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RESEARCH PAPER

Chemical Profile of *Malva Neglecta* and *Malvella Sherardiana* by LC-MS/MS, GC/MS and Their Anticholinesterase, Antimicrobial and Antioxidant Properties With Aflatoxin-Contents

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ABSTRACT

The purpose of the present work was to determine the phenolic and the fatty acids profiles by LC-MS/MS and GC/MS with their antioxidant, anticholinesterase and antimicrobial activities, and aflatoxin contents of *Malva neglecta* and *Malvella sherardiana*. The phenolic contents of *M. neglecta* and *M. sherardiana* were determined by LC-MS/MS. Malic and 4-OH benzoic acids were found to be the most abundant compounds in *M. neglecta* and *M. sherardiana*, respectively. On the other hand, essential oil and fatty acid compositions were determined by GC/MS analysis. The methanol extracts of the plants showed the highest effect in all antioxidant assays in this study. The methanol extract of *M. neglecta* showed the highest activity among the petroleum ether, acetone, methanol and water extracts against acetyl- and butyryl- cholinesterase enzymes (53.68% and 63.95% inhibition ratio, respectively). The acetone extracts of *M. neglacta* and *M. sherardiana* exhibited moderate activity against *C. albicans* with 18 and 17 mm inhibion zone diameter, respectively. The results of the present study is also showed that the *M. neglecta* and *M. sherardiana* can also be used as a food source due to its high phenolic acid content and good antioxidant property. Also, the samples were aflatoxin free.

Keywords: Anticholinesterase; antioxidant; *Malva neglecta*; *Malvella sherardiana*; LC-MS/MS, GC-MS

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INTRODUCTION

Malvaceae family includes the genera *Malva* L. and *Malvella* JAUB & SPACH. The genus *Malva* is represented by 26 species in all over the World and 9 species growing in Turkey (1). *M. neglecta* Wallr. is distributed in Europae, Africa and Asia. It is called as ebegümeci, develik, gömeç, kömeç, ebegömeci and tolık by local people in Turkey. Leaves and flowers of M. neglecta have a wide range of usage area in the treatment of constipation, sore throat, women sterility, wound, hemorrhoids, miscarriage swellings, rheumatic pain, stomachache, abdominal pain, abscess, renal diseases, ease cough, throat infection, peptic ulcer, common cold, stomachache, bronchitis, indigestion in Turkey (2-4).

In literature, there are some studies on *Malva* species. The anti-ulcerogenic (5), antibacterial (6), antioxidant properties and total phenolic contents of *M. neglecta* was investigated (7-8). Moreover, polyphenols and their antioxidant activity in callus-cultured *M. neglecta* cells under UV-B and UV-C irradiation were also reported (9). In vitro antioxidant and enzyme inhibitory properties and phenolic composition of only *M. neglecta* fruit were investigated (10). Macro and micro mineral content and characterization of phenolic compounds in flowers of *M. neglecta* and *M. sylvestris* were investigated (11- 12).

Genus *Malvella* is represented by 4 species in all over the world and a species growing in Turkey (1). *M. sherardiana* (L.) JAUB & SPACH. is distributed from Europe to Caucasia and Syria. It is known as ebekömeci, ebemkömeci, ebemgömeci in Turkey. Leaves and flowers of M. sherardiana are used as food and in the treatment of kidney disease and constipation (2-3). There is no report available in the literature on the chemical properties and bioliogical activities of *Malvella sherardiana* and *Malvella* species. The high content of polyprenols, which are chemotaxonomic markers, makes Malvaceae family plants exceptional among leafy plants (13).

The literature survey shows that there are no phenolic contents, essential oil and fatty acid composition or biological activity reports on *M. sherardiana*. Moreover, to our knowledge, fatty acid composition or anticholinesterase activity report on *M. neglecta* in the literature would not exist. The cooking and consumption pattern of both species which are the member of same family resemble each other (2-3), therefore we aimed to analyze both species and compare the results. Firstly, the essential oil and fatty acid compositions of *M. neglecta* and *M. sherardiana* were determined using GC/MS analyses, and then associated antioxidant, anticholinesterase, and antimicrobial activities, total phenolic and flavonoid contents, total aflatoxin and AFB1 levels were examined. Additionally, the phenolic and flavonoid contents of *M. neglecta* and *M. sherardiana* methanol extract were also determined using LC-MS/MS for quantitative and qualitative purposes. Also, aflatoxin contents were determined using HPLC –FLD.

MATERIALS AND METHODS

Plant Material

The whole plants in flowering stage of *M. neglecta* WALLR. and *M. sherardiana* (L.) JAUB. ET SPACH. were collected from southeastern Turkey (Diyarbakır) in July 2012 by Dr. Abdulselam Ertaş, and identified by Assist.Prof. Dr. Serpil Demirci (Çukurova University, Faculty of Pharmacy, Dept of Pharmaceutical Botany). These specimens have been stored at the Herbarium of Istanbul University (ISTE 97150 and ISTE 98928, respectively)

Preparation of the Extracts And Chromatographic Conditions For Aflatoxin Analysis With HPLC-FLD

25 g Aerial parts of plants were weighed and 300 mL of methanol (70%) was added**.** After shaking vigorously for thirty minutes in blender (Waring, 8011.S), the extract was filtered through Whatman No. 1 filter.10 mL of the obtained filtrate was diluted with 50 mL of PBS and 60 mL of the supernatant were passed through an immunoaffinity column. After the sample had passed, the column was washed with 10 mL of distilled water. Finally, the column was dried with air and mycotoxins were eluted with 1.5 mL of methanol and added 1.5 mL of water into the vial. 100 µL of this eluate was injected to HPLC for aflatoxin.

The instrument was used a Shimadzu LC-20 liquid chromatographic system equipped with a fluorescence detector (LC-20A) and controlled by Lab Solutions software. Separation was achieved on Cronusil-S ODS2 C18column (25 cm x 0.46 cm; 5 µm). A post column electrochemical derivatisation was used to enhance aflatoxins responses using KOBRA –cell, 100 mA. The injection volume was 100 µL and the flow rate was 1.00 mL/min. Cromatography was performed at 40°C with isocratic separation a mixture of acetonitril, methanol and water (2: 3: 6 v/v) with 0.132 g potassium bromide, acidified with nitric acid. The wavelenghts of excitation and emissions were fixed 365 and 435 nm. The LOD was 0.630 µg/kg for total aflatoxin and the recoveries were between % 83-96. Aflatoxins are toxic substances. They were always manipulated in solution, avoiding the formation of dust and aerosols. Nitrile gloves were used for all procedures.

Preparation of Extracts for Biological Activities, GC/MS and LC-MS/MS

The whole plant materials (including radix, herba, flower) were used for preparation of the extracts which were dried and powdered. 100 g of each plant material was sequentially macerated with petroleum ether, acetone, methanol and water for 24 hour at 25°C. To obtain the crude extracts, the solvents were evaporated after filtration. Afterwards, dry filtrates were reconstituted in methanol at a concentration of 250 mg/L and filtered through the 0.2 µm PTFE filter prior to LC-MS/MS analysis.

Esterification of Total Fatty Acids and GC/MS Conditions

Esterification of the petroleum ether extract and GC/MS procedure described by Ertaş et al. were applied (14). Thermo Scientific Polaris Q GC-MS/MS was used.

Preparation of Essential Oil and GC/MS Conditions

Essential oils were obtained using a Clevenger apparatus from the whole parts (including radix, herba, flower) of *M.*

neglecta and *M. sherardiana*, which were crumbled into small pieces and soaked in distilled water for 3 h. The obtained essential oils were dried over anhydrous Na_2SO_4 and stored at +4°C for a sufficient period of time. The essential oils were diluted using CH_2Cl_2 (1:3 volume/volume) prior to GC/FID and GC/MS analysis. GC/FID performed using Thermo Electron Trace GC FID detector and GC/MS performed using same GC and Thermo Electron DSQ quadrupole for MS. A nonpolar Phenomenex DB5 fused silica column (30 m ´ 0.32 mm, 0.25 µm film thickness) was used with helium at 1 mL/min (20 psi) as a carrier gas. The GC oven temperature was kept at 60°C for 10 min and programmed to 280°C at a rate of 4°C/min and then kept constant at 280°C for 10 min. The split ratio was adjusted to 1:50, the injection volume was 0.1 µL, and EI/MS was recorded at 70 eV ionization energy. The mass range was *m/z* 35–500 amu. Alkanes (C8-C24) were used as reference points in the calculation of Kovats Indices (KI) by the same conditions (15- 16). Identification of the compounds was based on comparing their retention times and mass spectra with those obtained from authentic samples and/or the NIST and Wiley spectra as well as data from the published literature. GC/FID and GC/MS were replicated three times (Mean RSD % <0.1).

Determination of Total Phenolic and Flavonoid Contents

Total phenolic and flavonoid amounts in the crude extracts expressing as pyrocatechol and quercetin equivalents were calculated according to the following equations (17-18):

Absorbance = 0.0164 pyrocatechol (µg) + 0.0266 (R^2 = 0.9969)

Absorbance = 0.1519 quercetin (µg) – 0.1294 (R^2 = 0.9986)

Antioxidant Activity of the Extracts

β-carotene-linoleic acid test system, DPPH free radical and ABTS cation radical scavenging activity and cupric reducing antioxidant capacity (CUPRAC) methods were used to determine the antioxidant activity (19-23).

Anticholinesterase Activity of the Extracts

A spectrophotometric method developed by Ellman *et al.* was used to indicate the acetyl- and butyryl-cholinesterase inhibitory activities (24- 25).

Determination of Antimicrobial Activity

The antimicrobial activities of the extracts against different microorganisms including yeast, Gram positive and Gram negative bacteria were assessed according to inhibition zone diameter by disc diffusion method (26). Imipenem (IPM) and Nystatin are used as positive controls for bacteria and yeast, respectively.

Identification and Quantitation of Phenolic Compounds

Instruments and Chromatographic Conditions

LC-MS/MS analyses of the phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatograph was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC autosampler. The chromatographic seperation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3μm) analytical column. The column temperature was fixed at 40°C. The elution gradient consisted of mobile phase A (water, 5mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5mM ammonium formate and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied t (min), %B: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The solvent flow rate was maintained at 0.5 mL/min and injection volume was settled as 4 μL.

Method Validation Parameters

In this study, twenty-four phenolic compounds (flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde, coumarin) and three non-phenolic organic acids that are widespread in edible plant materials were qualified and quantified in two edible plants. Rectilinear regression equations and the linearity ranges of the studied standard compounds were given in Table 1. Correlation coefficients were found to be higher than 0.99. The limit of detection (LOD) and limit of quantitation (LOQ) of the reported analytical method were shown in Table 1. For the studied compounds, LOD ranged from 0.05 to 25.8 µg/L and LOQ ranged from 0.17 to 85.9 µg/L (Table 1) (28). Moreover, the recoveries of the phenolic compounds ranged from 96.9% to 106.2%.

Statistical Analysis

The results of the antioxidant and anticholinesterase activity assays were expressed as means ± SD. The results were evaluated using an unpaired *t*-test and ANOVA variance analysis with the NCSS statistical computer package. The differences were considered statistically significant at $p< 0.05$.

RESULTS AND DISCUSSION

Quantitative Analysis of Phenolic and Flavonoid Compounds by LC-MS/MS

In the quantitative analysis of phenolic compounds various studies exist in literature reporting the use of LC-MS/MS or HPLC (29-30). Therefore, an accurate quantitative method was developed on a mass spectrometer equipped with a triple quadrupole analyzer for the analyses of twenty-seven compounds (Table 1). The methanol extracts of *M. neglecta* and *M. sherardiana* were screened to quantify their phenolic composition by using a Shimadzu LCMS 8040 model liquid chromatograph combined to a triple quadrupole mass spectrometer. This accurate method allowed us to qualify and quantify 27 different compounds including several phenolic acids, flavonoids and some none-phenolic compounds. Names and molecular ions of the investigated compounds, related daughter ions found in LC-MS/MS, collision energies for the corresponding ions and the quantified results for the studied plant species are shown in Table 2.

According to the present study, it is obvious that both *M. neglecta and M. sherardiana* are rich in terms of phenolic acids. Their phenolic acid profile are almost paralel to each other. However, malic acid content of *M neglecta* (13108.97± 694.77 µg/g) is considerably higher than that of *M. sherardiana* (1625.07± 86.12 µg/g) (Figure 1). Both species have considerable amounts of 4-hydroxy benzoic and salicylic acids. Additionally, *M. sherardiana* (1317.41± 63.21 µg/g) has higher amount of quinic acid than *M. neglecta* (155.93± 7.44 µg/g). On the other hand, according to the present study, both studied species are almost poor in terms of flavonoid compounds. But we might still say that, *M. sherardiana* is richer than *M. neglecta* in terms of flavonoid compounds. To illustrate, *M. sherardiana* (1429.29± 70.02 µg/g) contains good amount of hyperoside unlike *M. neglecta* that contains none of it. In addition, rutin (449.00± 22.45 µg/g) and hesperidin (591.25± 28.96 µg/g) content of *M. sherardiana* is much more than *M. neglecta*.

In literature, there are several sudies about *M. neglecta* but there is only one previous study related to the quantification of phenolic compounds in *M. neglecta*. In a previous study, the total phenolic, total proanthocyanidin, total 4-hydroxycinnamic acid, total flavonol and total anthocyanin contents of *M. neglecta* Wallr. was investigated (8). In another study a few phenolic compounds were quantified HPLC-DAD and HPLC-PDA-MS (10). To add up, two studies were also conducted about determination of phenolic compounds by LC-MS/MS on *Malva sylvestris* (11-12). To summarize, this is the first study to screen that many of 27 compounds in *M. neglecta* and that is the only study for the phenolic compound quantification of *M. sherardiana*.

Aflatoxin Content

In the present investigation, aflatoxin contents were determined using HPLC with flouresence detector (Figure 2). Total aflatoxin and AFB1 were not detected in these plants, so may be they can be used in public medicine as drug. Most of the analysed food can get contaminated with aflatoxins: pre-harvest, post-harvest, during the drying process, transportation and/or in storage. Mycotoxin which is a toxic secondary metabolite of fungus can cause significant health problems. So prevention of mycotoxin contamination has become an important issue in food control to protect human and animal health.The findings of the current work exhibit that control around the world should be regularly and effectively.

Fatty Acid Composition by GC-MS

The fatty acid composition of the petroleum ether extracts were ascertained by GC/MS analysis. Eleven components were identified as it can be seen in Table 3; constituting 97.9% of the petroleum ether extract of *M. neglecta*. The major constituents of the fatty acid obtained from the petroleum ether extract were identified as palmitic acid (C16:0) (36.8%), linoleic acid (C18:2 omega-6) (17.8%) and linolenic acid (C18:3 omega-3) (13.2%). The persentage of saturated and unsaturated faty acid is found to be 55.5 % and 42.4 %, respectively in *M.neglecta*. Twelve components were ascertained, constituting 99.2% of the petroleum ether extract of *M. sherardiana*. The major constituents of the fatty acid obtained from the petroleum ether extract of *M. sherardiana* were characterized as stearic acid (C18:0) (29.9%), palmitic acid (25.6%), and linoleic acid (15.6%). The persentage of saturated and unsaturated faty acid is found to be 65%and 34.2%, respectively in *M. sherardiana*.

When the fatty acid compositions of *M. neglecta* and *M. sherardiana* compared, they have palmitic acid (36.8% and 25.6%, respectively), oleic acid (4.6% and 14.5%), linolenic acid (13.2% and 3.2%) and stearic acid (6.8% and 29.9%). The persentage of saturated fatty acids is higher than unsaturated fatty acids in both species. This is the first report on the fatty acid compositions of *M. neglecta* and *M. sherardiana*.

There are some studies on fatty acid compositions of Malvaceae family in literature. The major component of

Table 1. Analytical parameters of LC-MS/MS method

Analyte no	Analytes	RT[a]	Equation	R²[b]	RSD%[c]	Linearity Range (mg/L)	LOD/LOQ (µg/L)[d]	Recovery (%)	U[e]
1	Quinic acid	3.36	f(x)=33.6626*x+25132.9	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8
2	Malic acid	3.60	f(x)=93.6102*x-5673.77	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3
3	tr-Aconitic acid	4.13	f(x)=79.2908*x-28416.2	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9
4	Gallic acid	4.25	f(x)=358.069*x+26417.5	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1
5	Chlorogenic acid	5.29	f(x)=48.9828*x+26779.7	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9
6	Protocatechuic acid	5.51	f(x)=36.8568*x+6197.38	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1
7	Tannic acid	6.30	f(x)=90.2704*x+30233.2	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1
8	tr- caffeic acid	7.11	f(x)=1585.16*x+83957.5	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2
9	Vanillin	8.57	f(x)=44.5478*x-574.867	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9
10	p-Coumaric acid	9.17	f(x)=73.5303*x+27064.3	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1
11	Rosmarinic acid	9.19	f(x)=18.0298*x-1149.86	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9
12	Rutin	9.67	f(x)=51.8835*x+3841.66	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0
13	Hesperidin	9.69	f(x)=195.773*x+105641	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9
14	Hyperoside	9.96	f(x)=0.978146*x+827.221	0.9549	0.2135	100-4000	12.4 / 41.4	98.5	4.9
15	4-OH Benzoic acid	11.38	f(x)=635.003*x+54284.6	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2
16	Salicylic acid	11.39	f(x)=915.178*x+72571.4	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0
17	Myricetin	11.42	f(x)=54.2823*x+5414.67	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9
18	Fisetin	12.10	f(x)=331.870*x+34409.0	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5
19	Coumarin	12.18	f(x)=236.639*x+34370.3	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9
20	Quercetin	13.93	f(x)=206.102*x+1693.14	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1
21	Naringenin	14.15	f(x)=1100.55*x+39055.7	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5
22	Hesperetin	14.80	f(x)=160.323*x+6545.07	0.9961	1.0164	25-1000	3.3/ 11.0	102.4	5.3
23	Luteolin	14.84	f(x)=111.474*x+3057.10	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9
24	Kaempferol	14.85	f(x)=20.9677*x+571.241	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2
25	Apigenin	16.73	f(x)=543.793*x+18525.6	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3
26	Rhamnetin	18.41	f(x)=110.091*x+632.444	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1
27	Chrysin	20.60	f(x)=698.787*x+23531.7	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3

[a]RT: Retention time, [b]R^2: coefficient of determination, [c]RSD: relative standard deviation, [d]LOD/LOQ(*µ*g/L): Limit of deteection/Limit of quantification, [e]U (%): Percent relative uncertainty at 95% confidence level (k=2).

the fatty acids was identified as linoleic acid (44.16-67.50%, bettwen) for *Thespesia populnea*, *Gossypium hirsutum* (31), *Malva sylvestris*, *M. sylvestris* var. *mauritiana*, *Althaea officinalis* (32) and *Hibiscus trionum* (33). It is found as palmitic acid (C16:0) (36.8%) and stearic acid (C18:0) (29.9%) for major constitutent in *M. neglecta* and *M. sherardiana* against the literature survey, respectively. Even if it is not a major constituent, linoleic acid was detected as 17.8% and 15.6% in both studied species.

Chemical Composition of the Essential Oils

Essential oils were obtained using a Clevenger apparatus by hydrodistillation from the whole parts of *M. neglecta* and *M. sherardiana*. The essential oils composition of both edible plants were determined by GC/FID and GC/MS analysis (Table 4). Forty one components were determined, constituting 95.0% of the essential oil composition of *M. neglecta*. The main constituents of the essential oil were

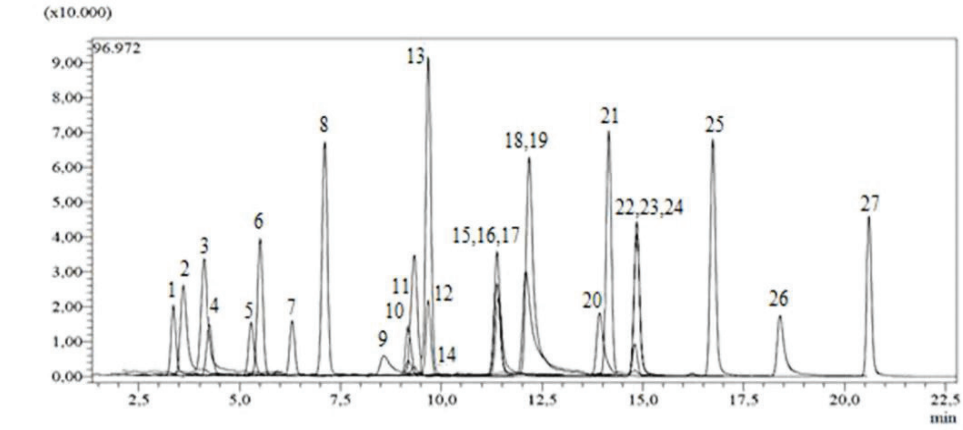
identified as cineole (18.8%), hexatriacontane (7.8%), tetratetracontane (7.8%) and α-selinene (4.2%) (Table 4). Eight components were determined, constituting 94.1% of the essential oil composition of *M. sherardiana*. The main constituents were 1-hexacosanol (19.1%), α-selinene (17.5%), 9-hexyl heptadecane (12.6%) and 2,5-di-tert octyl-p-benzoquinone (12.1%) (Table 4). When compared these two essential oil composition of Malvaceae family, *M. neglecta* is seemed to have richer content than *M. sherardiana*. It is found that monoterpene amount of *M. neglecta* essential oil was higher than *M. sherardiana*. On the other hand, essential oil of *M. sherardiana* was rich rich in fatty acid ester and hydrocarbons. Even among the species of a genus have different chemical contituents, this different chemical composition between different genus members is not surprising. In addition to this, cineole (18.8%) and

Table 2. Identification and quantification of phenolic compounds of methanol extracts of *M. neglecta* and *M. sherardiana*.

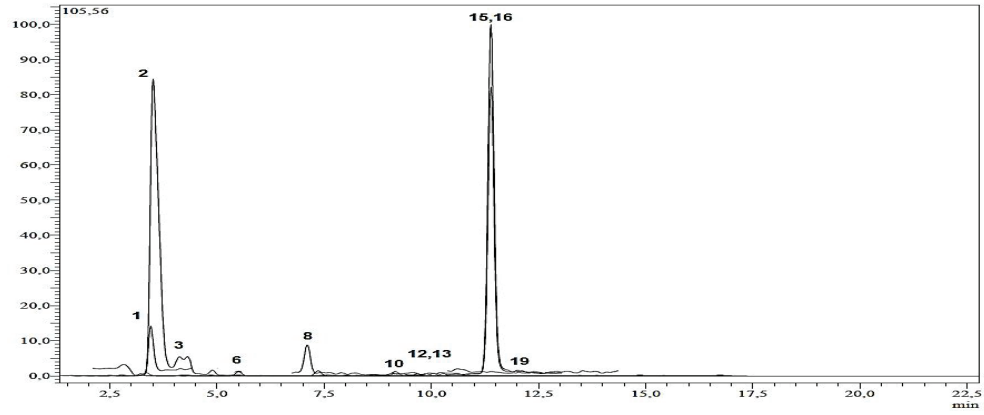
No	Compound	Parent ion (m/z)[a]	MS^2(Collision Energy)[b]	Quantification (µg analyte/g extract)[c]	
				MNM	MSM
1	Quinic acid	190,95	85 (22),93 (22)	155.93± 7.44	1317.41± 63.21
2	Malic acid	133,05	115 (14),71 (17)	13108.97± 694.77	1625.07± 86.12
3	tr-Aconitic acid	172,85	85 (12),129 (9)	284.98± 13.91	207.00± 10.14
4	Gallic acid	169,05	125 (14),79 (25)	N.D.[d]	N.D
5	Chlorogenic acid	353	191 (17)	N.D.	N.D.
6	Protocatechuic acid	152,95	109 (16),108 (26)	282.76± 14.38	233.61± 11.88
7	Tannic acid	182,95	124 (22),78 (34)	N.D.	N.D.
8	tr- caffeic acid	178,95	135 (15),134 (24),89 (31)	70,38± 3.65	N.D.
9	Vanillin	151,05	136 (17),92 (21)	N.D.	59.56±
10	p-Coumaric acid	162,95	119 (15),93 (31)	263,32± 13.41	311.58± 15.86
11	Rosmarinic acid	358,9	161 (17),133 (42)	N.D.	N.D.
12	Rutin	609,1	300 (37), 271 (51), 301 (38)	N.D.	449.00± 22.45
13	Hesperidin	611,1	303,465	N.D.	591.25± 28.96
14	Hyperoside	463,1	300,301	N.D.	1429.29± 70.02
15	4-OH Benzoic acid	136,95	93,65	1694.92± 88.09	2200.57±114.42
16	Salicylic acid	136,95	93,65,75	1456.04± 72.80	2014.27± 100.70
17	Myricetin	317	179,151,137	N.D.	N.D.
18	Fisetin	284,95	135,121	N.D.	N.D.
19	Coumarin	146,95	103,91,77	N.D	N.D.
20	Quercetin	300,9	179,151,121	N.D.	N.D.
21	Naringenin	270,95	151,119,107	N.D.	N.D.
22	Hesperetin	300,95	164,136,108	N.D.	N.D.
23	Luteolin	284,95	175,151,133	N.D.	20.01± 1.38
24	Kaempferol	284,95	217,133,151	N.D.	N.D.
25	Apigenin	268,95	151,117	N.D.	46.58± 2.99
26	Rhamnetin	314,95	165,121,300	N.D.	N.D.
27	Chysin	253	143,119,107	N.D.	3.99± 0.21

[a]Parent ion (*m/z):* Molecular ions of the standard compounds (mass to charge ratio), [b]MS^2(CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions), [c]Values in µg/g (w/w) of plant methanol extract, [d]N.D: not detected

A



B



C

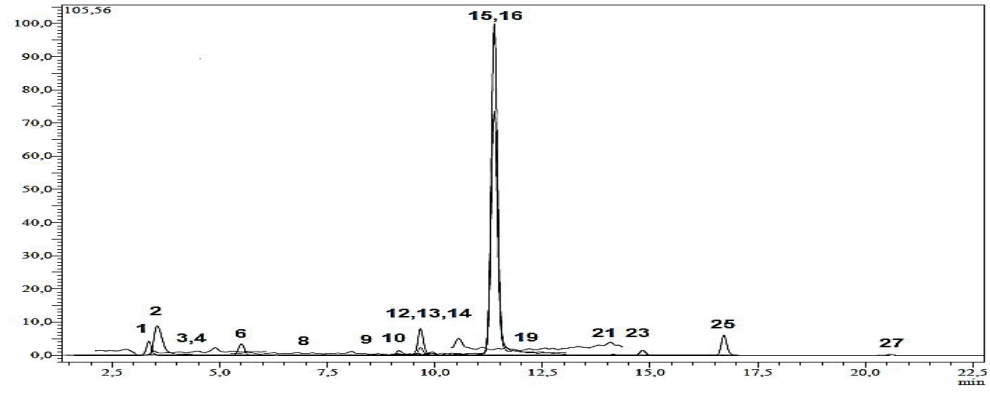
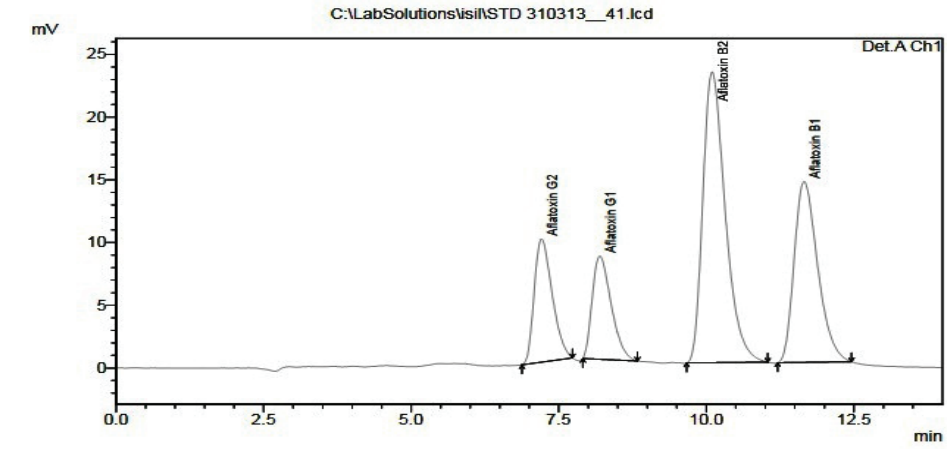
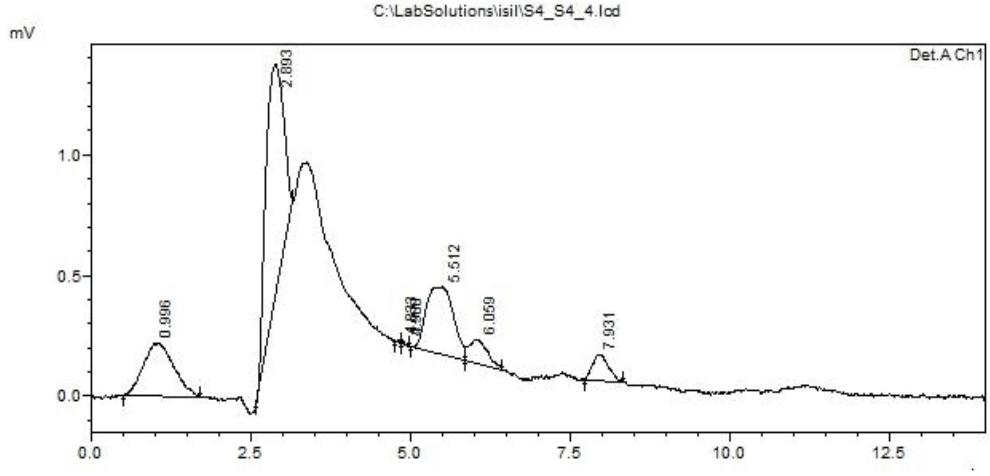


Figure 1. UHPLC-ESI-MS/MS chromatograms of A: 250 ppb standard mix, B: *M. neglacta* methanol extract (MNM), C: *M. sherardiana* methnol extract. (MSM). 1: Quinic acid, 2: Malic acid, 3: tr-Aconitic acid, 4: Gallic acid, 5: Chlorogenic acid, 6: Protocatechuic acid, 7: Tannic acid, 8: tr- caffeic acid, 9: Vanillin, 10: p-Coumaric acid, 11: Rosmarinic acid, 12: Rutin, 13: Hesperidin, 14: Hyperoside, 15: 4-OH Benzoic acid, 16: Salicylic acid, 17: Myricetin, 18: Fisetin,19: Coumarin, 20: Quercetin, 21: Naringenin, 22: Hesperetin, 23: Luteolin, 24: Kaempferol, 25: Apigenin, 26: Rhamnetin, 27: Chrysin.

A



B



C

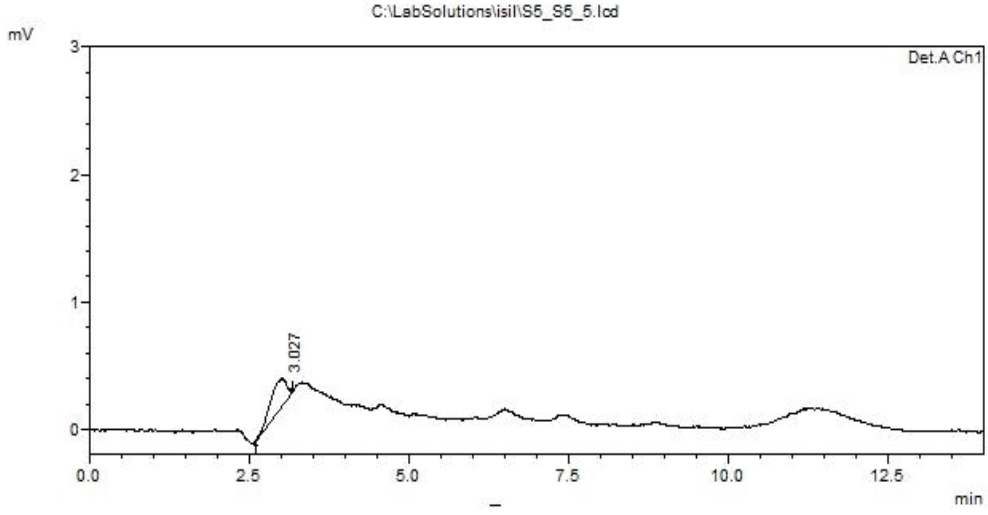


Figure 2. Aflatoxin chromatogram of A: standards, B: *M. neglacta* and C: *M. sherardiana*

Table 3. GC-MS Analysis of the petroleum ether extracts of *M. neglecta* and *M. sherardiana*

Rt (min)[a]	Constituents[b]	%Composition MN	%Composition MS
9.69	Octanedioic acid	-	0.3
12.00	Lauric acid	-	0.9
14.39	10-Undecenoic acid	1.6	-
18.60	Myristic acid	7.8	2.9
25.27	Palmitic acid	**36.8**	25.6
29.75	Phytol	2.9	0.9
30.64	Linoleic acid	17.8	15.6
30.77	Oleic acid	4.6	14.5
30.86	Linolenic acid	13.2	3.2
31.54	Stearic acid	6.8	**29.9**
37.38	Arachidic acid	2.3	3.5
38.19	6-Hexadecenoic acid	2.3	-
39.36	Docosane	-	0.7
43.82	Behenic acid	1.8	1.2
	Saturated fatty acids	55.5	65
	Unsaturated fatty acids	42.4	34.2
	Total	97.9	99.2

[a] Retention time (as minutes), [b] A nonpolar Phenomenex DB-5 fused silica colum.

numerous constituents were determined in Malvaceae family for the first time.

Antioxidant Activity and Total Phenolic and Flavonoid Content

The antioxidant activity of the petroleum ether (MNP and MSP, respectively), acetone (MNA and MSA), methanol (MNM and MSM) and water (MNW and MSW) extracts prepared from the whole plant of *M. neglecta* and *M. sherardiana* were studied by using *β*-carotene bleaching, DPPH free radical scavenging, cupric reducing antioxidant capacity and ABTS cation radical decolorisation assays. Also,the extracts were analyzed for total phenolic and flavonoid contents. The phenolic and flavonoid components of the MSM extract were found to be the richest among all of the extracts. (86.59 µg PEs/mg extract and 16.11 µg QEs/mg extract). The phenolic components are seen as higher than flavonoid components. Table 5 includes the results of total phenolic and flavonoid contents, antioxidant and anticholinesterase activities of *M. neglecta* and *M. sherardiana* extracts.

The current study is the first research about total phenolic and flavonoid content of *M. sherardiana* in literature; however there are two studies about *M. neglecta*. In the study of Dalar et al. (8), it is found that the phenolic content of *M. neglecta*'s different parts (root, stem, leaf, flower, fruit and whole plant) was between 3.4-17.4 mg GAE/g DW; and the total flavonoid content was between 0.97-7.21 mg RE/g DW. The report of Türker and Dalar, fruit of *M. neglecta* 12.8 mg GAE/g DW (10) and in the other study which was belong to Guder and Korkmaz, the total phenolic content of leaf and flower parts of *M. neglecta* was determined as 106.1-136.1 µg/mg cat.; and the total flavonid content is calculated as 22.9-46.7 µg/

Table 4. Chemical composition of the essential oil from *M. neglecta* (MN) and *M. sherardiana* (MS)

RI[a]	Constituents[b]	% Composition MN	% Composition MS
800	Octane	1.7	-
865	Isononane	1.3	-
939	α-Pinene	1.6	-
954	Camphene	1.1	-
979	β-Pinene	2.9	-
991	β-Myrcene	0.8	-
1029	Limonene	1.0	-
1031	Cineole	18.8	-
1063	2-methyl decane	0.8	-
1146	Camphor	1.4	-
1249	1,3-Di-tert butyl benzene	2.4	-
1276	Carvone oxide	0.8	-
1285	Anethole	2.2	-
1484	Valencene	1.7	-
1498	α-Selinene	4.2	17.5
1505	β-Himachalene	1.2	-
1528	α-Muurolene	0.9	-
1677	Cadalene	1.1	-
1746	2-Methyl heptadecane	1.6	-
1800	Octadecane	1.6	-
1890	2-Methyl-1-hexadecanol	2.3	-
1986	Hexadecanoic acid	0.9	-
2109	Heneicosane	1.8	-
2156	1-Nonadecanol	1.8	-
2171	Butyl phthalate	1.1	8.5
2172	2-Eicosanol	1.8	-
2185	Z-8-octadecen-1-ol acetate	1.4	8.1
2200	Docosane	1.3	-
2243	9-Hexyl heptadecane	0.7	12.6
2259	2,5-Di-tert octyl-p-benzoquinone	4.0	12.1
2366	Arachidic acid	1.3	8.3
2407	Tetracosane	2.3	7.9
2413	3-Ethyl-5-(2-ethylbutyl)octadecane	1.5	-
2700	Heptacosane	0.8	-
2852	1-Hexacosanol	2.8	19.1
2896	Choleic acid	0.9	-
2900	Nonacosane	1.3	-
3094	Ethyl iso-allocholate	0.8	-
3508	17-Pentatriacontene	1.5	-
3600	Hexatriacontane	7.8	-
4400	Tetratetracontane	7.8	-
	Total	95.0	94.1

[a] RI Retention indices (DB-5 column), [b] A nonpolar Phenomenex DB-5 fused silica column

mg cat, respectively (7). In our study; methanol extract of *M. neglecta* and water extract of *M. sherardiana* were found to be rich in phenolic content with 68.29 and 86.59 µg PEs/mg extract, respectively.

As indicated in Table 5, the MNA, MNM and MSM extracts showed good lipid peroxidation activity (IC_{50}: 45.92, 23.42 and 29.62 µg/mL, respectively) in β-carotene bleaching method. The other tested extracts indicated weak or no lipid peroxidation activity in β-carotene bleaching method. In literature there was a study about this activity of *M. neglecta*; however, the present study is a first report for *M. sherardiana*. Guder and Korkmaz reported that the hydroalcoholic extracts of flower and leaf of *M. neglecta* demonstrated antioxidant activities with 77.3% and 74.1% inhibition in lipid peroxidation activity respectively at 100 µg/mL concentration (7).

As indicated in Table 5, the MNM and MSM extracts showed moderate activity (IC_{50}: 60.51 and 72.37 µg/mL, respectively) in DPPH free radical scavenging activity. Other analyzed six extracts had weak or no activity in DPPH free radical scavenging activity test. Our results are in consistent with literature. Guder and Korkmaz published that the hydroalcoholic extracts of flower and leaf of *M. neglecta* demonstrated antioxidant activities with 59.00% and 62.10% inhibition in DPPH free radical scavenging activity, respectively at 100 µg/mL concentration (7). In a literature survey, it is going to be found that there hasn't been any study about *M. sherardiana*.

As shown in Table 5, the MNM, MNW, MSM and MSW extracts showed IC_{50}: 45.81, 55.02, 41.20 and 45.93 µg/mL in ABTS cation radical scavenging assay, respectively. Particularly, the MSM extract indicate good activity in ABTS cation radical scavenging assay. The other tested four extracts had weak activity in ABTS cation radical scavenging assay. Cupric reducing effect was indicated in none of the extracts. Because of that, data was not given. In a literature survey, it is going to be found that there have not been any study about *M. neglecta* and *M.sherardiana*.

Anticholinesterase Activity

As shown in Table 5, all of the extracts of *M. neglecta* exhibited moderate activity against acetyl-and butyryl-cholinesterase enzymes, excluding MNW extract. Especially, the MNM extract was determined to have the highest activity in all tested extracts against acetyl-and butyryl-cholinesterase

Table 5. Total phenolic and flavonoid contents, antioxidant and anticholinesterase activities of *M. neglecta* and *M. sherardiana* extracts

Samples	Inhibition % against AChE	Inhibition % against BChE	Phenolic content (µg PEs/mg extract)[y]	Flavonoid content (µg QEs/mg extract)[z]	Lipid Peroxidation	IC_{50} (µg/mL) DPPH Free Radical	ABTS Cation Radical
MNP	30.13± 1.32[a]	43.68± 1.12[a]	-	-	95.13± 0.65[a]	>200[a]	127.03± 1.03[a]
MNA	38.65± 1.39[b]	57.69± 0.63[b]	-	-	45.92± 0.29[b]	130.52± 1.60[b]	91.95±1.06[b]
MNM	53.68± 0.42[c]	63.95± 0.32[c]	68.29± 0.14[a]	15.58± 0.19[a]	23.42±0.48[c]	60.51±0.90[c]	45.81±0.82[c]
MNW	NA	9.68± 1.12[d]	-	-	121.65±1.11[f]	95.71±0.71[c]	55.02±0.61[g]
MSP	NA	15.45± 0.12[e]	-	-	>200[d]	>200[a]	106.93±0.23[h]
MSA	12.68± 0.46[a]	24.67± 1..46[f]	-	-	65.61±0.80[g]	155.69±0.91[b]	78.82±0.73[i]
MSM	21.68± 0.57[d]	17.09± 0.33[e]	86.59± 0.27[b]	16.11± 0.27[a]	29.62±0.89[h]	72.37±0.57[e]	41.20±0.69[j]
MSW	NA	07.07± 0.33[d]	-	-	135.82±1.91[i]	104.60±1.26[f]	45.93±0.12[k]
Galanth.[t]	84.59 ± 0.41[e]	82.39±0.59[g]	-	-	-	-	-
α-TOC[t]	-	-	-	-	16.11±0.18[j]	17.51±0.33[h]	9.08±0.08[m]
BHT[t]	-	-	-	-	8.61±0.19[k]	49.68±0.19[i]	10.59±0.19[n]

[x] Values expressed are means ± S.D. of three parallel measurements ($p<0.05$), [y]Standard drug, NA: Not active

[y] PEs, pyrocatechol equivalents ($y=0.0164x + 0.0266$ $R^2=0.9969$)

[z] QEs, quercetin equivalents ($y=0.1519x - 0.1294$ $R^2=0.9986$)

[t] Standarts, the letters from 'a' to 'n' express the statistically diffences between the values.

enzymes at 200 µg/mL (53.68 and 63.95% inhibition, respectively). In despite of the *M. neglacta*, all extracts of *M. sherardiana* showed weak activity or no activity against acetyl-and butyryl-cholinesterase enzymes at 200 µg/mL.

The current study has been the first study about *Malva* and *Malvella* genus anticholinesterase activty in literature. In this point of view, it is going to be an important data for this research area. In addition to that there are two studies about anticholinesterase activity of Malvaceae family that both of them relevant to *Thespesia populnea*. In this researches, it is reported that *T. populnea* bark is important for improving memory and determination of the potential of it in the management of Alzheimer patients would be beneficial (34).

Determination of Antimicrobial Activity

The antimicrobial activities of *M. neglacta* and *M. sherardiana* extracts against different microorganisms were assessed according to inhibition zone diameter. Results are presented in Table 6.

The petroleum ether and water extracts showed no activity at all against the five tested microorganisms (Data was not shown). The acetone and methanol extracts were active on all microorganisms tested with different zone diameters indicating weak (inhibition zone < 12) and moderate antimicrobial activity (inhibition zone < 20-12). The acetone extracts of *M. neglacta* and *M. sherardiana* showed moderate activity against *C. albicans* (inhibition zone 18 and 17 mm, respectively). The acetone extract of *M. sherardiana* showed weak activity against *E. coli, S. aureus, S. pyogenes* and *P. aeruginosa*. The methanol extract was showed moderate activity against *C. albicans*, *P. aeruginosa* and weak activity against *E. coli, S. aureus, S. pyogenes.*The acetone and methanol extracts of *M. neglacta* showed moderate active on all microorganisms tested. The study of Mansouret al. showed that the ethanol extract of *M. neglecta* had activity on *S. epidermidis*, *S. aureus* and *P. aeruginosa* (6). The highest inhibitory zone was seen in ethanolic extract of *M. neglecta* against *Staphylococcus epidermidis* (22 mm).The present study is a first report for *M. sherardiana*.

Table 6. Zones of growth inhibition (mm) showing the antimicrobial activity of the *M. neglecta*and *M. sherardiana* extracts

			Microorganisms				
			E. coli	***S. pyogenes***	***S. aureus***	***P. aeruginosa***	***C. albicans***
Inhibition zone diameter (in mm)	***M. neglecta*** **Acetone extract**	**10 mg/mL**	12±0.3	12±0.1	11±0.3	10±0.1	16±0.2
		20 mg/mL	14±0.2	13±0.3	12±0.2	11±0.2	16±0.3
		30mg/mL	14±0.5	12±0.4	12±0.4	13±0.4	18±0.4
	M. neglecta **Methanol extract**	**10 mg/mL**	10±0.2	11±0.1	10±0.2	12±0.1	12±0.3
		20 mg/mL	11±0.1	12±0.4	10±0.2	13±0.3	12±0.4
		30mg/mL	13±0.2	12±0.3	13±0.3	14±0.2	14±0.1
	M. sherardiana **Acetone extract**	**10 mg/mL**	9±0.1	11±0.2	10±0.2	9±0.2	15±0.2
		20 mg/mL	12±0.5	11±0.3	10±0.3	11±0.3	16±0.3
		30mg/mL	12±0.3	11±0.2	12±0.1	11±0.4	17±0.4
	M. sherardiana **Methanol extract**	**10 mg/mL**	10±0.3	11±0.2	9±0.3	12±0.3	11±0.1
		20 mg/mL	11±0.2	11±0.2	9±0.1	12±0.1	12±0.1
		30mg/mL	11±0.4	12±0.3	10±0.2	13±0.2	14±0.3
	IPM (10 µg)		19±1.4	39.5±0.7	27.5±0.7	12±0	-
	Nystatin (30 µg)		-	-	-	-	25±0.5

Briefly, being used as food source and food supplement, *M. neglacta* and *M. sherardiana* are known as Tolık and Ebegümeci in Anatolia. Present study also showed that the *M. neglacta* and *M. sherardiana* methanol extracts have good activity in β-carotene bleaching, DPPH free radical scavenging and ABTS cation radical decolorisation assays. The antioxidant capacities of these extracts were parallel to the antioxidant properties of 4-hydroxy benzoic and salicylic acids which were the main components of these extracts. Thus, the species may help to protect the people against lipid peroxidation and free radical damage, and its extracts will probably be used for the development of safe food products and additives. The *M. neglacta* methanol extract also demonstrated good acetyl- and butyryl-cholinesterase inhibitory activities. Therefore, consumption of *M. neglacta* may be useful as good cholinesterase inhibitory agents. However, further studies, particularly in vivo tests, are needed to understand the activity in biological systems.

Malva neglecta ve Malvella sherardiana'nın LC-MS / MS, GC / MS ile Kimyasal Profili ve Antikolinesteraz, Antimikrobiyal, Antioksidant Özellikleri ile Aflatoksin İçeriği

ÖZ

Bu çalışmanın amacı *Malva neglecta* ve *Malvella sherardiana* türlerinin antioksidan, antikolinesteraz ve antimikrobiyal aktiviterini, aflatoksin içeriği ile fenolik bileşik ve yağ asidi profillerini LC-MS/MS ve GC/MS ile belirlemektir. İncelenen yirmi yedi bileşikten, *M. neglecta* ve *M. sherardiana* türlerinde en bol bulunan bileşiklerin sırasıyla malik (13108.97 µg/g) ve 4-OH benzoik asit (2200.57 µg/g) olduğu saptanmıştır. *M. neglecta* türünün metanol ekstresindeki toplam flavonoid içeriğin (15.58 *µg* QEs/mg ekstre) toplam fenolik içerikten (68.29 *µg* PEs/mg ekstre) daha az olduğu belirlenmiştir. Bitkilerin metanol ekstresi çalışılan antioksidan aktivite belirleme yöntemlerinin tümünde en yüksek aktiviteyi göstermiştir. En iyi kolinesteraz enzim inhibisyonu ise *M. neglecta* türünün metanol ekstresi tarafından kaydedilmiştir (%53.68 AChE enzim inhibisyonu ve %63.95 BChE enzim inhibisyonu). Bitkilerin aseton ekstreleri ise *C. albicans* türüne karşı orta düzeyde antimikrobiyal aktivite göstermiştir. Bu çalışmadan elde edilen sonuçlar, *M. neglecta* ve *M. sherardiana* türlerinin yüksek fenolik asit içeriği ve antioksidan aktivitesinden dolayı besin kaynağı olarak kullanılabileceğini göstermektedir. Ayrıca ekstreler aflatoxin içermemektedir.

Anahtar kelimeler: Antikolinesteraz; antioxidan; *Malva neglecta*; *Malvella sherardiana*; LC-MS/MS, GC-MS

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