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Chemical composition and bioactive properties of the essential oil of *Rhinanthus angustifolius* subsp. *grandiflorus*

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In this study, the chemical composition and antimicrobial activities of the essential oil of the *Rhinanthus angustifolius* subsp. *grandiflorus* plant were investigated. Essential oil was obtained from all parts of the plant by hydrodistillation and was analyzed by GC-FID and GC-MS. 31 components representing 99.46% of the total oils were characterized. The main components of these species were found to be 2,3-dihydro-5-methyl-1H-indene (25.14%), α-cubebene (19.27%), 1-hexadecene (15.59%) and hexadecanoic acid (12.14%). The antimicrobial activity of the isolated essential oil was investigated. Antimicrobial activity was observed against *B. cereus, E. coli, E.coli* O157:H7 and *S. Aureus* microorganisms. Moreover, an effect on the ferments of *B. cereus, E. coli, E.coli O157:H7* and *S. Aureus* was detected.

Keywords: *Rhinanthus angustifolius* subsp. *grandiflorus*, Essential oil, GC-FID/MS, Antimicrobial activity, Phenolic content.

INTRODUCTION

Rhinanthus L. (Scrophulariaceae) consists of 30-40 species of hemi-parasitic annual herbs most of which are widespread in Europe [1,2]. *Rhinanthus* was formerly classified in Scrophulariaceae but, later it was suggested to be included into Orobanchaceae [3,4]. *Rhinanthus angustifolius* C. C. Gmelin is a very variable species and consists of three subspecies [1]. *R. angustifolius* subsp. *grandiflorus* is easily recognized from other subspecies by its leaf size (longer than 8 mm). The genus is represented by a subspecies (*Rhinanthus angustifolius* C. C. Gmelin subsp. *grandiflorus* (Wallr.) D. A. Webb) in the flora of Turkey [5]. The species of *Rhinanthus* is used in folk medicine for treating eye complaints caused by certain bacteria kinds [6]. An iridoid glucoside, 6′-*O*-benzoylshanzhiside methyl ester, was isolated from *R. angustifolius* [7]. Some scientists have found that alcoholic extracts of *R. angustifolius* have strong antibacterial activities [8].

Rhinanthus L. of Scrophulariaceae family has 40 natural species in Europe, North Africa, North Asia and North America. These are annual or perennial herbaceous or brier plants [9,10]. The *Rhinanthus angustifolius* subsp. *grandiflorus* plant used in this study is a non-endemic *Rhinanthus* taxon of the family mostly found in the northern parts of the Eastern Anatolia region and has bell-shaped yellow flowers. The flowers of the *R. angustifolius* subsp. *grandiflorus* plant are used as a medicine for treating ear complaints by people in Anatolia [11].

Studies in the literature show that some *Rhinanthus* species (*Rhinanthus angustifolius, Rhinanthus rumelicus* Velen. and *Rhinanthus serotinus* (Schönh.) Oborny contain natural compounds such as flavonoids, anthocyanines, anthraquinones and saponins which have certain biological activities [8,12,13]. However, no study was found on the chemical composition or biological activities of the essential oil of the *R. angustifolius* subsp. *grandiflorus* plant. This study investigates the chemical composition of *Rhinanthus angustifolius* found in Turkey and the antibacterial, antifungal and antioxidant properties of its essential oil extract.

EXPERIMENTAL

Studies conducted

Rhinanthus angustifolius subsp. *grandiflorus* was collected from the shores of Lake Limni, district of Torul, Province of Gümüşhane (approximately 1800 m) in July 2013. The plant material was identified by Dr. Mutlu Gültepe and stored under number KTUB-508 in the Karadeniz Technical University. Real samples were filtered through 0.45 μm nylon filter membranes (Varian, USA).

Clevenger-type hydrodistillation process

Collected plant material was shade-dried and weighed 76 g. Dried plants were broken into small pieces with a blender, put into a flask with 800 mL of pure water and heated for 3 h in a Clevenger-type water steam distillation apparatus. The resulting steam was cooled at -15 C^{o} and 20 mg (w/w 0.026%) of essential oil was obtained. The essential oil was taken into a brown vial with 1 mL hexane of HPLC

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quality, dried over anhydrous Na_2SO_4 and stored at -5 Cº for analysis [14].

GC and GC-MS analyses

GC-MS and GC-FID analyses were performed in accordance with the literature [15].

Identification of components

In order to determine the chemical content of the essential oil, each compound was firstly determined using the WILEY and NIST libraries of the computer performing the mass spectrum analysis and then by comparing their retention times with alkene standards (C_7-C_{30}) and literature data [16-18].

Antimicrobial activity analysis

All test microorganisms were obtained from the Food Engineering Laboratory of Gümüşhane University. Antimicrobial activities of the essential oil extract were determined using the agar diffusion method against *Aeromonas hydrophila* ATCC 7965, *Bacillus cereus* ATCC 33019, *Bacillus subtilis* ATCC 6633, *Enterobacter cloacea* ATCC 13047, *Escherichia coli* ATCC 11230, *Escherichia coli* O157:H7 ATCC 33150, *Klebsiella pneumoniae* ATCC 13883, *Listeria monocytogenes* ATCC 7644, *Proteus vulgaris* ATCC 13319, *Pseudomonas aeruginosa* ATCC 17853, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Saccharomyces cerevisiae* BC 5461, *Candida albicans* ATCC 1223, *Aspergillus niger, Aspergillus flavus and Penicillum*. Extracts were prepared from stock solutions by dissolving in hexane (1000 ppm) [19,20].

Agar diffusion method

All microorganisms were developed in a nutrient broth at 37ºC for 18 h. *C. albicans*, S. *cerevisiae*, *A.niger*, *A.flavus and Penicillum* were developed in a malt extract broth at 27ºC for 42 h. Microorganisms developed by cooling sterile nutrient agar and malt extract agar to 45ºC were inoculated (1%) and poured to petri plates. Once agars solidified, wells with a diameter of 5 mm were opened on agars. Pre-extracted samples were added to wells (50 µL). Hexane was used for control. Then, the petri plates were incubated at 37ºC for 24 h for bacteria and at 27ºC for 48 hours for fungi. Antimicrobial activity was determined by measuring the inhibition zones of test microorganisms [21] in mm.

Antioxidant activity tests

Determination of free radical scavenging activity

The free radical scavenging activity value was found by the DPPH method. A DPPH solution prepared with 4 mL of 0.1 mM methanol was added to the essential oil extract of *Rhinanthus angustifolius* subsp. *grandiflorus*. The mixture was kept at room temperature in dark for 30 min and measured against methanol at 517 nm. Measurements were repeated in five parallels and averaged [22]. % Inhibition results were calculated using the formula:

% Inhibition = (Control Absorbance-Sample Absorbance / Control Absorbance) ×10

Total Phenolic Content

The Folin-Ciocalteu method was used for determination of total phenolic content [20]. In summary, pre-extracted materials were taken to 40 µL test tubes and then 2.4 mL of pure water was added. 200 mL of Folin-Ciocalteu phenolic reagent, 600 mL of sodium carbonate solution and 760 mL of pure water were added to the mixture. The mixture was stirred for 15 sec. Reduction of Folin-Ciocalteu reagent results in a blue color in alkaline environment. The absorbance was measured at 765 nm after 2 h of incubation. All measurements were performed in five parallels and averaged. The chart used in the calculations is shown in Figure 1. The results are presented as GAE/L [23].

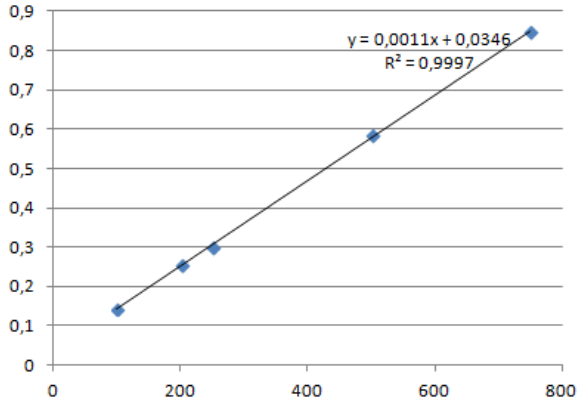

Fig. 1. Gallic acid curve used for total phenolic content determination

RESULTS AND DISCUSSION

As a result of the hydrodistillation applied to 76 g of dried *Rhinanthus angustifolius* subsp. *grandiflorus* plant, 20 mg of essential oil was obtained with a yield of 0.026%. 31 compounds at a rate of 99.46% were identified in the chemical structure of the essential oil by comparing the results of the GC-MS analysis with the literature. As seen in

Table 1, the identified compounds are divided into 6 groups as terpene or terpene-like, aldehydes, alcohols, esters, acids and other compounds. The main components of the essential oil were found to be 2,3-dihydro-5-methyl-1H-indene, (25.137%), *α*-cubebene (19.27%), 1-hexadecane (15.59%) and hexadecanoic acid (12.14%)

Table 1. Identified chemical content of the *Rhinanthus angustifolius* subsp. *grandiflorus* plant

No	Retention Time, min	Name of Compound	% Area	Experimental RI
		Terpene or terpene-like		
1	25.53	*α*-Cubebene	19.27	1309
2	27.02	Copaene	3.12	1344
3	28.97	*β*-Damascenone	0.45	1389
4	31.72	(E)-6,10-Dimethyl-5,9-undecadien-2-one,	0.54	1455
5	33.19	*β*-Cyclocitrylideneacetone	0.19	1490
6	34.76	(E)-3-(4,8-Dimethyl-3,7-nonadienyl)-furan	1.02	1536
7	46.40	Perhydrofarnesyl acetone	1.89	1847
8	61.50	Methyl dehydroabietate	0.59	2339
		Aldehydes		
9	26.14	2,4-Decadienal	1.18	1324
10	27.94	2-Undecenal	0.31	1365
11	59.33	1,2,3,4,4a,9,10,10a-Octahydro-1,4a-dimethyl-7-(1-methylethyl)-[1R-(1*α*,4a*β*,10a*α*)]-1-phenanthrenecarboxaldehyde	0.54	2262
		Alcohols		
12	9.75	1-Okten-3-ol	0.94	944
13	10.02	6-Methyl-2-heptanol,	0.38	952
		Esters		
14	37.54	2,2,4-Trimethyl-3-carboxyisopropyl pentanoic acid isobutyl ester	0.27	1599
15	47.26	Phthalic acid, diisobutyl ester	0.36	1873
16	51.29	Hexadecanoic acid, ethyl ester	0.09	1995
17	57.50	Phthalic acid, isohexyl 3-methylbut-3-enyl ester	0.67	2198
18	59.96	1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-1,4a,7-trimethyl methyl ester [1R-(1*α*,4a*β*,4b*α*,7*α*,10a*α*)]-	3.82	2286
		Acids		
19	33.59	Adamantane-1-carboxylic acid	1.05	1501
20	50.33	Hexadecanoic acid	12.14	1964
		Other compounds		
21	12.86	Indane	0.30	1026
22	16.33	2,3-Dihydro-5-methyl-1H-indene	25.13	1129
23	29.07	N-Methylbenzamid	0.63	1391
24	37.17	15-nor-Funebran-3-one	0.27	1591
25	38.02	1-Hexadecene	15.59	1613
26	41.74	3-Cyclohexyl-undecane	0.59	1717
27	46.14	Galaxolide	2.59	1839
28	48.20	*n*-Nonadecane	0.37	1900
29	54.55	Heneicosane	1.51	2100
30	60.37	Tricosane	2.92	2300
31	66.41	Pentacosane	1.26	2500
		Total	99.46	

Table 2. Antimicrobial activity results (in mm) of the essential oil of *Rhinanthus angustifolius* subsp. *grandiflorus*

Bacteria	1%	2%	5%	10%
A. hydrophila	-	-	-	-
B. cereus	12.01±0.10	10.24±0.10	-	-
B. subtilis	-	-	-	-
Ent. cloacae	-	-	-	-
E. coli	10.64±0.10	8.19±0.15	-	-
E.coli O157:H7	13.13±0.10	10.20±0.10	8.56±0.10	-
K. pneumoniae	-	-	-	-
L.monocytogenes	-	-	-	-
P. vulgaris	-	-	-	-
Pseu. aeruginosa				
Sal. typhimurium	-	-	-	-
S. aureus	8,14±0.10	-	-	-
Fungi				
Sac. cerevisiae	5.01±0.15	-	-	-
C. albicans	7.19±0.10	-	-	-
A.niger	-	-	-	-
A.flavus	-	-	-	-
Penicillum	-	-	-	-

Inhibition levels of the essential oil extracts of *Rhinanthus angustifolius* subsp. *grandiflorus* against test bacteria and fungi are given in Table 2. In the antimicrobial study, inhibition zones increased as the concentration of extracts increased. Essential oil extracts showed strong antimicrobial activity at concentrations of 1000 ppm and 500 ppm. Essential oil of *Rhinanthus* plant was found to show antimicrobial activity against *B. cereus*, *E. coli*, *E.coli O157:H7* and *S. aureus*. It was effective against *Sac. cerevisiae* and *C. albicans* fungi, whereas it showed no antifungal activity for the fungi studied in the present work.

It was found that the essential oil extract had high antioxidant activity. The essential oil extract of *Rhinanthus angustifolius* subsp. *grandiflorus* was found to have 53.92% DPPH radical scavenging activity against yellow-colored diphenylpicrylhydrazine.

The total phenolic activity of the essential oil extract of *R. angustifolius* subsp. *grandiflorus* was found to be 630.19 GGA/L. Due to its high phenolic content, the plant can be used in daily diet or in functional foods. It was found that antioxidants found in medicinal plants have biological effects in addition to being safe and effective.

CONCLUSIONS

In this work, we describe the chemical composition and antimicrobial activities of *Rhinanthus angustifolius* subsp. *grandiflorus* essential oil. 31 components obtained from all parts of the plant by hydrodistillation were analyzed by GC-FID and GC-MS. This is the first study in the literature on antioxidant and antimicrobial properties of the *Rhinanthus* plant found in the flora of Turkey. This study may contribute to future research on bioactive properties of the plant.

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ХИМИЧЕН СЪСТАВ И БИОАКТИВНИ СВОЙСТВА НА ЕСЕНЦИАЛНО МАСЛО ОТ *Rhinanthus angustifolius* subsp. *grandiflorus*

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(Резюме)

В това изследване са изследвани химичния състав и анти-микробните свойства на сенциално масло от *Rhinanthus angustifolius subsp. grandiflorus*. Маслото е извлечено от всички органи на растението чрез дестилация с водна пара и е анализирано по методите GC-FID и GC-MS. Охрактеризирани са тридесет и един компонента представляващи 99.46% от общото количество масло. Главните компоненти са 2,3-дихидро-5-метил-1H-инден (25.14%), α-кубебен (19.27%), 1-хексадекен (15.59%) and хексадеканова киселина (12.14%). Анти-микробната активност на изолираното есенциално масло е изпитана върху микроорганизмите *B. cereus, E. coli, E.coli* O157:H7 и *S. Aureus*. Освен това е определен и ефекта му върху ензимите на *B. cereus, E. coli, E.coli* O157:H7 и *S. Aureus*.