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Full Length Research Paper

Chemical composition and antimicrobial activity of *Teucrium arduini* essential oil and cirsimarin from Montenegro

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Essential oil from *Teucrium arduini* was analyzed by gas chromatography flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Fifty-two components were identified (99.52% of total) and quantified by calculating the response factors of FID. The oil was dominated by sesquiterpenes, which accounted for 55.57% of the oil. This study indicates the presence of a high percentage of sesquiterpene hydrocarbons (48.76%), of which the main constituents were germacrene D (16.98%) and β-caryophyllene (14.98%). The minimum inhibitory concentration (MIC) values of the oil obtained by using broth microdilution method were within the range of 50 to 6.25 µl/ml. Maximum activity was observed against gram negative bacteria *Klebsiella pneumonia* (6.25 µl/ml). In addition, at MIC value of 25 µl/ml, essential oil showed a good activity against gram positive bacteria *Staphylococcus aureus* (ATCC 25923) and *S. aureus* (FSB 30), as well as Gram negative bacteria *E. cloaceae* (FSB 22) and *Proteus mirabilis* (FSB 34). On the other hand, at the concentration of 31.25 µg/ml isolated cirsimarin showed a strong activity against *K. pneumonia*.

Key words: *Teucrium arduini*, essential oil, gas chromatography flame ionization detector, gas chromatography-mass spectrometry cirsimarin, antimicrobial activity.

INTRODUCTION

In recent years, multiple drug/chemical resistance, both in human and plant pathogenic microorganisms has been developed due to indiscriminate use of commercial antimicrobial drugs/chemicals commonly used in the treatment of infection diseases (Service, 1995). Resistance to chemotherapy has been noted in all categories of microorganisms including bacteria, fungi, viruses and parasites. Up to now, resistance in bacteria has been most prevalent (Cohen and Tatarsky, 1997). One of the methods that reduce resistance to antibiotics is using antibiotic inhibitors of plant origin (Iinuma et al., 1994; Khim et al., 1995). On the other hand, interest in biopreservation of food systems has recently been increased because of great economic costs of deterioration and poisoning of food products by food pathogens. Essential oils and compounds isolated from edible and medicinal plants present very potent natural, biologically active agents (Nychas et al., 2003). Use of essential oils as antimicrobial agents in food systems may be considered as an additional intrinsic determinant to increase the safety and shelf life of foods (Skandamis and Nychas, 2000).

Teucrium arduini L. belongs to the family Lamiaceae, subfamily Ajugoideae and section Stachyobotrys. Besides this species, *Teucrium lamifolium*, *Teucrium halacsyanum*, *Teucrium francisci-werneri* and *Teucrium heliotropifolium* belong to the section Stachyobotrys in the flora of Europe (Tutin et al., 1972). It is a perennial species with low branches, with half-ligneous stem, oval leaves with softly knobbed brims and compact terminal inflorescence, up 10 to 30 cm high. *T. arduini* is Mediterranean species distributed only along eastern Adriatic coast with status of regional endemic species of

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the western Balkan Peninsula. This is a thermophilic species in the category of xerophytes that inhabits arid limestone rocks up to 1400 m above sea level (Lakusic et al., 2007).

Previous phytochemical studies of *Teucrium* species revealed the presence of essential oil (Saroglou et al., 2007; Kaya et al., 2009; Baher and Mirza, 2003), diterpenoids (Bruno et al., 2002; Coll and Tandron, 2004), flavonoids (Harborne et al., 1986) and steroid compounds (Ulubelen et al., 1994; Kisiel et al., 1995). The genus shows certain similarity in essential oil composition, to the point of a high amount of sesquiterpene hydrocarbons (Saroglou et al., 2007; Kaya et al., 2009; Blazquez et al., 2003; Maccioni et al., 2007).

Teucrium species were found to possess a wide range of biological activities such as antioxidant (Saroglou et al., 2007), antimicrobial (Kucuk et al., 2006; Vukovic et al., 2007), antispasmodic, diuretic, antidiabetic (Gharaibeh et al., 1988), anti-inflammatory and antiulcer (Puntero et al., 1997) activities.

Some of these effects are attributed to the presence of the essential oil. Maccioni et al. (2007) noted the importance of *Teucrium* species as alimentary plants, as some of them are currently used in the preparation of herbal teas, flavoured wines, bitters and liqueurs and infusions of leaves and flowers are used for flavouring beer in some areas (Maccioni et al., 2007).

Since the herbal tea obtained from aerial parts of *T. arduini* is widely used in folk medicine, the aim of this study is to examine the composition of the essential oil and antimicrobial activities of isolated oil and major flavonoid glycoside compound cirsimarin against different microbial strains.

For the best of our knowledge, no reports on the composition of essential oil from the wild-growing population in Montenegro and its antimicrobial activity have been found. Also, for the first time, we presented here antimicrobial activity of cirsimarin.

MATERIALS AND METHODS

Collection of plant material

Aerial flowering parts of *T. arduini* L. were collected in July 2009 from natural populations in the region of Montenegro, Greben near Risan, Bay of Kotor (position: 42° 31' 46" N, 18° 41' 24" E, altitude: 509 m, exposition: SE, substratum: limestone, phytogeographycal belongs: Adriatic province of eu-Mediterranean region). The voucher specimen of *T. arduini* L. 1767, No 2-2208, Montenegro, Risan, Greben, UTM34 CM11, 01. July 2009., det.: Milan Stanković; rev.: Goran Anačkov, were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad.

Chemicals

For determination of retention indices, a hydrocarbon mixture (Supleco, PA, USA) ranging from heptane to triacontane was used. Other applied chemicals and reagents were of the highest purity available and purchased from the Sigma-Aldrich Chemical Company, Difco and BDH Laboratory Supplies.

Isolation of the oil

Air dried aerial parts of *T. arduini* were subjected to hydrodistillation for 3 h using a Clevenger type apparatus to produce oil. The oil was dried over anhydrous sodium sulphate overnight and stored in sealed vial at low temperature (4°C) before analysis. The oil yield was calculated on a dry weight basis as 0.15%.

Analysis of the essential oil

Gas chromatography

Gas chromatography flame ionization detector (GC-FID) analysis of the volatile components was carried out using an Agilent 6890N instrument coupled to an ionization flame detector (FID). Compounds were separated on a HP-5MS capillary column. Analytical conditions were injector and detector temperatures 280°C, respectively, oven temperature programmed at 60°C (isothermal for 5 min), with an increase of 4°C/min to 150°C (isothermal for 10 min), then 4°C/min to 260°C; carrier gas, helium at 1.4 ml/min; injection of 1 µl (10% hexane solution); split ratio 1:30.

Gas chromatography/mass spectrometry analysis

Analysis was carried out in a Agilent 6890N gas chromatograph fitted with a HP-5MS fused silica column (5% phenyl methyl polysiloxane 30 m x 0.25 mm i.d., film thickness 0.25 µm), interfaced with an Agilent mass selective detector 5975B (Agilent Technologies, USA) operated by HP Enhanced ChemStation software. Analytical conditions were injector and transfer line temperatures 250 and 280°C, respectively, oven temperature programmed at 60°C (isothermal for 5 min), with an increase of 4°C/min to 130°C (isothermal for 10 min), then 4°C/min to 240°C; carrier gas, helium at 1 ml/min; injection of 2 µl (10 % hexane solution); split ratio 1:50 whereas split flow was 50 ml/min; standard electronic impact (EI) MS source temperature: 230°C; MS quadruple temperature: 150°C; mass scan range: 35–500 amu at 70 eV; scan velocity: 3.12 scans s^{-1}; resulting EM voltage: 1200 V.

Calculation of Kovats Retention Indices (RI)

A mixture of aliphatic hydrocarbons (C7-C30) in hexane was directly injected into the GC injector under the above temperature programme. Retention indices were directly obtained by application of Kovats procedure (Kovats, 1965; Jennings and Shibamoto, 1980).

Qualitative and quantitative analyses

The constituents of the essential oil were identified by comparing their retention indices and their mass spectral fragmentation pattern with those reported in literature (Adams, 2007) and stored in the MS library (Wiely7Nist). In many cases, the essential oil was subjected to co-chromatography with authentic compounds. The quantification of the components was performed on the basis of their GC peak areas on HP-5MS column (Table 1).

Isolation of the cirsimarin

After hydrodistillation of the oil, water was separated from the plant

material, concentrated under reduced pressure up to volume of 100 ml and extracted with 3 x 70 ml of ethyl acetate.

Organic layers were combined and evaporated to dryness, to yield crude red solid (0.367 g). After addition of 10 ml of methanol, obtained insoluble yellow mixture was purified by column chromatography (toluene:acetone:methanol = 3:2:1) to yield cirsimarin (0.220 g). Compound was recrystallized from methanol as white prisms.

Analysis of cirsimarin

The compound was identified by determination of melting points (Kofler-hot stage apparatus), using elemental analysis (Carlo Erba 1106 microanalyser), IR (Perkin-Elmer Grating Spectrophotometers Model 137 and Model 337, KBr disc, ν in cm^{-1}), ^{1}H-NMR (at 200 MHz) and ^{13}C NMR (at 50 MHz) (Varian, Palo Alto, CA; Gemini 200 spectrometer, dimethyl sulphoxide-d_6 (DMSO-d_6), δ in ppm) techniques. GC/MS analysis of aglycon cirsimaritin obtained by procedure of Harborne (1973), was carried out at the same instrument and column listed above.

Analytical TLC was performed on Merck Silica gel 60 F_{254} TLC plates (layer thickness, 0.25 mm), and monitored by UV light (254 and 365 nm) or iodine vapours. Column chromatography was carried out using Merck 7734 (60 ± 200 mesh) silica gel and monitored by TLC.

White prisms; mp 158-160°C. IR ν_{max} (KBr) cm^{-1}: 3203-3411 (br), 1662, 1601, 1563, 1518, 1461. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.0-3.5 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″), 3.75 (3H, s, 6-OCH_3), 3.95 (3H, s, 7-OCH_3), 5.05 (1H, d, *J*=7.5 Hz, H-1″), 6.96 (1H, s, H-3), 6.99 (1H, s, H-8), 7.21 (2H, d, *J*=9.0 Hz, H-3′, H-5′), 8.08 (2H, d, *J*=9.0 Hz, H-2′, H-6′), 12.89 (1H, s, 5-OH). ^{13}C NMR (50 MHz, DMSO-d_6) δ 56.6 (7-OCH_3), 60.1 (6-OCH_3), 60.6 (C-6″), 69.8 (C-4″), 73.2 (C-2″), 76.4 (C-5″), 77.1 (C-3″), 91.5 (C-8), 99.6 (C-1″), 103.5 (C-3), 105.2 (C-10), 116.7 (C-3′ and C-5′), 123.9 (C-1′), 128.3 (C-2′ and C-6′), 131.9 (C-6), 152.1 (C-5), 152.5 (C-9), 158.7 (C-7), 160.2 (C-4′), 163.2 (C-2), 182.2 (C-4). MS data of aglycone cirsimaritin: m/z (%) 314 (100), 313 (13), 299 (95), 285 (15), 271 (25), 181 (17), 153 (27) (Figure 1).

Determination of antibacterial and antifungal activity

Cultures of microorganisms

Test microorganisms that were used in this experiment are bacteria *Bacillus subtilis* (FSB 2), *Enterobacter cloaceae* (FSB 22), *Enterococcus faecalis* (ATCC 29212), *Escerichia coli* (ATCC 25922), *Klebsiella pneumoniae* (FSB 26), *Micrococcus lysodeikticus* (ATCC 4698), *Proteus mirabilis* (FSB 34), *Staphilococcus aureus* (ATCC 25923), *Staphilococcus aureus* (FSB 30) and fungi *Candida albicans* (ATCC 10259). All test microbial strains were obtained from the Faculty of Biochemistry and Chemistry, University of Belgrade and Institute for Health Protection, Kragujevac, Serbia.

Bacteria were cultured for 24 h at 37°C in Mueller-Hinton broth. Suspensions of fungal spores were prepared from two-day old cultures that were grown on Saboraud Dextrose Agar (SDA) plates at 30°C.

The final inoculum size was 10^6 CFU/ml (according to 0.5 Mc Farland standard; turbidimetric method was applied) for the antibacterial assay and 10^4 CFU/ml for the antifungal assay.

Assay for in-vitro antibacterial activity

The MIC of the essential oil and cirsimarin against tested bacteria were determined by using a microdilution method in 96 multi-well microtiter plates (Sarker et al., 2007). The stock solutions of oil (in methanol, 100 µl/ml) and cirsimarin (in 5% DMSO, 1000 µg/ml) were first diluted to the highest concentration to be tested (50 µl/ml for oil and 500 µg/ml for cirsimarin), 50 µl of Mueller Hinton Broth (supplemented with Tween 80 at the final concentration of 0.5 % (v/v) for analysis of oil) was distributed from the 2nd to the 12th well, a volume of 50 µl from each of the solutions initially prepared was pipetted into the 1st test wells of each microtiter line, and then 50 µl of scalar dilution was transferred from the 2nd to the 12th well. To each well 10 µl of resazurin indicator solution (prepared by dissolving a 270 mg tablet in 40 ml of sterile distilled water) and 30 µl of Mueller Hinton Broth were added. Finally, 10 µl of bacterial suspension (10^6 CFU/ml) was added to each well to achieve a concentration of 10^5 CFU/ml.

The final concentrations of the oil and cirsimarin adopted to evaluate antibacterial activity were within the range of 50 to 0.244 µl/ml and 500 to 0.244 µg/ml, respectively. Three columns in each plate were used as controls: one column with a broad-spectrum antibiotic as a positive control (amracin in a serial dilution of 50 to 0.244 µg/ml) and two columns containing the solvents (methanol and 5% DMSO) as negative controls. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37°C for 18 to 24 h. Color change was then assessed visually.

Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated and that was the MIC for the tested compounds and standard drug.

Assay for in-vitro antifungal activity

Broth microdilution assays were performed in accordance with the guidelines in CLSI document M27-A2 (NCCLS, 2002). Stock solutions were prepared in water for ketoconazole, methanol for oil and 5% DMSO for cirsimarin. The final dilution was carried out in an RPMI 1640 medium, buffered to pH 7.0 with 0.165 M morpholenepropanesulfonic acid buffer. Each microdilution well containing 100 µl of the twofold concentrations of oil (50 to 0.244 µl/ml), cirsimarin (500 to 0.244 µg/ml) or standard drug ketoconazole (100 to 0.488 µg/ml) was inoculated with 100 µl of the diluted twofold inoculum suspension (the final volume in each well was 200 µl and inoculum size of 10^3 CFU/ml). Growth (drug free) and sterility controls were also included. Microdilution trays were incubated in ambient air at 35°C. MICs were determined visually after 48 h of incubation, as the lowest concentration of drug that caused no detectable growth. The average of 3 values was calculated and that was the MIC for the tested compounds and standard drug (Table 2).

RESULTS AND DISCUSSION

GC and GC-MS analyses of the oil from aerial parts of *T. arduini* led to the identification of fifty-two components, representing 99.52% of the total oil (Table 1). Identified compounds included 4 hydrocarbons (5.54%), two alcohols (4.90%), two aldehydes (0.28%), 5 ketones (3.27%), one phenylpropenes (tr), one norisporenoides (0.89%), 15 monoterpenes (24.88%), 17 sesquiterpenes (55.57%), two esters (2.34%) and 3 acids (1.85%). The most representative compounds of the essential oil were sesquiterpene hydrocarbons (48.76%) and among them the main constituents were germacrene D (16.98%),

Table 1. Qualitative and quantitative composition of *T. arduini* essential oil.

No.	Compound[a]	RI[b]	%[c]	Mode of Identification[d]
1	pentanal	698	tr	MS, RI
2	heptane	700	tr	MS, RI
3	3-pentanone	703	0.16	MS, RI
4	3-pentanol	710	0.21	MS, RI
5	2.4-pentanedione	783	0.19	MS, RI
6	(E)-2-hexenal	853	0.28	MS, RI
7	2-heptanone	889	tr	MS, RI
8	1-octen-3-ol	978	4.69	MS, RI
9	2-octanone	992	1.38	MS, RI
10	(Z)-β-ocimene	1040	tr	MS, RI, CoI
11	γ-terpinene	1062	2.89	MS, RI, CoI
12	α-terpinolene	1087	5.25	MS, RI
13	linalool	1098	7.05	MS, RI, CoI
14	Z-cinnamaldehyde	1214	tr	MS, RI
15	trans-carveol	1217	tr	MS, RI
16	citronellol	1226	tr	MS, RI
17	nerol	1228	tr	MS, RI, CoI
18	cis-carveol	1229	0.31	MS, RI
19	pulegone	1237	tr	MS, RI
20	cis-myrtanol	1251	0.11	MS, RI
21	geraniol	1255	2.62	MS, RI
22	vitispirane	1286	0.89	MS, RI
23	menthyl acetate	1294	2.66	MS, RI, CoI
24	trans-pinocarvyl acetate	1297	1.96	MS, RI
25	cis-pinocarvyl acetate	1309	1.16	MS, RI
26	citronellyl acetate	1357	0.87	MS, RI, CoI
27	α -copaene	1376	tr	MS, RI
28	β-burbonene	1384	5.59	MS, RI
29	longifolene	1401	1.36	MS, RI
30	di-epi-□-cedrene	1409	0.59	MS, RI
31	α -cis-bergamotene	1415	0.12	MS, RI
32	β-caryophyllene	1418	14.98	MS, RI, CoI
33	aromadendrene	1439	2.14	MS, RI
34	α -humulene	1454	1.29	MS, RI, CoI
35	germacrene D	1480	16.98	MS, RI
36	α -amorphene	1528	4.68	MS, RI
37	δ-cadinene	1530	1.03	MS, RI
38	α -cadinene	1539	tr	MS, RI
39	α -calacorene	1542	tr	MS, RI
40	caryophyllene oxide	1581	0.68	MS, RI, CoI
41	spathulenol	1585	0.56	MS, RI
42	τ-muurolol	1641	0.98	MS, RI
43	α -cadinol	1653	4.59	MS, RI, CoI
44	tetradecanoic acid	1780	1.13	MS, RI
45	(E.E)-farnesyl acetone	1921	1.54	MS, RI
46	methyl hexadecanoate	1926	0.99	MS, RI
47	hexadecanoic acid	1991	0.56	MS, RI
48	methyl oleate	2103	1.35	MS, RI
49	oleic acid	2141	0.16	MS, RI

Table 1. Continued.

50	pentacosane	2500	2.38	MS, RI
51	heptacosane	2700	2.49	MS, RI
52	nonacosane	2900	0.67	MS, RI

[a]Compounds are listed in order of their elution from a HP-5MS column. [b]RI, retention index on HP-5MS column, experimentally determined using homologous series of C7-C30 alkanes. [c]Percentage values are means of three determinations. tr-trace (<0.10 %). [d]Identification methods: MS, by comparison of the mass spectrum with those of the computer mass library Wiely7Nist; RI, by comparison of RI with those reported in literature; CoI, co-injection with authentic compound.

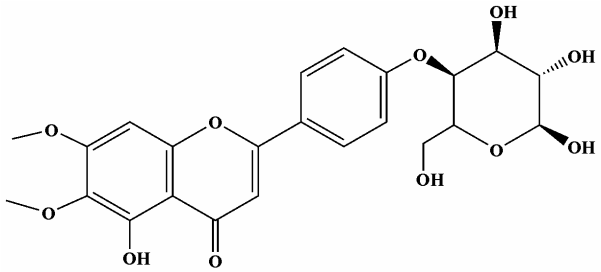

Figure 1. Structure of cirsimarin.

Table 2. Antimicrobial activities of the essential oil and cirsimarin.

	Minimal inhibitory concentration			
	µl/ml	µg/ml		
Microorganism	**Essential oil**	**Cirsimarin**	**A**	**K**
bacteria				
Gram +				
B. subtilis (FSB 2)	50	62.5	0.31	-
E. faecalis (ATCC 29212)	50	500	0.62	-
M. lysodeikticus (ATCC 4698)	50	500	0.62	-
S. aureus (ATCC 25923)	25	125	0.62	-
S. aureus (FSB 30)	25	250	1.25	-
Gram -				
E. cloaceae (FSB 22)	25	250	0.31	-
E. coli (ATCC 25922)	50	500	2.5	-
K. pneumonia(FSB 26)	6.25	31.25	0.62	-
P. mirabilis(FSB 34)	25	250	5	-
Fungi				
C. albicans (ATCC 10259)	50	500	-	1.25

β-caryophyllene (14.98%), β-burbonene (5.59%) and α-amorphene (4.68%). In amount of 7.05 %, linalool was the most abundant compound in the fraction of oxygenated monoterpenes, while α-terpinolene (5.25%) represents the major monoterpene hydrocarbon. Inside the class of alcohols, 1-octene-3-ol was presented in amount of 4.69%, while pentacosane (2.38%) and heptacosane (2.49%) are the main compounds of hydrocarbons fraction.

Since the sesquiterpene rich oils were observed in other *Teucrium* species (Saroglou et al., 2007; Kaya et al., 2009; Baher and Mirza, 2003), it is not surprising that

T. arduini essential oil contains a high level of this fraction, especially germacrene D and β-caryophyllene. On the other hand, α-pinene, a common constituent of other *Teucrium* essential oils (Blazquez et al., 2003; Cavaleiro et al., 2004; Ahmadi et al., 2002) is totally absent from our specimen.

Melting point, IR, ^{1}H NMR and ^{13}C NMR spectral data of cirsimarin were in accordance with those reported in literature (De Backer et al., 1997).), as well as the MS spectral data of aglycone cirsimaritin. Cirsimarin has been previously isolated from several species (De Backer et al., 1997; Morita and Shimuzu, 1963), and represents chemotaxonomic marker of *T. arduini* (Harborne et al., 1986). There have been no previous data that this compound is the most abundant flavonoid in water extract, nor the data on their antimicrobial activity. Literature states cirsimarin as a potent antilipogenic flavonoid that decreases fat deposition in mice intra-abdominal adipose tissue (Zarrouki et al., 2010), that is, the compound that triggers lipid metabolization (Girotti et al., 2005), which gives this plant a great practical value. Also, previous reports showed that flavonoids possess multiple biological effects, including antibacterial, antiallergic, antiviral, antioxidant, anti-inflammatory and antiaging activities (Hollman and Arts, 2000), and that cirsimarin has a high activity on adenosine A_1 receptors (acting against proteinuria) (De Backer et al., 1997).

Obtained results of MICs of *T. arduini* essential oil and flavonoid cirsimarin by broth microdilution method were presented in Table 2. The inhibitory properties of the oil were observed within the range of 50-0.244 µl/ml. In liquid medium the essential oil was active against all test microorganisms. Maximum activity was observed against the bacteria *K. pneumonia* (MIC 6.25 µl/ml). At MIC values of 25 µl/ml, the essential oil showed a strong antibacterial activity against *E. cloaceae*, *P. mirabilis*, *S. aureus* (ATCC 25923) and *S. aureus* (FSB 30), while at the highest tested concentration (50 µl/ml), growth of other four bacterial strains and fungus *C. albicans* was inhibited. Since the main constituents of the essential oil are germacrene D and β-caryophyllene, they can be attributed a significant contribution to antimicrobial activity. MIC values for cirsimarin against test microorganisms were within the range of 31.25-500 µg/ml. As shown in Table 2, at the concentration of 31.25 µg/ml the strongest activity was observed against bacteria *K. pneumonia*, while at twofold concentration (62.5 µg/ml) growth of bacteria *B. subtilis* was inhibited. On the other hand, bacteria *E. coli*, *E. faecalis* and *M. lysodeikticus*, as well as fungus *C. albicans*, were quite resistant to cirsimarin (MIC values 500 µg/ml).

Conclusion

Analysis of essential oil of dried aerial parts of *T. arduini* determined sesquiterpene hydrocarbons germacrene D and β-caryophyllene as main constituents.

On the other hand, analysis of residual water extract obtained after hydrodistillation of the oil showed a significant amount of flavonoid cirsimarin. Results of antimicrobial activity indicate that the oil, within the range of tested concentrations, inhibited growth of all test microorganisms, the strongest activity being observed against *K. pneumonia*. In addition, cirsimarin shows a strong activity, particularly against bacteria *K. pneumonia* and *B. subtilis*. These results indicate possibility of using *T. arduini* essential oil and cirsimarin as antimicrobial agents.

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