	1.21±0.07	0.57±0.02	1.98±0.13

Galanthamine (reference) 0.09 µg/µL.

According to Table 2,it was determined that extracts of *RO* had highest inhibitory activity among all of the plants, and its EtOH-extract showed the best inhibition activity with an IC_{50} value of 0.57±0.02 µg/µL, followed by the MeOH and water extracts with IC_{50} values of 1.21±0.07 and 1.98±0.13 µg/µL, respectively. Previously, Ozarowski et al. reported a study with *RO* L. leaf extract against AChE activity (Ozarowski *et al.,* 2013). They found that leaf extract prepared with 50% EtOH showed long-term inhibitory effect on AChE in rat's brain and they suggested that the *RO* leaf may be a possible option to prevent some neurodegenerative diseases (Ozarowski *et al.,* 2013). Our results were consistent with those findings. However, in another study, Orhan et al. reported that different extracts of *RO* prepared with methanol, petroleum ether, chloroform and ethyl acetate solvents were ineffective on AChE activity at 0.2 and 0.5 µg/µL concentrations (Orhan *et al*., 2008). However, in this study 25 µg/µL of *RO* extracts were used and strong inhibition was observed, so the difference between our results and Orhan *et al*. (Orhan *et al.,* 2008) may be occurred due to the differences of the doses.

According to the results given in Table 2, the extracts of *PT* also exhibited an effective inhibitory potential against AChE. The MeOH-extracts of *PT* exhibited the best inhibition potential (IC_{50}= 0.86±0.05µg/µL), followed by EtOH-extract (IC_{50}= 2.54±0.11µg/µL) and water extract (IC_{50}= 4.76±0.24µg/µL). The information in the literature is limited to compare with our data, but there is a study about the effect of *PT* extracts prepared with ethyl acetate and methanol on AChE activity (Orhan Erdogan *et al*., 2012). Researchers found that the *PT* extracts (25, 50, 100, and 200 µg/mL) did not show inhibitions against AChE but they selectively inhibited butyrylcholinesterase (BChE) activity at the tested concentrations (Orhan Erdogan *et al.*, 2012). In fact, it is an expected result to have different inhibition potentials at lower concentrations.

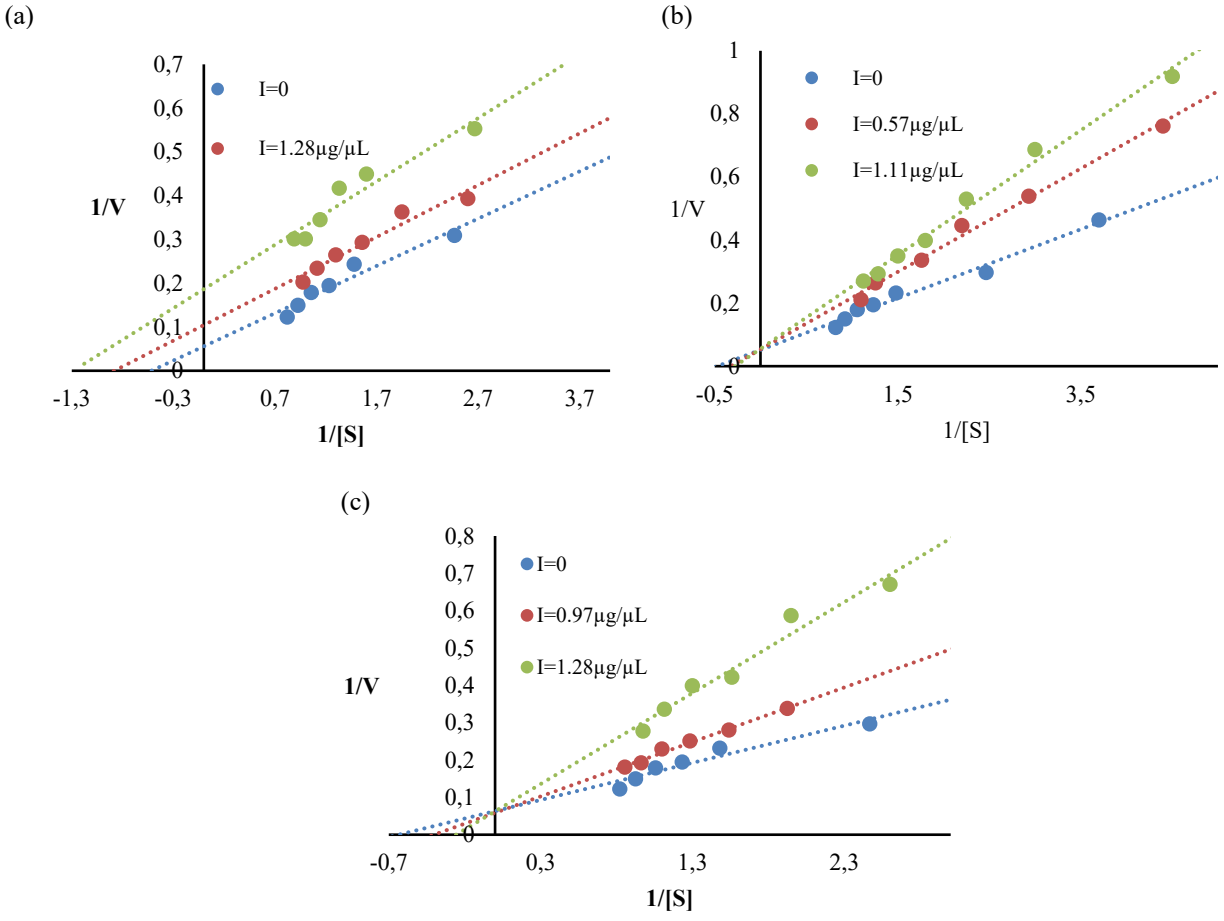
Table 3. Inhibition types and Ki values of *Drosophila melanogaster* AChE.

Inhibitors	I (µg/µL)	Ki (µg/µL)	Ki' (µg/µL)	Type of inhibition
RO (EtOH-extract)	1.28 1.88	--- ---	1.47 0.80	Uncompetitive
SD (EtOH-extract)	0.57 1.11	1.14 1.37	--- ---	Competitive
PT (MeOH-extract)	0.97 1.28	2.07 0.89	--- ---	Competitive

SD is a herbal tea consumed by local people because of its anti-inflammatory, antirheumatic, digestive and antimicrobial activities (Wang *et al*., 2006; Bahadori *et al*., 2016). In our study, all of the extracts of *SD* effectively reduced the AChE activity. EtOH-extract of *SD* showed the best inhibition activity against to AChE enzyme, and its IC_{50} value was found as 2.01±0.08µg/µL. IC_{50} values of MeOH and water extracts of *SD* were also determined as 2.19±0.25 and 2.54±0.14µg/µL, respectively. However, there isn't any research in the literature about the effects of *SD* on cholinergic system enzymes.

In addition, one of the results that make our study different from the literature is the determination of the inhibition types seen in Figure 1.Lower IC_{50} values result from the higher inhibition of AChE. Therefore, extracts with the lowest IC_{50} values were used to determine the inhibition types of each plant sample. Figure 1 shows the effects of *PT*-MeOH, *RO*-EtOH, and *SD*-EtOH inhibitors on *D. melanogaster* AChE using ACh as substrates. According to the results given in Figure 1(a), inhibition type of *RO* was determined as uncompetitive. Uncompetitive inhibition occurs when an inhibitor binds only to the complex formed between the enzyme and the substrate (ES complex). On the other hand, the competitive inhibitions were observed in the reactions between the *PT* and *SD* inhibitors and substrate catalysed by AChE enzyme (Figure 1(b), (c)). Moreover, Ki and Ki' values for inhibitors used are shown in Table 3. The inhibition constants given for the plant extracts (inhibitors) were obtained by fitting the experimental data with Lineweaver–Burk equation for competitive and uncompetitive inhibition. As a result, from Ki values in Table 3, it can be said that *RO* is a more effective inhibitor among the others due to lower Ki values. *SD* and *PT*, respectively, follow the inhibition efficiency.

Figure 1. Inhibition types of *RO*-EtOH (a), *SD*-EtOH (b) and *PT*-MeOH (c) extracts on AChE enzyme of *Drosophila melanogaster.*



3.2. Antioxidant capacity test results

It is known that oxidative stress has important roles both in early stages and the development of AD by activating multiple cell signalling pathways that contribute dangerous lesions (Feng & Wang, 2012). Thus, antioxidant therapies are considered an alternative or supplementary therapy option for AD (Feng & Wang, 2012). In fact, a great number of studies have examined the positive benefits of antioxidants to reduce or block neuronal death occurring in the pathophysiology of neurodegeneretive disorders like AD (Ramassamy, 2006).

In this study, DPPH and ABTS methods were used to determine the antioxidant properties of the plant extracts. According to the results, all extracts of *RO* and *SD* showed DPPH and ABTS radical scavenging activities equal or more than 90% (Table 4). Thus, it was obvious that the solvent types studied did not affect their antioxidant properties so their AChE inhibition capacities were not only dependent on their great antioxidant properties but also dependent on some unknown enzyme-specific inhibition mechanisms. Although DPPH and ABTS radical scavenging activities in EtOH-extract of *PT* were less than 90% (Table 4), they were quite high in MeOH-extract of *PT* (96.94±1.84 and 98.01±2.10%, respectively). These results were consistent with our findings showing that AChE inhibition capacity (Table 2) and natural anti-cholinesterase compositions (Table 5) were high in MeOH-extract of *PT*. In literature, researchers also have mentioned that *PT* extracts might provide neuro-protection to some extent with their strong antioxidant effects by metal-chelation (Orhan *et al*., 2012).

Table 4. Antioxidant scavenging activity and total phenolic content of the extracts.

	DPPH Scavenging Activity (%)			ABTS Scavenging Activity (%)			Total Phenolic Content (g/100g)		
Sample	MeOH extract	EtOH extract	Aqueous extract	MeOH extract	EtOH extract	Aqueous extract	MeOH extract	EtOH extract	Aqueous extract
PT	96.94±1.84	77.87±1.72	94.18±2.28	98.01±2.10	90.57±2.18	97.10±1.83	1.53±0.04	1.63±0.03	1.33±0.02
SD	93.47±2.37	94.41±1.51	90.83±1.53	98.18±2.36	97.41±2.26	96.58±1.86	1.74±0.04	1.31±0.03	2.05±0.05
RO	94.93±1.90	95.15±2.15	90.51±1.15	97.90±2.15	96.4±2.03	97.04±1.66	2.17±0.05	1.51±0.06	1.86±0.05

3.3. Total phenolic content results

It is well known that there is a linear correlation between total phenolic content values and antioxidant capacities (Johari & Khong, 2019). The total phenolic contents of all extracts of *RO*, *SD* and *PT* were determined in this study and expressed as g equivalent of gallic acid in 100g of extract. According to the findings given in Table 4, MeOH-extract of *RO* had the highest concentration of phenolic content (2.17±0.05 g/100g) among all extracts. It was followed by *SD* water extract as 2.05±0.05 g/100g and EtOH-extract of *PT* as 1.63±0.03 g/100g. The other extracts results were detected between 1.31±0.03 and 1.86±0.05 g/100g.

3.4. GC-MS results

Terpenes, the largest single class of compounds found in essential oils, have been shown to provide relevant protection under oxidative stress conditions like neurodegenerative disorders due to their antioxidant behaviors (Gonzalez-Burgos & Gomez-Serranillos, 2012). Therefore, to be able to identify the terpene compositions (monoterpenes, diterpenes and sesquiterpenes) of the plants that showed AChE inhibition, GC/MS analyses were performed. When the antioxidant effects were compared with respect to solvents (Table 4), it was clearly seen that MeOH-extracts of *PT* and *SD* showed the highest antioxidant capacities (above 95%) and the same extract of *RO* showed the highest phenolic content (2.17±0.05 g) which is also related to the antioxidant capacity. Therefore, MeOH-extracts were chosen in GC/MS analyses.

As seen in Table 5, twenty derivatives of terpenes were observed in *RO* extracts and among these compounds some monoterpenes like limonene (8.41%), borneol (7.49%), verbenone (6.19%) and camphor (4.68%) were at high concentrations. It was also clearly seen that acetylcholinesterase inhibitors such as 1,8-cineole, α-pinene, limonene, borneol, terpinene and verbenone, which are monoterpenes, were found in *RO* extracts. These results were consistent with the high AChE inhibition effects of *RO* extracts observed in this study.

Table 5. Composition of some terpene derivatives in *RO, PT* and *SD* extracts.

Compounds	*RO* (Area %)	*PT* (Area %)	*SD* (Area %)
1,8-cineole	0.77	0.19	1.60
α-pinen	0.01	0.09	0.001
Camphor	4.68	0.41	2.11
Borneol	7.49	0.51	1.19
Terpinene	0.12	0.28	0.09
α-terpineol	0.01	0.39	1.24
Verbenone	6.19	0.11	ND
Carvacrol	0.46	12.19	7.84
Viridiflorol	0.19	1.15	1.59
Caryophyllene	0.04	ND	0.33
Terpinolene	ND	0.43	0.67
Manool	ND	ND	1.72
Thymol	0.50	12.78	8.78
Limonene	8.41	0.01	ND
Linalool oxide	0.004	ND	ND
Linalool	0.013	0.03	0.02
Phytol	0.006	4.06	0.02
Dihydrocarveol	0.005	ND	ND
Totarol	1.61	ND	2.59

ND: non-detected

According to our GC/MS results with *PT* extracts (Table 5), thymol (12.78%) and carvacrol (12.19%) were found as main terpenes, followed by phytol (4.06%). It was also observed that *PT* extracts have many monoterpenes such as camphor, borneol, viridiflorol, α-terpineol, α-pinene and 1,8-cineole are natural anti-cholinesterase molecules (especially against to AChE) (Dave *et al*., 2000; Ozarowski *et al*., 2017). Thus, the GC/MS results of *PT* were consistent with our findings with AChE inhibition data.

GC/MS results showed that monoterpenes were predominant among numerous derivatives of terpenes in *SD* extracts. Among the compounds, thymol and carvacrol were found highest concentrations as 8.78 and 7.84%, respectively. Moreover, many monoterpenes such as totarol, camphor, manool, 1,8-cineole, borneol, α-terpineol and viridiflorol were determined in high concentration, too. The total concentration of terpene derivatives observed in *SD* extracts were approximately 30% of the extract and this can explain the strong inhibition activity of *SD* extracts observed in this study.

3.5. Antibacterial activity results

Antibacterial characteristic and the antioxidant activity of plant extracts are one of the most studied features for control of human and animal diseases of bacterial origin (Zhang *et al*., 2016). In addition, there is a hypothesis about the pathogenic bacteria invading the intestine can lead to brain dysfunctions by changing the flora of the intestine and AD may be associated with

that (Angelucci *et al.,* 2019). Therefore, the antibacterial effect of *RO, PT* and *SD* extracts were investigated in this study by disc diffusion method. According to the results given in Table 6 and Figure 2, all extracts showed antibacterial effects with obvious inhibition zones. However, the strongest antibacterial activity against *S. aureus* was found with the EtOH-extract of *RO* (25.77 mm inhibition zone) and the one against *E. coli* was found with the EtOH-extract of *SD* (19.52 mm inhibition zone). When the solvent types were compared, EtOH-extracts were found as more effective against *S. aureus* and *E. coli* than other extracts. Previous studies related to the different extracts of *RO, PT* and *SD* showed different antibacterial activity values (Bozin *et al*., 2007; Dhifi *et al*., 2012; Durak & Uçak, 2015; Fernández-López *et al*., 2005; Kilic *et al*., 2003). Compared with the literature values, the results of this study showed higher activities against *S. aureus* and *E. coli* than most of the others. All of the plants studied here can be regarded as natural antibiotics because they showed strong activities against both gram-positive and gram-negative bacteria. Therefore, *RO, PT* and *SD* should be considered as potential candidates for AD pharmaceutical applications with their important capacities to reduce AChE activity, their important phytochemical ingredients, and their prevention capacities from pathogenic bacteria.

Figure 2. Antibacterial activity results of the plants extracted in solvents against *S. aureus* and *E. Coli* performed by disc diffusion method (a- EtOH-extracts, c- MeOH-extracts, e- water-extracts against to *S. aureus* and b- EtOH-extracts, d- MeOH-extracts, f- Water-extracts against to *E. coli*) (C-Positive Control (Ampiciline), *RO-Rosmarinus officinalis*, *SD- Sideritis dichotoma*, *PT- Pistacia terebinthus*).

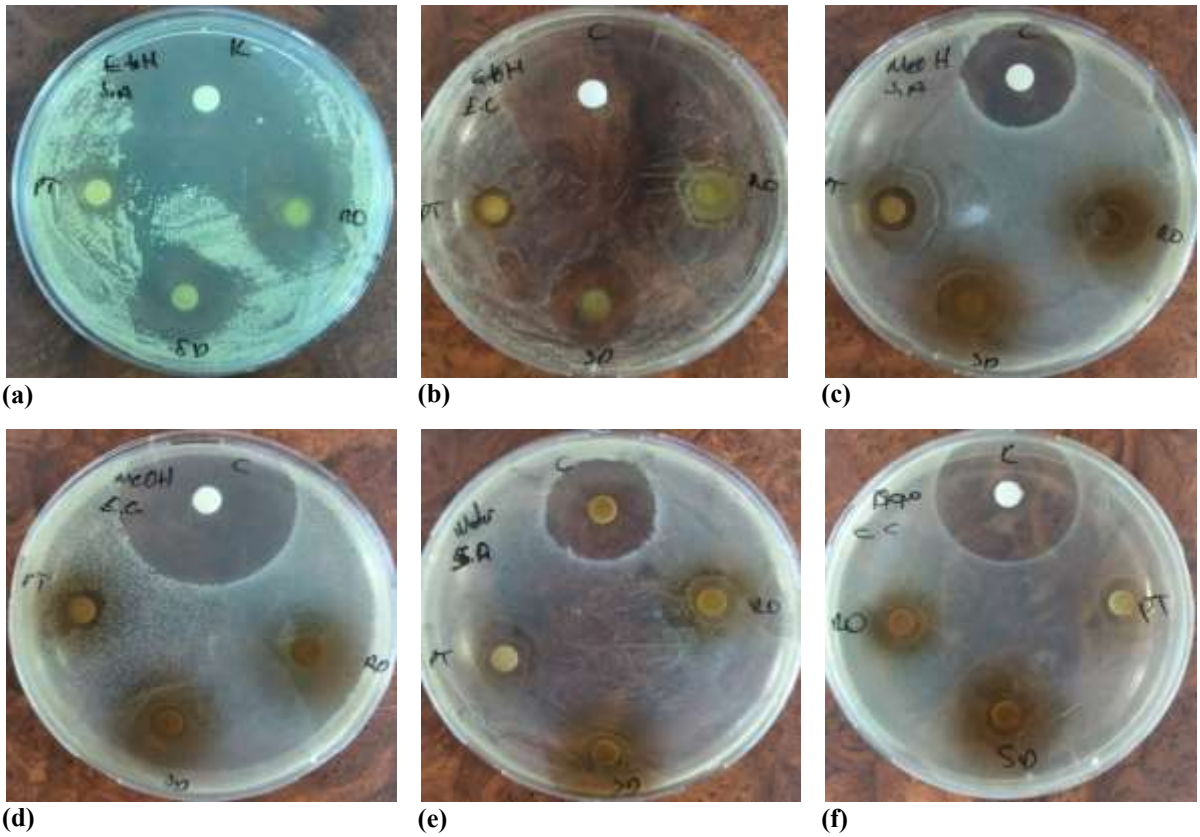


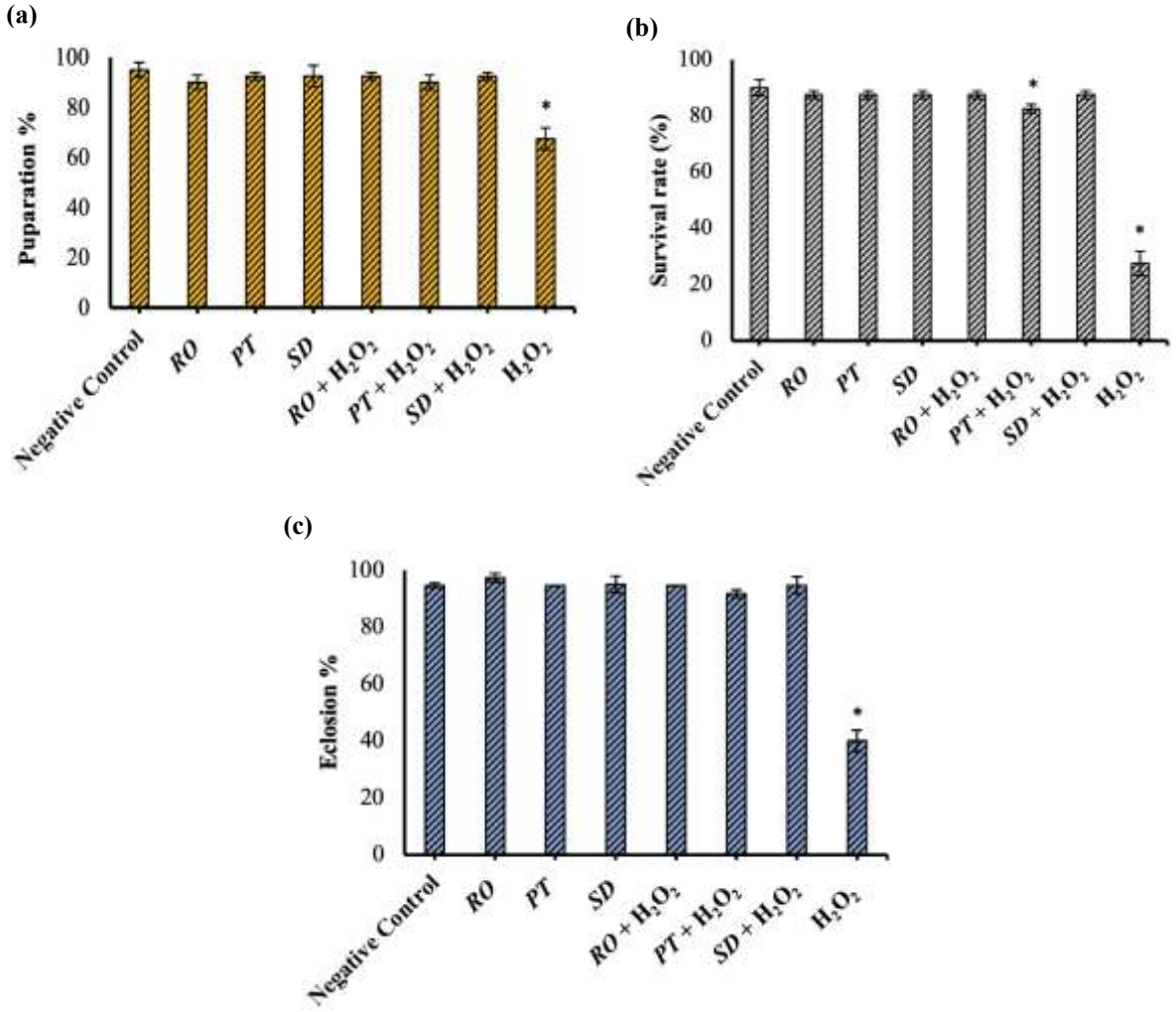
Table 6. Antibacterial activity results of the extracts against *S. aureus* and *E. coli* bacteria.

	Inhibition zone diameter (mm)					
	MeOH extract		EtOH extract		Aqueous extract	
Samples	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
PT	11.54	10.32	9.07	11.22	10.38	9.03
SD	15.08	13.94	19.52	20.58	11.60	11.92
RO	12.04	15.03	12.69	25.77	12.14	13.84
Control (Ampicilin)	28.96	24.22	33.39	34.97	26.42	23.02

3.6. Results of *in vivo* toxicological analyses

The larval (eclosion) assays are recent techniques used to screen the effects of developmental susceptibility or tolerance to toxicants *in vivo* (Rand *et al.*, 2014). The assay is based on the relationship between toxic compounds and the metamorphosis process of *Drossophila* which is regulated by activation of four hormones (ecdysis triggering hormone, eclosion hormone, crustacean cardioactive peptid and bursicon) (Macedo *et al*., 2017). In order to determine the toxicologic effects of *RO, PT* and *SD* extracts *in vivo*, the larval (eclosion) assay was performed in this study and the results were given in Figure 3. The extracts that showed highest AChE inhibition values in this study (EtOH extract of *RO,* MeOH extract of *PT* and EtOH extract of *SD*) were analyzed in this assay. According to the results, it was clearly seen that H_2O_2 caused significant decreases ($p< 0.05$) in puparation %, survival rate % and eclosion % (Figure 3(a), (b), (c)). However, when the *RO, PT* and *SD* extracts were applied there was no significant change in puparation %, survival rate % or eclosion % (Figure 3(a), (b), (c)). In addition, extracts co-administered with H_2O_2 (*RO* + H_2O_2, *PT* + H_2O_2 and *SD* + H_2O_2) showed similar puparation and eclosion % values like negative control (Figure 3(a), (c)). Although there is a decrease in survival rate (%) of cultures treated with *PT* + H_2O_2, the value was significantly higher than the ones treated with H_2O_2 alone ($p<0.05$). Therefore, it can be concluded that none of the extracts used in this study was toxic for *Drossophila* and they ameliorated the H_2O_2 induced decrease of puparation %, survival rate % and eclosion % values. To date, no study has demonstrated the developmental susceptibility or tolerance to *RO, PT* and *SD* extracts *in vivo*. However, there are some studies in literature about *in vivo* toxicological effects of different plant species on *Drosophila (Liu et al*., 2015; Macedo *et al*., 2017; Riaz *et al*., 2018). For example, Liu et al. studied the effects of *Coriandrum sativum*, *Nardostachys jatamansi*, *Polygonum multiflorum*, *Rehmannia glutinosa* and *Sorbus commixta* on *Drosophila* strains and found significant increases in survival rate % with those plant extracts compared to the ones with AD phenotypes *(Liu et al*., 2015). In another study, it was investigated that hydroalcoholic extract from leaves of *Senecio brasilienis* (Spreng) Less. caused significant decrease in the eclosion rate of flies at higher concentrations (1mg/ml) (Macedo *et al*., 2017). The toxicity of petroleum extract of *Euphorbia prostrata*, *Parthenium hysterophorus*, *Fumaria indica, Chenopodium murale* and *Azadirachta indica* against *D. melanogaster* were also studied (Riaz *et al*., 2018). According to Riaz et al., *E. prostrata* was the only one with high mortality (51.64%) at 30% concentration and it was significantly higher than the negative control after 72 h of incubation.

Figure 3. EtOH extract of *R. officinalis* (*RO*), MeOH extract of *P. terebinthus* (*PT*) and EtOH extract of *S. dichotoma* (*SD*) extracts ameliorated the decreased **a-**puparation %, **b-**survivalrate (%) and **c-**eclosion % of *Drosophila.* * indicates that $p< 0.05$ compared to negative control.



4. DISCUSSION and CONCLUSION

Although the pathogenesis of AD has not been fully deciphered yet, increased activity of AChE and oxidative stress are considered the main reasons for (Cavdar *et al.,* 2019; Zhao & Zhao, 2013). Natural compounds have become an emerging and promising area of research for the therapy of neurodegenerative diseases like AD because of their strong antioxidant capacities (Ramassamy, 2006). Therefore, this study identified the inhibition capacities of *RO*, *PT* and *SD* extracts on AChE, the antioxidant properties, phenolic contents, terpene compositions, antibacterial effects, and *in vivo* toxicities of the plants. All of the plant extracts showed strong inhibitory effects on AChE activity. The inhibition type of *RO* was uncompetitive, while *SD* and *PT* extracts showed competitive inhibition on AChE activity. Moreover, GC/MS results showed that carvacrol and thymol were the major monoterpenes of *PT* and *SD* extracts, while limonene and borneol were the main monoterpenes of *RO* extracts. The strongest antibacterial activities were observed with EtOH extract of *RO* (25.77 mm) against *S. aureus* and with EtOH extract of *SD* (19.52 mm) against *E. coli*. To conclude, all of the plant extracts studied were capable of inhibiting the AChE activity and this observation was compatible with their important biochemical compositions revealed in this study. It was also determined that their great potential as antibacterial agents and non-toxic characteristics make them important

candidates for pharmaceutical applications like anticholinesterase drugs or starter compounds for synthesizing more effective AChE inhibitors.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Mehmet Emin Diken: Investigation, Methodology, Supervision, Resources, and Writing - original draft. **Begumhan Yilmaz Kardas**: Investigation, Methodology, Resources, and Writing -original draft.

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Research Article

Comparison of three different protocols of alkaloid extraction from *Glaucium corniculatum* plant

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Abstract: Alkaloids, plant secondary metabolites, have a wide variety of biological effects. For this reason, the extraction of alkaloids from plants is of strategic importance. Different extraction protocols for the extraction of alkaloids from plants have been described by many authors. The objective of this study was to compare the efficacy of three different protocols for the extraction of alkaloids from *Glaucium corniculatum*. This article compares the Soxhlet and ultrasonication protocol, used in previous studies, to a modified Soxhlet protocol. While the alkaloid amount in the extract was determined by the spectrophotometric method, the qualitative estimation of the compounds in the extract was determined by Gas chromatograph-Mass spectrometer (GC-MS). The alkaloid amount and diversity in the extract, obtained through the recommended modified Soxhlet protocol, were higher than that of any extract obtained through other protocols. Thus, a new modified alkaloid extraction protocol was established, which is better than the other protocol.

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1. INTRODUCTION

Plant secondary metabolites have become the focus of many studies due to their therapeutic effects. For this reason, extraction protocols that allow the extraction of secondary metabolites have gained importance. One of the most important groups of secondary metabolites is alkaloids. These are organic compounds containing nitrogen and heterocyclic rings and having significant pharmacodynamic activity even in very small doses (Hesse, 2002). About 20% of plant species contain alkaloids, which play a role in defense against herbivores and pathogens. Archaeological and historical records reveal that alkaloid-containing plants have been used as empirical drug sources since ancient times (Amirkia & Heinrich, 2014). Alkaloids have a wide variety of biological effects, including anti-microbial, anti-diabetic, anti-ulcer, anti-viral, anti-inflammatory, anti-arrhythmic, anti-oxidant, anti-diarrheal , anti-mutagen, hypolipidemic, anti-tumor and neuroprotective (Cushnie *et al*., 2014; Chaves *et al*., 2016; Yu *et al*., 2005). The extraction of alkaloids with high yield and variety is of strategic importance due to the foregoing medicinal properties. The plant selected for this study is *G. corniculatum*, the subject matter of

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the study, is rich in alkaloid content (Doncheva et al., 2014; Kintsurashvili & Vachnadze, 2000; Phillipson *et al*., 1981; Shafiee *et al*., 1985). Therefore, it is a good source of alkaloids.

Recent advancements in Soxhlet and ultrasonic extractions have increased the interest in the evaluation and optimization of the extraction of alkaloids. These techniques tend to be accurate and generally practicable and therefore, can replace the old standard protocols used for the extraction of alkaloids. Soxhlet method is one of the most widely used methods for the extraction of total alkaloids. This is mainly because it is very easy to carry out, and a formally recognized method used for the determination of a wide range of alkaloid content. In the Soxhlet extraction method, the solvent contacts the soluble sample directly, and the bioactive compounds in the sample are extracted directly into the solvent (Webster, 2006). Khamtache-Abderrahim *et al*. (2016) recommended a Soxhlet protocol for the extraction of alkaloids from plants (Khamtache-Abderrahim *et al*., 2016).

Ultrasonic extraction involves the transfer of bioactive components from a permeable solid matrix to the solvent through sound energy (Webster, 2006). Recently, innovative extractions, such as ultrasound extraction, have been shown to be an alternative to conventional procedures for the extraction of bioactive alkaloids from plants because they are more efficient and faster than conventional procedures. Moreover, they are more ecologically friendly, and solvents used therein are less toxic (Desgrouas *et al*., 2014). Existing studies have concluded that ultrasonic energy is absolutely beneficial for the extraction of alkaloids from plants, provided that ultrasound is sufficiently intense and correctly applied (Demaggio & Lott, 1964). Sarikaya *et al*. (2014) proposed a protocol based on ultrasonic method for the extraction of alkaloids from plants by methanolic extraction. Application time, temperature and chemicals affect the extraction yield and variety both in Soxhlet protocol and ultrasonication protocol *(Sarikaya et al.*, 2014).

This article compared two protocols, recommended by Khamtache-Abderrahim *et al*. and Sarikaya *et al*. for the extraction of alkaloids, to a modified Soxhlet protocol. After extraction, the total amount of alkaloids in three protocols was determined using spectroscopic method. The alkaloid yields of these protocols were also determined. In addition, the alkaloid diversity of the extracts, obtained by different protocols, was analyzed by GC-MS.

2. MATERIAL and METHODS

2.1. Plant Material

G. corniculatum (L) RUD. subsp. *refractum* (NAB.) CULLEN was collected from Beypazari district in the northwest of Ankara on 27.07.2015 by Prof. Dr. Zeki Aytaç. The aerial parts of the plant were dried and powdered. Gazi University herbarium material number of this plant is ZA10700.

2.2. Total Alkaloid Extraction

Three total alkaloid extraction protocols were evaluated in this study. For protocol A, B and C, the *G. corniculatum* was subjected to different extraction protocols.

Protocol A: Extraction by Soxhlet method

15 g of powdered plant samples were separately extracted with 150 mL of methanol solvent for 4 hours in a Soxhlet apparatus (LabHeat). The solvent was removed in the evaporator (Heidolph Laborator 4000) at controlled temperature (60-100 °C) and low pressure. The plant extracts were taken up in 10 mL of hydrochloric acid (HCl) (2.5%) and 150 mL of diethyl ether ($(C_2H_5)_2O$) was added to dissolve the oils in the extracts. The pH of the aqueous acid solution was adjusted to 8 with ammonium hydroxide (NH_4OH). Then 150 mL of dichloromethane (CH_2Cl_2) was added. The extracts were dried over magnesium sulphate ($MgSO_4$). The solvent was evaporated at controlled temperature (60-100 °C) and using a low pressure evaporator

(Heidolph Laborat 4000) to obtain the crude solids of the total alkaloids. The residue was stored at + 4 °C for use in experimental studies (Khamtache-Abderrahim *et al*., 2016).

Protocol B: Extraction by ultrasonication method

1 g of powdered plant samples were separately sonicated for 30 minutes with 10 mL of methanol solubilizer. The liquid portion was filtered, and the solvent removed at the controlled temperature (40 °C) and using a low pressure evaporator. The plant extracts were taken up in 10 mL of sulfuric acid (2%) and passed through 3 × 50 mL of $(C_2H_5)_2O$. It was separated from the oil in the separation funnel. The pH of the aqueous acid solution was adjusted to 9 with NH_4OH. 3 x 50 mL chloroform was then added. The extract was dried in sodium sulphate and evaporated in a rotary evaporator under controlled temperature (40 °C) and low pressure. The residue was stored at + 4 °C for use in experimental studies (Sarikaya *et al*., 2014).

Protocol C: Recommended modified Soxhlet method

10 g of powdered plant sample was extracted with 150 mL of methanol solvent in a Soxhlet apparatus (LabHeat) for 8 hours. The solvent was removed at the controlled temperature (40 °C) and using a low pressure evaporator. The plant extracts were taken up in 10 mL of sulfuric acid (2%) and passed through 3 × 50 mL of $(C_2H_5)_2O$. It was separated from the oil in the separation funnel. The pH of the aqueous acid solution was adjusted to 9 with NH_4OH. 3 x 50 mL chloroform was then added. The chloroform extract was dried in sodium sulphate and evaporated in a rotary evaporator under controlled temperature (40 °C) and low pressure. The residue was stored at + 4 °C for use in experimental studies.

Spectroscopic method and GC-MS were used to compare the amount, ratio and variety of alkaloid obtained by the different protocols.

2.3. Determination of Total Alkaloids Using Spectroscopic Methods

Briefly, bromocresol green (BCG) solution was prepared by heating 69.8 mg BCG (Sigma-Aldrich, Italy) with 3 ml of 2 N NaOH and 5 ml distilled water until completely dissolved and the solution was diluted to 1000 ml with distilled water. 1 mg of plant extract was dissolved in 1 mL of HCl (pH 2.5). Then, 5 mL of bromocresol green solution (0.04%) and 5 mL of phosphate buffer (pH 4.7) was added to the extract solution which was transferred to a separation funnel. The mixture was shaken with 5 mL chloroform. A set of reference standard solutions of boldine (25 to 250 µg/mL) were prepared. The yellow complex in chloroform was finally recovered and the absorbance at 470 nm was measured against blank (Novelli *et al*., 2014). The experiment was performed in three replicates and 10 parallels (Novelli *et al*., 2014).

2.4. Gas Chromatograph-Mass Spectrometer (GC-MS) Analysis

Compound analyses were performed by employing GC-MS using Thermo GC -Trace Ultra Ver 2.0 Thermo MS DSQ II (Thermo Fisher Scientific, San Jose, CA, USA) instrument at Ege University Faculty of Pharmacy Pharmaceutical Sciences Research Centre. The temperature conditions followed the program: The initial temperature was 100 °C. It was increased from 100 °C to 180 °C at the rate of 15°C per minute. It was increased from 180 °C to 300 °C at the rate of 5 °C per minute. Then the temperature was held at 300 °C for 10 minutes. The injector temperature was 250 °C. Helium was used as the carrier gas at a flow rate of 0.8 mL / min. HP-5 MS column (30 m × 0.25 mm × 0.25 µm) was used. The spectra of chromatographic peaks were investigated using Xcalibur (version 2.07, Thermo Fisher Scientific, San Jose, CA, USA). Compounds were defined by comparing the mass spectral fragmentation with the standard reference spectra of the Wiley 7N library database (Kaya *et al*., 2017).

2.5. Statistical Analysis

Plant uptake was repeated three times for each sample and the average of the three replications was calculated. Data were analyzed with the use of SPSS software (version 21.0) using the one-way ANOVA test. Values are shown as mean ± standard deviation (SD). Statistical significance was taken at a p value <0.01.

3. RESULTS

3.1. Extract and Alkaloid Amount, and Alkaloid Yield

The extract amount from 1 g plant, the alkaloid amount in 1 g extract and the alkaloid yield of the dry plant are shown in Table 1. According to the results, the highest alkaloid content (153 ± 6 mg alkaloid/g extract) and the highest alkaloid yield (0.765 mg alkaloid/g dry plant) in the dry plant were obtained through Protocol C. In Protocols A and B, a higher amount of extract was obtained than Protocol C, although the amount of alkaloid in the extract was lower than the amount in Protocol C. The extract amount, obtained in Protocol B, was statistically significant at a level of 0.01 compared to the extract amount obtained in Protocols A and C ($^{*}p$ <0.01). The difference between the protocols of total alkaloid extract was also explained statistically. A statistically significant difference was found between Protocol C and other protocols at the level of 0.01 ($^{\#}p$ <0.01) (Table 1).

Table 1. Amount of extract and alkaloid, and the alkaloid yield of *G. corniculatum.*

Protocols	Extract amount (mg extract/g plant)	Alkaloid amount (mg alkaloid/g extract)	Alkaloid yield (mg alkaloid/ g plant)
A	7±1	71±2	0.355
B*	41±2	40±4	0.200
C#	5±0	153±6	0.765

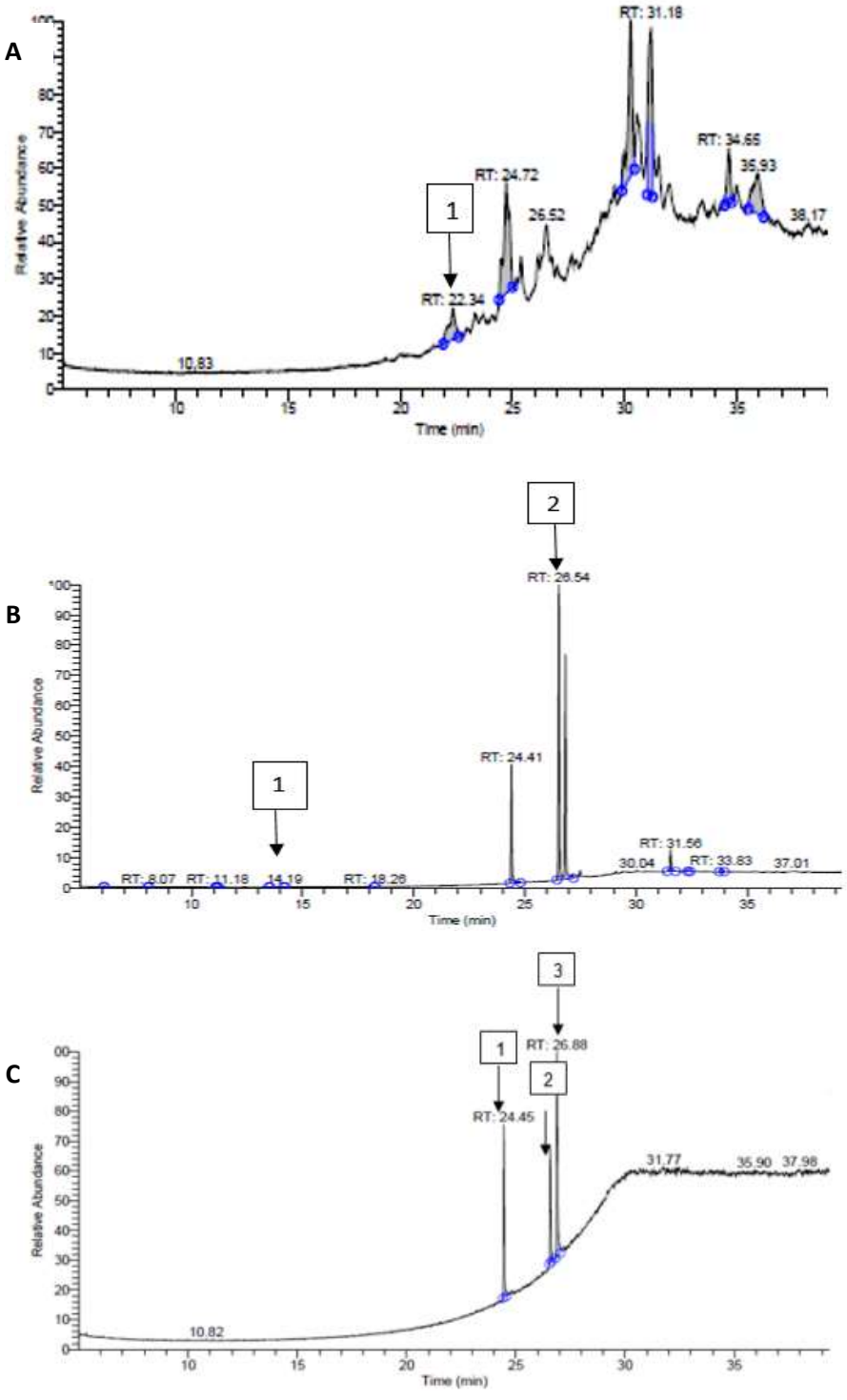
*p < 0.01 difference between protocols in terms of the amount of extract
#p < 0.01 difference between protocols in terms of the amount of alkaloid

3.2. Alkaloid Diversity

The alkaloid diversity of alkaloid extracts, obtained from *G. corniculatum* by different extraction protocols, was determined by GC-MS method. GC-MS chromatograms of the extracts are shown in Figure 1. Six different peaks were identified in the chromatogram of the extract in Protocol A but only one of them was alkaloid. The ratio of alkaloids, obtained through Protocol A, was 7.6%; and non-alkaloid compounds were 92.4% (RT:22.34: 6H-Dibenzo[a,g]quinolizine, 5,8,13,13a-tetrahydro-2,3,9,10-tetramethoxy-, (ñ)- (Tetrahydropalmatine) (alkaloid), RT:24.72, 30.26 and 31.18: 9-Hexadecenoic acid, eicosyl ester, (Z) (Fatty acid), RT:34.65 and 35.93: Quercetin 7,3',4'-trimethoxy (flavone)). Twelve different peaks were identified in GC-MS chromatogram of Extract B, but only two of them were alkaloid (RT:6.09: Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl- (CAS) (Phenol), RT:8.07 and 11.10 Trans-2-phenyl-1,3-dioxolane-4-methyl octadec-9,12,15-trienoate (Phenol), RT:11.18 and 18.26: Quercetin 7,3',4'-trimethoxy (flavone), RT:13.52: Isochiapin B (Terpenoid), RT:14.19: [1,3]Benzodioxolo[5,6-e][2]benzazecin-14(6H)-one, 5,7,8,15-tetrahydro-3,4-dimethoxy-6-methyl- (Allocryptopine) (alkaloid), RT:24.41: 9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)- (fatty acid) RT:26:54: (+)-Canadine (alkaloid), RT:31.56: 9-Hexadecenoic acid, eicosyl ester, (Z) (Fatty acid), RT: 32.39 and 33.83: 1,1,3,3,5,5,7,7,9,9,11,11-Dodecamethyl-hexasiloxane (Siloxane derivative)) (Nigdelioglu Dolanbay *et al.*, 2021). The ratio of alkaloid compounds was 77.0%; and non-alkaloid compounds were 23%. Three alkaloid peaks were identified in the chromatogram of the extract obtained by the modified Soxhlet protocol. The ratio of alkaloids in Protocol C was 100%

(RT:24.45: 6H-
Dibenzo[a,g]quinolizine, 5,8,13,13a-tetrahydro-2,3,9,10-tetramethoxy-, (ñ)- (Tetrahydropalmatine) (alkaloid), RT: 26.58: Tetrahydroberberine N-oxide (Canadine) (alkaloid), RT: 26.88: [1,3]Benzodioxolo[5,6-e][2]benzazecin-14(6H)-one, 5,7,8,15-tetrahydro-3,4-dimethoxy-6-methyl- (Allocryptopine) (alkaloid)).

Figure 1. GC-MS chromatograms of total alkaloid extracts obtained from *G. corniculatum* by Soxhlet (A), ultrasonication (B), and recommended modified Soxhlet (C) protocols *(Nigdelioglu Dolanbay et al*., 2021).



4. DISCUSSION and CONCLUSION

Isolation and purification of alkaloids are crucial for medical and chemical studies due to their bioactivity. This emphasizes the importance of protocols for alkaloid extraction from plants. In this study, total alkaloid extracts were obtained from three different protocols (preceding Soxhlet (A) and ultrasound (B) protocol and recommended modified Soxhlet protocol (C)) from *G. corniculatum*, a member of the *Papaveraceae* family. The alkaloid amount, purity and diversity in the extracts obtained were compared.

The *Papaveraceae* family is distinguished by the richness of their alkaloid contents (Almousawi & Alwan, 2017). It has been shown in the literature that about 165 *Papaveraceae* species contain alkaloids (Preininger, 1985; Yu *et al*., 2014), and *G. corniculatum* is one of these species. More than 20 alkaloids have been identified from *G. corniculatum* extracts prepared with various solvents (Shafiee *et al*., 1985; Slavík & Šantavý, 1972). Accordingly, *G. corniculatum* is a good source for total alkaloid extraction.

The main factors affecting the extraction efficiency and the amount and variety of bioactive compounds in the extract were the extraction protocol, duration, temperature and chemicals used. In the modified Soxhlet protocol, the plant was extracted in the Soxhlet apparatus for 8 hours and diethyl ether administration was carried out in three steps for greater separation of the oils, unlike Protocol A. In addition, increasing the amount of acid in the extraction process helped the alkaloid to decompose highly from other compounds. In the protocol, chloroform was preferred as organic solvent instead of dichloromethane (Kutchan, 1995). Thus, alkaloids in base form were obtained in a purer form. In the Soxhlet method, it was reported that the components passed to the solvent better than the ultrasonication method (Schmeck & Wenclawiak, 2005). The most obvious advantage of using the Soxhlet method is that the sample phase repeatedly comes into contact with a new part of the solvent, thereby allowing the components to separate from the plant (Luque de Castro & García-Ayuso, 1998). Our study also supported this data.

In Protocols A and B, higher amounts of extract were obtained compared to Protocol C; however, the alkaloid amount in the extract was lower than the amount obtained in Protocol C. This may be due to the fact that the amount of the extracts is higher than the amount in Protocol C because of the presence of other non-alkaloids in Protocols A and B. This may be associated with the presence of purer alkaloids with more variety in Protocol C.

Various methods with different sensitivities, such as gravimetric, titrimetric and spectrophotometric, have been developed for the determination of alkaloids in plant materials. However, gravimetric and titrimetric methods lack sufficient sensitivity. On the other hand, spectrophotometric determination of total alkaloids by bromocresol green is a simple and sensitive method and does not require any special equipment (Novelli *et al*., 2014). The highest amount of alkaloids were determined in 1 g extract and 1 g plant in Protocol C according to our data obtained by spectrophotometric analysis (153 ± 6 mg/g extract and 0.765 mg/g dry plant, respectively), and the total amount of alkaloids in 1 g dry plant, belonging to the *Papaveraceae* family, was found as 0.02-25 mg (Dittbrenner, 2009; Jimoh *et al*., 2010). According to these data, the total amount of alkaloids, obtained in Protocol C, was similar to the total amount of alkaloids obtained from different species in the same family.

Another method for the determination of alkaloids is chromatographic analysis. Since alkaloids appear in solutions in ionized and combined forms, chromatographic peaks of alkaloids are difficult to determine and have low system yields (Petruczynik, 2012). GC-MS is a useful and reproducible technique in eliminating this problem. This technique can be used to identify and quantify ionized compounds that have a low molecular weight in complex mixtures. In addition, GC-MS provides fast and reliable identification of compounds since

spectra of compounds can be compared to library data (Järnberg *et al*., 1994; Villas-Bôas *et al*., 2005). Alkaloid content and diversity of extracts were determined by GC-MS method for the above reason.

A good alkaloid extraction protocol should not only give high alkaloid content, but also high alkaloid diversity. The number of alkaloids identified in these three different protocols and their ratios in the extract were different in qualitative GC-MS analyses. Only one alkaloid (7.6%) was identified in the extract that is obtained in Protocol A, while two (92.4%) alkaloids were identified in the extract obtained in Protocol B. Non-alkaloid compounds were identified in both the extracts. The extract that is obtained by the modified Soxhlet protocol (C), had no peaks other than three alkaloid peaks. These results proved that high purity and variety of alkaloids can be obtained using Protocol C, the recommended extraction method. For this reason, Protocol C is a recommended method for alkaloid extraction from plants due to high extraction amount and advantages of a wide variety of alkaloids.

The study confirms that the proposed modified Soxhlet protocol is more efficient than previous Soxhlet and ultrasonication protocols for the extraction of alkaloids. This protocol is advantageous because it allows for obtaining greater amounts of extract, more diverse and purer alkaloids compared to other methods.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Fatma Gonca Kocanci: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Serap Nigdelioglu Dolanbay**: Investigation, Resources, Visualization, Software, Formal Analysis. **Belma Aslim**: Methodology, Supervision, and Validation.

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Research Article

Synthesis of Some Alkyl Polyglycosides

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Abstract: Surfactants are indisputably more important today, in terms of their use in industry and daily life, as well as the tasks they perform. In recent years, due to the driving force of environmental concerns, orientations to alternatives of surfactants that cause less or no harm to the environment have accelerated. One of these trends is the synthesis of alkyl polyglycosides (APG) and their use in the detergent industry. In this study, different acidic catalysts were used for the synthesis of APGs and the highest yield was achieved with sulfuric acid. APGs with different carbon numbers were obtained using octanol, decanol, dodecanol, and octanol/cetyl alcohol (w/w 80/20). The FTIR spectra of the structures of APG products obtained with these fatty alcohols and some commercial APG products were compared, and their structures were elucidated. In addition, the foam quality of the obtained APGs and the hydrophilicity properties they impart to the textile material were compared with some commercial surfactants, and the results were interpreted and evaluated.

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1. INTRODUCTION

Surfactants are chemicals that change the surface tension in the solution to which they are added and often reduce the surface tension. The molecules of a liquid attract each other due to dispersion, dipole-dipole, dipole-excited dipole, and hydrogen bonds. A molecule in a liquid mass exhibits the same attractive and repulsive forces in all directions. But on the surface, a direction of these forces is missing. This asymmetry of forces is the source of surface energy, or surface tension (Pispanen, 2002).

At the molecular level, surfactants are organic compounds containing at least one lipophilic (solvent-loving) and one lipophobic (solvent-loving) group. If the solvent in which surfactants will be used is water or an aqueous solution, these terms are called hydrophilic and hydrophobic, respectively (Rosen & Dahanayake, 2000).

Surfactants classified according to the sign of the charge at the hydrophilic ends are grouped as anionic, non-ionic, cationic, and amphoteric and are demanded by users considering the unique characteristics of each group. Alkyl polyglycosides, which we have been working on, non-ionic APGs are surfactants. The use of non-ionic surfactants accounts for 40% of the world's use of surfactants (Schmitt, 2001).

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Alkyl polyglycoside (APG) is a surfactant made from renewable natural ingredients, namely carbohydrates and fatty alcohols. APG can be used as an additive in the formulation of several products such as herbicides, personal care products, cosmetics, and fabric/textile bleaching. Alkyl polyglycosides are non-ionic surfactants because the polar (hydrophilic) and non-polar (hydrophobic) groups have no charge. Its hydrophobic nature is found in the alkyl groups of fatty alcohols and its hydrophilic nature is found in the glucose molecule. This APG surfactant is harmless to the eyes, skin, and membranes, reduces the irritant effect, and can decompose well aerobically and anaerobically (Mehling *et al*., 2007).

APG surfactants can be produced by the Fischer method directly (acetalization) and indirectly through two stages, namely butanolysis and trans acetalization, and then through the stages of neutralization and distillation. The synthesis of APG through a two-step process using glucose and fatty alcohols with different chain lengths has been carried out by Ware *et al*. (2007) with 5 different carbon chains, namely octanol (C8), decanol (C10), dodecanol (C12), hexadecanol (C16), and octadecanol (C18) and El-Sukkary *et al*., (2008) with different alkyl chain lengths, namely octanol (C8), nonanol (C9), decanol (C10), dodecanol (C12) and tetradecanol (C14).

Generally, the catalyst used is *p*-toluene-sulfonic acid (PTSA) (Ware *et al.*, 2007; El-Sukkary *et al.*, 2008). In this study, an experiment was conducted using the MESA catalyst as an alternative catalyst that is more environmentally friendly and renewable than palm oil.

The saccharides that can be used to produce APG include glucose, fructose, mannose, galactose, xylose, starch, sucrose, lactose, and so on, both in liquid and solid form. The use of glucose and starch is more widely used for reasons of availability and low cost (O'Lenick, 2007). The process of making APG is still dominated by the use of potato and corn starch as hydrophilic groups and C14-C18 fatty alcohols as a source of hydrophobic groups (Hill, 2009). Research using sago starch has been carried out by Suryani *et al*. (2008) and tapioca by Bastian *et al*. (2012).

In this study, fatty alcohols of different chain lengths and alkyl polyglycoside surfactants were synthesized using the two-step trans acetylation method and these products were compared with their counterparts, which are widely used in the industry.

2. MATERIAL and METHODS

2.1. Materials

All chemicals used in the study are of analytical purity. Butanol, octanol, decanol, dodecanol, cetyl alcohol, *D*-(+)-glucose, sodium hydroxide, potassium hydroxide, sulfuric acid, meta-phosphoric acid, *p*-toluene sulfonic acid, methanol, and potassium hydroxide chemicals were obtained from Merck and Aldrich companies.

2.2. Preparation of APGs

A number of APGs were synthesized using fatty alcohols of different alkyl chain lengths to produce surfactant compounds.

2.2.1. *Indirect method*

Three different chemical substances (**s**ulfuric acid, meta-phosphoric acid, and *p*-toluene sulfonic acid) were used in the catalyst selection. Anhydrous glucose and butanol were mixed in different proportions and mixed at a constant speed with a mechanical mixer in the apparatus shown in Figure 1, in the presence of a catalyst, in the temperature range of 80-120^{o}C. During this time, Fehling's solution was used to determine the amount of glucose in the sample taken from the reactor balloon. Unreacted fatty alcohols were distilled under a vacuum in a rotary evaporator. In the synthesis of APG, catalysts such as *p*-toluene sulfonic acid, sulfuric acid, and meta-phosphoric acid were used for each separate experimental process**.** In the first step, the

reaction method was selected for the convenience of our experimental studies in the APG synthesis process. In general, from the information we obtained from our literature research, it was determined that two types of methods were used, namely, one-step and two-step synthesis methods. The single-step reaction method requires less equipment. However intermediate product, butyl glycoside, is a stable compound to start to combine long-chain fatty alcohol and glycose part, and the yield of the overall reaction is higher in two-step synthesis. Therefore, in this study for the synthesis of APGs, it was decided to work with the two-step synthesis method.

Figure 1. Experimental setup used in the synthesis of butyl-glycoside and alkyl polyglycosides.

2.3. Tests Applied to Synthesized Products

2.3.1. *Structure confirmation of APGs*

The structure of the prepared compounds was confirmed by: Fourier transform infrared spectroscopy (FTIR) spectra using a Perkin Elmer UATR Two Model spectrophotometer.

2.3.2. *Foam test*

Each surfactant solution was prepared as 1 g/L. 50 mL of this solution was taken in a 100 mL mixing cylinder and shaken 50 times in 30 seconds. By removing the cover of the cylinder, the foam level was measured with a ruler. Foam strength was checked by measuring the foam level at 1-minute intervals.

2.3.3. *Hydrophility test*

Wetting power can be defined as the effective reduction of surface tension under dynamic conditions. During wetting, surfactant molecules must diffuse rapidly to the boundary between the moving liquid and the surface (Holmberg *et al.*, 2002). The wetting abilities of surfactants are examined by the Draves test (Draves & Clarkson, 1931).

500 mL of 0.70 g/L wetting solution is poured into a 500 mL mixing cylinder and it is waited for a while for the solution to become inactive. If there was foaming on the solution surface, waited until it disappeared. The textile sample, together with the weight, is kept at the mouth level of the mixing cylinder, left on the solution surface, and the chronometer is started as soon as it is released. When the fabric is completely wet and sinks to the bottom, the stopwatch is stopped and the result is recorded.

3. RESULTS and DISCUSSION

3.1. Catalyst Effect and Selection in Butyl Glycoside Synthesis

As a result of all these trials, it was seen that sulfuric acid was the most advantageous catalyst in terms of homogeneous mass transfer and in various temperatures. The FPG yields obtained for these three mineral acids were found to be around 70%. The results of the studies on this

subject support the results of our experiments. Xinping *et al*., 1999, reported that the best catalyst was sulfuric acid when the reaction times and yield were considered together. However, when *p*-toluene sulfonic acid is used, the amount of alkali required for neutralization is less, which means a product containing a lower concentration of salt (Xinping *et al*., 1999).

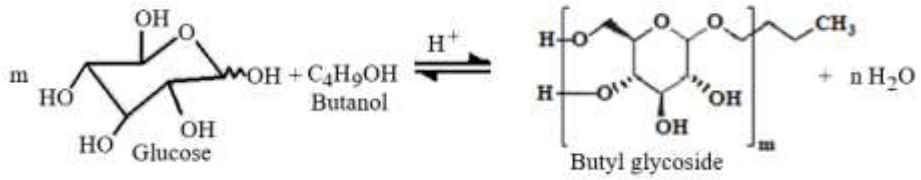
3.2. Butyl polyglycoside synthesis

In the APG synthesis study, 44.4 g anhydrous *D*-(+)-glucose ($C_6H_{12}O_6$,) and 56.6 g n-butanol ($C_4H_{10}O$) were used as basic raw materials. *D*-(+)-glucose and n-butanol were placed in a four-necked balloon placed in a jacketed heater. After connecting the mechanical stirrer, thermometer, and reflux condenser (cooler) to the four-necked flask, the mixture containing *D*-(+)-glucose and n-butanol was mixed at 350 rpm (Renhua et al., 1999). It is expected to reach the desired constant temperature (105°C).

Temperature control is one of the most important parameters in the synthesis of butyl glycoside and APG. While the reaction mixture is in dispersion, glucose can be cooked at 120°C and above and caramelized. Xinping *et al*. (1999) emphasized that the reaction efficiency of working at high temperatures (115°C) is high, but it should be taken into account that a sudden increase in temperature in the solution environment may cause glucose to cook (Xinping *et al*. 1999). At the beginning of our experimental studies, we started our reaction by starting from low values in order to keep the temperature stable, and by increasing the temperature over time and setting it to 105°C, where the reaction can be realized most efficiently. After the mixture of *D*-(+)-glucose and n-butanol in the balloon were brought to a constant temperature, 0.13 mL of sulfuric acid was added into the balloon as a catalyst. The vacuum pump was operated at 200 mmHg and the water formed in the environment was taken with the help of reflux.

In this process, which was carried out at constant pressure and temperature, after about 30 minutes, the cloudy color of the solution lightened completely and became a transparent yellow solution. Fehling marker was used to detect the presence of glucose in the reaction medium. In order to terminate the reaction, KOH solution was added into the solution and allowed to cool so that the pH value of the solution, which was approximately pH 4, was carried to between 8-10. The synthesized butyl-glycoside reaction mixture was filtered through a Buchner funnel with the help of a vacuum pump. The solvent in the obtained product was removed with the help of a rotary evaporator under a vacuum. The reaction of the synthesized butyl-glycoside is given in Figure 2 and the FTIR spectrum is given in Figure 3.

Figure 2. The reaction of butyl polyglycoside synthesis.



It is seen that there are characteristic different peaks in the FTIR spectrum of butyl glycoside (Figure 3). The characteristic signal for the O-H group was between 3600 and 3200 cm^{-1}, the asymmetric stretching vibration frequency of the CH_3 and CH_2 group, the symmetrical stretching vibrations of the other CH_2 group were observed at 2960, 2933, and 2873 cm^{-1}, respectively.

The vibration signal of the unbonded C-C bond can be observed around 1639 cm^{-1}. Asymmetric bending vibration of CH_2 group, asymmetric bending vibration of CH_3 group and symmetrical bending vibration of CH_3 group were detected at 1462, 1416, and 1378 cm^{-1}, respectively (Kurashima *et al*., 2003; El-Sukkary *et al*., 2008). The vibration frequency seen at 1727 cm^{-1} indicates the presence of the butyl methyl group and the presence of hemiacetal H

(where H is attached to the glycosylated carbon). The peak seen at 1100 cm^{-1} confirms that the glycoside product contains apo-glucosidase identified by etherification (Yu *et al*., 2008). It is seen that the ether formation in the structure is at 1170 cm^{-1} with a typical signal (Kurashima *et al*., 2003; El-Sukkary *et al*., 2008). Some properties of the synthesized butyl glycoside are given in Table 1.

As can be seen from Table 1, the melting point also increased, which may be due to the increase in the glycoside alkyl chain length and the van der Waals force between this chain.

Figure 3. FTIR spectrum of butyl glycoside.

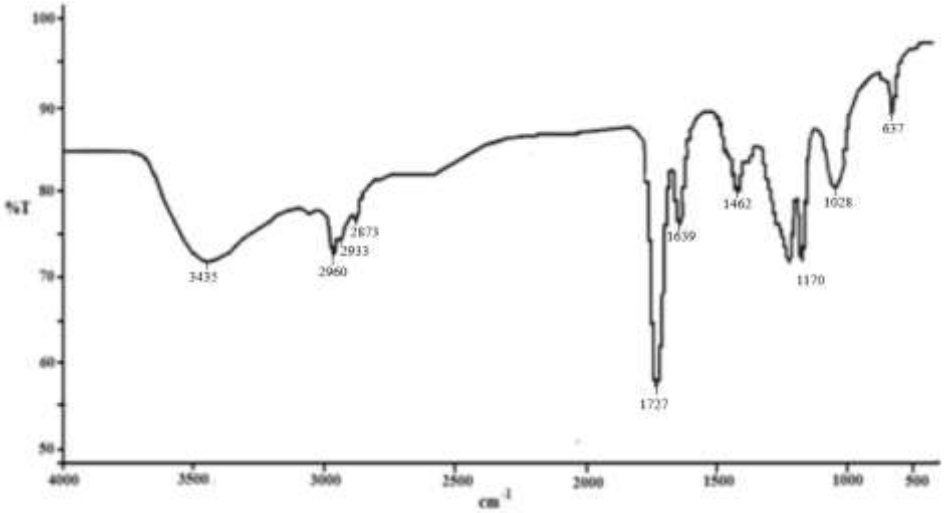


Table 1. Properties of experimentally synthesized butyl glycosides.

Physical characteristics	Solvent type	Butyl glycoside
Organoleptic analysis	-	Solid, light yellow, odorless, sticky, creamy
Melting point	-	52-58°C
Dissolution 25°C	Water	>% 20
	Pyridine	> %20
	Glycerine	%20 - %1
	Carbon tetra chloride	< 1%
	Acetone	Stratified
	Petroleum ether	< %1

3.3. Synthesis of Dodecyl Polyglycoside

In the synthesis study of 1-dodecanol polyglycoside, butyl polyglycoside, which was previously synthesized and subjected to the necessary separation and purification processes, was used as the basic raw material. For the synthesis of dodecyl polyglycoside, 50 g of butyl polyglycoside and 100 g of 1-dodecanol were put into the experimental setup given in Figure 1. After the necessary connections (refrigerant, thermometer, etc.) of the experimental setup were made, the temperature of the mixture was adjusted to be between 105-120°C. The mixture containing butyl polyglycoside and 1-dodecanol was mixed at 500 rpm and waited for the system to reach the desired constant temperature (105-120°C). After the mixture of butyl polyglycoside and 1-dodecanol in the balloon was brought to a constant temperature, 0.40 mL of sulfuric acid was added into the balloon as a catalyst. After the catalyst was added, the temperature in the balloon was adjusted to 110±5°C and a vacuum distillation connection was made at the same time to

separate the water in the system. The vacuum pump was operated at a pressure of 200 mmHg and the water formed in the environment was taken with the help of reflux. It was studied for four hours to complete the reaction. At the end of this period, potassium hydroxide dissolved in methanol was used to neutralize the pH of the solution. Base addition was continued until the pH value of 7.0 was reached. In order to purify the product, the unreacted fatty alcohols were removed from the environment by vacuum distillation in a way that the temperature would not exceed 140°C in the system where the vacuum pump was connected to the reflux. When the advent of the fatty alcohol was stopped by vacuum distillation, the process was terminated and the synthesized dodecyl polyglycoside mixture was left to cool. The synthesis reaction of the obtained dodecyl polyglycoside is given in Figure 4 and the FTIR spectrum is given in Figure 5.

Figure 4. Two-step APG synthesis process.

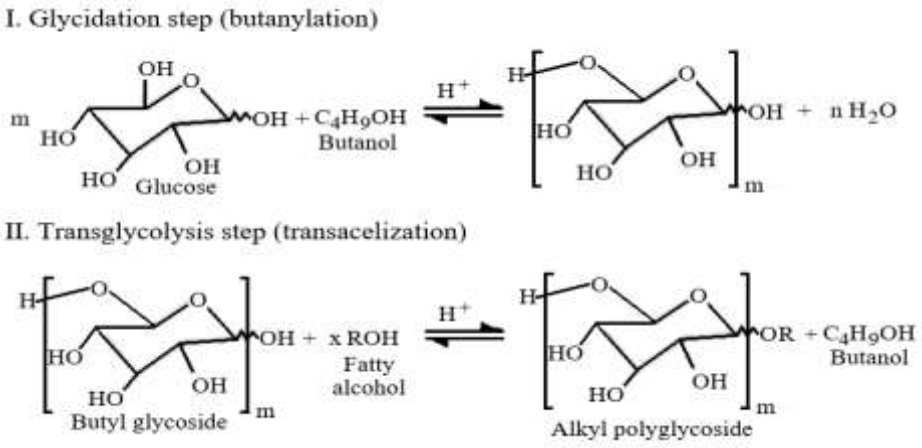
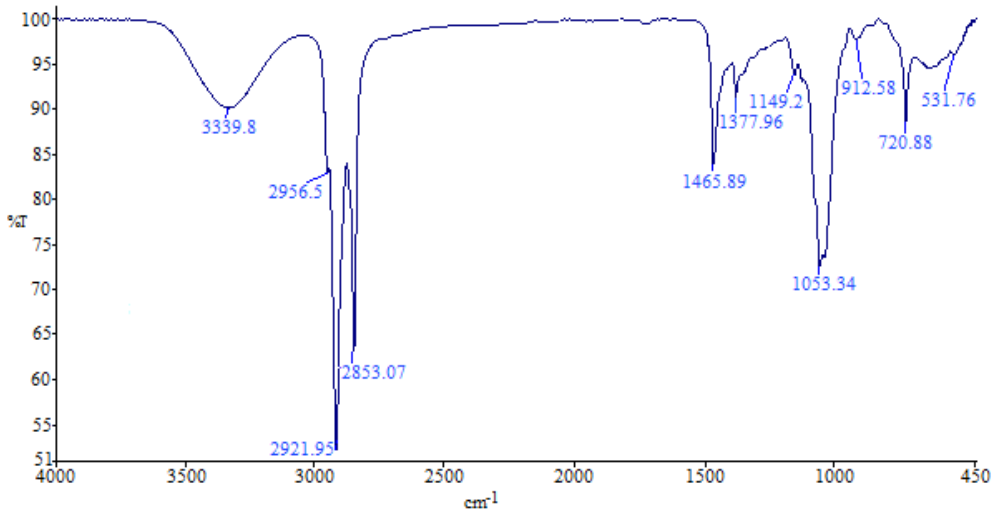


Figure 5. FTIR spectrum of synthesized dodecyl polyglycoside.



From the FTIR spectrum of the obtained alkyl polyglycoside (Figure 5), 2924 cm^{-1} shows the C-H and 3361 cm^{-1} shows the vibration peak of the O-H group. It is observed from the FTIR spectrum that it belongs to the polyglycoside alkyl formation from the C-O vibration peak at 1032 cm^{-1}. Looking at the spectrum of C-O-C ether formation, it is understood that dodecyl poly glycoside surfactants, acetylated glucose, and 1-dodecanol can be obtained by this synthesis method. The wavenumbers of both the OH and ether groups of the synthesized dodecanol polyglycoside are within the limits specified in the literature. According to the FTIR results of this APG obtained, it shows that ether groups (C-O-C) functional groups are formed

together with fatty alcohols, the OH group has a hydrophobic structure, and APG is synthesized through the hydroxyl groups of glucose. Some physical and chemical properties of dodecyl polyglycoside are given in Table 2.

Table 2. Properties of experimentally synthesized dodecyl alkyl polyglycoside.

Physical characteristics	Solvent type	Dodecyl polyglycoside
Organoleptic analysis	-	Solid, brown, odorless, sticky, creamy
Melting point	-	108-114ºC
Dissolution 25ºC	Water	>% 20
	Pyridine	> %20
	Glycerine	%20 - %1
	Carbon tetra chloride	< 1%
	Acetone	Stratified
	petroleum ether	< %1

As can be seen from Table 2 as the glycoside alkyl chain length increased, the melting point increases due to the van der Waals force between the chains.

In Table 3, some other properties of experimentally synthesized butyl glycoside and dodecyl polyglycoside with other surfactants are given comparatively. It is seen from Table 3 that dodecyl polyglycoside has lower surface tension and a higher foam height and excellent surface activity than other surfactants. On the other hand, it is seen that butyl glycoside basically does not have surface activity properties.

Table 3. Comparison of experimentally synthesized butyl and dodecyl polyglycoside with other surfactants (Test conditions: 1% aqueous solution, 30ºC).

Surfactants	Bubble height	Surface tension (Dyn/cm)	Foam resistance	Bubble state
Butyl polyglycoside	35	0.6451	Not good	Elegant, small quantities
Dodecyl polyglycoside	195	0.3461	Good	Elegant, large quantities
Sodium dodecyl sulfate	220	0.4889	Good	Bubble big, large quantities
OP-10	220	0.4283	Good	Bubble big, large quantities

3.4. APG Synthesis with Different Fatty Alcohols and Fatty Alcohol Mixtures

As mentioned in Section 3.2.3, the procedures applied in the dodecyl polyglycoside synthesis method were repeated with octanol, decanol, and an octanol/cetyl alcohol mixture adjusted to 80/20 (w/w) by weight, instead of 1-dodecanol. The synthesis of four different alkyl group polyglycosides was made by taking the ratios of butyl glycoside and other alcohols as ½ by weight, respectively. The FTIR spectra of these four different alkyl polyglycosides are given in Figure 6, respectively.

FTIR spectroscopy analysis provides information about the presence of functional groups present in the molecule. The vibration of each functional group is observed at different wavelengths. The approximate frequencies at which organic functional groups (such as C=O, CH3, C≡C) absorb IR radiation can be calculated from the atomic masses and the bond constant between them. These are called group frequencies and can change when one or both atoms in the group are affected by other vibrations. The frequency ranges and bond vibration properties of organic groups of synthesized APGs are given in Table 4.

Figure 6. FTIR spectrum of octyl, decyl, and cetyl/octyl polyglycoside.

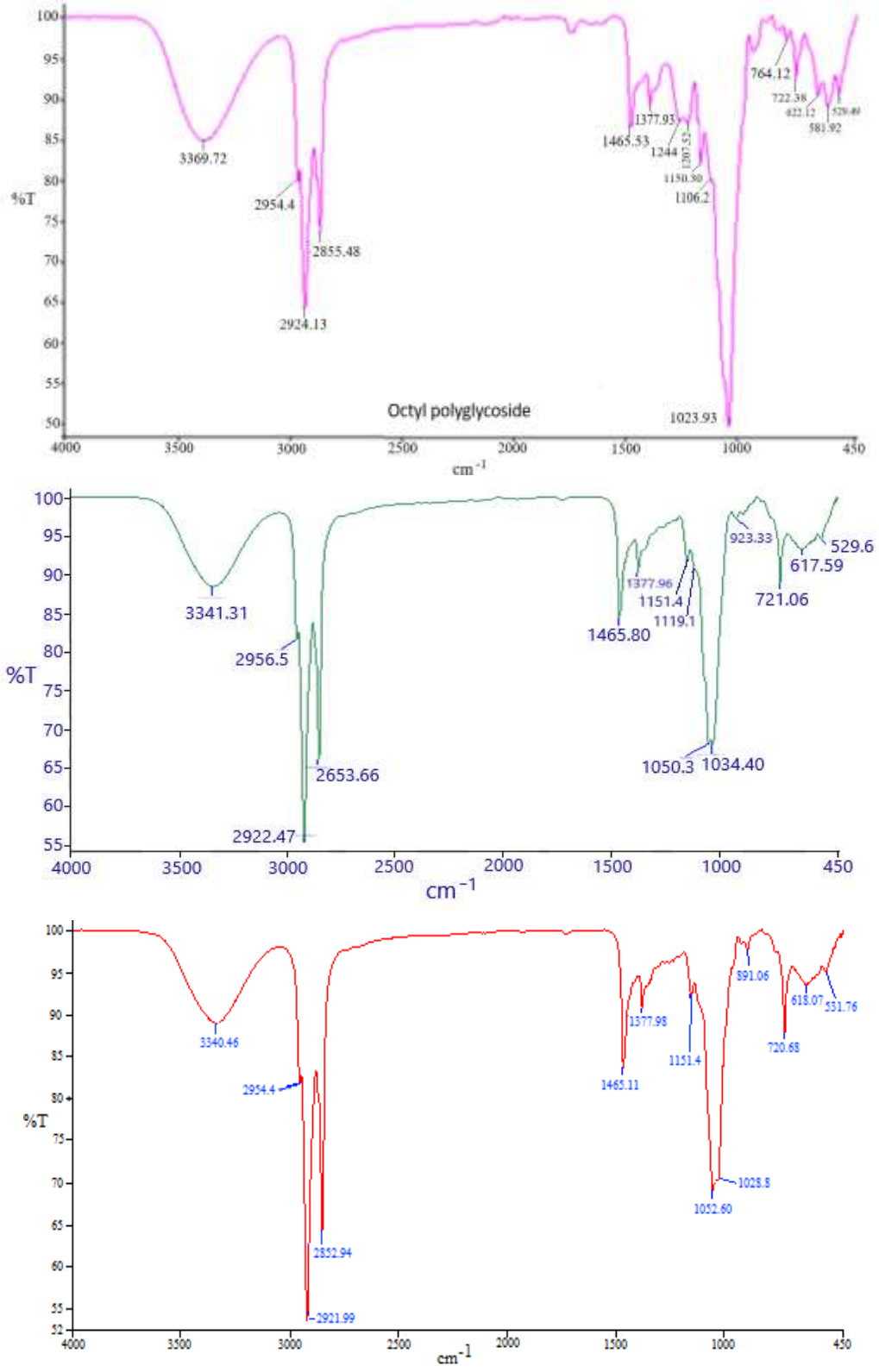


Table 4. Characteristic peaks of the prepared alkyl polyglycosides (APGs).

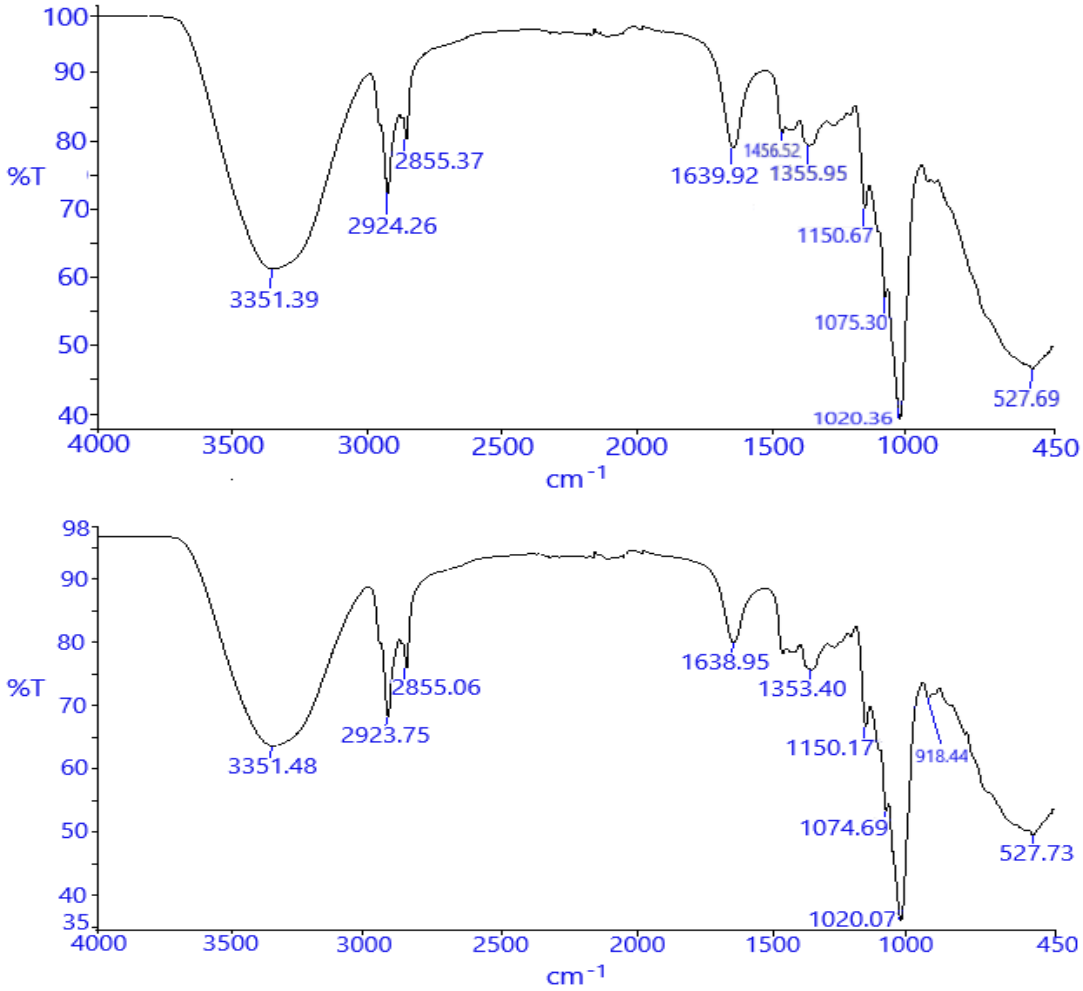
Function group	Wave Number (cm^{-1})		
	Octyl polyglycoside	Decyl polyglycoside	Cetyl/Octyl polyglycoside
CH_2			
Multiple $(CH_2)_n$ rock	722.38	721.06	720.68
Asymmetric bending	1465.3	1465.80	1465.11
Symmetric stretch	2855.48	2653.66	2852.94
Asymmetric stretch	2924.13	2922.47	2921.99
CH_3			
Symmetric bending	1377.98	1377.96	1377.97
Asymmetric bending	1465.13	1465.80	1465.11
Symmetric stretch			
Asymmetric stretch	2954.4	2956.50	2954.4
O-H	3369.72	3341.31	3340.46
C-O	1023.93	1034.40	1052.60
Ether linkage	1150.30	1151.40	1151.40

It was observed that the O-H absorption wave number of octyl polyglycoside was 3369.72 cm^{-1}, the O-H group of decyl polyglycoside was 3341.31 cm^{-1} and the OH group of cetyl/octanol polyglycoside was 3340.45 cm^{-1}. Sukkary *et al*., 2007, stated that the wavenumber of this absorption O-H group varies between 3200-3400 cm^{-1} and the absorption wavenumbers of the ether groups (C-O-C), which is the main component in the group of alkyl-polyglycosides, occur within 1120-1170 cm^{-1}. The wavenumbers of both O-H and ether groups of these three synthesized alkyl polyglycosides are within the limits specified in the literature. The FTIR results of these three APGs show that ether groups (C-O-C) have functional groups together with fatty alcohols, the O-H group has a hydrophobic structure, and APG is synthesized through the hydroxyl groups of glucose.

Two commercially available APG samples were provided to compare the alkyl polyglucose structure we synthesized. FTIR spectra of two different products, whose trade names are Triton and Milcoside, were taken and it was investigated whether there was a structural similarity with the products we synthesized. The FTIR spectra of these products are given in Figure 8, respectively.

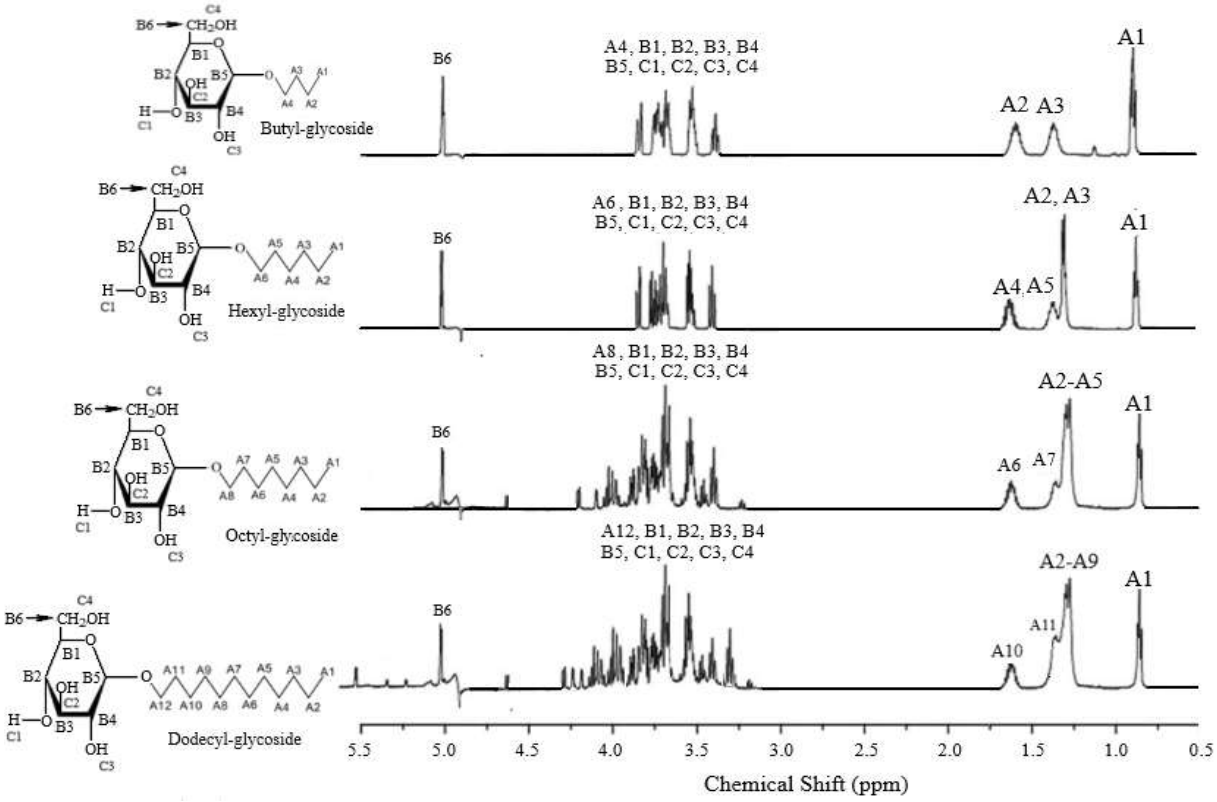
It is seen that there are very close similarities between the commercial alkyl polyglycosides and the FTIR spectra of the synthesized alkyl polyglycosides. While the ether (C-O-C) groups in the APG we have synthesized have a wave absorption of 1149.20, the ether (C-O-C) groups of the commercial APGs have a wave absorption of 1150.17 and 1150.67 cm^{-1}. When the wave absorptions of the OH groups are compared, the wave absorptions of commercial APGs are 3351.39 and 3351.48 cm^{-1}, while the wave absorption of the synthesized APGs is 3339.8 cm^{-1}. The formation of ether groups indicates that the synthesis between glycosides and fatty alcohols has taken place and the structure of hydrophobic groups has been formed, whereas the OH groups indicate the hydrophilic groups of APG.

Figure 8. FTIR spectrum of Triton BG 10 and Milkoside 101 alkyl polyglycoside.



Four different poly alkyl polyglycoside (Butyl, Hexyl, Octyl, and Dodecyl polyglycoside) products were dissolved in chloroform-d ($CDCl_3$) solvent and their ^1H NMR spectrum is shown in Figure 9. The peaks of δ 0.875, 1.250, and 1.600 ppm belong to the three protons of the methyl group (A1), the sixteen protons of methylene group (A2, A3, A4, A5, A6, A7, A8, A9), and the four protons of the methylene group (A10, A11), respectively. The one proton of the glucose ring (B6) linked to the hydroxyl group of 1-dodecanol is shown in the peak of δ 5.080 ppm. The peaks in the range from δ 3.840 to 4.310 ppm are a result of the other twelve protons (A12, B1, B2, B3, B4, B5, C1, C2, C3, C4). The peaks of the ^1H NMR spectrum are in accordance with the molecular structure of dodecyl polyglycosides (Chen *et al*., 2019).

Figure 9. [1]H NMR spectrum of the butyl polyglycoside, hexyl polyglycoside, octyl polyglycoside, and dodecyl polyglycoside poliglucosides.



3.5. Tests Applied to Synthesized Products

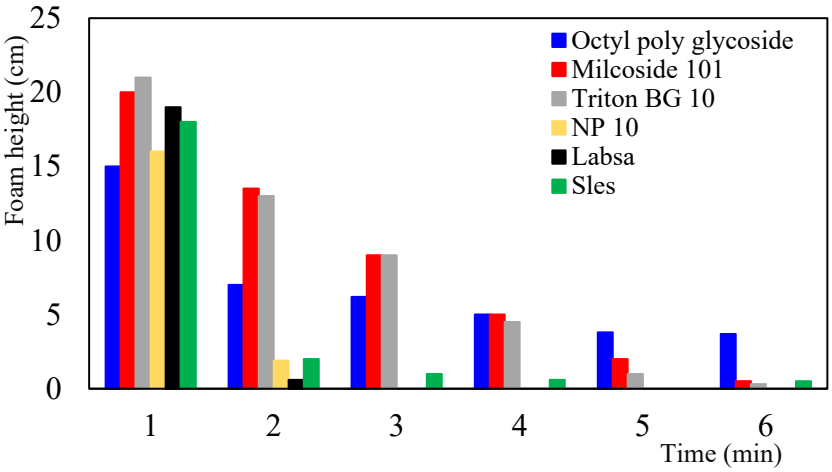
3.5.1. *Foam test*

In this test, the foam characteristics of the synthesized alkyl polyglycoside and five different surfactants were compared. The tests were carried out on the same day and time zone, with the same measuring tape, using distilled water from the same container. Surfactants used in the tests; SLES (Sodium lauryl ether sulfate) Galaxy Surfactant Ltd. company, Milcoside 101 (APG produced with the octanol-decanol mixture) Elotan brand commercial purity product, Triton BG 10 (APG produced with octanol-decanol mixture) Triton brand commercial purity product, NP10 (Nonylphenol ethoxylate) Tergitol brand commercial pure product, LABSA (Linear alkylbenzene sulfonate) Hayat Kimya is commercially pure products. The results obtained as a result of the tests are given in Table 5 and Figure 10.

Table 5. Variation of foam amounts according to time as a result of foam test.

Surfactants	Time (min)						
	0	1	2	3	4	5	Foam height (cm)
Oktyl polyglikoside	15	7.0	6.2	5.0	3.8	3.7	
Milcoside 101	20	13.5	9.0	5.0	2.0	0.5	
Triton BG 10	21	13	9.0	4.5	1.0	0.3	
NP 10	16	1.9	0.0	0.0	0.0	0.0	
LABSA	19	0.6	0.0	0.0	0.0	0.0	
SLES	18	2.0	1.0	0.6	0.5	0.5	

Figure 10. Graphical comparison of foam test results.



Considering the foam formations in the first moment of our tests, commercial APG samples gave the highest results. When the foam structure is compared with the well-known and most widely used alternatives such as SLES, LABSA, NP10, the foams of APGs are more stable, small, and late-extinguishing, while the foam structures of the others are large and less durable.

Although the foam heights of the products we obtained as a result of our synthesis studies are not as high as the commercial alternatives, better results were obtained in terms of foam permanence. The reason for the foam height difference between commercial APGs and synthesized APGs is thought to be the residual alcohols they contain. Alcohols generally affect the effects of foaming agents negatively and prevent foam formation.

The foam structure and the suitability of its permanence vary according to the sector and the product in which surfactants are used. While it is important that the foam is very permanent in products such as hand washing detergents, shampoos, and lipstick, the same situation is undesirable in textile chemicals and machine detergents used at home.

Shampoo formulations can be given as an example of situations where foam is particularly desired. In such products, the consumer wants a dense and creamy foam. Ether sulfates (especially SLES) have a foamy structure that goes out quickly. Therefore, a second co-surfactant is used to increase foam and viscosity properties in shampoos formulated with SLES. These foam boosters interact with the primary surfactant, reducing the electrostatic repulsion between the foam molecules by affecting the micelle structure. Thus, more permanent foams are formed.

3.5.2. *Hydrophility test*

Hydrophility is an important property sought in the fabric to be processed, especially in the textile industry. It is essential to add hydrophilicity to the fabric, which is handled as raw, in the pre-treatment processes. Because cotton, which is woven in its natural state and strengthened with sizing agents, is in a hydrophobic state due to natural oil residues and sizing agents. During the pre-treatment process, surfactant combinations, which are used under the name of "Wetting agents", take part in ensuring the penetration of caustic and peroxide into the fabric with water. Wetting agents are produced and used as a mixture of several surfactants, not from a single surfactant. In many biological and industrial applications, surfactant mixtures exhibit superior properties and superior micelle aggregation compared to their individual components.

The octyl glycoside product we obtained as a result of our synthesis studies was compared with SLES, NP10, LABSA, and the commercial equivalents of our product, Milcoside 101 and

Triton BG 10. The data obtained as a result of the study are given in Table 6 below. Considering the ionic character from the values given in the table, it is obvious that the products we obtained have a wetting effect similar to NP10 in a non-ionic structure.

Table 6. Hydrophility test results.

Surfactant	Time (sn)
SLES	65
NP10	30
LABSA	37
Milcoside 101	143
Triton BG 10	96
Decyl polyglycoside	30
Dodecyl polyglycoside	290
Octyl polyglycoside	23
Octyl/cetyl polyglycoside	<300

If we compare the synthesized products among themselves, as the carbon number of the alkyl group increases, the wetting ability decreases. The reason for this can be thought of as the difficulty of penetrating the large-molecule surfactant molecules, which facilitate the relationship between these two, by penetrating the oil-water interface.

4. DISCUSSION and CONCLUSION

The products obtained by this study are examined in terms of foam quality, which is the main feature possessed by surfactants, and their contribution to the hydrophility in the area they are used. All results show that their behaviors comply with described surfactant behaviors in the literature and they have properties close to other ready-made surfactants in the comparisons.

First of all, the structures of the obtained products were determined by FTIR measurements, and the results were compared with some commercial APGs. As a result of the comparison, it was seen that the synthesized products gave FTIR values close to the commercial products, and their structures were confirmed. Afterward, the synthesized products were evaluated with other commercial surfactants in terms of foam quality and hydrophilicity to the textile material. As a result of the foam tests, it has been observed that the foams of APGs are more stable, small, and late extinguishing, while the foam structures of other surfactant materials are coarse and their strength is less.

If the synthesized products are compared among themselves in terms of the hydrophilicity imparted to the textile material, it can be concluded that the wetting ability of the alkyl group decreases as the carbon number increases. As a result of the comparison of commercial APGs with other commercial surfactants, it was observed that they were weaker in terms of wetting ability.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Ramazan Donat: Investigation, Resources, Visualization, Software, Formal Analysis. **Volkan Demirel**: Writing-original draft, Methodology, Supervision.

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Research Article

Curvularia lunata: A fungus for possible berberine transformation

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Abstract: The prevalence of multidrug-resistant microorganisms results in an urgent need for the development of new antimicrobial agents or new treatment strategies. In this sense, plants serve different alternatives. Berberine, a plant-derived compound, is one of the alkaloids known to display antimicrobial activity against several types of microorganisms, while its being a substrate of various efflux pumps causes a decrease in its efficacy. Biotransformation makes it possible to obtain novel or more effective compounds with only minor structural modifications using enzyme systems. In this study, biotransformation of berberine by *Curvularia lunata* was examined. The working concentration of berberine was determined by observing the microbial growth on agar plates. The concentration of residual berberine in the media was analyzed by HPLC. In addition, laccase and beta-glucosidase enzyme activities were followed for their possible roles during the biotransformation of berberine. The results show that at the end of 14 days, *C. lunata* consumed 99% and 87% of berberine with the initial concentrations of 0.35 mg/mL and 0.5 mg/mL, respectively. Enzyme activities were not affected significantly. Since the concentration of berberine decreased, the biotransformation of berberine by *C. lunata* could be mentioned. Monitoring of biotransformation products plays a crucial role in discovering novel antimicrobial compounds and new valuable molecules.

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Antimicrobial resistance.

1. INTRODUCTION

Biotransformation is defined as the process in which biological systems (cells or enzymes) convert chemical compounds into structurally related products (Eliwa *et al.*, 2021; Liu & Yu, 2010; Sultana, 2018). It has numerous advantages over chemical methods/organic synthesis such as having high regio-/stereo-/enantiospecificity, mild process conditions, and lower costs and being environmentally friendly (Rozzell, 1999; Sultana, 2018). Biotransformations are generally composed of acetylation, esterification, glycosylation, hydrolysis, hydroxylation, isomerization, methylation, oxidation, and reduction reactions which can result in the formation of several intermediates and final compounds. These products of biotransformation have been used in agrochemical, food, pharmaceutical, and other industries for centuries (Fura, 2006; Giri *et al.*, 2001; Pervaiz *et al.*, 2013). Additionally, biotransformation reactions are applied for the

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specific conversion of natural compounds such as alkaloids, steroids, and terpenoids using different catalysts like plant cells, microbial cells, or isolated enzymes to obtain their derivatives (Liu & Yu, 2010). Microbial biotransformation is an important part of white biotechnology and gains prominence in the pharmaceutical industry due to its numerous advantages including low-cost and simple repetitive processes, large amounts of biomass production in a short time, novel, more active, or less toxic products from natural or synthetic compounds, and ease of scale-up (Bianchini *et al.*, 2015).

Antimicrobial resistance has been regarded as one of the most important health concerns and natural products are promising candidates to overcome this problem with their divergent structures and multi-target properties (Avci *et al.*, 2018). In addition, natural products are valuable sources of drug leads. Biotransformations have become crucial for the structural diversification of natural compounds and led to optimization in drug discovery and development (Venisetty & Ciddi, 2003). The biotransformation of many natural products including phytosterols, steroids, terpenes, alkaloids, and flavonoids by different bacteria and fungi has been reported in the literature (Bukvicki *et al.*, 2021).

In the light of given information, this current study aims to examine the biotransformation of berberine by the fungus *Curvularia lunata*, a microorganism preferred for biotransformation due to its capacity to transform natural substrates (Collins *et al.*, 2001; Schmeda-Hirschmann *et al.*, 2004). No work about the biotransformation of berberine by *C. lunata* has been found during our literature research.

2. MATERIAL and METHODS

2.1. Chemicals and Microorganism

Berberine chloride hydrate (CAS No. 141433-60-5) and all other chemicals were purchased from Sigma-Aldrich.

Curvularia lunata ATCC 12017 was obtained from the American Type Culture Collection (Manassas, Virginia, US).

2.2. Effect of Berberine on *C. lunata* Growth

To determine the effect of berberine on the growth, the radial growth of *C. lunata* was followed. Cut mycelial discs (1x1 cm) from 7-days grown fungi were placed at the center of potato dextrose agar (PDA) plates containing different berberine concentrations (0, 0.1, 0.35, 0.5, 1, 2 mg/mL). The growth was followed for 14 days at 24 ^{o}C and expressed in mm by measuring the diameter of the colony.

2.3. Biotransformation of Berberine

The biotransformation experiments were carried out in 50 mL of potato dextrose broth (PDB) inoculated with 7-days grown *C. lunata* on 1x1cm agar discs. Berberine was added to 3-days grown cells in PDB with a final concentration of 0.35, 0.5, and 1 mg/mL. Cells were incubated at 24 ^{o}C and 114 rpm for 14 days. The control culture was grown without berberine under identical conditions.

2.4. Analysis of Residual Berberine Amount Using HPLC

The concentration of the residual berberine in media was monitored by high-performance liquid chromatography (HPLC) system with a reverse-phase Poroshell 120® C18-EC (50 × 4.6 mm i.d. and 2.7-µm-film thickness) column. The column temperature was 30 °C and the injection volume was 20 µL. A solution of Acn:H_2O (1:9) was used as the mobile phase at a flow rate of 0.6 mL/min. Samples collected after the 0^{th}, 8^{th}, and 14^{th} days of biotransformation were filtered through a 0.22 µm pore size syringe filter and injected into HPLC. The analyses were carried out using at least three replicates.

2.5. Enzymatic Studies

Laccase and beta-glucosidase activities were measured to examine their effects on the biotransformation of berberine at different concentrations (0-1 mg/mL). Samples were collected after 0, 8, and 14 days of incubation. The mycelia were separated from the fungal culture using Whatman filter paper and then the culture was filtered through a 0.22 µm pore size filter. Culture broth without berberine was used as the control. Enzyme activities were measured using at least three replicates.

2.5.1. *Laccase activity assay*

Laccase activity was determined by measuring the oxidation of the substrate 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) - ABTS. The assay mixture containing 950 µL acetate buffer (0.1 M, pH 4.5), 200 µL ABTS (15 mM), and 50 µL sample was incubated at room temperature for 30 min. The absorbance was read at 420 nm using spectrophotometer. One unit (U) of laccase activity was defined as the enzyme amount which oxidizes 1 µmol of ABTS per minute under the assay conditions.

Laccase activity was calculated through the equation below:

$$U/L = [(\Delta A/t) / \varepsilon.d] \times (1 \times 10^6 \text{ µmole/mole}) \times (V_t/V_s)$$

ΔA: Absorbance change at 420 nm (ΔOD: OD_{assay}-OD_{blank})
t: Reaction time (30 min)
ε: Extinction coefficient of the substrate (36000 M^{-1} cm^{-1})
d: Lightpath (1 cm)
V_t: Total reaction volume (1.2 mL)
V_s: Sample volume (0.05 mL)

2.5.2. *Beta-glucosidase activity assay*

Beta-glucosidase activity was measured using 4-Nitrophenyl β-D-glucopyranoside – pNPG – as the substrate. The assay mixture containing 800 µL acetate buffer (0.1 M, pH 4.5), 100 µL pNPG (10 mM), and 100 µL sample was incubated at 45 °C for 15 min. After the addition of 1 mL Na_2CO_3 (1 M) to the mixture to stop the reaction, the absorbance was read at 420 nm. One unit (U) of beta-glucosidase activity was defined as the enzyme amount required to release 1 µmole of pNP (p-Nitrophenol) per minute under the assay conditions.

Beta-glucosidase activity was calculated through the equation below:

$$U/mL= [(\Delta A/t) / \varepsilon.d] \times (V_t/V_s)$$

ΔA: Absorbance change at 420 nm
t: Reaction time (15 min)
ε: Extinction coefficient of the substrate (18.1 cm^2/µmole)
d: Lightpath (1 cm)
V_t: Total reaction volume (1 mL)
V_s: Sample volume (0.1 mL)

3. RESULTS and DISCUSSION

The prevalence of multidrug-resistant microorganisms causes a serious worldwide health crisis. The development of new antimicrobials or improvement of the effectiveness of current ones might be a solution to this alarming problem. Plant-derived substances are promising sources in antimicrobial drug design. Berberine is a valuable alkaloid in the search for effective and novel antimicrobial compounds with its antimicrobial activity against several types of

microorganisms. However, being a substrate of many multidrug efflux pumps in microorganisms reduces its efficacy.

Biotransformation is a process used to develop metabolites with greater pharmacological activities. Minor structural modifications in the substances can be done through different reactions performed by enzyme systems (Bianchini *et al.*, 2015). Biotransformation is also considered to decrease the toxicity of a drug and transform it into a more polar and easily excreted metabolite in the pharmaceutical industry (Pervaiz *et al.*, 2013). In the current study, experiments for the biotransformation of berberine using *C. lunata* were performed.

3.1. Determination of Berberine Working Concentration and Addition Time

3.1.1. *Effect of berberine on C. lunata growth*

To determine the berberine working concentration, fungal growth on PDA plates containing 0, 0.1, 0.35, 0.5, 1, and 2 mg/mL concentrations of berberine was observed.

Figure 1. Radial growth of *C. lunata* in the presence of different berberine concentrations.

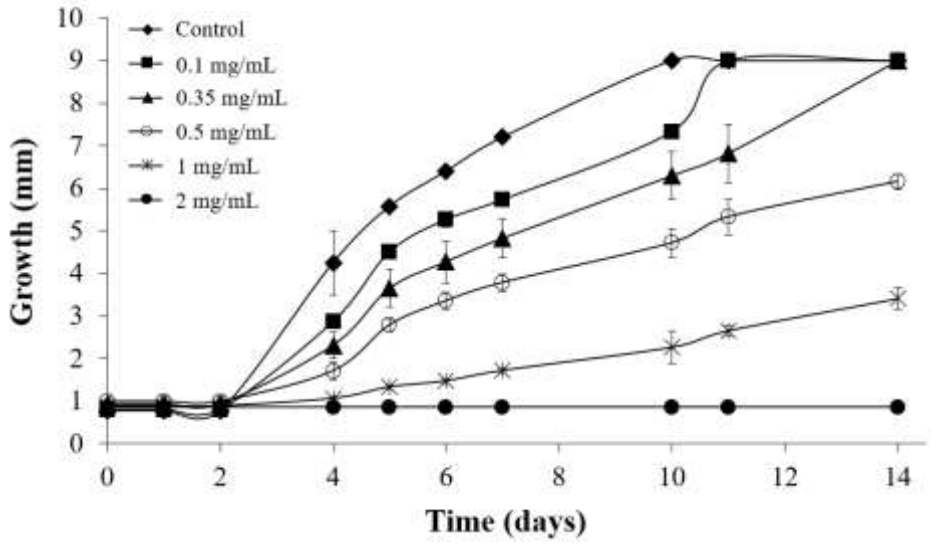


Figure 1 and Table 1 show that the growth (rate) in PDA plates decreased with the increasing berberine concentrations. At the end of 14 days of incubation, the PDA plates with 0.1 and 0.35 mg/mL berberine concentrations were covered with *C. lunata* completely although the growth was initially slower. The growth rate dropped to 50% with 0.5 mg/mL berberine and it was seen that the whole PDA plate was not covered with the fungal cells. It was observed that *C. lunata* growth was inhibited much when berberine concentration was ≥ 1 mg/mL. The radial growth was very slow at 1 mg/mL concentration while 2 mg/mL berberine completely inhibited the cell growth. The radial growth of the fungus increased with the longer incubation periods.

Table 1. Radial growth rates ($mm.day^{-1}$) of *C. lunata* at different berberine concentrations.

Berberine Concentration (mg/mL)	Radial Growth Rate ($mm.day^{-1}$)
0	0.818
0.1	0.724
0.35	0.634
0.5	0.464
1	0.176
2	0

3.1.2. *Determination of the berberine addition time*

The chemicals used in biotransformation may inhibit cell growth. Thus, if these compounds were added to the media with the inoculum simultaneously, there would be no biomass to carry out the biotransformation. Therefore, the process initiation time was investigated. As expected, there was no growth when berberine was added to the fresh PDB together with the *C. lunata* cells. A reasonable growth was observed when berberine was added to 3-days grown cells.

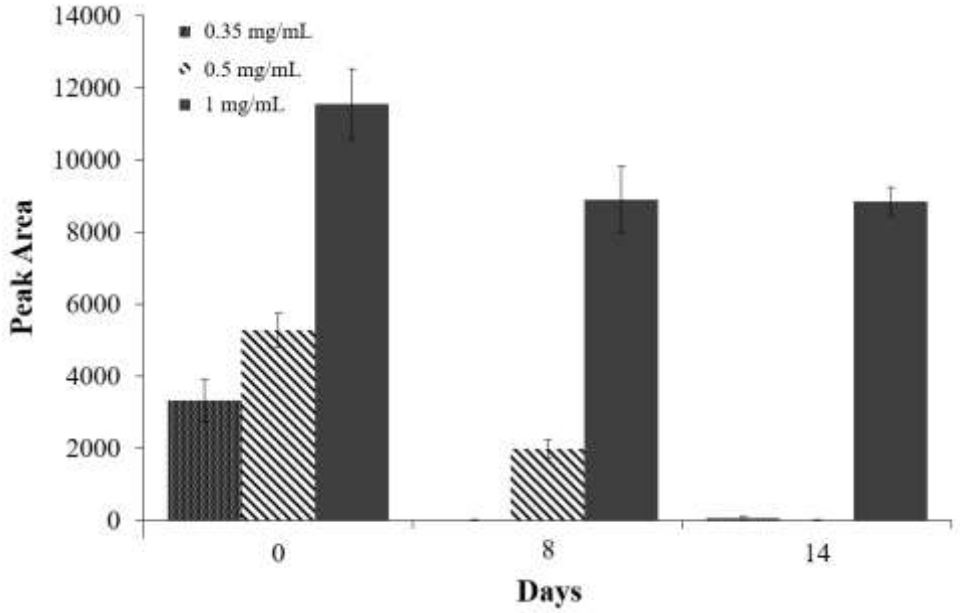
According to the results obtained, three concentrations, 0.35, 0.5, and 1 mg/mL, were selected as the working concentrations for the biotransformation experiments. Berberine was added to 3-days grown *C. lunata* cells and the disappearance of berberine was followed for 14 days.

3.2. Analysis of Biotransformation

The disappearance of berberine was monitored using HPLC. Samples with different berberine concentrations were prepared and they were injected into the HPLC system using Acn: H_2O (10:90) as the mobile phase. The retention time of berberine was determined to be between 3.5 – 4 min. Residual berberine amounts of the biotransformation reactions were monitored using the same procedure.

HPLC results showed that the concentration of berberine in PDB was effectively reduced by *C. lunata* if it was below 1 mg/mL. *C. lunata* cells degraded 99% and 87% of berberine with the initial concentrations of 0.35 mg/mL and 0.5 mg/mL, respectively, after 14 days of incubation. The change in berberine concentration was negligible with 1 mg/mL berberine because of the slow cell growth at this concentration. Only 15.7% of the berberine was degraded in the same period (Figure 2). The intracellular accumulation of berberine was negligibly small for all working concentrations.

Figure 2. HPLC analysis of residual berberine amounts for 0.35, 0.5, and 1 mg/mL berberine concentrations.



Additionally, thin layer chromatography (TLC) was applied to 14th day samples with initial concentrations of 0.35 and 0.5 mg/mL berberine. The results of the study confirm that *C. lunata* can degrade the available berberine. However, more interestingly, although berberine was consumed by the cells, no other alkaloid was observed as the biotransformation product after the HPLC analysis and TLC.

3.3. Enzyme Activities During Biotransformation

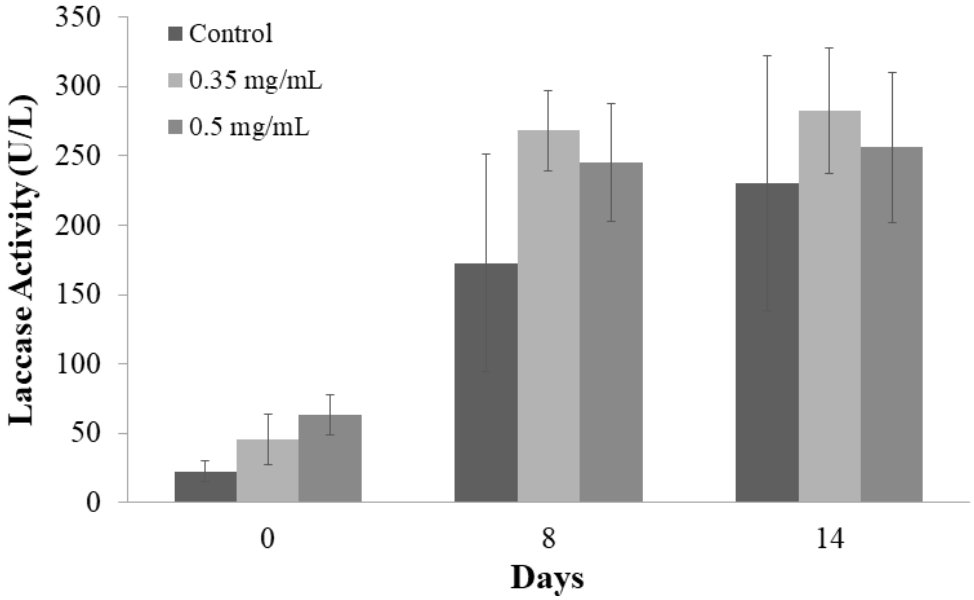
Biotransformation of chemicals is commonly achieved with the help of different enzymes synthesized by the cells and their concentrations/activities can give clues about the transformation pathway. Sing et al. (2017) investigated the biodegradation of ciprofloxacin by *Pleurotus ostreatus* through examining the effect of ciprofloxacin on the growth rate and enzyme activity. It was observed that ciprofloxacin had stimulated the enzymatic activity of the fungus (Singh *et al.*, 2017). *C. lunata* produces several extracellular enzymes including beta-glucosidase and laccase (Banerjee, 1992). In the light of this information, enzymatic assays were performed to research laccase and beta-glucosidase activities in our study. Since there was no significant change in the concentration for 1 mg/mL berberine, the effects of 0.35 and 0.5 mg/mL of berberine on laccase and beta-glucosidase activities in *C. lunata* were determined after 8 and 14 days of incubation.

3.3.1. *Laccase activity*

In a previous study, Coman et al. (2013) searched for laccase inducers in the *Chelidonium majus* extract including berberine (26 µg/mL). The results showed that berberine did not show any effects on the laccase activity of *Sclerotinia sclerotiorum* at all concentrations between 1% and 4% *C. majus* extract (Coman *et al.*, 2013). Motivated by this work, the change in laccase activity was investigated in our specific study as well.

When the results of the laccase activity assay were examined (Figure 3), no significant change in activity was observed at different concentrations of berberine. However, at 0.5 mg/mL berberine concentration, although there was a decrease in the growth rate up to 50%, the laccase activity was relatively higher than that in other samples. This might point out a correlation between laccase and berberine degradation. Besides, it should be kept in mind that laccase could be a part of the defensive mechanism of the microorganism, as reported previously (Coman *et al.*, 2013).

Figure 3. Effect of berberine on laccase activity.



3.3.2. *Beta-glucosidase activity*

The color interference of berberine during the measurement of beta-glucosidase activity has led to inconsistent results with high standard deviations. However, in general, these results indicate no significant changes in the extracellular beta-glucosidase activity.

4. CONCLUSION

In this study, berberine biotransformation by *C. lunata* was evaluated. 0.35 and 0.5 mg/mL berberine concentrations were selected as working concentrations based on the growth experiments. The change in the concentration of berberine was followed using HPLC. Since biotransformation reactions were carried out by the enzymes, laccase and beta-glucosidase activities were measured for their effects on the biotransformation of berberine. In addition, the samples were checked by TLC for the formation of possible products.

The results show that *C. lunata* consumed almost 100% of berberine after 14 days of incubation. No significant changes were observed in the laccase or beta-glucosidase activities. Although berberine was consumed by the cells, no spots regarding biotransformation products were detected on the TLC plates. Nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS) analyses could be performed to search for different biotransformation products of berberine. Monitoring these products will be helpful to enlighten the berberine biodegradation/biotransformation pathway(s) with the key enzymes which play important roles in the discovery of new valuable products and bioactive compounds.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Deniz Yilmaz: Performing the experiments and Writing. **Fatma Gizem Avci**: Writing, Editing, and Validation. **Berna Sariyar Akbulut:** Design of the study, Supervision, and Editing.

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Research Article

The effect of salinity stress on germination parameters in *Satureja thymbra* L. (Lamiaceae)

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Abstract: Salinity is an important problem all over the world. The destructive effect of salinity is observed from the seed germination stage. In this study, it was aimed to determine the effect of salinity on seed germination of the medically important *Satureja thymbra* L., whether pre-treatments are a factor in breaking the salinity stress, and to determine the level of salinity tolerance of this species. In the research, firstly, the seeds were exposed to two pre-treatments (80°C (5 minutes) + 10 ppm GA_3 (24 hours), 80°C (5 minutes) + 100 ppm GA_3 (24 hours)) and then 8 different NaCl concentrations (0.1 g/l, 1 g/l, 2.5 g/l, 5 g/l, 7.5 g/l,10 g/l, 15 g/l and 30 g/l) were tried. Germination seeds were counted every day and the effects of salinity on germination characteristics were investigated. The highest germination percentage (90%) was obtained at 0.1 g/l NaCl after 80°C (5 min.) + 100 ppm GA_3 (24 h.) pre-treatment. The results showed that the effect of salinity was significant on germination parameters in $p < 0.05$. Obtained results showed that the highest NaCl concentration at which *Satureja thymbra* seed could germinate was 10 g/l.

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Salinity,
Stress,
Pre-treatment.

1. INTRODUCTION

The world population is growing rapidly and is estimated to reach 9.7 billion by 2050. With the increasing population, the need for agricultural production also increases, it is inevitable that there will be an increase in the agricultural land allocated for food production, and it becomes necessary to produce even in unproductive lands. Today, only 37% of agriculture can be done due to problems such as drought, salinity and mineral deficiency (Bensidhoum & Nabti, 2021; Godoy *et al*., 2021; Leonardi *et al*., 2021; Turcios *et al*., 2021). Salinity is a major hazard in arid and semi-arid climatic regions and is an important limiting factor in global food production (Ahmed *et al*., 2020; Tolay, 2021). Desertification and high evaporation rate in arid and semi-arid areas cause rapidly salinization of soil and water (Bensidhoum & Nabti, 2021). Today, it is stated that there is a significant decrease in crop yield due to soil salinization worldwide and approximately 1125 million hectares of land are adversely affected by salt (Asgari & Diyanat, 2021; Karle *et al*., 2021). In many arid and semi-arid areas, groundwater aquifers are also saline. The trace amount of NaCl in the irrigation water increases the salinity in the arable land and

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salinity becomes more and more a problem every day (Chandel *et al.*, 2021; Neji *et al*., 2021; Tolay, 2021).

Salinity tolerance and response mechanisms differ according to many parameters such as salt exposure time, salt concentration, plant genotypes and environmental factors. In some plant species and varieties, stress factors cause a lot of damage, while in others, the level of this damage is less (Babalik & Göktürk Baydar, 2021; Tlahig *et al*., 2021; Tokarz *et al*., 2021). Since most crops are sensitive to salinity, an increase in salt content causes yield loss. Depending on the salt concentration in the environment, the decrease in yield varies between 10-50% (Godoy *et al*., 2021). Salinity also changes the physicochemical and biological properties of the soil. Both the osmotic stress that causes water scarcity and the ionic effect caused by the accumulation of ions have a negative effect on plants (Karabay *et al*., 2021).

Drought and high salinity have negative effects on important parameters such as seed germination, seedling growth, crop yield, food quality, etc. (Kang *et al.*, 2021; Liu *et al.*, 2021; Neji *et al*., 2021). Seed germination includes the process that begins with the seed's absorption of water and ends with the formation of radicles and is a critical stage in the reproduction of plant species (Jiang *et al*., 2021). Seed germination and seedling formation stages, which have vital importance in the plant, are the stages most affected by salinity in most plants (Fos *et al*., 2021; Khaldi *et al*., 2021). Salinity effects seed germination and plant growth as a result of biochemical events such as osmotic pressure imbalance, inhibition of the uptake of important plant nutrients, ion toxicity and production of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anions (Babaei *et al*., 2021; Luo *et al*., 2021; Mwando *et al*., 2021). The increase in salinity stress causes a delay in germination of the seed and a decrease in the germination percentage, and even concentrations above the tolerance threshold cause complete inhibition of germination (Moghaddam *et al*., 2020). Finding salinity-tolerant plants is one of the best solutions for improving agriculture in areas where salinity is detrimental to production (Al-shoaibi & Boutraa, 2021). In addition, it is necessary to develop appropriate methods to reduce the negative effect of salinity on seed germination in plants with low salinity tolerance. It has been stated that pretreatment of the seed with some substances such as plant growth regulators can stimulate some metabolic processes in germination and increase the performance of the seed under various environmental conditions (Ren *et al*., 2020).

Lamiaceae is a family with approximately 7173 species, mostly distributed in the Mediterranean basin. Many plants belonging to this family are used a lot in fields such as medicine, pharmacy, perfumery and culinary culture due to their fragrance and medicinal properties (Bouriah *et al*., 2021; Li *et al*., 2021; Sarıkaya *et al*., 2021). The genus *Satureja* L. belongs to the Lamiaceae family and consists of more than 200 species (Khalil *et al*., 2020). One of these species, *Satureja thymbra* L., is a xerophilous and heliophilous plant mostly distributed in the Eastern Mediterranean Basin (Pinna *et al*., 2021). *S. thymbra* is widely consumed as a tea by applying the infusion method, it is used in the form of decoction in gingivitis and in the kitchen due to its antiviral effect (Gürdal & Kültür, 2013; Roviello & Roviello, 2021). In the researches, it was also stated that this species is used in traditional medicine due to its antiseptic, antimicrobial, antifungal, anti-inflammatory, antidiarrheal, cardiotonic and blood purifying properties (Khoury *et al*., 2016). The feature that gives plants their medicinal properties is the essential oil contained in these plants and the substances found in their composition.

The essential oil of *S. thymbra* contains important chemical components such as *p*-cymene, *γ*-terpinene, thymol, carvacrol, β-caryophyllen, α-humulene (Khalil *et al*., 2020). It has been stated in studies that the essential oil obtained from this species has antifungal effects against *Mycogone perniciosa*, acaricidal effects against *Hyalomma marginatum*, insecticidal effects against *Culex pipiens*, and antibacterial effects against *Aeromonas salmonicida* (Cetin *et al*.,

2010; Dawood *et al*., 2021; Gea *et al*., 2021; Reis *et al*., 2021). It was emphasized that the essential oil of this species also showed an in vitro inhibitory effect against SARS-CoV and HSV-I replication (Khalil *et al*., 2020). In addition, it has been stated that the essential oil of this species has antimicrobial effect especially against *Chryseomonas luteola* and *Stenotrophomonas maltophilia* (Jafari *et al*., 2016). In another study, it was revealed that the essential oil was highly effective against *Pseudomonas fragi* and *Escherichia coli* when grown as a mixed biofilm with *Staphylococcus aureus* and *Listeria monocytogenes* (Chorianopoulos *et al*., 2008). It has been reported that emulsions enriched using *S. thymbra* maintain their phenolic content and oxidative stability at refrigeration temperatures (Choulitoudi *et al*., 2021). In another study, it was stated that the essential oil obtained from this species has an antinociceptive effect (Scuteri *et al*., 2021). Another study using *S. thymbra* essential oil concluded that this species can protect people against oxidative stress and amnesia without any side effects (Abd Rashed *et al*., 2021). The essential oil of this species is also used in muscle and joint pain, in the treatment of rheumatism, in arthritis and as a wound healer (Khalil *et al*., 2020). In addition to all these, it has antioxidant, cytotoxic, antidiabetic, insect repellant, herbicidial, antiplasmoidal and ovicidal effects (Giweli *et al*., 2012; Tepe & Cilkiz, 2016).

There is no study that determines the effect of salinity on seed germination of *Satureja thymbra,* which has high medicinal value. In this study, it was aimed to reveal the effect of salinity on seed germination of *S. thymbra*, to determine whether gibberellic acid seed priming reduces the negative effect of salinity and to contribute to future studies.

2. MATERIAL and METHODS

2.1. Sample Collection

The research was conducted in February 2021 at Department of Plant and Animal Production, Alaşehir Vocational School, Manisa Celal Bayar University, Turkey. The seeds of the *Satureja thymbra*, which constitute the study material, were collected from their natural habitats in Milas, Muğla, in July 2019. The collected seeds were stored in paper envelopes at +4°C in the refrigerator until the study was conducted.

2.2. Germination experiments

Before starting the experiment, seeds with similar characteristics were selected and surface sterilization was performed with 0.5% sodium hypochlorite solution for two minutes. Then the seeds were rinsed with deionized water and dried at room temperature. This study was planned in two groups, as the best results in the previous study (Oz, 2020) conducted with the same seed were obtained by exposing the seeds to 80°C for 5 minutes and keeping them in 10 ppm GA_3 and 100 ppm GA_3 for 24 hours. All seeds were kept in an oven at 80°C for 5 minutes and then the first group was kept in 10 ppm GA_3 solution for 24 hours, and the second group was kept in 100 ppm GA_3 for 24 hours. In order to determine the effect of salinity on germination, solutions containing 8 different concentrations of NaCl (0.1 g/l,1 g/l, 2.5 g/l, 5 g/l, 7.5 g/l, 10 g/l, 15 g/l and 30 g/l) were prepared after temperature and gibberellic acid pretreatments. Germination experiment was carried out at room temperature with 3 replicates and 10 seeds in each replicate. 10 seeds in each experiment were inserted to the three-layer filter paper and 5 ml of the salt concentrations to be applied were dropped on to them. Then the filter papers were rolled up and placed in a sealed plastic bag to prevent moisture loss. The seeds in the control group were only soaked with distilled water. Each roll of paper was changed every two days to prevent salt accumulation and the same procedures were repeated (Ergin *et al*., 2021). Germination was checked every day and all seeds with a radicle length of 2 mm were considered germinated. The obtained data were recorded every day. The germination experiment was continued for 28 days.

The following parameters were calculated using the necessary equations based on the data obtained. Germination percentage (GP): (Number of germinated seeds/Total number of seeds incubated)× 100 (Mwando *et al*., 2021), Germination speed (GS): $\sum(\frac{Gt}{Dt})$, Gt is the number of seeds newly germinated on day t and Dt is the number of days (Mzibra *et al*., 2021), Mean germination time (MGT): $\sum(D \times n) / \sum n$, n is the number of seeds newly germinated on day D and D is the number of days counted from the beginning of the test, and expressed as days (Mzibra *et al*., 2021), The first day of germination (day): was the day on which the first germination is observed (Adilu & Gebre, 2021), The last day of germination (day): was the day on which the last germination is observed (Adilu & Gebre, 2021), Germination tolerance index (GTI): (Number of seeds germinated under NaCl stress/Number of seeds germinated under deionised water) × 100 (Mwando *et al*., 2021).

2.3. Statistical Analyzes

The obtained data were subjected by one-way analysis of variance (ANOVA), the means were analyzed by Duncan's test at 5% level of significance and the correlation between the NaCl treatments and studied parameters was evaluated with Pearson's correlation coefficient using IBM SPSS Statistics 25 software.

3. RESULTS

This study was carried out in two groups and the data of each group is explained in detail with tables and graphs below.

3.1. 80°C (5 min) + 10 ppm GA_3 (24 h) treatment

In this treatment, 8 different NaCl concentrations were used and the effects of these concentrations on germination parameters such as germination percentage, germination speed, mean germination time, the first day of germination, the last day of germination and germination tolerance index were observed (Table 1 and Figure 1). As a result of the germination test, it was determined that there was no germination at 15 g/l and 30 g/l. The highest germination percentage (70%) was obtained from the second replicate of the control . When we compared the averages of germination percentages, it was determined that the highest germination percentage was in the control and the least germination percentage was in 10 g/l NaCl. The highest germination speed (57%) was observed in the second replicate of the control, and when the average germination speed were examined, the highest germination speed was in the control group and the lowest germination speed was obtained in 10 g/l NaCl.

Table 1. The effect of NaCl concentrations on seed germination of *Satureja thymbra* in 80°C (5 min) + 10 ppm GA_3 (24 h) treatment

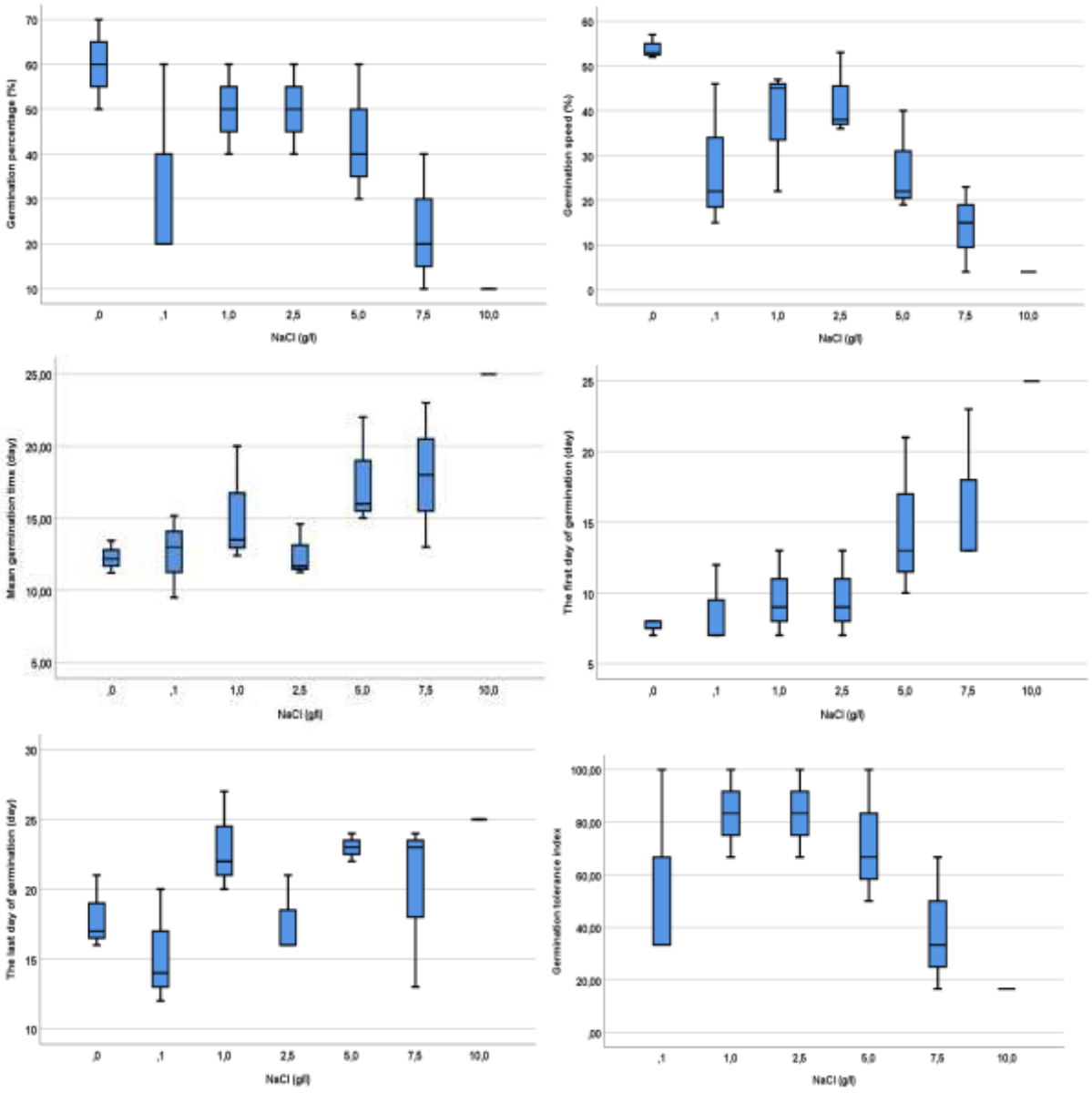
NaCl (g/l)	Germination percentage (%)	Germination speed (%)	Mean germination time (day)	The first day of germination (day)	The last day of germination (day)	Germination tolerance index
0.1	33.33±23.09a-c	27.67±16.26bc	12.56±2.86a	8.67±2.89a	15.33±4.16a	55.55±38.49ab
1	50±10cd	38±13.89cd	15.30±4.11a	9.67±3.06ab	23±3.61bc	83.33±16.66b
2.5	50±10cd	42.33±9.29cd	12.51±1.82a	9.67±3.06ab	17.67±2.89ab	83.33±16.66b
5	43.33±15.28b-d	27±11.36bc	17.67±3.78a	14.67±5.69ab	23±1bc	72.22±25.46b
7.5	23.33±15.28ab	14±9.54ab	18±5a	16.33±5.77b	20±6.08a-c	38.89±25.46ab
10	10a	4a	25b	25c	25c	16.67a
Control	60±10d	54±2.65d	12.27±1.12a	7.67±0.58a	18±2.65ab	-

*The data in the table are represented as the mean ± standard deviation, and different lowercase letters in the same column indicate significant differences between NaCl concentrations at $p<0.05$.

When compared in terms of mean germination time, the lowest mean (9.5) was determined at the second replicate of 0.1 g/l NaCl treatment. When we consider the mean of the replicates, it was observed that the lowest mean germination day was in the control and the highest was in 10 g/l NaCl. The first germination was observed on day 7 at the first replicate of the control, the first and second replicates of 0.1 g/l NaCl, the second replicate of 1 g/l NaCl and 2.5 g/l NaCl. When we examined in terms of the mean of replicates, the earliest germination was determined in the control group, and the latest was in 10 g/l NaCl. Final germination was observed on day 27, at the first replicate of 1 g/l NaCl treatment.

When we examined the average of replicates of the last day of germination, it was revealed that the last day of germination was earlier in the control and the latest in the treatment of 10 g/l NaCl. The highest germination tolerance index (100) was determined at the first replicate of 0.1 g/l NaCl treatment, and at the third replicates of 1 g/l NaCl, 2.5 g/l NaCl and 5 g/l NaCl treatments. When we consider the average values, the highest germination tolerance index was determined at 1 g/l NaCl and 2.5 g/l NaCl, and the lowest at 10 g/l NaCl.

Figure 1. The effect of NaCl concentrations on germination parameters of *Satureja thymbra* in 80°C (5 min) + 10 ppm GA_3 (24 h) treatment.



3.2. 80°C (5 min) + 100 ppm GA_3 (24 h) treatment

In this group, the effects of 8 different salt concentrations on seed germination parameters such as germination percentage, germination speed, mean germination time, first and last germination days and germination tolerance index were observed (Table 2 and Figure 2). As a result of the germination test, it was determined that there was no germination at 15 g/l and 30 g/l. The highest germination percentage (90%) was observed in the first replicate of 0.1 g/l NaCl treatment. When evaluated in terms of average germination percentage, the highest germination was determined at 0.1 g/l NaCl and the lowest at 10 g/l NaCl.

Germination speed was the highest (159%) in the control group. When compared in terms of average germination speed, the highest value was observed in 0.1 g/l NaCl treatment, and the lowest value was observed in 10 g/l NaCl. When compared in terms of mean germination day, it was determined that the lowest mean (4.17) was obtained from the third replicate of the control. In addition, when analyzed as the average of the replicates, it was observed that the lowest mean germination day was in the control group, and the highest was in the treatment of 10 g/l NaCl.

The first germination was observed at the first replicate of 1 g/l NaCl treatment and the third replicate of the control on the 3rd day. When we examined in terms of the mean of replicates, the earliest germination was determined in the control group, and the latest was in 10 g/l NaCl. Final germination was determined on the 28th day in the treatment of 10 g/l NaCl . When the data were evaluated in terms of the average of the replicates, it was observed that the last germination day was earlier at 0.1 g/l NaCl and later at 10 g/l NaCl . The highest germination tolerance index (168.86) was determined at the first replicate of 0.1 g/l NaCl treatment. When we consider the average values, the highest germination tolerance index was determined at 0.1 g/l NaCl, and the lowest at 10 g/l NaCl.

Table 2. The effect of NaCl concentrations on seed germination of *Satureja thymbra* in 80°C (5 min) + 100 ppm GA_3 (24 h) treatment.

NaCl (g/l)	Germination percentage (%)	Germination speed (%)	Mean germination time (day)	The first day of germination (day)	The last day of germination (day)	Germination tolerance index
0.1	76.67±11.55c	109.33±30.27c	6.89±1.23a	5.33±0.58ab	9±1.73a	143.84±21.67c
1	56.67±5.77bc	101±42.53c	6.55±2.54a	5±2ab	9.33±4.16a	106.32±10.83bc
2.5	63.33±20.82bc	92.33±31.94bc	7.67±1.40a	5ab	13.33±6.51ab	118.82±39.05bc
5	43.33±15.28b	45±1.73ab	10.36±4.36ab	7.33±2.52ab	13.67±6.03ab	81.30±28.66b
7.5	46.67±15.28b	39±17.58ab	13.79±2.95bc	9.33±2.08bc	18.67±2.31ab	87.55±28.66b
10	13.33±5.77a	10±6.93a	16.50±0.87c	13±5.20c	20±6.93b	25.01±10.83a
Control	53.33±11.55bc	108.33±44.79c	5.89±1.94a	4±1a	10±5.20a	-

*The data in the table are represented as the mean ± standard deviation, and different lowercase letters in the same column indicate significant differences between NaCl concentrations at $p < 0.05$.

After pre-treatment of 10 ppm GA_3 and 100 ppm GA_3, the effect of different NaCl doses on germination parameters was observed and it was determined that 100 ppm GA_3 pre-treatment slightly reduced the negative effect of NaCl (Figure 3).

Figure 2. The effect of NaCl concentrations on germination parameters of *Satureja thymbra* in 80°C (5 min) + 100 ppm GA_3 (24 h) treatment.

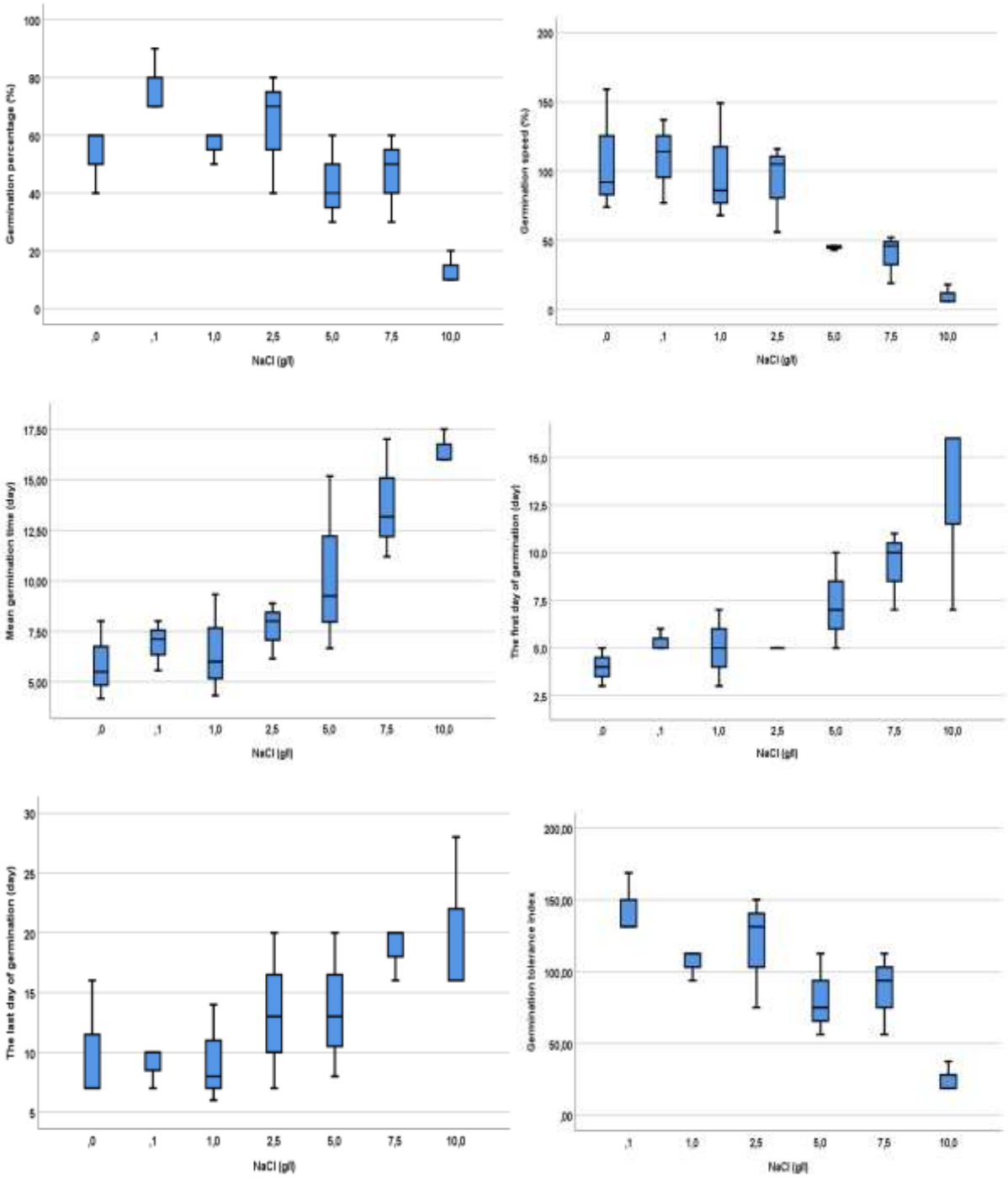
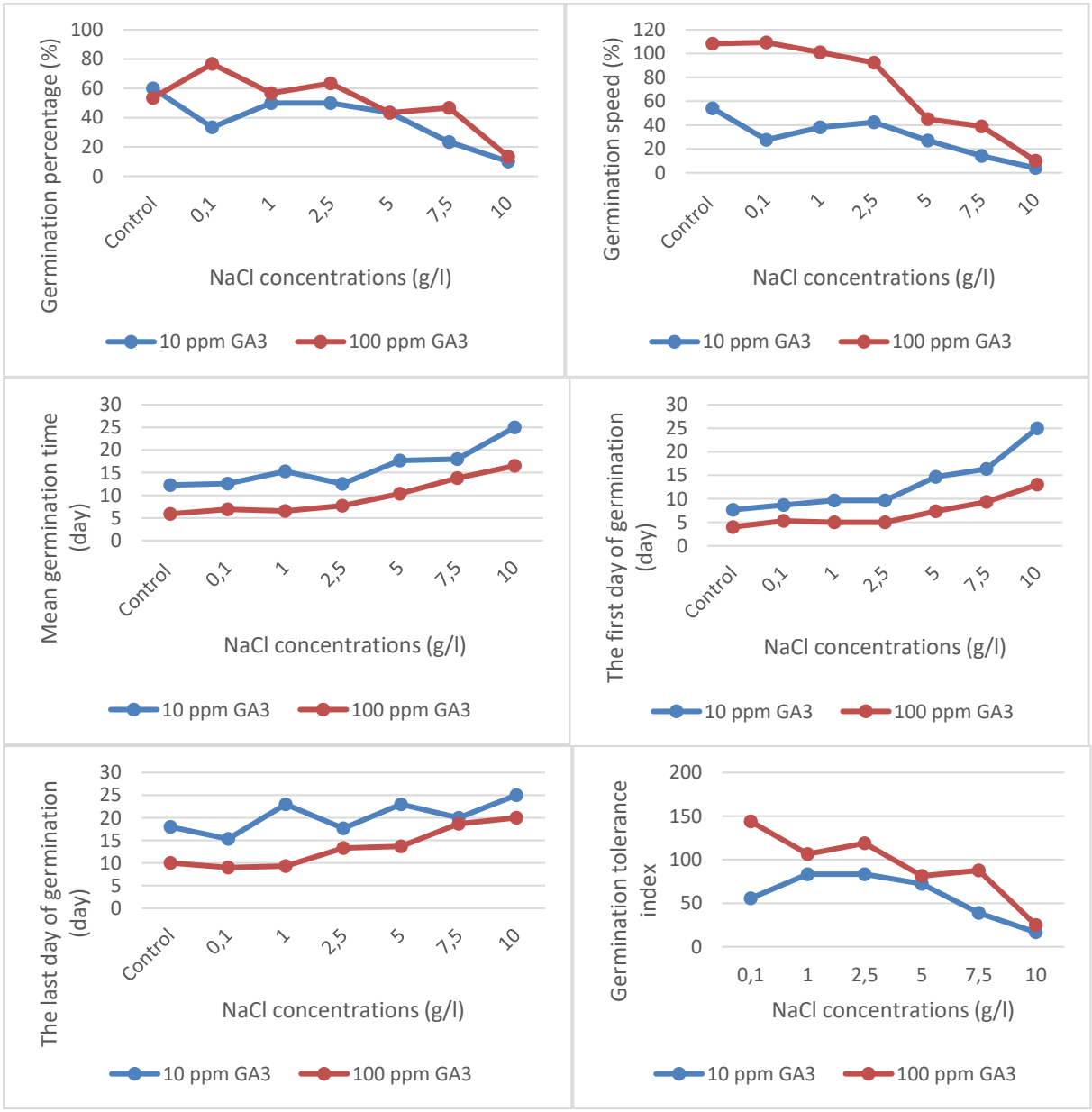


Figure 3. Comparison of the effects of different NaCl doses applied together with 10 ppm GA_3 and 100 ppm GA_3 pre-treatments on germination parameters.



3.3. Correlation Analyzes

According to the Pearson correlation coefficients between the NaCl treatments and studied parameters, there were positive and negative correlations. At 80°C (5 min) + 10 ppm GA_3 (24 h) treatment, a strong negative correlation was found between salt concentration and germination speed (r=-0.76; p<0.01), and also a moderate negative correlation was determined between salt concentration with germination percentage and germination tolerance index (r=-0.68; p<0.01, r= -0.62; p<0.01, respectively). In addition, while a strong positive correlation was determined between salt concentration with mean germination time (r= 0.79; p< 0.01) and the first day of germination (r= 0.85; p<0.01), a positive, moderate correlation was observed between salt concentration and the last day of germination (r= 0.50; p<0.05).

At 80°C (5 min) + 100 ppm GA_3 (24h) treatment, a strong negative correlation was determined between salt concentration with germination percentage (r=-0.74; p<0.01), germination speed (r=-0.82; p<0.01) and germination tolerance index (r=- 0.80; p<0.01). On

the other hand, there was a strong positive correlation between salt concentration and mean germination time (r= 0.87; p<0.01) and the first day of germination (r= 0.80; p<0.01), and a moderate positive correlation between salt concentration and the last day of germination (r=0.69; p<0.01).

4. DISCUSSION

With the rapid increase in the world population, the demand for food is increasing day by day. For this reason, it has become one of the urgent needs to increase the productivity of plants grown in salty areas and used for both food and treatment purposes and to find a solution to salinity stress (Shahid *et al*., 2021). Salinity tolerance during the germination and emergence stages is an important indicator of salt tolerance in other subsequent growth stages (Feghhenabi *et al*., 2021).

It is thought that seed priming can be used technically to increase the salt tolerance of plants (Bahmani Jafarlou *et al*., 2021). Seed priming treatment using hormone solutions is important in seed metabolism and it has been revealed that this application using herbal hormones such as auxin, cytokinin, gibberellic acid plays a role in the functioning of biochemical and molecular metabolisms that are involved in creating tolerance against abiotic stress (Rhaman *et al.*, 2021).

Gibberellic acid is an important hormone in defending the plant against stress conditions (Alharby *et al*., 2021). Studies have shown that externally applied gibberellic acid can increase seed germination and salt tolerance of seeds (Chauhan *et al*., 2019; Oral *et al*., 2019; Ali *et al*., 2021). In this study, it was determined that the negative effect of salinity could be reduced by gibberellic acid pre-treatment. In *Satureja thymbra*, it was observed that 100 ppm GA_3 application had more positive effects than 10 ppm on seed germination parameters and 100 ppm GA_3 was more effective in coping with salinity stress condition. In addition, even if the salt concentration increased (up to a certain concentration), the germination percentage was found to be higher in seeds with 100 ppm giberrelic acid pre-treatment compared to the control. It was observed that even giving 100 ppm giberrelic acid and 2.5 g/l NaCl to this species gave better results in terms of germination percentage compared to the control group. In a study conducted in *Hordeum vulgare* L. (Adjel-Lalouani *et al*., 2021), it was reported that when NaCl was applied together with GA_3, GA_3 attenuated the inhibitory effect of salinity on germination percentage and germination rate.

In present study, when the data were examined in terms of germination percentage, it was observed that as the salt concentration increased, the germination percentage decreased and germination was inhibited in *Satureja thymbra* at 15 g/l and 30 g/l NaCl concentrations. In *Satureja hortensis* L., which is in the same genus as the species used in the study, it was stated that increasing the salt concentration decreased the germination percentage (Nejatzadeh, 2021). The effect of salinity on seed germination of *Salvia hispanica* L., another species belonging to the Lamiaceae family, was tested and it was revealed that salinity stress minimized the germination percentage compared to the control (Younis *et al*., 2021). In the studies conducted with *Marrubium vulgare* L. and *Mentha pulegium* L., which are also members of the same family, it was concluded that the increase in salinity decreased the germination percentage (Nedjimi *et al*., 2020; Azad *et al*., 2021). In other studies on salinity (Akram *et al*., 2020; Dadaşoğlu *et al*., 2020; Dehnavi *et al*., 2020; Mondal *et al*., 2020; Singh *et al*., 2020; Wang *et al.*, 2020; Zhumabekova *et al*., 2020; Bahrabadi *et al*., 2021; De Rossi *et al*., 2021; El Hamdaoui *et al*., 2021; Shariatinia *et al*., 2021; Tonguç *et al*., 2021; Zeng *et al*., 2021), it has been reported that increasing the salt concentration decreases the germination percentage.

High salt concentration is a limiting factor for the germination process, reducing the amount of water available, affecting both germination percentage and germination speed *(*dos Santos *et al.*, 2019). In this study, it was observed that seeds exposed to NaCl concentrations generally

had a lower germination speed than control; however, when we compare the salt concentrations with each other, it was observed that the germination speed increased as the salinity increased up to 2.5 g/l NaCl in 10 ppm GA_3 application, and the germination speed decreased after this concentration. In 100 ppm GA_3 application, it was determined that the germination speed decreased as the salinity increased. If we compare the 10 ppm GA_3 and 100 ppm GA_3 treatments, it is seen that the germination speed is higher at 100 ppm, as expected. The reason for this is the breaking effect of gibberellic acid on salinity stress. It has been reported that the germination speed decreases as NaCl and KCl concentrations increase in *Camelina sativa* (L.) Crantz. (Yohannes *et al.*, 2020). It has been stated that increasing salinity decreases the germination speed in *Lens culinaris* Medic. (Ceyhan & Çakır, 2021). In a study with *Vigna umbellata* (Thunb.) Ohwi & H. Ohashi, 50 mM, 100 mM and 200 mM NaCl levels were tested and it was concluded that as the salinity increased, the germination speed decreased (Atta *et al*., 2021).

In present study, it was determined that the mean germination time increased as the salt concentration increased. When we examine Table 1, the reason for the sudden increase in the mean germination time at 1 g/l NaCl is that germination is observed even on the 27th day. When we examined the other data, it was concluded that the increase in salinity increased the mean germination time. In addition, it was observed that 100 ppm GA_3 pre-treatment reduced the negative effect of salinity and reduced the mean germination time compared to 10 ppm GA_3 pre-treatment. The reason for this, gibberellic acid is involved in inducing hydrolytic enzymes such as α-amylase and hydrogenase to initiate the germination process in the seed and accelerate the germination process (Aziz & Pekşen, 2020). In the study in which the effect of salinity on the germination of *Carthamus tinctorius* L. was observed (Tonguç *et al*., 2021), it was stated that the mean germination time increased as the salinity increased. In a study using *Lactuca sativa* L. (Alves *et al*., 2020), it was concluded that salinity increased mean germination time in seeds exposed to salt stress (NaCl) without any pretreatment. In another study, it was revealed that increasing salinity in *Chloris gayana* Kunth. had a negative effect on germination percentage and mean germination time (Daba *et al*., 2019). In a study conducted in *Avena sativa* L., it was concluded that salinity stress greatly effects parameters such as germination percentage and mean germination time (Kumar *et al*., 2021). Other similar studies (Melendo & Giménez, 2019; Ceritoğlu & Erman, 2020; Ku-Or *et al*., 2020; Székely *et al*., 2021) on this subject also support our results.

In this study, it was determined that generally increasing NaCl doses decreased the germination tolerance index and the germination tolerance index was higher in 100 ppm GA_3 treatment. The reason for this is the stress breaking effect of GA_3 as we mentioned in the previous parameters. In a study on this subject, it was revealed that increasing NaCl dose caused a significant decrease in the germination stress tolerance index (Ergin *et al*., 2021).In a study in which the effect of salinity on the germination of *Lolium perenne* L. was observed, it was concluded that the salt tolerance index decreased as the salinity increased (Kusvuran *et al*., 2015). In another study on this subject, it was determined that as the NaCl concentration increased, the Germination Stress Tolerance Index decreased (Marium *et al*., 2019).

It is thought that there is mostly a positive relationship between the response to salinity during the germination and seedling stages and the response to salinity in other growth stages of the plant, and therefore, the results in the germination and seedling stages can provide information about the salinity resistance of that plant (Güldüren & Elkoca, 2012). When the data were examined, it was observed that when 80°C (5 min) + 100 ppm GA_3 (24 h) was applied as a pre-treatment, even at 7.5 g/l NaCl concentration (0.75% NaCl), it was observed that approximately control germination was achieved, and it is thought that this species can be resistant to salinity.

5. CONCLUSION

In the study, it was determined that salinity has a negative effect on seed germination, but appropriate doses of gibberellic acid reduce the negative effect of salinity on parameters related to seed germination. In addition, it was observed that the resistance of *Satureja thymbra* seed to salinity was up to 10 g/l NaCl and higher amount of NaCl prevented germination.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

Authorship contribution statement

Ummahan Oz: Investigation, Methodology, Resources, Visualization, Software, Formal Analyzes, Validation and Writing original draft.

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Research Article

Acute toxicity, phenol content, antioxidant and postprandial anti-diabetic activity of *Echinops spinosus* extracts

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Abstract: *Echinops spinosus,* belonging to Asteraceae family, is used in folk medicine as an abortifacient and diuretic and for blood circulation, diabetes, stomach pain, indigestion and spasmolytic problems. The objective of this work is the study of acute toxicity, the content of phenolic compounds (polyphenols, flavonoids and tannins), antioxidant activity (DPPH, ABTS, FRAP, H_2O_2 and xanthine oxidase) and antidiabetic (α-amylase, α- glucosidase and lipase) *in vitro* and *ex-vivo* by studying the starch tolerance test. The phytochemical assay showed that the ethanolic extract is the richest in polyphenols, flavonoids and tannins with 77.01 mg GEA/g extract; 544.33 mg RE/g extract, and 32.20 mg EC/g extract, respectively. The ethanolic extract showed better antioxidant activity compared to the aqueous extract with (IC_{50}=13±0.25 µg/mL; IC_{50}=75.11±0.34 mg TE/g extract; IC_{50}=51.1±1.2 mg AAE/g extract; IC_{50}=28.2±2.87 µg/mL and 16.83 ± 0.72 µg/mL) in DPPH, ABTS, FRAP, H_2O_2 and xanthine oxidase. Extracts of *E. spinosus* have shown a remarkable inhibitory effect α-amylase and interesting inhibitory effect of α-glucosidase and lipase. The aqueous and ethanolic extract also lowered blood sugar levels to 0.96 and 0.93g/L, respectively, after 90 minutes in starch-loaded rats. Acute toxicity results indicate that *E. spinosus* extracts are non-toxic with an LD_{50} greater than 2 g/kg in female Swiss mice. Therefore, the antioxidant and anti-diabetic activity may be at the origin of the bioactive compounds contained in the plant *E. spinosus.* However, *in vivo* studies on the mechanism of action are needed against oxidative stress associated with diabetes.

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1. INTRODUCTION

Diabetes mellitus is a complex metabolic disease characterized by impaired metabolism of carbohydrates, fats and proteins (Soliman and Abd El Raheim, 2015). They most commonly affect children and adolescents in developed and developing countries. Type 1 diabetes is the

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result of a deficiency in insulin production due to dysfunction of the pancreatic β cells and type 2 diabetes is a consequence of a low sensitivity to insulin in the target tissues and/or insufficient insulin secretion (Musabayane, 2012). Several scientific reports showed that hyperglycemia is a risk factor for micro-vascular complications (nephropathy, retinopathy and neuropathy) and macro-vascular (stroke) (Burton-Freeman 2010; Marc *et al*. 2008). Moreover, among these common symptoms are frequent urine, thirst, and overeating (Bahmani *et al*., 2014)**.**

Postprandial hyperglycemia is a factor in the development of type 2 diabetes and complications, it has been shown to cause glucose toxicity and damage the function of pancreatic beta cells (Shelly *et al*., 2010). The acute changes in blood sugar during the postprandial phase cause a state of oxidative stress, and diabetes management should adjust these postprandial changes as well (Shihabudeen *et al*., 2011). On the other hand, among the therapeutic strategies to control the development of diabetic complications is to delay the absorption of glucose from the intestine by suppressing the activity of digestive enzymes namely α-amylase and intestinal transporters of α-glucosidase and glucose such as SGLT 1 and GLUT 2 (Yusoff *et al*., 2015)**.**

Currently, the mode of action of some antidiabetic drug are available, such as inhibiting hepatic production of glucose (biguanides), triggering insulin secretion (sulfonylureas and glinides), slowing down digestion and absorption of intestinal carbohydrates to adjust the postprandial glucose level (α-glucosidase and α-amylase inhibitors), restore the sensitivity of the insulin receptor and peripheral uptake of glucose (thiazolidinediones and metformin) or insulin (Elya *et al*., 2015). Studies have reported that the use of plants (rich in active compounds such as polyphenols) in food to prevent chronic disease can help to regulate biological pathways and antioxidant balance (Etoundi *et al*., 2010)**.**

E. spinosus of the Asteraceae family contains about 120 species distributed throughout the Mediterranean regions, Central Asia and tropical Africa. The species *spinosus* thrives in desert conditions with rainfall between 20 and 100 mm, and a wide range of soil, widespread on coastal dunes, sandy and gravelly to rocky surfaces (Bouzabata *et al*., 2018)**.** It is a perennial herbaceous plant, reaching nearly 1 m, characterized by upright brownish to reddish stems with few leaves, 10 to 15 cm long, is hairy, arachnoid, and bears very long spines (Helal *et al.*, 2020). In folk medicine, it is used as an abortifacient, diuretic and for blood circulation, diabetes, gastric disorders, indigestion and sposmolytic problems (Khedher *et al*., 2014). In Morocco, it is used to facilitate childbirth. A decoction of the roots in water or olive oil is applied to help pregnant women to expel the placenta. Also administer before birth to stimulate contractions. In Marrakech and Salé, a root decoction is used for stomach pains, indigestion and lack of appetite, as well as diabetes. In Casablanca, the whole plant, in powder or decoction, is used as a diuretic or depurative and to treat liver diseases (Agyare *et al*., 2013). Here, the aim of this study was to examine acute toxicity of *E. spinosus* extracts and determine phenolic content as well as to evaluate antioxidant and postprandial antihyperglycemic activities of these extracts.

2. MATERIAL and METHODS

2.1. Standards and Reagents

α-glucosidase from *Saccharomyces cerevisiae*, α-amylase from *Bacillus licheniformis,* p-Nitrophenyl-α-D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich (France), phlorizin hydrate (Sigma Aldrich, USA), Acarbose.

2.2. Plant Material and Extraction

The roots of *E. spinosus* were collected from Oujda Region, Morocco. The collected plant was deposited under the voucher number HUMPOM 10051 in the herbarium at University Mohammed I., Oujda (Morocco). The roots of *E. spinosus* were dried at room temperature, ground into a powder and kept in the shade until use. In this study, two types of extraction

(infusion and maceration) were used. Indeed, the aqueous extract was prepared by the infusion method, by which 30g of *E. spinosus* powder was infused with 300 mL of distilled water for 1 hour and left to cool. The extract was filtered and evaporated at 50 °C using a rotary evaporator. Subsequently the extract was lyophilized and stored for later use.

For the ethanolic extract, 30 g of the root powder was macerated for 48 hours with stirring and at room temperature. The extract was filtered and evaporated at 40 ° C using a rotary evaporator.

2.3. Determination of Phenolic Contents

2.3.1. *Total phenolic content*

The determination of the total polyphenols is carried out by the method of Folin-Ciocalteau reagents described by Spanos and Wrolstad (1990). The gallic acid standard range has been evaluated at different concentrations ranging from 1.95 to 31.25 µg / mL, and the results are expressed in milligrams of gallic acid equivalent per one gram of extract (mg EAG / g extract). Briefly, 2.5 mL of 10% (v / v) of Folin Cioalteu reagent was mixed with 0.5 mL of sample solution. Subsequently 4 ml of sodium carbonate Na_2CO_3 at 7.5% (W / V) are added. The reaction mixture was incubated at 45 ° C for 30 minutes and the absorbance against the blank was determined by at 765 nm.

2.3.2. *Total flavonoid content*

The flavonoid content was evaluated by the aluminum trichloride ($AlCl_3$) colorimetric method described by Dewanto *et al*. (2002). Briefly, 0.5 mL of the sample at a concentration of 1 mg / ml was mixed with 3.2 mL of distilled water and 0.15 mL of 5% (w / v) sodium nitrite solution $NaNO_3$. The mixture is left to stand for 5 minutes. Then 0.15 mL of $AlCl_3$ is added. After standing for 6 minutes, 1 mL of 1M NaOH was added, then the mixture was incubated at room temperature for 30 minutes. Absorbance was determined at 510 nm. Rutin was used as a standard at final concentrations ranging from 50 to 400 g/mL and results are expressed in milligrams of gallic acid equivalent per gram of extract (mg RE/g of extract).

2.3.3. *Total tannin content*

The tannin content was quantified by the vanillin method described by Julkunen-Tiitto (1985). Indeed, 50 µL of the sample or standard were mixed with 1.5 mL of 4% vanillin (prepared with methanol) then 750 µL of concentrated HCL were added. The mixture was stirred and incubated at temperature in the dark for 20 minutes. The absorbance was measured at 500 nm. The standard curve of the catechin was carried out under the same conditions and the results were determined in mg equivalent of catechin per gram of dry weight of extract (mg EC/g of extract).

2.4. Antioxidant Activity

2.4.1. *DPPH radical scavenging assay*

Antioxidant activity by the method of DPPH (2,2-Diphenyl-1-pierylhydrazyl) was achieved by the protocol of Huang *et al*., (2011). This method is based on the reduction of DPPH to DPPHH in the presence of radical scavengers. The extracts or the standard (BHT) were dissolved in a methanol solution of DPPH (0.02 M) and then incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517nm against a blank. The percentage of DPPH radical scavenging was calculated according to the following formula:

I% = [(Absorbance Negative Control- Absorbance Sample) / Absorbance Negative Control)] x 100

2.4.2. *Ferric reducing power assay* (FRAP)

The measurement of the ability to reduce ferric iron to ferrous iron was estimated according to the protocol described by Amarowicz *et al*. (2004). Briefly, 0.5 mL of the extracts were mixed with 1.25 mL of phosphate buffer solution (0.2M, pH 6.6) and 1.25 mL of 1% potassium

ferricyanide. The mixture was incubated at 50 °C for 20 minutes then 1.25 mL of 10% trichloroacetic acid was added in order to stop the reaction. The tubes are then centrifuged at 3000 rpm for 10 minutes. Then 1.25 mL of the supernatant was mixed with 1.25 mL of distilled water and 0.25 mL of $FeCl_3$ (0.1%, w/v). The absorbance was determined at 700 nm. Ascorbic acid is used as a standard and the final results are expressed in milligrams of ascorbic acid equivalent per gram of extracts (mg AAE/g of extract).

2.4.3. *Trolox equivalent antioxidant capacity* (TEAC) *assay*

The evaluation of the antioxidant capacity by the ABTS cation decolorization assay was estimated according to the method described by Tuberoso *et al*. (2013). The cationic radical ABTS was generated by oxidation of ABTS (2mM) with potassium persulfate (70mM) then the mixture was kept for 16 hours. The resulting solution was diluted with methanol to an absorbance of 0.70 at 734 nm. Subsequently, 2 mL of the diluted ABTS solution was mixed with 200 µL of each sample and allowed to react for 1 minute and the absorbance was measured at 734 nm. Trolox is used as a reference and sample results were expressed as milligram equivalent of Trolox per gram of extract (mg TE/g extract).

2.4.4. H_2O_2 *trapping test*

The antioxidant activity of H_2O_2 was estimated by the method described by Muruhan *et al*. (2013). Briefly, 1 mL of the sample or ascorbic acid was mixed with 0.6 mL of H_2O_2 (40 mmol / L), then the mixed were incubated for 10 min and the absorbance was measured at 230nm. The percent inhibition of H_2O_2 was calculated using the following equation:

$$\% = [(A_0 - A_1) / A_0] \times 100$$

Where A0 was the absorbance of the control, and A1 was the absorbance in the presence of the sample or standard.

2.4.5. *Xanthine oxidase inhibition test*

The method of Umamaheswari *et al*. (2007), was used to determine the percent inhibition of xanthine oxidase (xo). Allopurinol was used as a positive control. Indeed, 1mL of the sample was mixed with 1.9 mL of phosphate buffer (pH 7.5), 0.1 mL of enzymatic solution (0.2 unit / mL) and 1.0 mL of 0.5 mM xanthine solution. Subsequently, 1mL of 1M HCl was added after incubation for 15 minutes at 25 ° C. The absorbance was read at 295 nm and the results were calculated using the following formula:

$$I (\%) = [((Ac-Acb) - (As-Asb)/ (Ac-Acb)) \times 100]$$

Where Ac was the absorbance of control; Acb was the absorbance of control blank; as was the absorbance of sample; and Asb was the absorbance of sample blank.

2.5. Antihyperglycemic Activity

2.5.1. *α-Amylase inhibitory assay*

The inhibition of α-amylase was evaluated by the starch-iodine method as described by Chakrabarti *et al*. (2014) with certain modifications. Briefly, 250 µL of the sample or standard (Acarbose) and mixed with 100 µL of the phosphate buffer solution (20mM, PH 6.9) containing the α-amylase enzyme. The mixture was incubated at 37 °C for 10 min, then 600 µL of starch substrate (1%) was added and the mixture was re-incubated at 37 °C for 10 min. At the end of the reaction, 250 µL of the HCl solution and 100 µL of iodine were added. Absorbance was determined by spectrophotometer at 630 nm. The results were calculated as a percentage according to the following formula:

$$\text{Inhibition } (\%) = (1-(OD_{\text{Test sample}}/OD_{\text{Control}})) \times 100$$

2.5.2. *α-Glucosidase inhibition assay*

The inhibitory activity of α-glucosidase was determined by the protocol described by Kee *et al*. (2013). In fact, 150 µL of sample solution or acarbose were mixed with 100 µL of the α-glucosidase enzyme. (0.1U), the reaction is incubated for 10 minutes, 200 µL of ρ-nitrophenyl-α-D-glocopyranoside (pNPG) substrate were added, Then, a second incubation was carried out for 30 minutes, and at the end of the reaction 1 ml of 0.1 M Na_2CO_3 was added. The absorbance was determined at 405 nm.

The percentage of inhibition was calculated according to the following formula:

$$\text{Inhibitory activity (\%)} = [OD_{Control}\text{-}OD_{Test\ sample})/OD_{Controle}] \times 100$$

2.5.3. *Lipase inhibition assay*

Lipase inhibitory activity was determined by Hu *et al*. (2015) with some modifications. Briefly, 150 µL of the extract or orlistat were mixed with 500 µL of Tris-HCl buffer (1mM, pH8) containing the lipase enzyme (2U), the reaction was incubated for 30 minutes at 37 °C, then 450 µL of 1 mM of -4-Nitrophenyl butyrate substrate were added followed by a second incubation at 30 minutes for 37 ° C. Absorbance was determined at 405 nm. The percentage inhibition of lipase was calculated using the following formula:

$$\text{Inhibition (\%)} = [((Ac - Acb) - (As - Asb)) / (Ac - Acb)] \times 100$$

Where Ac refers to the absorbance of the control, Acb refers to the absorbance of the control blank, As the absorbance of the sample, and Asb is the absorbance of the blank sample.

2.6. Experimental Animals

Male and female Wistar rats weighing 150-250 g were used in the experiments. The animals were kept in cages at the Faculty of Medicine and Pharmacy in Rabat. They were maintained under standard conditions. The experiment was carried out according to the principles described in the "Guide to the care and use of laboratory animals", 8th edition prepared by the National Academy of Sciences (National Research Council of the National Academies). Every effort has been made to minimize animal suffering and the number of animals used. Ethics approval was obtained from Mohammed V University in Rabat

2.7. Oral Starch Tolerance in Normal Rats

The *ex-vivo* antihyperglycemic activity of the *E. spinosus* samples was determined according to the method as described by Beejmohun *et al*. (2014). Briefly, six groups of rats each composed of five rats (n = 5) were put on an empty stomach for 18 hours with free access to water. The control group received the vehicle (distilled water); the negative control group treated with the vehicle (starch); the positive control group treated with the acarbose vehicle at 50 mg / kg and the other two groups were treated with the aqueous and ethanolic extract of *E. spinosus* at 150 mg/kg orally (p.o). Thirty minutes later, all animals were loaded with starch orally at a dose of 2.5 mg/kg. Blood was drawn from the tail vein before (t = 0), and at 30, 60, 90 and 120 min after starch administration.

2.8. Acute Oral Toxicity

Acute oral toxicity was achieved using the method described in European guideline OECD-425 (Guideline, 2012). Swiss albino mice weighing 20 to 30 g were used in this experiment. Each group received the extracts orally at a dose of 2 g/kg. After treatment, the animals were observed for 14 days in order to assess the behavioral toxic effects.

2.9. Statistical Analysis

Data were expressed as mean± SEM. Statistical analysis and comparison of means were evaluated by one-way analysis of variance (ANOVA). The differences were considered statistically significant at $p< 0.05$. Analysis was performed with GraphPad Prism 6.

3. RESULTS

The results of the assays of polyphenols, tannins and flavonoids are summarized in the Table 1. In the aqueous extract, TPC is (34±0.58 mg GEA/g extract), TFC (10.33 ± 4.2 mg RE/g extract) and TCC (42 ± 13.3 mg EC / g extract). Thus, the ethanolic extract was found to be richer with TPC (77.01±2.25 mg GEA/g extract), TFC (544.33 ± 26.33 mg RE / g extract) and TCC (32.20±2.49 mg EC/g extract). These results reveal the richness of *E. spinosus* in polyphenols, tannins and in particular in flavonoids with a difference in variability between the two extracts. The comparison with other study reveals that the content of polyphenols and tannins in the ethanolic extract of *E. spinosus* in this study is higher than those obtained in the same species with TPC (19.3 mg GAE / 100g), TCC (10.5 mg EC/100g) (Khedher, Moussaoui and Salem, 2014).

Table 1. Total phenolic, flavonoid and condensed tannin content of *E. spinosus* extracts.

Aqueous extract			Ethanol extract		
TPC (mg GEA/g extract)	TFC (mg RE/g extract)	TCC (mg CE/g extract)	TPC (mg GEA/g extract)	TFC (mg RE/g extract)	TCC (mg CE/g extract)
34±0.58	10.33± 4.2	42± 13.3	77.01±2.25	544.33±26.33	32.20± 2.49

TPC : total phenolic content
TFC: total flavonoid content
TCC: total condensed tannins
mg GEA/g extract: mg Galic Acid equivalent per gram of extract
mg RE/g extract: mg of Rutin equivalent per gram of extract
mg CE/g extract: mg Catechin equivalent per gram of extract

The antioxidant activity of the different extracts was evaluated using five methods: anti-radical power with (DPPH), reducing power (FRAP), antioxidant power by ABTS, trapping of peroxide dismutase (H_2O_2) and inhibition of xanthine oxidase from the aqueous and ethanolic extracts of *E. spinosus* is shown in the Table 2. The study of the antioxidant activity by the DPPH test shows that the aqueous and ethanolic extract have significant anti-free radicals with IC_{50} respectively of (25 ± 10.69 and 13 ± 0.25 µg / mL). The study reported by Khedher *et al.* (2014) of the same species showed lower activity than the extracts of *E. spinosus* obtained by our study with an IC_{50} of 147 µg/mL using the DPPH test. Likewise, the extracts exhibited an interesting effect towards the ABTS cation, with better activity of the ethanolic extract (75.11±0.34 mg TE/g extract). The mode of action of antioxidants towards the DPPH radical is particularly linked to the hydroxyl group responsible for this action. We can see that this anti-free radical activity of the ethanolic extract is due to their richness in substance with a hydroxyl group (Boylan *et al.*, 2015). Likewise, the study of the reducing power of Fe^{3+} into Fe^{2+} has shown that the aqueous and ethanolic extracts have a reducing effect respectively of (20.61±2.72 and 51.1±1.2 mg AAE/g extract). It has been reported that flavonoids not only react as an antioxidant, some of them have the power to break down deoxyribose as a result of the reduction of Fe^{3+} to Fe^{2+} (Schinella *et al.*, 2002).

Chemical compounds in plants are electron donors, they help speed up the conversion of H_2O_2 to H_2O. The results of *E. spinosus* extracts on H_2O_2 scavenging showed that the aqueous extract exhibited antioxidant activity with an IC_{50} of (36.04±1.65 µg/mL) and the ethanolic extract with an IC_{50} of (28.2 ± 2.87 µg / mL). Likewise, extracts of *E. spinosus* showed lower

activity than that of ascorbic acid (IC_{50}=5.98±0.47µg / mL). For xanthine oxidase inhibitory activity, the ethanolic extract (IC_{50}=16.83±0.72 µg / mL) showed a higher inhibitory activity than that of the aqueous extract (IC_{50}=20.14±1.28 µg/mL). This minor variation between the different antioxidant methods may be due to the intrinsic mode of action of the antioxidant reactions, or to certain factors namely the stereoselectivity of the radicals and the solubility of the antioxidant components (Yahyaoui *et al*., 2018).

Table 2. Antioxidant activity by DPPH, FRAP, ABTS, H_2O_2 and xanthine oxidase (XO) methods of *E. spinosus*; Average of three replicates.

	DPPH IC_{50} (µg/mL)	ABTS (mg TE/g extract)	FRAP (mg AAE/g extract)	H_2O_2 IC_{50} (µg/mL)	Xanthine oxydase IC_{50} (µg/mL)
Aqueous extract	25±10.69	38.13±1.76	20.61± 2.72	36.04±1.65	20.14±1.28
Ethanol extract	13±0.25	75.11± 0.34	51.1± 1.2	28.2±2.87	16.83±0.72
BHT	3.28± 0.79	-	-	-	-
Ascorbic acid	-	-	-	5.98± 0.47	-
Allopurinol	-	-	-	-	0.78±0.01

E. spinosus extracts have also been evaluated for their inhibitory effect on α-amylase, α-glucosidase and lipase. Salivary and pancreatic α-amylase hydrolyzes the α-1,4-glucosidic bonds of polysaccharides, such as starch. Subsequently, α-glucosidase located in the brush borders of intestinal cells hydrolyzes the resulting oligosaccharides into glucose, which is then transported in the blood. Moreover, the main function of pancreatic lipase is the breakdown of triacylglycerides into glycerol and free fatty acids (Loizzo *et al.*, 2008). The results obtained have been summarized in the Table 3. The macerated ethanolic extract showed a better inhibitory effect against the three anti-diabetic enzymes with IC_{50} of 371±0.62, 18.6±1.2, and 10.44±1.08 µg/mL, respectively. The aqueous extract was less effective against the enzymes α-amylase, α-glucosidase and lipase compared to the ethanolic extract with IC_{50} of 668.8 ± 1.45; 19.68 ± 0.46 and 24.96 ± 1.52 µg / mL, respectively. Likewise, the ethanolic extract exhibited an inhibitory power greater than that of orlistat (12.55 ± 4.17 µg / mL) for lipase and an almost similar activity of acarbose (18.01 ± 2.00 µg / mL) for α-glucosidase. The study of Dammak et al (2020) showed that *E. spinosus* has lipid-lowering activity in mice. The inhibition of digestive enzymes (α-amylase, α-glucosidase and lipase) responsible for the degradation of carbohydrates and lipids can therefore be one of the strategies in the management of the postprandial state in diabetics and their ability to prevent type 2 diabetes. Similarly, the different phenolic compounds have been identified for their ability to inhibit the enzyme α-amylase due to their action to bind to proteins (Shobana, Sreerama and Malleshi, 2009).

Table 3. IC_{50} values of ESA and ESE extracts on α-amylase, α-glucosidase and lipase inhibition.

	IC_{50} (µg/mL)		
	α-amylase	α-glucosidase	Lipase
ESA	668.8±1.45	19.68±0.46	24.96±1.52
ESE	371±0.62	18.6±1.2	10.44±1.08
Acarbose	44.75±0.54	18.01±2.00	-
Orlistat	-	-	12.55±4.17

ESA: aqueous extract of *E. spinosus*

ESE: ethanolic extract of *E. spinosus*

The *ex vivo* oral starch tolerance test study showed that groups increased blood glucose 30 minutes after starch loading, ESA significantly increased (p<0.05) hyperglycemia at 0.83g/L and ESE at 0.91g/L while acarbose increased insignificantly at 1.03g/L. Also, acarbose lowered blood sugar to 0.98 mg/dL after 60 minutes, and then gradually reduced to 0.88 g/L after 120 minutes. Rats treated with starch caused an increase in blood sugar of 1.26g / L after 30 minutes. Then, it is reduced to 0.97g/L after 120 minutes. On the other hand, ESA and ESE reduced blood sugar to 0.96 and 0.93g / L, respectively, only after 90 minutes. Thus, all groups decreased blood sugar compared to control. Furthermore, the area under the curve (AUCglucose) for the ESA and ESE treated groups was significantly lower than that of the control group. Likewise, the AUC values of the acarbose group were significantly lower than those of the control group (Figure 1). The hypoglycemic results obtained *ex vivo* confirm the results obtained in vitro. Therefore, this can be explained by the inhibition of α-amylase and α-glucosidase observed *in vitro*. Thus, more experimentation will be necessary in order to validate the same thing and to elucidate other diabetic pathways as well. Natural resources have been known by these therapeutic effects some of them have been confirmed by its action to slow down absorption of glucose by reversibly modulating the action of enzymes (α-amylase and α-glucosidase) responsible for the breakdown of complex carbohydrates into monosaccharides. On the other hand, some have the ability to slow gastric emptying to the stomach (Ali *et al.*, 2013). The decrease in blood sugar levels can be caused by the excessive secretion of insulin causing the deposition of intracellular glycogen (Uddin *et al*., 2014).

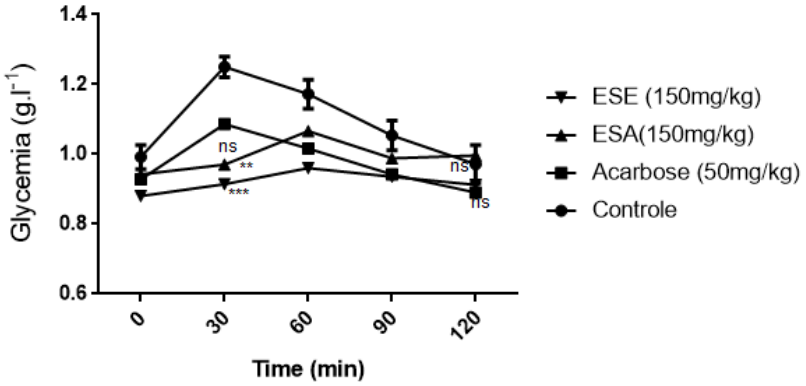
There is evidence that the generation of oxidative stress occurs as a result of depletion of antioxidants and may contribute to pancreatic cell apoptosis and hence increased diabetic complications (Khadayat *et al*., 2020).

The study of the phytochemical profile showed that *E. spinosus* is rich in terpenoids, thiophenes and sterols namely lupeyl acetate, taraxasterile acetate, lupeol, stigmasterol-bD-glucoside and b-sitosterol -bD-glucoside and two sesquiterpenoids and Echinopines A as well as fatty acids and alkanes. In addition, phytochemical analysis by HPLC-UV identified phenolic acids, the most abundant being p-coumaric (8.59 mg / kg DW), cinnamic (4.68 mg / kg DW). The most abundant flavonoids are kaempferol (30.37 mg/kg DM), quercetin and rutin in ethanol extract (Khedher *et al*., 2020).

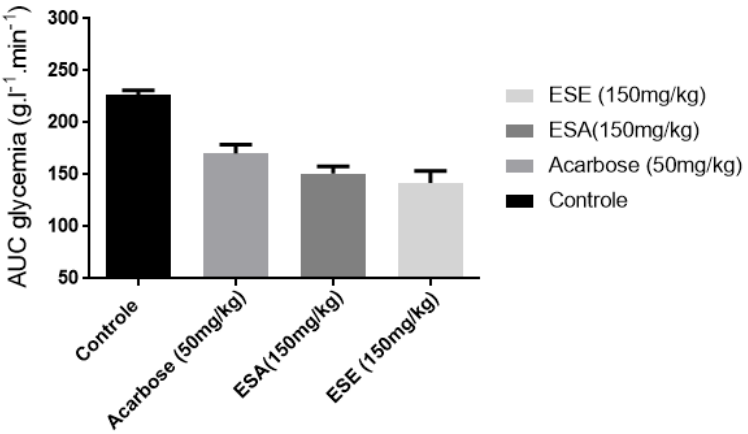
Cinnamic acid and its derivatives have been reported to be known antioxidants for their contribution to free radical scavenging, restoration of beta cell function, increased expression of glucose transporters (GLUT) and a the regulation or inhibition of enzymes involved in glucose metabolism (Ferreira *et al*., 2019). Terpenoid has been reported to have the same function of insulin, promotes intracellular glycogen deposition by stimulating glycogen synthesis and blocking glycogen phosphorylase, also improves glycogen metabolism when hepatic glycogen level is reduced (Uddin *et al*., 2014). Rutin induces reduced absorption of carbohydrates from the small intestine, suppression of tissue gluconeogenesis and formation of sorbitol, reactive oxygen species and advanced glycation end product precursors (Ghorbani, 2017).

The study of the acute toxicity of aqueous and ethanolic extracts of *E. spinosus* at a dose of 2 g/kg, no mortality was recorded and no behavioral or other changes were observed. Therefore, the oral LD_{50} of *E. spinosus* is greater than 2000 mg/kg.

Figure 1. Effect of *Echinops spinosus* extracts and acarbose on glycemia after starch intake in normal rats (a) and with presentation in the area under curve (b). The values are means ± SEM (n = 5). *** p <0.001; ** p <0.01 compared with normal controls. Ns = not significant to the normal controls. ESA: aqueous extract of *E. spinosus*; ESE: ethanolic extract of *E. spinosus* and AUC: area under the curve.



(a)



(b)

4. CONCLUSION

This work aims to evaluate the acute toxicity, the content of phenolic compounds, the antioxidant activity with five methods (DPPH, FRAP, ABTS, H_2O_2 and xanthine oxidase), antihyperglycemic with three methods (α-amylase and α-glucosidase and lipase) and *ex-vivo* by the starch tolerance test of aqueous and ethanolic extracts of *E. Spinosus*. The study of *E. spinosus* extracts has demonstrated their richness in phenolic compounds, in particular flavonoids. The extracts have also demonstrated antioxidant power in vitro, postprandial antihyperglycemic. Further studies and *in vivo* antioxidant and antidiabetic pathways must be performed to confirm the effect. Moreover, a chronic toxicological and phytochemical study is necessary.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Kaoutar Benrahou: Investigation, Resources, and Writing - original draft. **Otman El Guourrami**: Methodology, Supervision, and Validation. **Hanaa Naceiri Mrabti**: Visualization, Software, Formal Analysis. **Gokhan Zengin**: Validation. **Abdelhakim**

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Review Article

A review on essential oil analyses and biological activities of the traditionally used medicinal plant *Thymus vulgaris* L

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Abstract: Since the old times, seeds producing plants have played a vital role in the progress of human culture to treat diseases. Medicinal plants are used traditionally by the local communities to treat diseases. Recently, a report has shown that more than 250,000 flowering plant species are available globally. Scientists are continuously working on higher plants to help and understand plant poisonousness and to defend humans and animals from natural toxins. A plant`s toxicity and its medical use are dependent on the plant's volatile phytochemicals. *Thymus vulgaris* L is a common aromatic plant used widely as a folk medicine to treat various diseases by different ethnic communities around the globe including the Sultanate of Oman. Previous studies in Oman showed that the selected plant species contains several groups of phytochemicals such as essential oils and secondary metabolic compounds they can enhance their biological and toxicological activities. Therefore, the aim of the present review is to explore the volatile phytochemicals, biological and toxicological features of *Thymus vulgaris* grown in Oman. The results can be helpful for discovering new drugs to treat asthma, cough, chronic bronchitis and other infectious diseases. In conclusion, this review provides information on the volatile phytochemicals, pharmacological and toxicological aspects of the selected plant species.

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1. INTRODUCTION

Due to the increasing demands for daily foods that contain bioactive constituents such as volatile oils and secondary metabolic compounds, which may occur as health assistance, nutrition, as well as herbal. Nowadays, medicinal plants are used as sources in most countries of alternative medicines in many countries. Different traditional systems are revived to treat diseases instead of using synthetic drugs (Hossain, 2019). As drug resistance is becoming a global health issue, the main target of scientists is to discover herbal extracts or pure ingredients that may act as microbial, antifungal or anticancer agents. Numerous spices and their extracts are used for food preservation as antimicrobial agents and natural antioxidants. Some of the species are used in natural healing and as appetizers. Now, many local plant species are used by the local ethnic communities as safe medicine to treat aliments (Ait M'barek *et al*., 2007).

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The World Health Organization (WHO) reported that a large percentage of the population in most developing countries depend on plants and herbal medicine for their primary healthcare needs (Aziz & Rehman, 2008). In the process of drug discovery, medicinal plants played a significant part due to the presence of safe bioactive ingredients including essential oils (Hossain, 2019). The ingredients normally present in the plants and their crude extracts are essential oils, saponins and steroids derivatives, phenolics and flavonoids derivatives, lignans and glycosides complexes, terpenes, and alkaloids. Due to their powerful therapeutic and biological properties, all the ingredients have been used for a long time to discover modern pharmaceutical drugs (Wayne *et al.*, 2004; Cronquist, 1988; Cruz *et al*., 1989). Among the aromatic plants, some plants are economical and have played a crucial part in human illness (Cruz *et al.,* 1989). In addition, a good number of medicinal plants can be used as fuel for the survival of all of living things. All the higher plants contain ingredients with synergistic effects that can neutralize toxicity.

1.1. Synonyms

More than fifteen synonyms of the selected plant species are identified and available globally (Dob *et al.,* 2006). Some significant synonyms are as follows: *Origanum thymus, Origanum webbianum, Thymus baeticus* var. *prostrates, Thymus chinensis, Thymus collinus, Thymus ilerdensis, Thymus sublaxus, Thymus vulgaris* var. *Thymus vulgaris* var. *latifolius, Thymus vulgaris, Thymus vulgaris subsp. Thymus vulgaris* var. *palearensis, Thymus vulgaris* var. *verticillatus, Thymus vulgaris* subsp. *Thymus webbianus, Thymus webbianus* var. *prostrate, Thymuswelwitschii subsp. Ilerdensis Thyymus zygis subsp ilerdensis*.

1.2. Taxonomic Classification

Kingdom Plantae-plantes, Planta, Vegetal, plants; Subkingdom: Viridiplantae-green plants; Infrakingdom: Streptophyta-land plants; Superdivision; Embryophyta; Division: Tracheophyta–vascular plants, tracheophytes; Class: Magnoliopsida; Superorder: Asteranae; Family: Lamiaceae– mints, menthes; Genus: *Thymus* L; Species: *T. vulgaris* L.

1.3. Plant Description

Thymus vulgaris (*T. vulgaris*) is one of the tiny plant species with flowers. Several species including *T. vulgaris* are available in Oman. Locally, the plant species is called “kekik” (Al Hashmi *et al.,* 2013). The species grow up to 40 cm. It has a branch of stems and the stems are woody when the plant is matured (Hossain *et al.,* 2013). Figure 1 shows different parts of *T. vulgaris* L. (Hazzit *et al.,* 2009). The leaves of this species is approximately not more than 5 mm and it is covered by white hair. The shape of the leaves is oval or rectangular. The whole aerial parts including leaves are fleshy and they contain maximum essential oils. The species have a strong smell, but the smell may differ due to the chemical ingredients of different types (Houmania *et al.,* 2002).

Figure 1. Pictures of *T. vulgaris*

1.4. Traditional Use

Traditionally, the selected thyme species have been used widely to treat heart failure, chest infections, and encourage saliva production (Al Hashmi *et al.,* 2013; Hudaib *et al*., 2002). All parts of this plant are medicinally important and used widely to treat human diseases because of their medicinal values. In addition, several prescription drugs contain ingredients from this plant species. In Oman, local ethnic communities used it as a juice to kill worms (Hossain *et al.,* 2013; Hwang *et al.,* 2004). Leaves paste is taken to release sore throats (Hossain *et al.,* 2013; Hwang *et al.,* 2004). The flowers are edible with an acceptable taste. It also has a powerful capability to kill bacteria, fungi and viruses (Karaman *et al.,* 2001). The dried aerial part of this species is used by the local communities as a tea to treat sore throats. Thyme species have a very rich flora in all over the world including the Sultanate of Oman. Based on the traditional uses of the plant species, scientists and researchers are highly interested in working on the selected plant species for the isolation of active ingredients and to use them to treat diseases.

1.5. Pharmaceutical Importance of T. vulgaris

Since ancient times, people have been using thyme in alternative traditional systems to treat numerous respiratory diseases, especially chronic cough, bronchitis, and asthma (Hossain et al., 2013). Based on the active ingredient, the plant species are also used to treat vascular problems, diseases of the urinary tract, teeth pain and indigestion (Hudaib *et al.,* 2002; Karaman *et al*., 2001). The plant contains the highest percentage of thymol (approximately 60%); thymol has a good ability to increase appetite as well as to kill bacterial infections. Recently, the plant has been used for treating asthma. In the last decades, the authors carried out several studies and concluded that the essential oil and plant crude extracts showed significant biological activities. The plant is also a good source of Fe, Ca, Mg and vitamin K that can increase blood flow (Hudaib *et al.,* 2002; Karaman *et al.,* 2001; Hwang *et al.,* 2004).

1.6. Sample Process and Extraction

Several methods such as steam distillation, solvent extraction, supercritical fluid extraction, and pressurized liquid extraction procedure are extensively used for the extraction of essential oils, and other secondary metabolic constituents (Hossain *et al.,* 2019; Marino *et al.,* 1999; Maryam *et al.,* 2022). All these methods are efficient and can provide a great percentage of bioactive constituents. Mainly two methods, such as simple steam distillation and extraction with the solvent method, are used for the extraction of essential oils (Markovic, 2011). Nowadays, supercritical carbon dioxide and pressurized liquid extraction are relatively recent solvent-solvent extraction techniques to minimize the degradation of the active compounds because both processes can function in the absence of light and air (Miura *et al.,* 2002).

1.7. Distribution of The Plant

T. vulgaris is a perennial plant species that belongs to the Lamiaceae family. Globally, its common name is thyme (Al Hashmi *et al.,* 2013). The selected plant species is indigenous to some parts of EU countries, and indigenous in several South Asian and Gulf countries (Farooqi *et al.,* 2005). In addition, this plant is also native to Northern Africa, parts of Africa. Some of the countries such as Egypt, Cameroon, Algeria, Tunisia, Nigeria, South Africa, and Libya have cultivated the plant species due to its and economic medicinal values (Ghasemi, 2009; Giordiani *et al.,* 2008; Giweli *et al.,* 2013; Guillen & Manzanos, 1998).

2. BIOCHEMICAL STUDIES

Several secondary bioactive compounds were isolated and identified from the selected plant species described by several authors (Pina-Vaz *et al.,* 2004; Naghdi-Badi, 2004). The types of compounds such as polar and non-polar compounds isolated and identified by chromatographic

and spectroscopic methods from this plant species are presented in Table 1. Some of the compounds are used as herbal medicine as well as modern medicine; another group of them are used as food nutrients, natural antioxidants and food preservatives (Naghdi-Badi, 2004). Normally, it is a continuous process to isolate the bioactive compounds from the traditionally used plants that are used by the local communities to treat diseases. Scientists and researchers are working on pure isolated compounds to explore their *in-vitro* and *in-vivo* biological and pharmacological activities to enhance formulation of drug for treatments of human diseases (Nickavar *et al.,* 2005; Nikolić *et al.,* 2012).

Table 1. List of phytochemicals of different leaves extracts of T. vulgaris.

Hexane Extract	Ethyl acetate extract	Butanol extract
Linalyl anthranilate	o-Cymol	4-Heptanone
Bicyclo[3.1.0]hexan-2-ol	Linalyl anthranilate	n-Butyl ether
α-Terpineol	1,5-Octadiene-3,7-diol	Hexanal
Thymol	α-Terpineol	4-Heptanone
O-Thymol	Thymol	Butanoic acid, butyl ester
2.Thymol acetate	o-Thymol	5-Methyl-3-heptanone
Bicyclo[7.2.0]undec-4-ene, 4,11,11-trime	4-Methoxy-2,3,6-trimethylphenol	o-Cymol
O-methoxy-α,α-dimethylbenzyl	Spathulenol	Linalool
Spathulenol	Phytol	4-Terpineol
α.-farnesene	Naringenin	Terpineol
1-octadecyne	1-Iodo-2-methylundecane	Thymol
n-Hexadecanoic acid	3,7-Octadiene-2,6-diol	o-Thymol
Naringenin	2,4-Dimethylbenzaldehyde	Thymol acetate
	Thymol	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trime
	o-Thymol	Aromadendrene
		Spathulenol
		Alpha.-farnesene
		3,7,11,15-Tetramethyl-2-hexadecen-1-ol
		n-Hexadecanoic acid

2.1. Essential Oil

Based on the essential oil, *T. vulgaris* plant is used by different ethnic communities to treat asthma, bronchitis and cough. The essential oil was isolated from various parts of the selected plant species by using different methods such as steam distillation method, cohobating method and many others (Nickavar *et al.,* 2005; Nikolić *et al.,* 2012; Markovic, 2011; Ozguven & Tansi, 1998; Akhtar *et al.,* 2012). The percentage of essential oils varies due to the extraction methods, the parts of the plants used and the different environmental conditions. Our previous study showed that the selected species contains more than 80 compounds. However, most of the authors identified more than 70 compounds. The major compounds are presented in Table 2. They are mainly oxygenated monoterpenes and sesquiterpenes. Some of them show significant antimicrobial, cytotoxic and antioxidant activities. Most of the researchers reported that the highest percentage ingredient was thymol approximately (56-60%).

Table 2. List of phytochemicals in the essential oil of *T. vulgaris.*

Sl. No	Name of Phytochemicals	Percentage
1	Tetra hydro-3-methylfuran	12.76
2	Cyclohexane	0.15
3	Camphene	0.13
4	α-Pinene	0.71
5	β-Myrcene	0.32
6	Octanol-3	0.18
7	Carene	0.28
8	*p*-Cymene	2.27
9	o-Cymene	0.39
10	γ-Terpinene	1.21
11	Terpinen-4-ol	0.35
12	α-Terpineol	0.33
13	Thymol	9.91
14	o-Thymol	41.90
15	2-Methyl-5-(1-methylethyl) phenolacetate	0.58
16	Caryophyllene	1.01
17	Humulene	0.11
18	Caryophyllene oxide	0.61

2.2. Nutritional Value

The selected thyme species showed remarkable health benefits that can be endorsed due to its nutritional value. The main nutrients in this species are namely vitamins, minerals, volatile oils and antioxidants. Most of them have strong disease-preventing activities as well as health - promoting properties (Naghdi-Badi *et al.,* 2004; Ozguven & Tansi, 1998; Penalver *et al.,* 2005). The selected plant species contain different phytonutrients, natural minerals and vitamins that are energetic for maintaining good health (Ozguven & Tansi, 1998). Thyme is a natural source of vitamins C and A and carbohydrates. In addition, the plant also contains vitamin B-complex and vitamin B_6. All these vitamins are essential for maintaining healthy skin and protecting the infectious diseases. The selected plant also contains several minerals such as K, Ca, Mg, Fe, and Se. These minerals are essential to maintain the electrolyte balance in the human body (Penalver *et al.,* 2005).

3. PHARMACOLOGICAL ACTIVITIES

The oil and extracts of the selected species showed significant antiseptic, antibacterial, anticancer, and anti-cough properties that can enhance the healing of different diseases (Kizil & Uyart, 2006).

3.1. Antioxidant Properties

Generally, *in-vivo* and *in vitro* methods were used to determine antioxidant properties of various extracts and essential oils from the plant species as described by several authors (Penalver *et al.,* 2005; Pirbalouti *et al.,* 2013; Raal *et al.,* 2004). Our previous experimental results showed that various polarities extracts and essential oils at different concentrations showed significant activity against DPPH (2,2-diphenyl-1-picrylhydrazyl), superoxide and hydroxyl radical scavenging activity and bestows protection (Pirbalouti *et al.,* 2013; Raal *et al.,* 2004; Al-Matani et al., 2015). Among the existing phytochemicals in this plant, thymol is the main ingredient about 60% and it shows powerful antioxidant activity against superoxide, DPPH radical scavenging and reducing capacity at various concentrations (Penalver *et al.,* 2005; Pirbalouti *et*

al., 2013; Raal *et al.,* 2004). The thymol ingredient also showed modest activity against V79 Chinese hamster lung fibroblast cells (Rasooli & Mirmostafa, 2002). It also showed strong antioxidant activity against lipid system in gamma-ray induced V79 Chinese hamster cells (Raal *et al.,* 2004). The carvacrol is the isomer of thymol that also showed better antioxidant capacity in lipid systems due to its synergetic effect.

3.2. Antimicrobial Activity

A good number of scientists evaluated the antimicrobial activities at various concentrations of essential oils and different polarities crude extracts of thyme plant species against various bacterial and fungi strains (Reis *et al.,* 2004; Rota *et al*., 2004; Soliman & Badeaa, 2002). They revealed that both the essential oils and crude extracts showed significant activity against the applied bacterial and fungi strains. These significant biological activities of the plant extracts and essential oil are due to the main chemical ingredients. Among the phytochemicals present in the selected plant, two phytochemicals, carvacrol and p-cymene, are significant components that have very weak antibacterial properties due to their synergy effect with carvacrol (Stahl-Biskup, 1991). Some scientists reported previously that polar extracts such as ethanol and aqueous extracts demonstrated high antimicrobial activity against gram-positive bacterial strains (Thompson *et al.,* 2003; Al-Matani et al., 2015). Tsukatani *et al.* (2012) conducted a comparative study of the antibacterial activity of the essential oils of cultivated *T. vulgaris* L. and wild thyme species against gram (+ and -) bacterial strains and the results revealed that wild thyme essential oil showed less activity compared the essential oil of the cultivated plant (Tsukatani *et al.,* 2012). Other work by Verma et al. reported that the microbial activity of the essential oil, either it is from the wild or the cultivated thyme species, depends on the phenolic compounds and their derivatives (Verma *et al.,* 2009 & 2011). Furthermore, the antimicrobial activity of essential oils also depends on incubation, synergistic ingredient effects, and the other ingredients.

3.3. Cytotoxic Activity

The preliminary screening of cytotoxic activity of various polarity plant extracts and essential oil of the thyme herb is assessed against the Brine Shrimp Lethally (BSL) and 96-cell wall described by several authors ((Vichai *et al.,* 2006; Vukovic-Gacic & Simic, 1993; Zaidi & Crow, 2005). The majority of reports showed that both plant extracts and essential oil at different concentrations attribute potential cytotoxic activity against (BSL) and 96-cell walls. However, some of the researchers mentioned that the cytotoxic activity only showed when the concentration of chemical ingredients is high of the extracts and essential oil. They mentioned that low concentration extracts and essential oil of the selected species did not show any activity. In addition, the non-polar extracts showed high activity compared to polar extracts which mean that the toxic compounds are present only in the non-polar extract. Similar results were also obtained by the 96-cell wall method.

4. CONCLUSION

T. vulgaris L. is a small plant that has been used as a spice, remedy, drug, and cosmetics. The essential oil of this plant is used in medicine, food, and cosmetics industries as preservative and antioxidant. This current review focuses on the latest status of phytochemicals, pharmacological and toxicological activities reported on *T. vulgaris.* The selected plant species contains a high amount of phytochemicals named phenolic derivatives and flavonoids; therefore, the plant exhibits antioxidant and antibacterial activity, anticancer and larvicidal effects. Thyme native to Oman can be used as a natural antioxidant in food products, supplements and drugs so that more clinical and pathological studies must be conducted to investigate the unexploited potentials of the *T. vulgaris* plant grown in Oman before using the plant for treating different diseases.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Mohammad Amzad Hossain: Data curation; Design study; Data analysis; Wrote a first draft of the review. **Yahya Bin Abdullah Alrashdi**: Literature survey; Data collection; Edit data. **Salem Said Jaroof Al-Touby**: Contributed to the design and results interpretation; Review manuscript.

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Research Article

LC-MS/MS analyses and biological activities of *Onosma sintenisii* and *O. mutabile*

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Abstract: This study was aimed to investigate the chemical compositions, *in vitro* antioxidant and enzyme inhibitory activities of methanol extracts from *O. sintenisii* and *O. mutabile*. Spectrophotometric analyzes showed that the total phenolic and flavonoid content of *O. mutabile* was higher than *O. sintenisii*. Findings from the chromatographic analyzes also confirmed the spectrophotometric analyses. It was determined that *O. mutabile* contains high levels of apigenin 7-glucoside and rosmarinic acid. *O. mutabile* extract exhibited higher activity in all of the antioxidant activity tests. *O. sintenisii* exhibited higher inhibitory activity on other enzymes except for α-amylase. It was understood that there was a close relationship between the antioxidant activities of the extracts and their chemical compositions. However, it was concluded that more detailed tests should be done to determine the phytochemicals responsible for the enzyme inhibitory activities of the extracts in question.

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1. INTRODUCTION

The origin of the word 'onosma' is based on the Latin word 'osma'. The word 'osma' has been used by Latin communities to mean fragrance (Stearn, 1993). Since *Onosma* species are among the plant species that have just begun to be discovered in their biological activities, the number of studies on these species is limited. It has been reported that *Onosma* species characteristically contain some phenolic compounds, alkaloids, and naphthoquinones (Mehrabian *et al.,* 2012). In addition, it has been found that the alkanines and shikonins in *Onosma* species are also found in other members of Boraginaceae and are responsible for interesting biological activities such as wound healing, pain relief, anti-inflammatory, anti-microbial, etc. (Zhou *et al.,* 1992; Kumar *et al.,* 2013).

Antioxidants are essential compounds in the food industry. These agents protect the lipids in foods against oxidation, preventing the formation of toxic oxidation products and the bitterness of the food. Due to these properties, antioxidants extend the shelf life of food and prevent commercial losses (Serafini & Peluso, 2016; Bi *et al.,* 2017). Some synthetic

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antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used in the food industry over the past decades. However, interest in natural antioxidant compounds of plant origin has increased over time due to the researchers' concerns about the side effects of these compounds on health (Surh, 2006; Choi *et al.,* 2014). Oxidative stress also triggers many health problems in organisms, such as cancer, cardiovascular system disorders, and rapid aging (Yashin *et al.,* 2017). Researchers working in both medicine and pharmacy agree that phytochemicals can significantly contribute to the relief of health problems associated with oxidative stress (Yesiloglu *et al.,* 2013; Samah *et al.,* 2017; Yashin *et al.,* 2017).

Alzheimer's disease (AD), a progressive neurodegenerative disease, primarily affects older people. The risk of contracting the disease doubles every five years after the age of 65. Authorities suggest that more than 130 million of the world's population will be in the grip of AD by 2050 (Prince *et al.,* 2016). The most prominent clinical symptom is neuronal loss and a decrease in acetylcholine (ACh) levels in the patients' forebrain, cortex, and hippocampus. In parallel with these molecular changes, cognitive disorders, learning difficulties, and memory loss occurs in patients. Researchers suggest that this is caused by disruption of signal transmission in cholinergic neurons (Selkoe, 1996; de la Torre, 2004; Ferri *et al.,* 2004). Since two main cholinesterases (ChE)s [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] are responsible for the regulation of ACh level in the brain, the most effective treatment approach in AD is to inhibit the activities of these enzymes (Genç *et al.,* 2016). Progression could be delayed in AD treatment with some ChE inhibitors (tacrine, galantamine, rivastigmine, etc.) used today (Rampa *et al.,* 2001). However, due to the short half-lives, low bioavailability, limited therapeutic efficacy, and toxicity of these compounds, researchers are making intense efforts to discover new ChE inhibitors. Plants are one of the primary sources used for this purpose (Almansour *et al.,* 2020).

Today, phytochemicals are also under scrutiny for their tyrosinase inhibitory activities. Excessive tyrosinase activity, which catalyzes the melanogenesis process, causes browning of foods and thus deterioration of their flavor (Sasaki & Yoshizaki, 2002; Fattahifar *et al.,* 2018). Tyrosinase hyperactivity also causes excessive accumulation of melanin in skin cells. Inhibition of this enzyme prevents browning in fruits and vegetables and provides skin whitening in organisms (Pillaiyar *et al.,* 2017). Therefore, tyrosinase inhibitors are among the favorite agents of the cosmetic industry. However, the cytotoxic and mutagenic properties of some synthetic tyrosinase inhibitors used today are of concern to health authorities (Baurin *et al.,* 2002). Therefore, there is a need for new tyrosinase inhibitors that do not have harmful side effects on the body (Guo *et al.,* 2020).

Diabetes is one of the most common diseases that afflict human beings and are common around the world. Since diabetes treatment is costly and complex, it can sometimes create difficulties for the functioning of medical systems (King *et al.,* 1998; Kameswararao *et al.,* 2003). The most effective way to prevent the increase in blood sugar level, especially immediately after meals (postprandial hyperglycemia), is to inhibit carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase) (Balan *et al.,* 2017). Researchers suggest that plants are rich sources of α-amylase and α-glucosidase inhibitors (Chokki *et al.,* 2020).

In this study, it was aimed to investigate the chemical compositions, antioxidant activities, and inhibitory activities of the methanol (MeOH) extracts obtained from the aerial parts of two *Onosma* species (*O. sintenisii* Hausskn. ex Bornm., *O. mutabile* Boiss. & Hausskn.) distributed naturally in Turkey on AChE, BChE, tyrosinase, α-amylase, and α-glucosidase.

2. MATERIAL and METHODS

2.1. Plant Material

The aerial parts of *O. sintenisii* (635 m., 37° 31' 13" N 30° 52' 26" E, herbarium number: OC. 5039) and *O. mutabile* (1550 m., 38° 24' 49.05" N 36° 27' 21.96" E, herbarium number: OC.5040) were collected from Todurge lake, Hafik, Sivas-Turkey, and Ayranlik village, Sariz, Kayseri-Turkey in 2019, respectively. The plants were identified and deposited by Dr. Olcay CEYLAN from the Department of Biology, Mugla Sitki Kocman University, Mugla-Turkey.

2.2. Preparation of The Extracts

Methanol extracts of both plants were prepared by maceration. Extract yields of *O. sintenisii* and *O. mutabile* were measured as 13.51% and 3.96% (w/w), respectively. Details of the extraction procedure can be found in the supplementary file.

2.3. Determination of The Phenolic Compositions of The Extracts

The chemical compositions of *Onosma* extracts were determined qualitatively and quantitatively using spectrophotometric and chromatographic methods (Zengin *et al.,* 2015; Cittan & Çelik, 2018). Experimental details for determining chemical composition are provided in the supplementary file.

2.4. Antioxidant and Enzyme Inhibition Capacity

Phosphomolybdenum, radical scavenging, reducing power, and ferrous ion chelating assays were used to determine the antioxidant activities of the extracts. (Apak *et al.,* 2006; Tepe *et al.,* 2011; Zengin *et al.,* 2015). On the other hand, inhibitory activities of the extracts on AChE, BChE, tyrosinase, α-amylase, and α-glucosidase were also performed by following the methods specified in the literature (Ozer *et al.,* 2018). Details on the tests performed can be found in the supplementary file.

2.5. Statistical Analysis

Details of the statistical analyzes applied to the data obtained from the tests are given in the supplementary file.

3. RESULTS

3.1. Chemical Compositions of The Extracts

Total phenolic and flavonoid contents of the MeOH extract obtained from *O. sintenisii* and *O. mutabile* are given in Figure 1. As in many studies published previously by our research group, the amounts of phenolics in the extracts were higher than the amounts of flavonoids in the current study. When the species are compared with each other, it is seen that both phenolic and flavonoid contents of *O. mutabile* are higher than *O. sintenisii*. Total phenolic and flavonoid contents of O. mutabile were 38.95 mg GAEs/g and 25.49 mg QEs/g, respectively.

In addition to the spectrophotometric analyses applied to the extracts, quantitative chromatographic analyses were also performed to determine the concentrations of the compounds in Table 1 in the extracts. Analyzes showed that both extracts were significantly higher in apigenin 7-glucoside and luteolin 7-glucoside, the flavonoid glycosides, apigenin, a flavonoid aglycone, rosmarinic acid, and pinoresinol. *O. mutabile* was richer in these compounds than *O. sintenisii*. This finding was found to be consistent with those obtained from spectrophotometric analyses. The concentrations of apigenin 7-glucoside, rosmarinic acid, luteolin 7-glucoside, pinoresinol and apigenin in *O. mutabile* were 112284.57, 47562.37, 8446.38, 5005.55 and 3114.73 µg/g, respectively. On the other hand, both extracts did not contain (+)-catechin, pyrocatechin, (-)-epicatechin, verbascoside, taxifolin, 2-hydroxycinnamic acid, and kaempferol.

Figure 1. Antioxidant capacities, total phenolics and flavonoids contents of *O. sintenisii* and *O. mutabile* extracts [GAEs, QEs, TEs, EDTAEs: Gallic acid, quercetin, trolox, and ethylenediaminetetraacetic acid (disodium salt) equivalents]. There is no statistical difference between the values marked with the same superscripts on the bars.

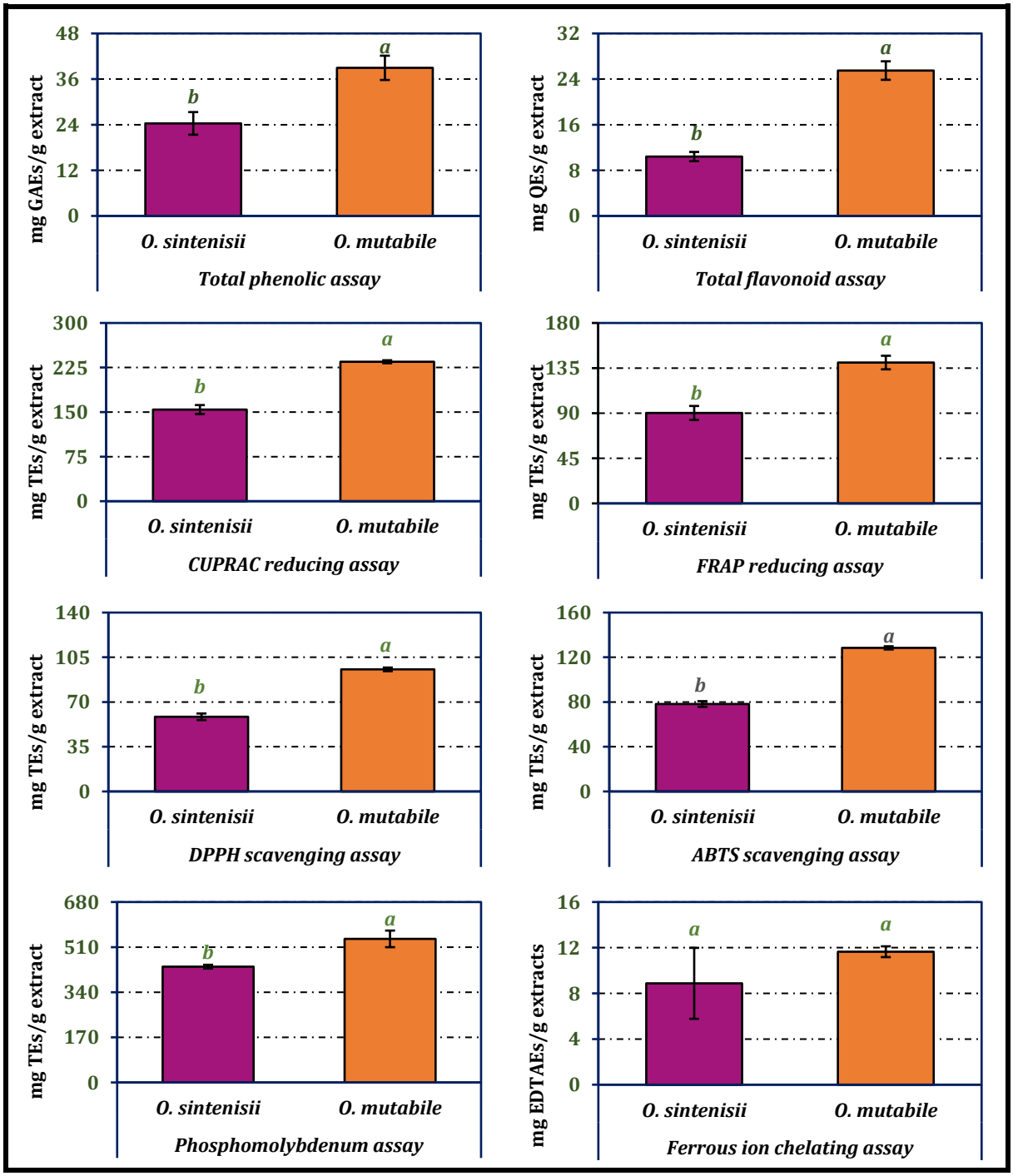


Table 1. Concentration (µg/g extract) of selected phytochemicals in *O. sintenisii* and *O. mutabile* extracts.

Compound	*O. sintenisii*	*O. mutabile*
Gallic acid	2.43 ± 0.02^b	11.55 ± 0.45^a
Protocatechuic acid	66.04 ± 1.00^b	117.27 ± 7.70^a
3,4-Dihydroxyphenylacetic acid	3.33 ± 0.24	nd
(+)-Catechin	nd	nd
Pyrocatechol	nd	nd
Chlorogenic acid	4990.28 ± 55.61^a	34.66 ± 2.19^b
2,5-Dihydroxybenzoic acid	15.07 ± 0.53^b	325.71 ± 11.11^a
4-Hydroxybenzoic acid	241.21 ± 0.74^b	1105.12 ± 3.44^a
(-)-Epicatechin	nd	nd
Caffeic acid	256.38 ± 10.30^b	833.43 ± 18.75^a
Vanillic acid	171.82 ± 1.48^b	901.90 ± 49.30^a
Syringic acid	12.02 ± 0.93^b	49.52 ± 0.65^a
3-Hydroxybenzoic acid	nd	13.01 ± 0.25
Vanillin	27.49 ± 0.69^b	81.04 ± 2.20^a
Verbascoside	nd	nd
Taxifolin	nd	nd
Sinapic acid	4.81 ± 0.44^b	73.62 ± 0.63^a
p-Coumaric acid	30.62 ± 4.04^b	221.51 ± 7.93^a
Ferulic acid	117.98 ± 0.22^b	474.74 ± 8.33^a
Luteolin 7-glucoside	2346.37 ± 1.36^b	8446.38 ± 137.54^a
Hesperidin	71.73 ± 1.80^b	226.16 ± 2.90^a
Hyperoside	8.89 ± 0.26^b	664.89 ± 9.32^a
Rosmarinic acid	20610.01 ± 113.72^b	47562.37 ± 127.59^a
Apigenin 7-glucoside	3253.85 ± 67.76^b	112284.57 ± 2262.44^a
2-Hydroxycinnamic acid	nd	nd
Pinoresinol	61.74 ± 0.63^b	5005.55 ± 527.22^a
Eriodictyol	0.17 ± 0.01^b	0.28 ± 0.03^a
Quercetin	1.93 ± 0.08^b	5.85 ± 0.16^a
Luteolin	300.81 ± 9.73^b	442.61 ± 33.42^a
Kaempferol	nd	nd
Apigenin	585.48 ± 3.18^b	3114.73 ± 22.44^a

There is no statistical difference between values marked with the same superscripts on the same row. nd, not detected.

3.2. Antioxidant Activities of The Extracts

The antioxidant activities of the extracts are given in Figure 1 in terms of positive control equivalents and Table 2 IC_{50} or EC_{50}. To elucidate the antioxidant activity potential of the extracts, various methods in which different antioxidant activity mechanisms were tested were used together. Thus, the extracts' total antioxidant and radical scavenging activities, chelating, and reducing powers were documented. In all test systems, *O. mutabile* exhibited higher activity

than *O. sintenisii*. *O. mutabile*'s activity values in reducing power (CUPRAC and FRAP), radical scavenging (DPPH and ABTS), phosphomolybdenum, and ferrous ion chelating assays were 234.71, 140.53, 95.56, 128.42, 541.13 mg TEs/g, and 11.65 mg EDTAEs/g, respectively. The extracts exhibited more potent activity in the CUPRAC test than they did in the FRAP test. Although the activity values were close to each other, the ABTS radical scavenging activities of the extracts were higher than the DPPH scavenging activities. The main reason why *O. mutabile* exhibits higher activity than *O. sintenisii* is thought to be closely related to its phytochemical composition. Because the concentration of the significant components given in Table 1 was higher in *O. mutabile*.

Table 2. Antioxidant capacities of standards and *O. sintenisii* and *O. mutabile* extracts.

Assays	*O. sintenisii*	*O. mutabile*	Trolox	EDTA
1	2.54 ± 0.04^{c}	2.05 ± 0.12^{b}	1.09 ± 0.04^{a}	-
2	1.78 ± 0.09^{c}	1.17 ± 0.01^{b}	0.29 ± 0.04^{a}	-
3	1.11 ± 0.08^{c}	0.71 ± 0.03^{b}	0.10 ± 0.01^{a}	-
4	4.27 ± 0.19^{c}	2.61 ± 0.04^{b}	0.27 ± 0.04^{a}	-
5	3.75 ± 0.10^{c}	2.22 ± 0.07^{b}	0.33 ± 0.05^{a}	-
6	6.44 ± 2.48^{b}	4.44 ± 0.19^{ab}	-	0.05 ± 0.003^{a}

1: Phosphomolybdenum (EC50: mg/mL), 2: CUPRAC reducing power (EC50: mg/mL), 3: FRAP reducing power (EC50: mg/mL), 4: DPPH radical scavenging (IC50: mg/mL), 5: ABTS radical scavenging (IC50: mg/mL), 6: Ferrous ion chelating (IC50: mg/mL). There is no statistical difference between values marked with the same superscripts on the same row.

3.3. Enzyme Inhibitory Activities of The Extracts

In the current study, ChEs, α-amylase, α-glucosidase, and tyrosinase inhibitory activity tests were applied to determine the anti-Alzheimer's, anti-diabetic and skin-whitening activities, in addition to the antioxidant activities of the extracts. Results are given in Figure 2 in terms of positive control equivalent and Table 3 in terms of IC_{50}.

Table 3. Enzyme inhibitory capacities of standards and *O. sintenisii* and *O. mutabile* extracts.

Assays	*O. sintenisii*	*O. mutabile*	Galanthamine	Kojic acid	Acarbose
1	1.11 ± 0.03^{b}	1.37 ± 0.07^{c}	0.0036 ± 0.0004^{a}	-	-
2	2.92 ± 0.12^{b}	8.63 ± 0.43^{c}	0.0057 ± 0.0004^{a}	-	-
3	2.30 ± 0.0^{b}	2.30 ± 0.07^{b}	-	0.30 ± 0.04^{a}	-
4	3.13 ± 0.14^{b}	2.67 ± 0.09^{b}	-	-	1.10 ± 0.14^{a}
5	1.02 ± 0.01^{a}	2.54 ± 0.02^{c}	-	-	1.67 ± 0.07^{b}

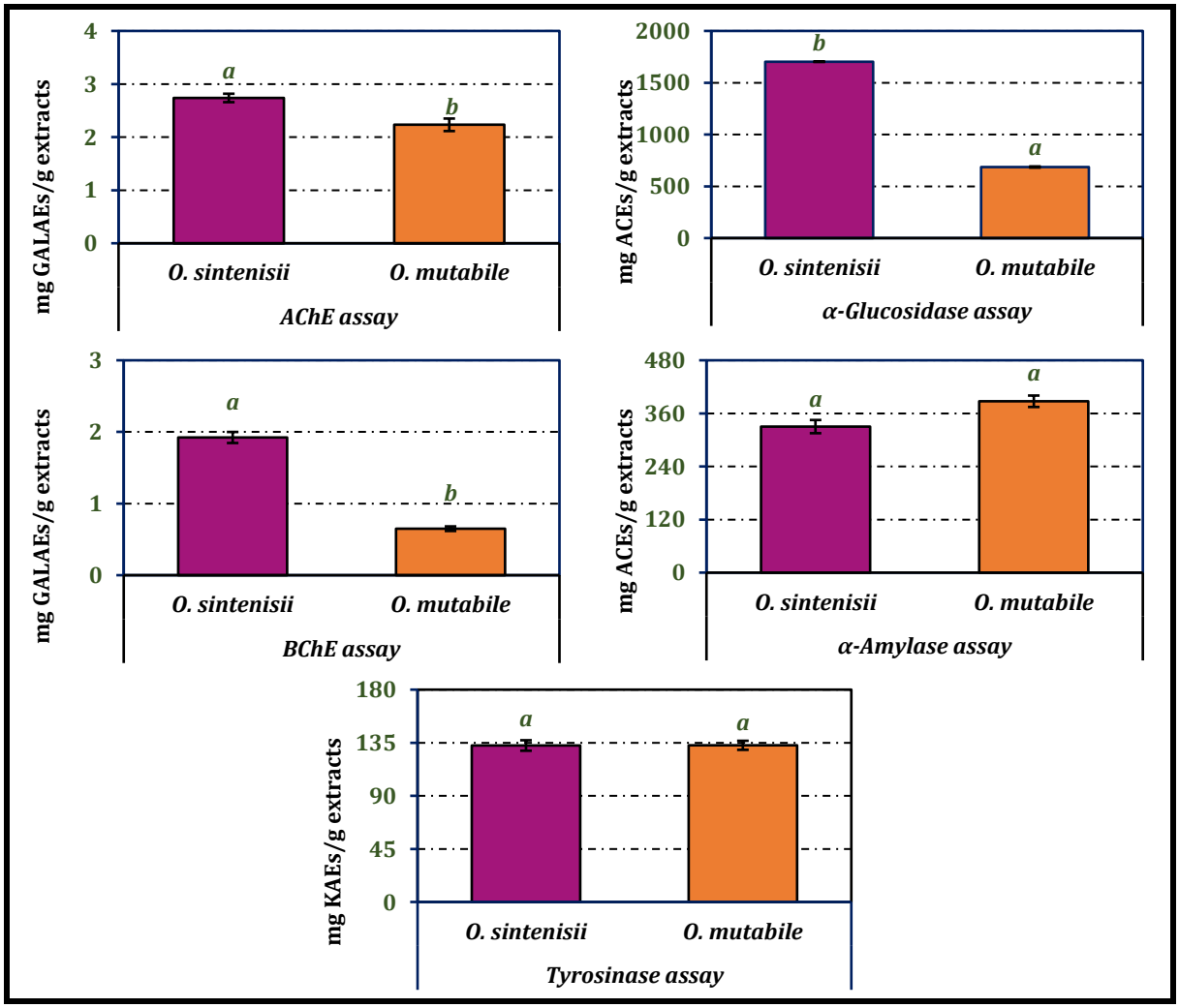
1: AChE inhibition (IC50: mg/mL), 2: BChE inhibition (IC50: mg/mL), 3: Tyrosinase inhibition (IC50: mg/mL), 4: α-Amylase inhibition (IC50: mg/mL), 5: α-Glucosidase inhibition (IC50: mg/mL). There is no statistical difference between values marked with the same superscripts on the same row.

According to the data in Figure 2 and Table 3, the extracts exhibited higher inhibitory activity on AChE than on BChE. The ChE inhibitory activity of *O. sintenisii* was higher than that of *O. mutabile*. The AChE and BChE inhibitory activity of *O. sintenisii* were 2.74 and 1.92 mg GALAEs/g, respectively, while *O. mutabile* exhibited 2.23 and 0.65 mg GALAEs/g inhibitory activity on the enzymes in question. In both test systems, the inhibitory activities of the extracts were statistically different from each other.

O. sintenisii showed higher activity in both α-amylase, and α-glucosidase inhibitory activity tests performed to reveal the anti-diabetic activity potential of the extracts. The extracts were more effective on α-glucosidase than α-amylase. The α-amylase and α-glucosidase inhibitory

activities of *O. sintenisii* were 330.08 and 1702.44 mg ACEs/g, respectively. On the other hand, the inhibitory activity of *O. mutabile* on these enzymes was determined as 387.38 and 686.04 mg ACEs/g, respectively. As can be understood from the findings, the inhibitory activities of the extracts were statistically different from each other.

Figure 2. Enzyme inhibitory capacities of *O. sintenisii* and *O. mutabile* extracts (GALAEs: galanthamine equivalent, KAEs: kojic acid equivalent, ACEs: acarbose equivalent). There is no statistical difference between the values marked with the same superscripts on the bars.



In the case of tyrosinase inhibitory activity assay, it was understood that the skin whitening activity potentials of the extracts were almost equal to each other. The activity potentials of *O. sintenisii* and *O. mutabile* were 132.66 and 132.82 mg KAEs/g, respectively. This finding means that the tyrosinase inhibitory activity of both extracts is statistically indistinguishable from each other.

4. DISCUSSION and CONCLUSION

Researchers have begun to focus on the chemical composition and biological activities of *Onosma* species in recent years. Therefore, there is no sufficient data in the literature regarding the chemical composition and activity potential of many *Onosma* species. This also applies to the *Onosma* species analyzed in the current study. In the literature, there are studies on pollen and/or nutlet morphologies of *O. sintenisii* and *O. mutabile* (Akcin, 2007; Binzet, 2011). However, the researchers revealed some phytochemicals such as alkaloids, naphthoquinones,

alkannins, and shikonins, which are characteristic of this genus (Zhou *et al.,* 1992; Mehrabian *et al.,* 2012; Kumar *et al.,* 2013). However, the phytochemicals documented in detail above in *O. sintenisii* and *O. mutabile* have been brought to the literature for the first time with the present study.

As stated in Section 3.1, there are no reports in the literature regarding the antioxidant activities of *Onosma* species analyzed in the current study. However, from the data presented in Table 1, it is possible to infer which major compounds may contribute to the antioxidant activity of *O. mutabile*. Some researchers have reported that extracts rich in some flavonoid glycosides, such as apigenin 7-glucoside and luteolin 7-glucoside, exhibit remarkable antioxidant activities (Pavlenko-Badnaoui *et al.,* 2021; Salamatullah *et al.,* 2021). In addition, in some studies conducted by our research group on the antioxidant activities of some other *Onosma* species, antioxidant activities of extracts rich in these compounds were found to be high (Sarikurkcu *et al.,* 2020a, 2020b; Sarikurkcu *et al.,* 2020c; Sarikurkcu *et al.,* 2020d). Literature data confirm that rosmarinic acid can also contribute significantly to antioxidant activity (Tzima *et al.,* 2021; Wang *et al.,* 2021; Zhuang *et al.,* 2021). There are also some reports in the literature that pinoresinol or some derivatives of this compound, or some extracts containing high amounts of this compound, alleviate the oxidative stress suppression and the severity of the symptoms developing accordingly (Youssef *et al.,* 2020; Lei *et al.,* 2021). The same is also true for apigenin, a flavonoid aglycone. In a study by Wu *et al.* (2021), it was reported that apigenin ameliorates doxorubicin-induced renal injury via inhibition of oxidative stress and inflammation. The literature data above confirm that the compounds in question may have contributed significantly to the antioxidant activity of *O. mutabile*.

There are no studies in the literature on the ChE inhibitory activity of *O. sintenisii* and *O. mutabile*. However, based on the data in Table 1, it is possible to know the compounds that contribute to the ChE inhibitory activity of *O. sintenisii*. According to the data in the table, rosmarinic acid is found in high amounts in *O. sintenisii* extract. Some reports in the literature show that this compound or extracts containing high amounts of rosmarinic acid show significant ChE inhibitory activity. Asghari *et al.* (2019) reported that the MeOH extract obtained from *Echium amoenum* showed significant inhibitory activity on both ChEs. The researchers suggested that the plant extract in question contained high amounts of rosmarinic acid and that the compound contributing to the activity was probably rosmarinic acid. In another study by Georgy & Maher (2017), it was reported that rosmarinic acid reduced doxorubicin-induced ChE activity. There are also some studies in the literature that chlorogenic acid has ChE inhibitory activity. In a study investigating the effects of chlorogenic and caffeic acids on systolic blood pressure, angiotensin-1-converting enzyme (ACE), and CHEs in cyclosporine-induced hypertensive rats, it was reported that chlorogenic acid significantly reduced the activity of both ChEs (Agunloye *et al.,* 2019). These findings are thought to be extremely useful in establishing a relationship between the phytochemical compositions of the extracts and their enzyme inhibitory activities.

In an *in silico* study investigating the inhibitory activities of certain flavonoids and phenolic acids on α-amylase and α-glucosidase, it was reported that rosmarinic acid exhibited an IC_{50} value equivalent to acarbose (Tolmie *et al.,* 2021). McCue & Shetty (2004) also obtained findings supporting these results. According to these researchers, rosmarinic acid has an *in vitro* inhibitory effect on porcine pancreatic amylase. Some reports in the literature show that some extracts were containing chlorogenic acid as a major compound exhibit significant inhibitory activity on digestive enzymes (Chokki *et al.,* 2020; Liu *et al.,* 2020a; Liu *et al.,* 2020b;Si *et al.,* 2020). These findings support those from the present study.

As stated in the above section, tyrosinase inhibitory activity of the extracts analyzed in the present study was brought to the literature for the first time with this study. In line with the data

in Figure 2 and Table 3, since it was understood that both extracts showed similar inhibitory activity on tyrosinase, it is helpful to examine the contribution of the primary compounds found in both extracts to the activity. Apigenin 7-glucoside, a flavonoid glucoside, and rosmarinic acid, a phenolic acid, are significant compounds in both extracts. In a study investigating the tyrosinase inhibitory activities of some compounds isolated from *Lepechinia meninii*, rosmarinic acid inhibited the monophenolase and diphenolase activities of tyrosinase at a rate of 4.14 and 8.59 µM, respectively (Crespo *et al.,* 2019). The data presented in the study reported by Lin *et al.* (2011) also supports the literature data above. These researchers suggested that rosmarinic acid inhibits tyrosinase in a non-competitive manner. There are also some reports in the literature that apigenin 7-glucoside may show tyrosinase inhibitory activity. In an *in silico* study by Istifli *et al.* (2021), it was stated that the binding energy of apigenin 7-glucoside to tyrosinase is vital and can be a potential tyrosinase inhibitory agent. It is thought that the literature mentioned above findings may help to establish the relationship between chemical composition and tyrosinase inhibitory activity in the current study.

This study documented the chemical compositions, antioxidant and enzyme inhibitory activities of *O. sintenisii* and *O. mutabile*. The results obtained from the antioxidant activity tests revealed that the activity in question depends on the chemical composition of the extracts. However, in enzyme inhibitory activity tests, an activity profile different from the antioxidant activities of the extracts was determined. Although there are some reports in the literature that the compounds found in high amounts in extracts may contribute to the inhibitory activities of the extracts on these enzymes, it is thought that more detailed tests should be done to detect bioactive phytochemicals.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Mehmet Sabih OZER: Methodology, Resources, Visualization, Software, Formal Analysis. **Kemal Erdem SENCAN:** Investigation, Resources, Validation, and Writing -original draft. **Cengiz SARIKURKCU:** Methodology, Formal Analysis, Software. **Bektas TEPE:** Investigation, Resources, Validation, and Writing -original draft.

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