© by PSP Volume 31– No. 05/2022 pages 4951-4957 Fresenius Environmental Bulletin

In vitro ANTIMICROBIAL ACTIVITY AND BIOCHEMICAL COMPOSITION OF *Hibiscus syriacus* FROM TURKEY

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ABSTRACT

Hibiscus syriacus is an ornamental plant, and it also has uses in traditional medicine. This study aimed to investigate *in vitro* antimicrobial activity and the biochemical composition of *Hibiscus syriacus*. In this study, the biochemical composition of ethanol extracts of *H. syriacus* flowers (HSFEt) and *H. syriacus* leaves (HSLEt) was analyzed by GC-MS. The antimicrobial activity was investigated using a disk diffusion test against 39 bacteria (including 11 multidrug-resistant strains) and 2 fungi strains. 21 and 12 components were identified in HSFEt and HSLEt, respectively. The main component is 2,3-Dihydroxypropyl stearate in both extracts. HSFEt and HSLEt have antimicrobial activity against most of the tested microorganisms. It has been detected the highest activities against *Staphylococcus aureus* ATCC 25923 (ST11) with a 15 mm, *Staphylococcus aureus* (CI1) and *Staphylococcus aureus* MRSA+MDR (MDR9) with a 14 mm inhibition zones in HSFEt, and *Staphylococcus aureus* ATCC 25923 (ST11) with an 11 mm inhibition zone in HSLEt. All these results showed that *H. syriacus* has antistaphylococcal potential.

KEYWORDS:
Hibiscus syriacus, disk diffusion, GC-MS, ethanol extract, antistaphylococcal

INTRODUCTION

The use of medicinal plants for the treatment of diseases before chemical drugs is an ancient order that took place with the transition of mankind to a sedentary life. Herbal medicines constitute a significant part of the customs of rural communities in developing countries [1].

According to WHO reports, 80% of the population in developing countries relies on non-chemical drugs, usually of plant origin, to ensure the sustainability of their health. It is noted that usually at least 25% of the active substances of drugs produced pharmacologically by modern methods are obtained from plants. In addition, the active substances of many synthetically produced drugs are also structural analogs of chemicals isolated from plants for the first time. The demand for plants used as pharmaceutical raw materials is increasing at a high level in both developed and developing countries due to their low cost, low side effects compared to synthetic drugs, very low toxicity, and being produced organically [2].

Medicinal plants are a natural source of compositions that can be used against many diseases today [3]. These plants contain many chemicals and active substances of a wide variety that have significant effects on humans [1]. Chemicals such as flavonoids, alkaloids, terpenoids, tannins, berberine, quinine, and emetine synthesized by plants are used quite widely in maintaining health and treating most diseases [4].

The reason why it has become increasingly difficult to treat infections in recent years is the increase of microorganisms with multiple antibiotic resistances. Drug resistance gradually increases in bacteria that develop resistance to all known antibiotics and is passed on from generation to generation. That is why it is recommended to use medicinal plants as an alternative to synthetic drugs, and some of them are even used as antimicrobials [5]. It is believed that the therapeutic properties of plants are due not to a single active substance, but the mutual interaction of a large number of compositions. Therefore, it is noted that herbal compositions provide a more effective healing process against resistant microorganisms that are difficult to kill with a single synthetic drug [6, 7]. This circumstance pushes researchers to study the compositions of active substances obtained from plant extracts that have an inhibitory property [8].

Essential oils, tannins, alkaloids, and bitter substances are the active substances usually used in plant metabolism. They strengthen the body's defense systems, support the functioning of organs, and promote healing. Thus, they have a positive effect on the functioning of certain organs and tissues in the organism. Throughout the history of mankind, many diseases such as diabetes, jaundice, shortness of breath have been tried to be treated using plants [9].

Although the possible effects vary according to the active ingredients, it is known that most essential oils have effects such as antimicrobial, carminative, choleretic, sedative, diuretic, and antispasmodic [5, 10].

Hibiscus syriacus is a native species of Southern China and Taiwan. Nowadays it is widely cultivated as ornamental plants and hedges in Japan, Korea, Malaysia, and other warm-temperate and subtropical regions of the world. It is called Common Hibiscus, Rose of Sharon, and Shrub Althea. It has antimicrobial, antioxidant, anticancer, antiulcer, anti-aging, and wound healing properties. Flowers decoction or infusion is used as a diuretic, stomachic, and ophthalmic in traditional medicine. It is also used for treating itches, other skin ailments, dizziness, dysentery, and bloody stools with much gas. In addition, leaves are used as a diuretic, stomachic, and expectorant [11].

We have determined that the antimicrobial activity of *H. syriacus* against a wide range of microorganisms, including multidrug-resistant strains, has not been adequately studied at the end of the literature review. Therefore to avoid toxic effects and to serve as the basis for plant-based drug molecule determination studies in the later stages, ethanol was preferred as a solvent when preparing plant extract in this study.

In this study, we investigated *in vitro* antimicrobial activity and the biochemical composition of HSFEt and HSLEt.

MATERIALS AND METHODS

Plant samples. *H. syriacus* samples were collected from near Faculty of Arts and Science, Canakkale Onsekiz Mart University, Canakkale, Turkey (40°6'39" N, 26°25'7" E) and identified by Dr. Bozyel. The plant aerial parts were placed in sample bags and transported to our laboratory. The samples were air-dried at room conditions. The voucher specimens were deposited at the Fauna and Flora Research and Application Center, Dokuz Eylül University, Buca, Izmir, Turkey (Personel herbarium number FFDEU-ERA1734).

Active compound extraction. Dried *H. syriacus* flowers and leaves samples were ground to obtain a fine powder, to increase the surface area for extraction. The active compounds were extracted by ethanol (Sigma Aldrich) through shaking at room conditions for two days [12]. After filtering through filter paper (Whatman No.1), the ethanol in the extract was evaporated at 45°C under vacuum by using a rotary evaporator (Heidolph Hei-Vap Value HL/HB-G1) [13]. The remnant was weighed and an extract stock was prepared for each plant using a defined volume of ethanol, and 50 µL, 90 µL, and 200 µl of the extracted stock of HSFEt and 50 µL, 100 µL, and 200 µl of the extracted stock of HSLEt were transferred to empty sterile antibiotic disks. The disks were loaded with 5.78, 10.41, and 23.13 mg of HSFEt and 1.15, 2.3, and 4.6 mg of HSLEt, respectively.

Antimicrobial activity. The disk diffusion test, which was described previously in detail by Yakan et al. [14] was used for testing the antimicrobial activity of HSFEt and HSLEt against 11 standard (ST), 11 multidrug-resistant (MDR), 10 clinical isolated (CI), and 7 food isolated (FI) bacteria, and 1 standard (ST) and 1 clinical isolated (CI) fungi strains. The incubation conditions for microorganisms excluding fungi strains were 37°C for 24 hours, but 27°C for 48 hours for *C. albicans* and *C. tropicalis*. Inoculum for each microorganism was prepared in 0.9% sterile saline solution and the turbidity of all inocula was adjusted by comparing with 0.5 McFarland standard. The Petri dishes containing disks, on which the ethanol extract was loaded, incubated according to the suitable time-temperature combinations mentioned above, and the inhibition zones were observed and recorded in millimeters. Empty antibiotic disk and ethanol-loaded disk were used as negative controls. A wide range of antibiotics was used as positive controls.

GC-MS Analysis. The biochemical composition of HSFEt and HSLEt were determined by GC-MS analysis according to the protocol given in previous studies [15].

Statistics. All tests were applied as triplicates. One-way analysis of variance (ANOVA), which is a parametric method was performed (P = 0.05) [16]. Pearson correlation coefficient was determined for any possible correlation between the intensity of antimicrobial activity and concentration. Statistical analysis was performed using R Studio (version 3.3.2) [17].

RESULTS AND DISCUSSION

Antimicrobial activity. The data obtained from the study about the inhibition zone diameters are shown in Table 1. According to the results, negative controls show no activity [18]. Additionally, statistical analysis verified that the differences between the results of three replicates of each extract volume were statistically non-significant (p>0.05). In addition, obtaining a Pearson correlation coefficient of 0.1986 and 0.1703 presented a very weak positive correlation between the antimicrobial activity and the volumes of extracts used. Also, thirteen antibiotics in standard, food isolated, and clinical isolated strains (Table 2) and thirty-three antibiotics in multidrug-resistant strains were used as positive controls (Table 3).

TABLE 1
Antimicrobial activity of HSFEt and HSLEt

Lab. Code	Microorganisms	HSFEt			HSLEt		
		50 µL*	**90** µL*	**200** µL*	**50** µL*	**100** µL*	**200** µL*
ST1	*Bacillus subtilis* DSMZ 1971	11,00 ± 0,00	7,00 ± 0,00	8,00 ± 0,00	8,00 ± 0,00	8,00 ± 0,00	9,00 ± 0,00
ST2	*Candida albicans* DSMZ 1386	10,00 ± 0,00	10,00 ± 0,00	10,00 ± 0,00	-	-	-
ST3	*Enterobacter aerogenes* ATCC 13048	-	-	-	-	-	-
ST4	*Enterococcus faecalis* ATCC 29212	7,00 ± 0,00	8,00 ± 0,00	8,00 ± 0,58	-	-	7,00 ± 0,00
ST5	*Escherichia coli* ATCC 25922	-	-	7,00 ± 0,00	-	-	-
ST6	*Listeria monocytogenes* ATCC 7644	11,00 ± 0,00	11,00 ± 0,00	11,00 ± 0,00	8,00 ± 0,00	8,00 ± 0,00	9,00 ± 0,00
ST7	*Pseudomonas aeruginosa* DSMZ 50071	-	7,00 ± 0,00	9,00 ± 0,00	-	-	-
ST8	*Pseudomonas fluorescens* P1	7,00 ± 0,00	8,00 ± 0,00	10,00 ± 0,00	-	-	-
ST9	*Salmonella enteritidis* ATCC 13076	-	7,00 ± 0,00	8,00 ± 0,00	-	-	7,00 ± 0,00
ST10	*Salmonella typhimurium* SL 1344	-	-	-	-	7,00 ± 0,00	7,00 ± 0,00
ST11	*Staphylococcus aureus* ATCC 25923	15,00 ± 0,00	9,00 ± 0,00	12,00 ± 0,58	10,00 ± 0,00	11,00 ± 0,00	11,00 ± 0,00
ST12	*Staphylococcus epidermidis* DSMZ 20044	11,00 ± 0,00	12,00 ± 0,00	13,00 ± 0,00	-	-	-
FI1	*Enterococcus durans*	8,00 ± 0,00	9,00 ± 0,00	9,00 ± 0,00	8,00 ± 0,00	8,00 ± 0,00	8,00 ± 0,00
FI2	*Enterococcus faecium*	10,00 ± 0,00	11,00 ± 0,00	12,00 ± 1,15	9,00 ± 0,00	10,00 ± 0,58	10,00 ± 0,00
FI3	*Klebsiella pneumoniae*	-	-	-	-	7,00 ± 0,00	7,00 ± 0,00
FI4	*Listeria innocua*	7,00 ± 0,00	8,00 ± 0,00	9,00 ± 0,00	-	-	-
FI5	*Salmonella infantis*	-	-	-	-	-	-
FI6	*Salmonella kentucky*	-	-	-	-	-	-
FI7	*Escherichia coli*	-	-	-	-	-	-
CI1	*Staphylococcus aureus*	14,00 ± 0,00	14,00 ± 0,00	14,00 ± 0,00	9,00 ± 0,00	9,00 ± 0,00	10,00 ± 0,58
CI3	*Staphylococcus hominis*	-	-	-	-	-	-
CI4	*Staphylococcus haemolyticus*	-	-	-	-	-	-
CI5	*Staphylococcus lugdunensis*	-	-	-	-	-	-
CI6	*Shigella boydi*	-	-	-	-	-	-
CI7	*Acinetobacter baumannii*	-	-	-	-	-	-
CI8	*Shigella flexneri*	-	-	-	-	-	-
CI9	*Staphylococcus aureus*	-	-	-	-	-	-
CI10	*Enterococcus faecalis*	-	-	-	-	-	-
CI11	*Klebsiella pneumoniae*	-	-	-	-	-	-
CI12	*Candida tropicalis*	-	-	-	-	-	-
MDR1	*Escherichia coli*	-	-	-	-	-	-
MDR2	*Klebsiella pneumoniae*	-	-	-	-	-	-
MDR3	*Acinetobacter baumannii*	-	-	-	-	-	-
MDR4	*Enterobacter aerogenes*	-	-	-	7,00 ± 0,00	7,00 ± 0,00	7,00 ± 0,00
MDR5	*Serratia odorifera*	-	-	-	-	-	-
MDR6	*Proteus vulgaris*	-	7,00 ± 0,00	8,00 ± 0,00	-	-	-
MDR7	*Streptococcus pneumoniae*	7,00 ± 0,00	8,00 ± 0,00	10,00 ± 0,00	7,00 ± 0,00	7,00 ± 0,00	8,00 ± 0,00
MDR8	*Staphylococcus aureus* MRSA	9,00 ± 0,00	7,00 ± 0,00	12,00 ± 0,00	-	-	-
MDR9	*Staphylococcus aureus* MRSA+MDR	11,00 ± 0,00	13,00 ± 0,00	14,00 ± 0,58	-	-	-
MDR10	*Providencia rustigianii*	-	-	-	-	-	-
MDR11	*Achromobacter* sp.	-	-	-	-	-	-

"-": No information, *: The data is given as the mean values of three replicates with standard errors

TABLE 2
Positive controls against all strains except MDR strains

Lab. Code	1	2	3	4	5	6	7	8	9	10	11	12	13
ST1	30	26	36	44	34	37	38	41	36	44	20	52	42
ST2	12	13	-	-	-	-	-	-	-	-	-	-	-
ST4	12	8	19	14	-	19	29	14	-	-	15	28	29
ST5	22	20	7	18	-	22	-	6	6	6	6	16	12
ST6	28	24	27	24	11	25	-	23	-	-	23	34	32
ST7	15	22	18	-	-	9	-	-	-	-	-	-	-
ST8	13	12	19	10	8	22	-	14	-	-	16	26	26
ST9	21	15	24	23	-	28	27	16	-	16	-	28	31
ST10	24	15	35	22	-	27	27	13	-	14	-	26	21
ST11	21	14	22	31	24	21	16	25	22	29	16	30	27
ST12	22	20	34	37	35	33	26	24	26	32	21	45	32
FI1	11	13	24	36	30	8	29	28	22	26	24	37	32
FI2	28	15	28	40	30	11	31	32	24	33	26	43	34
FI3	19	23	30	-	-	22	6	6	6	6	-	9	6
FI4	13	15	18	14	-	23	-	13	-	-	16	28	33
CI1	22	18	23	-	-	-	-	-	-	-	-	-	-

"-": No activity; 1: Gentamicin; 2: Tobramycin; 3: Ciprofloxacin; 4: Cefazolin; 5: Clindamycin; 6: Chloramphenicol; 7: Ceftriaxone ; 8: Ampicillin; 9: Cephalothin; 10: Cefuroxime; 11: Vancomycin; 12: Amoxicillin/Clavulanic acid; 13: Trimethoprim/Sulfamethoxazole

TABLE 3
Positive controls against MDR strains

Lab. Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
MDR4	16	18	32	11	-	31	32	-	-	18	-	-	30	15	33	15	-
MDR6	11	11	42	-	9	22	23	9	-	20	-	9	30	10	37	32	12
MDR7	10	8	42	-	9	22	26	9	-	19	9	10	8	10	40	31	15
MDR8	-	7	-	-	45	27	8	12	-	-	26	11	24	14	-	11	12
MDR9	22	21	27	26	38	30	19	22	28	31	19	25	30	15	-	23	23

"-": No activity; 1: Gentamicin; 2: Tobramycin; 3: Ciprofloxacin; 4: Cefazolin; 5: Clindamycin; 6: Chloramphenicol; 7: Ceftriaxone ; 8: Ampicillin; 9: Cephalothin; 10: Cefuroxime; 11: Vancomycine; 12: Amoxicillin/Clavulanic acid; 13: Trimethoprim/Sulfamethoxazole; 14: Clarithromycin; 15: Aztreonam; 16: Piperacillin/Tazobactam; 17: Ampicillin/Sulbactam

TABLE 3 (Continued)
Positive controls against MDR strains

Lab. Code	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
MDR4	31	8	8	-	-	8	29	14	-	28	9	13	25	8	8	30
MDR6	25	13	-	24	11	8	26	12	12	26	16	-	12	8	-	22
MDR7	27	11	-	24	13	9	29	10	10	30	13	9	13	8	-	14
MDR8	8	-	-	9	40	19	14	-	-	9	7	8	23	-	-	-
MDR9	19	36	17	21	33	18	25	9	20	56	20	8	17	-	8	22

"-": No activity; 18: Ceftazidime; 19: Rifampicin; 20: Oxacillin; 21: Piperacillin; 22: Linezolid; 23: Teicoplanin; 24: Amicasin; 25: Polymyxin B; 26: Cefoxitin; 27: İmipenem; 28: Sulbactam/Cefoperazone; 29: Colistin sulfate; 30: Furazolidone; 31: Optochin; 32: Bacitracin; 33: Cefotaxime.

According to Table 1, HSFEt shows antimicrobial activity against 18 of 41 strains. One of them has high susceptibility (≥ 15 mm), ten of them have moderate susceptibility (14-10 mm), and seven of them have low susceptibility (9-7 mm). HSLEt has antimicrobial activity against 12 of 41 strains. Three of them have moderate susceptibility (14-10 mm) and nine of them have low susceptibility (9-7 mm) [19]. The most susceptible gram-positive bacteria strain is ST11 with a 15 mm inhibition zone and the gram-negative bacteria strain is ST8 with a 10 mm inhibition zone in HSFEt. The most susceptible gram-positive bacteria strain is ST11 with an 11 mm inhibition zone and the gram-negative bacteria strains are ST9, ST10, FI3, and MDR4 with a 7 mm inhibition zone in HSLEt. In additionally the HSFEt has shown highly effective results against ST12, FI2, CI1, MDR8, and MDR9. Also, the HSLEt has shown highly effective results against ST1, ST6, FI1, FI2, CI1, and MDR7.

ST5 has more effective results than Ampicillin, Cephalothin, Cefuroxime, and Vancomycin, while ST8 showed more effective results than Clindamycin in HSFEt. ST11 showed more effective results than Gentamicin, while FI1 and FI2 have more effective results than Chloramphenicol in HSFEt. It was determined that MDR7 and MDR8 are more effective than 8 antibiotics, and MDR9 is more effective than 3 antibiotics in HSFEt. Only FI3 showed more effective results than Ceftriaxone, Ampicillin, Cephalothin, Cefuroxime, and Trimethoprim/Sulfamethoxazole in HSLEt. According to these results, it is observed that HSFEt has a higher antimicrobial activity than HSLEt.

Punasiya et al. [20] reported the antimicrobial activity of dichloro-methane, isopropyl alcohol, and petroleum ether extracts of *Hibiscus syriacus* leaves against *B. cereus* (MTCC 430), *E. coli* (MTCC433), *K. pneumoniae* (MTCC432), and *S. aureus* (MTCC 3160). Petroleum ether and isopropyl alcohol extracts showed antimicrobial activity against these strains. According to our results, HSLEt is more effective against *S. aureus* (ST11) than petroleum ether extract of *H. syriacus* leaves.

Panaitescu and Lengyel [21] showed the antimicrobial activity of aqueous, ethanol 50%, and ethanol 96% extracts of flowers of *Hibiscus sabdariffa* against *E. coli* ATCC 25922, *E. cloacae* ATCC 13047, *K. pneumoniae* ATCC 13833, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 29213, and *Y. enterocolitica* ATCC 9610. When we compared two studies with each other, it appears that HSFEt has high antimicrobial activity against similar strains as *E. coli* ATCC 25922 and *S. aureus* ATCC 29213.

Mak et al. [22] investigated the antimicrobial activity of aqueous and ethanolic extracts of flowers of *Hibiscus rosa-sinensis* against *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumonia*, *L. monocytogenes*, *S. typhimurium*, *S. enteritidis*, and *S. aureus*. The ethanolic extract showed antimicrobial activity against only *S. aureus*. Our results presented that HSFEt is more effective results against *S. aureus*. In the light of all these results, it has been determined that both HSFEt and HSLEt have a high antimicrobial potential.

Biochemical composition of HSFEt and HSLEt. The major components of HSFEt and HSLEt, which were observed higher than 1%, and their composition percentages are given in Table 5

TABLE 4
Biochemical composition of HSFEt

No	Retention Time	Components	Formula	Molecular Weight (g/mol)	Area (%)
1	22.028	Nonanoic acid	$C_9H_{18}O_2$	158.238	1.79
2	22.739	Ethyl nonanoate	$C_{11}H_{22}O_2$	186.291	1.61
3	41.705	Palmitic acid	$C_{16}H_{32}O_2$	256.424	12.18
4	42.389	Ethyl palmitate	$C_{18}H_{36}O_2$	284.477	2.59
5	45.785	Linoleic acid	$C_{18}H_{32}O_2$	280.445	9.81
6	45.915	Linoleic acid	$C_{18}H_{32}O_2$	280.445	7.76
7	45.843	Ethyl linoleate	$C_{20}H_{36}O_2$	308.499	6.20
8	46.551	Ethyl linolenate	$C_{20}H_{34}O_2$	306.483	1.74
9	54.762	2-Methoxyaniline	C_7H_9NO	123.152	1.69
10	59.694	2,3-Dihydroxypropyl stearate	$C_{21}H_{42}O_4$	358.556	20.38
11	59.911	2,3-Dihydroxypropyl stearate	$C_{21}H_{42}O_4$	358.556	2.10
12	63.697	1-Heptacosene	$C_{27}H_{54}$	378.718	1.03
13	63.961	1-Tetracosanol	$C_{24}H_{50}O$	354.653	2.64
14	64.087	Eicosane	$C_{20}H_{42}$	282.547	1.04
15	68.563	UNKNOWN	-	-	1.50
16	71.483	UNKNOWN	-	-	2.57
17	73.040	UNKNOWN	-	-	1.15
18	74.769	UNKNOWN	-	-	6.18
19	75.483	UNKNOWN	-	-	1.60
20	76.950	UNKNOWN	-	-	1.05
21	78.936	UNKNOWN	-	-	1.36

"-": No information

TABLE 5
Biochemical composition of HSLEt

No	Retention Time	Components	Formula	Molecular Weight (g/mol)	Area (%)
1	43.578	Palmitic acid	$C_{16}H_{32}O_2$	256.424	4.74
2	47.868	Phytol	$C_{20}H_{40}O$	296.531	1.97
3	48.697	Linoleic acid	$C_{18}H_{32}O_2$	280.445	3.71
4	48.882	α-Linolenic acid	$C_{18}H_{30}O_2$	278.430	4.81
5	49.574	Ethyl linolenate	$C_{20}H_{34}O_2$	306.483	1.25
6	60.714	2-Methoxyaniline	C_7H_9NO	123.152	4.14
7	67.986	2,3-Dihydroxypropyl stearate	$C_{21}H_{42}O_4$	358.556	50.33
8	68.182	2,3-Dihydroxypropyl stearate	$C_{21}H_{42}O_4$	358.556	6.21
9	85.784	UNKNOWN	-	-	3.01
10	89.536	UNKNOWN	-	-	5.17
11	90.202	β-Amyrin acetate	$C_{32}H_{52}O_2$	468.754	1.95
12	91.856	UNKNOWN	-	-	1.89

"-": No information

and 6, according to the data obtained from the GC-MS analysis. According to Table 4, 2,3-Dihydroxypropyl stearate (22.48%), Linoleic acid (17.57%), and Palmitic acid (12.18%) are major components in the biochemical composition of HSFEt. According to Table 5, 2,3-Dihydroxypropyl stearate (56.54%), α-Linolenic acid (4.81%), and Palmitic acid (4.74%) are major components in the biochemical composition of HSLEt.

CONCLUSIONS

H. syriacus is a medicinal plant with antimicrobial activity. HSFEt and HSLEt showed antimicrobial activity against 18 and 12 microorganism strains, respectively. Purification and further studies are recommended to determine whether the antimicrobial activity is caused by a single known biochemical compound, such as 2,3-Dihydroxypropyl stearate, or by a synergistic effect. Besides, some compounds found in the extracts are not matching with the library. As a reason for that, this medicinal plant is proposed to contain some unknown molecules and they should be identified and their 3D structure should also be determined. The unknown compounds, which consist of 15.41% and 10.07%, respectively, should be analyzed in detail. Also, the mode of action(s) of the extract should be determined in further studies.

ACKNOWLEDGEMENTS

The authors acknowledge the Dokuz Eylül University Scientific Research Coordination Unit for funding this research (project number 2018.KB.FEN.021).

Author contributions KC and EMA planned the study. KC, MEB, AB, DT, MAY, EMA designed, performed the experiments, and analyzed the results. MEB conducted literature retrieval and drafted the manuscript. KC and EMA supervised the experiments, critically reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Received: **25.11.2021**
Accepted: **11.01.2022**

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