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Antibacterial Activity and Chemical Composition of Essential Oils from Some Galium (Rubiaceae) Species Against Pathogenic Bacteria

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ABSTRACT

In this work, chemical composition and antimicrobial activity of the essential oils of *Galium incanum*, *Galium dieckii* ve *Galium aladaghense* were firstly reported. The essential oils were obtained from the all parts of the plant by hydrodistillation and analyzed by using GC-MS. Antimicrobial activity of synthezied essential oils was carried out against 5 pathogen bacteria *Escherichia coli* (*E. coli*) ATCC 25922, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, *Pseudomonas syringae pv. tomato* (*P. syringae*) DC300, *Salmonella enterica serotype Typhmurium* (*S. typhmurium*) SL 1344 and *Streptococcus mutans* (*S. mutans*) ATCC 25175. According to the results, it was determined that isolated essential oils comprised of 61 compounds. Compounds of essential oils included that structure monoterpene (8.2%), monoterpenoid (14.75%) and sesquiterpene (14.75%). Unclassified compounds have been identified as other compounds. From the antimicrobial activity was observed that the isolated essential oil from *Galium incanum*, *Galium dieckii* ve *Galium aladaghense* exhibited a potent inhibitory effect against all gram negative and gram positive bacteria with diameter of inhibition zones ranging from 4.3 to 12.3 mm. Essential oil of *Galium aladaghense* indicated that high antimicrobial activity on all bacteria than *Galium incanum* and *Galium dieckii.*

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Introduction

Plants have been used medicinally all over the world for many centuries. The intake of fresh fruits, vegetables and tea rich in natural antioxidants has been connected with prevention of cancer and cardiovascular diseases (Willcox et al, 2004). Approximately 60% of the commercially available anti-tumor and anti-infective agents are of natural origin (Cragg et al., 1997).

Galium species are one of the most important species of *Rubiaceae* family. The genus *Galium* is comprised of approximately 1300 species and 101 species are represented in Turkey by divided into 10 sections. *Galium* species called "Yogurt herb" due to contain an enzyme that coagulate the milk (Başer at al., 2004). Galium species include flavonoids, alkaloids and iridoids (Iavarone et al, 1983; de Rosa et al., 2000). Iridoids are considered as a group of terpenoids which has biological activities. Extract of this plants is used for the treatment of stomach disorders, neuroprotective, epilepsy, hysteria and gout (Menković et al., 2011). Therefore, the analysis of the oil components of Galium species is considered important.

In our study have been reported that composition of essential oils of *Galina incanum*, *Galium dieckii* and *Galium aladaghensis* which are endemic plants with relative percentage amount of any of the components and biological activity of isolated essential oil obtained *Galium* species against five pathogenic bacteria. To the best of our knowledge, the composition and antimicrobial activity of essential oil of *Galium incanum*, *Galium dieckii* and *Galium aladaghensis* has not previously been reported.

Materials and Methods

Luria Bertoni (LB) agar, Mueller Hinton agar, DMSO, n-hexane, Na_2SO_4 were purchased from Sigma-Aldrich. All plant samples (*Galina incanum*, *Galim dieckii* and *Galim aladaghensis)* were collected in Niğde Region. The tested microorganisms (*Escherichia coli* (*E. coli*) ATCC 25922, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, *Pseudomonas syringae pv. tomato* (*P. syringae*) DC300, *Salmonella enterica serotype Typhmurium* (*S. typhmurium*) SL 1344 and *Streptococcus mutans* (*S. mutans*) ATCC 25175) were provided by Biology Department of Niğde Ömer Halisdemir University.

Plant Material and Isolation of Essential Oils

From the tested plant samples, *Galium dieckii* and *Galium aladaghense* are endemic plants and also located in the Central Anatolia Region. Plants *(Galina incanum*, *Galim dieckii* and *Galim aladaghensis)* were collected in 2013 and identified by Dr. Ahmet SAVRAN. The dried and powdered plant sample (100 g) was subjected to hydro-distillation using a Clevenger-type apparatus for 4 h. The resulting essential oils were dissolved in 0.5 mL n-hexane (HPLC grade) and dried over anhydrous sodium sulfate (Na_2SO_4) and stored at 4°C in a sealed vial until use. All experiment was performed triplicate (Hammami et al., 2015).

GC-MS Analysis Conditions

The essential oil was analysed by using Thermo-DSQII GC-MS equipped with a T-WAXMS capillary column (60mx0.32mmx0.25m) For gas chromatography–mass spectrometry (GC-MS) detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas at a flow rate of 1 mL/minute. Injector and MS transfer line temperatures were set at 220° and 290°C, respectively. The programme was used at 50–150°C at a rate of 3°C/minute. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 µL were injected manually and in splitless mode. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, MAINLAB, Wiley 7N and Replic library data of the GC–MS system and literatüre data. The results were also confirmed by the comparison of the compounds' elution order with their relative retention indices on non-polar phases reported in the literature (Sarikurkcu et al., 2013).

The Tested Microorganisms

The following microorganisms including *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. syringae* DC300, *S. typhmurium* SL 1344 and *S. mutans* ATCC 25175 were used as the tested microorganisms. Bacteria were maintained on Luria Bertani (LB) agar culture medium at 4°C. All the tested microorganisms were sub-cultured on LB broth medium at 37°C for 24 h before the antimicrobial activity assay.

Antibacterial Activity Test

Antimicrobial activities of the synthesized essential oils of *Galium incanum*, *Galium dieckii* ve *Galium aladaghense* against the sensitive organisms *Staphylococcus aureus* and *Streptococcus mutans* as Gram-positive bacteria and *Escherichia coli*, *Pseudomonas syringae pv. tomato* and *Salmonella enterica serotype Typhmurium*. as Gram-negative bacteria were determined by using disc-diffusion method according to Clinical and Laboratory Standards Institute with small modifications (CLSI, 2012). 0.5 McFarland standard was used as a reference to adjust the turbidity of microorganisms. The tested compounds were dissolved in dimethylsulfoxide [(DMSO) which has no inhibition activity]. The essential oil concentration in DMSO were adjusted to 15 mg/mL. The samples were sterilized by UV irradiation for 30 min before incubation. Microorganism suspensions of 100 µl were inoculated (10^6 cells/mL) onto Müller-Hinton medium. Filter discs (6 mm in diameter) containing with essential oils (*Galium incanum* (A), *Galium dieckii* (B), *Galium aladaghense* (C)) were placed on an inoculated petri plate and incubated at 37°C for 24 h. Only DMSO solvent containing discs was used as a negative control. The diameter of inhibition zone around of the disc was calculated after incubation. All experiments were repeated triplicate and mean values were calculated.

Determination of Minimum Inhibition Concentration (MIC)

A microdilution broth susceptibility assay was used as recommended by Clinical and Laboratory Standards Institute (CLSI, 2010). with small modifications for the determination of the Minimum inhibition concentrations (MICs). Each strain was tested with sample that was serially diluted in LB broth to obtain concentrations ranging from 0.1 - 10 mg/mL in DMSO. The samples were previously sterilized by UV irradiation was inoculated with 50 mL suspension of 10^6 CFU/mL of the tested microorganisms and incubated for 24 h at 37°C. Another culture medium without adding microorganisms suspension was prepared as the negative control. The MIC value was determined as the lowest concentration (highest dilution) of the sample showing no visible growth at which the tested microorganisms did not demonstrate any visible growth after incubation. Cells from the tubes showing no growth were subcultured on LB agar plates to determine if the inhibition was reversible or permanent.

Results and Discussion

Characterization of Essential Oils

61 compounds were identified from results of GCMS analyses of essential oils of *Galium incanum* (A), *Galium dieckii* (B), *Galium aladaghense* (C) (Table 1). Compounds of essential oils included that structure momoterpen (8.2%), momoterpenoid (14.75%) and sesquiterpene (14.75%). From the results, which was observed that five compounds are monoterpenes (α-terpinene, α-pinene, β-pinene, limonene, tymole), nine compounds are monoterpenoid (linalool, camphor, L-borneol, borneol, myrtenol, 1,8-cineole, carvacrol, eugenol, pinocarvone), nine compounds are sesquiterpene (cipren, α-cedrene, β-caryophyllen, trans-β-pharnesene, α-humulene, caryophyllen oxide, cubenol, zerumbone, cis-cis-calamenene). Excluding of this compounds were identified as other compounds (Shahzad, 2015).

The essential oil of *Galium incanum* was predominated by monoterponoid (13.2%), sesquiterpene (9%) and monoterpenes (8.1%), respectively. On the other hand, the oil of *Galium dieckii and Galium aladaghense* was comprised of monoterponoid (12.2 and 11.3%), monoterpenes (5.9 and 22.7%) and sesquiterpene (5.7 and 5%), respectively. Major components of the oils of

Galium incanum, *Galium dieckii* and *Galium aladaghense* were eugenol (7.8, 8.1 and 6.9%), α-humulene (7.8, 5.3, 3.2%) and thymol (7.4, 5.7 and 21.4%). The essential oil of *Galium aladaghense* can be considered as an aromatic plant more than *Galium incanum* and *Galium dieckii* due to comprise high content of thymol.

In vitro antimicrobial activity

The in vitro antimicrobial activity of *Galium incanum* (A), *Galium dieckii* (B), *Galium aladaghense* (C) essential oil was evaluated by disc diffusion method against Gram-positive (*Staphylococcus aureus* and *Streptococcus mutans*) and Gram negative bacteria (*Escherichia coli*, *Pseudomonas syringae* and *Salmonella t*yphmurium). According to the results shown in Table 2, the essential oil exhibited a potent inhibitory effect against all gram negative and gram positive bacteria (*E. coli*, *P. syringae*, *S. t*yphmurium, *S. aureus* and *S. mutans*) with diameter of inhibition zones ranging from 4.3 to 12.3 mm. The essential oil exerted a broad antimicrobial spectrum and showed a high antimicrobial effect on *S. mutans*, *E.coli* and *S. typhmurium* with the diameter of inhibition zones of 12.3, 11.5 and 11.3 mm, respectively. Essential oil of *Galium aladaghense* indicated that high antimicrobial activity on all bacteria than *Galium incanum* and *Galium dieckii.*

Antimicrobial activity of essential oils increased by increasing content of thymol component. The essential oil of *Galium aladaghense* was comprised of more thymol content than others (Baser et al., 2004).

All tested microorganisms are of clinical importance. The usefulness of this method is limited to the generation of preliminary quantitative data only, as the hydrophobic nature of most essential oils and plant extracts components prevents their uniform diffusion through the agar medium. Based on this, it is recommended to use an emulsifier such as DMSO, to assure contact between the microorganism and the possible antimicrobial agent (Samusenko, 2008). Essential oil exhibited antimicrobial activity against the tested strains, but in variable degree (Table 2).

Table 1 Compounds of essential oils.

Compound	RI	%A	%B	%C	Compound	RI*	%A	%B	%C
trans-2-hexenal	801	1.5	2.3	-	β-ionene	1487	-	-	0.2
Heptanal	903	-	1.8	0.7	α-campholene aldehyde	1500	1.1	0.9	0.2
Cyclopentene	931	0.9	0.6	-	Benzaldehyde	1541	0.3	0.1	0.2
3-octanone	984	-	0.2	0.2	Caryophyllen oxide	1555	-	-	0.6
α-terpinene	1019	0.3	-	0.7	Elemicin	1558	-	-	0.2
α-pinene	1032	0.1	0.2	-	Octanol	1562	2.7	9.7	0.6
n-octanol	1063	-	-	0.3	5-metyl furfural	1585	0.9	-	0.2
Hexanal	1093	0.7	0.3	0.4	Pinocarvone	1586	-	-	0.9
Linalool	1098	-	0.2	-	Cubenol	1648	0.6	-	0.2
β-pinene	1118	0.2	-	0.6	Carvenone	1737	0.4	0.3	0.3
Camphor	1147	1.2	0.2	0.5	Zerumbone	1754	-	-	-
L-Borneol	1165	3.2	1.8	-	Octadecane	1800	0.2	1.9	0.4
Borneol	1171	-	-	0.3	cis-calamenene	1853	0.5	-	-
Myrtenol	1194	-	0.7	0.5	Hexanoic acid	1870	0.4	6.8	0.3
Limonene	1203	0.1	-	-	1-hexadecanol	1874	0.6	0.4	1.6
İsoamyl alcohol	1212	0.3	-	0.5	hexadekanoic acid	1929	21.6	-	6.4
1,8-cineole	1213	0.6	-	-	Tetradecanal	1933	1.7	-	0.6
Nonanoic acid	1267	0.1	0.3	-	Eicosane	1999	0.7	0.3	0.4
Carvacrol	1298	0.4	1.2	2.4	Phytol	2102	-	-	1.5
2-heptanol	1320	0.1	-	0.3	Thymole	2205	7.4	5.7	21.4
Eugenol	1360	7.8	8.1	6.9	Tricosane	2300	-	6.8	0.4
(Z)-3-hexenol	1391	0.2	-	-	Decanoic acid	2300	1.7	2.8	-
Cipren	1398	-	-	0.4	Pentacosane	2499	-	-	0.2
Nonanal	1400	1.2	2.4	0.8	Metyl linoleate	2509	0.2	-	0.7
Dodecanal	1409	11.5	-	2.6	Heptacosane	2700	0.9	0.2	-
α-cedrene	1411	-	-	0.6	Tetradecanoic acid	2713	-	3.6	-
β-caryophyllen	1426	-	0.2	-	Pentadecanoic acid	2822	0.3	-	0.1
Geranyl acetone	1445	-	-	0.7	Nonacosane	2900	1.7	-	-
Trans-β-farnesene	1449	0.1	0.2	-	Oleic acid	3200	1.3	0.2	-
1-octene-3-ol	1452	0.7	-	0.4	Linoneic acid	3290	-	2.1	4.7
α-humulene	1454	7.8	5.3	3.2					

RI: Retention Index A:Galium incanum, B: Galium dieckii, C: Galium aladaghense

Table 2 Antimicrobial activity of *Galium incanum* (A), *Galium dieckii* (B), *Galium aladaghense* (C) in DMSO against the sensetive gram negative and gram positive bacteria

Microorganisms	Strain	Inhibition zone (mm)			Minimum inhibitory concentrations (MIC) (mg/mL)		
		A	B	C	A	B	C
E. coli	ATCC 25922	9.8±0.6	9.3±0.7	11.5±0.8	0.4±0.02	0.4±0.02	0.8±0.02
P. syringae	DC 3000	4.3±0.4	7.9±0.5	10.2±0.6	3.2±0.06	1.6±0.03	1.2.±0.03
S. typhmurium	SL 1344	7.1±0.5	8.2±0.6	11.3±0.7	2.4±0.04	1.6±0.03	1.6±0.03
S.aureus	ATCC 25923	7±0.5	9.1±0.7	10±0.6	2.4±0.04	1.6±0.02	1.2±0.02
S. mutans	ATCC 25175	8.9±0.6	9.8±0.7	12.3±0.9	0.8±0.04	0.4±0.03	0.4±0.02

Minimum Inhibitory Concentration (MIC)

MIC values of essential oil against the testes strains are shown in Table 2. These Riaz et al. 2012 results demonstrated that this oil displayed potential antibacterial and microbicidal property. In general, the MIC values of the essential oil against the tested microorganisms ranged from 0.4 mg/mL to 3.2 mg/mL. By considering the results of inhibition zone assay in Table 1, *S. mutans* was the most sensitive one in the tested microorganisms. The data indicated that the Gram-negative *E. coli* and the Gram-positive *S. mutans* were the most sensitive strains tested to the essential oils of *Galium incanum*, *Galium dieckii*, *Galium aladaghense*. Gram-negative *E. coli* is known to highly resistant even to synthetic drugs due to a very restrictive outer membrane barrier (Piggot et al., 2011). However, essential oil of *Galium aladaghense* inhibits growth of this pathogen bacterium. Gram-positive *S. mutans* is the most common species as a human pathogen. Tested essential oils of all *Gallium* species showed prominent antimicrobial activity against this pathogen bacterium.

Conclusions

To the best of our knowledge, this is the first report of the composition and antimicrobial activity of essential oil of *Galium incanum*, *Galium dieckii*, *Galium aladaghense*. Considering the results of the GCMS analysis of essential oils from *Galium incanum*, *Galium dieckii*, *Galium aladaghense*, the data are suitable for the evaluation of the components of oils and compare the components content to antimicrobial affectivity. In general, the results show that with increasing monoterpen components, especially thymol groups, increased inhibition of growth of bacteria (Baser et al., 2004). The essential oil of this *Galium* species can be used as antimicrobial agent in medicine, pharmacy or biotechnological area for the future applications.

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