Essential Oil of *Hypericum perforatum*

The chemical composition and antimicrobial activity

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The chemical composition and antimicrobial activity of the essential oil isolated by steam distillation from Hypericum perforatum L. (St John's wort) growing wild in western Romania have been studied. The extraction yield was 0.41% (v/w) based on the dry plant material. The essential oil was analyzed by GC–MS, and a total of twenty-two components were identified. The major components were alpha-pinene (30.92%), beta-pinene (18.32%) and caryophyllene (15.26%). The antimicrobial activity of the H. perforatum essential oil was screened using the disk diffusion method against 7 common food-related bacteria and fungus. The analyzed EO possesses strong antimicrobial activity. Klebsiella pneumoniae and Pseudomonas aeruginosa were the most resistant species. The analyzed oil can represent an inexpensive natural source of antiseptic compounds, an alternative to synthetic preservatives.

Keywords: Hypericum perforatum, essential oil, GC-MS, alpha-pinene, beta-pinene, antimicrobial activity

Hypericum L. (*Hypericaceae*) is a large genus of herbaceous or shrubby plants, containing about 400 species and occurring widely in temperate regions around the world [1,2]. The Romanian spontaneous flora contains 12 species of *Hypericum*, including two very rare species (*H. rumeliacum* Boiss. and *H. rochelii* Griseb. & Schenk) [3]. *H. perforatum*, locally known as *sunatoare*, is a medicinal plant with a long history of use in the Romanian traditional medicine (antiseptic, astringent and cicatrizing activity) [4]. This particular species is the most widely spread *Hypericum* species, especially in the hilly regions of Romania [5].

A large number of biologically active components have been isolated from *H. perforatum*: naphtodianthrones (hypericin, pseudohypericin), phloroglucinol derivatives (hyperforin and adhyperforin), procyanidins, flavonoids and essential oil (EO) [6–11]. *H. perforatum* is known as a plant with a low EO content, generally the extraction yield being between 0.05-0.9% [7,8].

Previous investigations of the *H. perforatum* EO's chemical composition conducted in Romania [6], Serbia [9–11], Kosovo [12], Turkey [13], Greece [2,14] and Portugal [15] did not give homogeneous results. Some studies have reported as a major component 2-methyloctane [2,11,12,16]; others *n*-nonane, along with smaller amounts of 2-methyldecane and *n*-undecane [10,17], α- and β-pinene [6,15], or caryophyllene and germacrene [12,16], respectively. These results can be explained by the postulate that the chemical composition of EOs is influenced by genetic and environmental factors, harvesting time, stage of development of the plant and plant part analyzed [11,12,16].

In addition to its pharmacological activities (anti-depressant and antiviral effects), *H. perforatum* extracts present antimicrobial [6,9,10,18,19] and antioxidant properties [20,21].

The aims of our study were to determine: i) the chemical composition and ii) the antimicrobial properties of the EO isolated from *H. perforatum* growing wild in western Romania.

Experimental part

Materials and methods

Raw materials

The influorescences, on full flowering, were harvested manually in July 2015, from the following location: Lipova, Arad County, Romania. The influorescences was dried under natural conditions (shielded from light radiations) for 14 days. A voucher specimen was deposited in the herbarium of the Victor Babes University of Medicine and Pharmacy, Timisoara, Romania.

Isolation of essential oil

The EO was extracted, using a Clevenger-type apparatus, by steam distillation for 4 h, as prescribed in the 5th European Pharmacopoeia [22]. The EO obtained was dried over anhydrous sodium sulfate (Sigma-Aldrich, Germany) and stored at -18°C for further analyses.

Physical analysis

Refractive index and the specific gravity of the EO were measured according to the method described by the Food Chemical Codex [23]. For the determination of the specific gravity a 2-mL Gay-Lussac pycnometer (DURAN) was used, and for the refractive index a DR6100 digital

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refractometer (Krüss Optronic GmbH, Germany) was used. The assays were performed in triplicate at temperatures of 20°C (refractive index) and 25°C (specific gravity), respectively.

Gas chromatography–mass spectroscopy (GC–MS)

The chemical composition of the *H. perforatum* EO was determined by GC–MS, using a CLARUS 500 PerkinElmer gas-chromatograph coupled with a quadrupole mass spectrometer, fitted with FID and a 15-m Elite-1701 capillary column (0.53 mm i.d. and 1.00 μm film thickness, PerkinElmer, USA). The FID temperature was 250°C, for the injector 70-260°C (5°C/min) and for the oven 60-250°C (5°C/min). Helium was the carrier gas (6 mL/min). The chemical composition of the EO was calculated as a percentage. The EO components were identified by comparison of the obtained mass spectra with mass spectra from the NIST 98 library (USA National Institute of Science and Technology software).

Antimicrobial activity

Reference strains of 7 common food-related bacteria and fungus: *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 13882), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 10231) were obtained from the Department of Microbiology, Victor Babe' University of Medicine and Pharmacy, Timisoara. The *H. perforatum* EO was tested using filter paper discs (Whatman No 1 filter paper – 6 mm diameter) by the disk diffusion method, as previously described [24]. Briefly, a culture suspension of the tested microorganisms (10^6 cells mL^{-1}) was spread on the solid media plates. The filter paper discs were impregnated with 5, 10, 15 and 20 μL EO and placed on the solid medium plates. The diameter of the inhibition zone (in millimeters) was measured after 24 h at 37°C for the bacteria and 48 h at 30°C for the fungus, respectively. Ciprofloxacin (30 μg/disk) was used as positive control for the bacterial strains and fluconazole (10 μg/disk) for the fungus, respectively.

Statistical analysis

We analyzed the effect of various amounts of *H. perforatum* EO (5, 10, 15 and 20 μL, respectively) on 7 types of food-related bacteria and fungus. Both the interspecific comparisons at a given amount and the comparisons of the effects of different amounts of oil for each species were performed via ANOVA, followed by post-hoc Tukey tests to identify significant pairwise differences. The results of these comparisons were visualized using grouped boxplots. All the analyses were conducted using the R software, version 3.3.0, and the database was created using Microsoft Excel.

Results and discussions

The extraction yield was 0.41% (v/w) based on the dry plant material. Comparatively, the contents of essential oils from Romania were 0.23% [6], Serbia 0.03–1.93 [25], Portugal 0.15% [15], Turkey 0.04–0.5% [26] and Kosovo 0.04–0.26% [12]. The *H. perforatum* EO's specific gravity at 25°C was 0.703 ± 0.006 g/cm^3; the refractive index at 20°C was 1.431 ± 0.041, respectively.

The chemical composition of the *H. perforatum* EO determined by GC–MS is presented in table 1. Twenty-two components representing 99.91% of the total area were identified. The major components were alpha-pinene (30.92%), beta-pinene (18.32%) and caryophyllene (15.26%). The EO is also rich in germacrene D (9.23%) and β-cis-ocimene (7.85%). The studies of *H. perforatum*

Table 1

CHEMICAL COMPOSITION OF THE *H. PERFORATUM* ESSENTIAL OIL

RT	Area % of Total	Name of Compound
5.40	1.33	alpha-Thujene
5.62	**30.92**	**alpha-Pinene**
6.19	0.24	Camphene
6.79	0.42	Sabinene
6.89	**18.32**	**beta-Pinene**
6.98	1.67	beta-Phellandrene
7.50	2.53	Decane, 2-methyl
7.77	0.50	4-Carene
8.04	0.74	D-Limonene
8.26	1.53	p-Menth-3-ene
8.40	0.67	Undecane
8.47	**7.85**	**β-cis-Ocimene**
8.95	0.92	gamma-Terpinene
12.10	0.48	Dodecane, 2-methyl
17.32	**15.26**	**Caryophyllene**
18.23	0.84	1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl
18.63	0.79	alpha-Muurolene
18.84	**9.23**	**Germacrene D**
19.03	1.94	beta-Bisabolene
19.28	1.85	gamma-Elemene
19.60	1.35	gamma-Cadinene
19.70	0.53	gamma-Muurolene
	99.91	**Total**

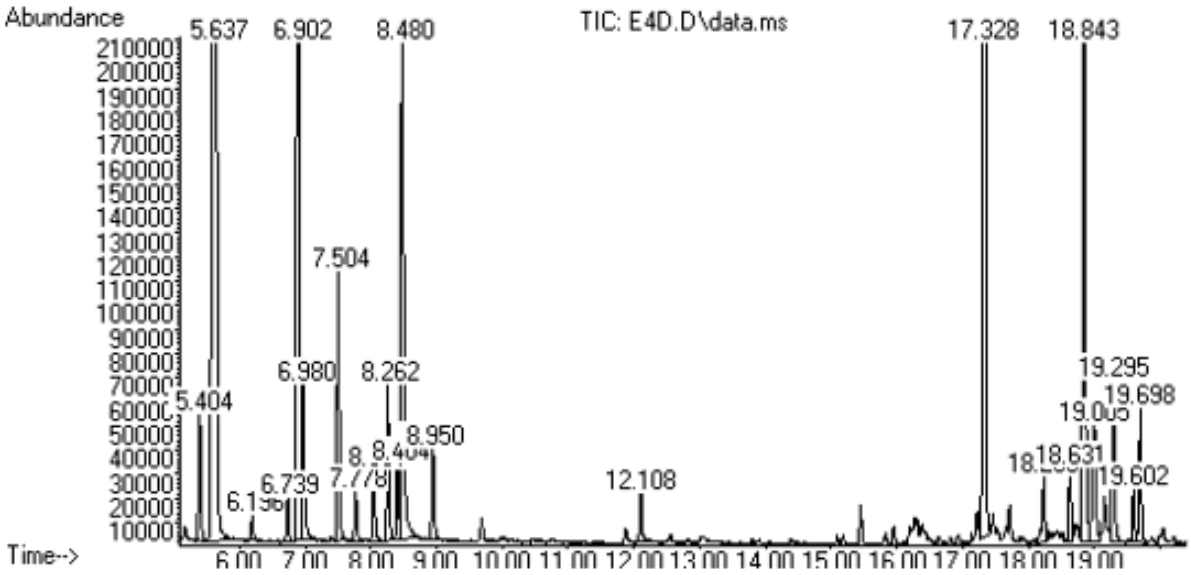

Fig. 1. GC chromatogram of the essential oil fromm H. perforatum

populations originating from Greece [14] and Portugal [15], respectively, showed a higher content of alpha-pinene and beta-pinene than those reported in our study. The wild populations in Kosovo showed a high content of alpha-pinene along with other major components: 2-methyloctane, beta-caryophyllene, germacrene D [12]. In contrast, a Serbian sample [10] showed a different chemical composition, in this study nonane (63.8%) being the most abundant component. These differences in the *H. perforatum* EO compositions may be due to the different chemotypes as previously reported [2,11–13,15,16].

For all our data we computed a basic descriptive statistic (an average value and a standard deviation value), calculated for each bacteria for all four concentrations (table 2). Because we observed that there are some differences between the average values of our bacteria, we decided to run a statistical analysis in order to decide if the differences seen are statistically significant or not.

For all the bacterial species considered, there were significant differences between the effects of different amounts of *H. perforatum* EO ($p < 0.001$ in all cases), revealing that the inhibition zone clearly increases with the concentration. More in-depth analyses performed via post-hoc tests showed that in general responses are significantly greater for larger amounts and this trend is consistent for all species (table 3).

Regarding interspecific comparisons, significant differences between species were observed at each amount ($p < 0.001$ in all cases). At 5μL, the *H. perforatum* EO was found to be most effective against *K. pneumoniae*, and least effective against *C. albicans*. At 10μL, the EO performed significantly better in the case of *K. pneumoniae* than for all the other investigated species, and was least effective against *E. faecalis*. At 15μL, the ranking of species in decreasing order of effectiveness is *K. pneumoniae, S. aureus, S. typhimurium, E. coli, C. albicans, E. faecalis* and *P. aeruginosa*, with significant pairwise differences in all cases except for the comparisons of *S. typhimurium* and *E. coli*, and *P. aeruginosa* and *E. faecalis*, respectively, which displayed similar effects. The same behaviour was observed at 20 μL, however in this case no significant differences were found between the effects on *Salmonella, E. coli* and *C. albicans* (table 4).

In previous studies, the *H. perforatum* EO was reported to have antibacterial activity against several bacterial strains such as *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *E. coli* and *C. albicans* [6,9,10,19]. In contrast, Saddiqe *et al* (2010) [18] citing Gudzic *et al* (1997) [27] points to the inefficiency of the *H. perforatum* EO against *P. aeruginosa*. In the present study the EO tested demonstrated a lower efficiency against *P. aeruginosa* (table 2) compared to those previously reported by Jianu *et al.* (2016) [6]. The biological activity recorded can be attributed to the high content of terpenes present in the tested *H. perforatum* EO: caryophyllene, alpha- and beta-pinene (table 1), the antimicrobial properties of which having been previously reported [28–30]. However, the mechanism of action of terpenes is not yet fully understood, but is speculated to involve membrane disruption by the lipophilic compounds [31].

Table 2

EFFECTS OF *H. PERFORATUM* OIL AGAINST BACTERIA EXPRESSED BY THE MEAN SIZE OF THE INHIBITORY ZONES

Test microorganism	Amount of essential oil [µL]							
	5 µL		10 µL		15 µL		20 µL	
	Mean	Stdev	Mean	Stdev	Mean	Stdev	Mean	Stdev
S. aureus	11.09	0.53	11.61	0.58	13.14	0.41	15.84	0,19
S. typhimurium	8.90	0.13	11.23	0.35	11.42	0.35	12.09	0.22
P. aeruginosa	NA	NA	NA	NA	6.82	0.16	7.34	0.19
E. coli	9.01	0.19	10.60	0.31	11.27	0.31	12.13	0.24
K. pneumoniae	12.47	0.34	14.14	0.30	14.76	0.19	16.93	0.22
E. faecalis	NA	NA	6.70	0.10	6.87	0.23	7.97	0.13
C. albicans	7.97	0.17	8.89	0.40	10.66	0.21	12.04	0.21

Inhibitions (expressed in mm) include the diameter of filter paper disc (6 mm). Data distributions were expressed as mean values and standard deviations (SD) (n = 9). Ciprofloxacin (30 µg/disk) was used as positive control for the bacteria and fluconazole (10 µg/disk) for the fungus, respectively; NA: no activity.

Table 3

RESULTS (MEAN DIFFERENCES AND p-VALUES) OF TUKEY TESTS FOR PAIRWISE COMPARISON OF RESPONSED TO DIFFERENT CONCENTRATIONS OF *H. PERFORATUM* EO, FOR EACH ANALYZED BACTERIAL SPECIES

S. aureus ATCC25923			
Concentration	5 µL	10 µL	15 µL
10 µL	**diff=0.522 *p=0.087***		
15 µL	diff=2.056 *p<0.001*	diff=1.533 *p<0.001*	
20 µL	diff=4.756 *p<0.001*	diff=4.233 *p<0.001*	diff=2.700 *p<0.001*

K. pneumoniae ATCC 13882			
Concentration	5 µL	10 µL	15 µL
10 µL	diff=1.678 *p<0.001*		
15 µL	diff=2.289 *p<0.001*	diff=0.611 *p<0.001*	
20 µL	diff=4.467 *p<0.001*	diff=2.789 *p<0.001*	diff=2.178 *p<0.001*

S. typhimurium ATCC14028			
Concentration	5 µL	10 µL	15 µL
10 µL	diff=2.333 *p<0.001*		
15 µL	diff=2.522 *p<0.001*	**diff=0.189 *p=0.493***	
20 µL	diff=3.189 *p<0.001*	diff=0.856 *p<0.001*	diff=0.667 *p<0.001*

E. faecalis ATCC 29212			
Concentration	5 µL	10 µL	15 µL
10 µL	NA		
15 µL	NA	**diff=0.167 *p=0.365***	
20 µL	NA	diff=1.267 *p<0.001*	diff=1.100 *p<0.001*

P. aeruginosa ATCC 27853			
Concentration	5 µL	10 µL	15 µL
10 µL	NA		
15 µL	NA	NA	
20 µL	NA	NA	diff=0.522 *p<0.001*

C. albicans ATCC 10231			
Concentration	5 µL	10 µL	15 µL
10 µL	diff=0.922 *p<0.001*		
15 µL	diff=2.689 *p<0.001*	diff=1.767 *p<0.001*	
20 µL	diff=4.078 *p<0.001*	diff=3.156 *p<0.001*	diff=1.389 *p<0.001*

E. coli ATCC 25922			
Concentration	5 µL	10 µL	15 µL
10 µL	diff=1.589 *p<0.001*		
15 µL	diff=2.256 *p<0.001*	diff=0.666 *p<0.001*	
20 µL	diff=3.122 *p<0.001*	diff=1.533 *p<0.001*	diff=0.867 *p<0.001*

The *diff* values designate mean differences between line and column concentrations; significance value $\alpha < 0.05$.

Table 4

RESULTS (MEAN DIFFERENCES AND *P*-VALUES) OF TUKEY TESTS FOR PAIRWISE COMPARISONS OF THE RESPONSES OF THE BACTERIAL SPECIES TO THE SPECIFIED CONCENTRATION OF *H. PERFORATUM* ESSENTIAL OIL

5 µL	A	B	C	D	E	F	15 µL	A	B	C	D	E	F
B	diff=-2.189 *p<0.001*						B	diff=-1.722 *p<0.001*					
C	NA	NA					C	diff=-6.322 *p<0.001*	diff=-4.600 *p<0.001*				
D	diff=-2.078 *p<0.001*	**diff=0.111** ***p=0.939***	NA				D	diff=-1.878 *p<0.001*	**diff=-0.156** ***p=0.899***	diff=4.444 *p<0.001*			
E	diff=1.380 *p<0.001*	diff=3.567 *p<0.001*	NA	diff=3.455 *p<0.001*			E	diff=1.611 *p<0.001*	diff=3.333 *p<0.001*	diff=7.933 *p<0.001*	diff=3.489 *p<0.001*		
F	NA	NA	NA	NA	NA		F	diff=-6.278 *p<0.001*	diff=-4.556 *p<0.001*	**diff=0.045** ***p=0.999***	diff=-4.400 *p<0.001*	diff=-7.889 *p<0.001*	
G	diff=-3.12 *p<0.001*	diff=-0.933 *p<0.001*	NA	diff=-1.044 *p<0.001*	diff=-4.500 *p<0.001*	NA	G	diff=-2.489 *p<0.001*	diff=-0.767 *p<0.001*	diff=3.833 *p<0.001*	diff=-0.611 *p<0.001*	diff=-4.100 *p<0.001*	diff=3.789 *p<0.001*
10 µL	**A**	**B**	**C**	**D**	**E**	**F**	**20 µL**	**A**	**B**	**C**	**D**	**E**	**F**
B	**diff=-0.378** ***p=0.331***						B	diff=-3.756 *p<0.001*					
C	NA	NA					C	diff=-8.500 *p<0.001*	diff=-4.744 *p<0.001*				
D	diff=-1.011 *p<0.001*	diff=-0.633 *p=0.016*	NA				D	diff=-3.711 *p<0.001*	**diff=0.044** ***p=0.999***	diff=4.789 *p<0.001*			
E	diff=2.533 *p<0.001*	diff=2.911 *p<0.001*	NA	diff=3.544 *p<0.001*			E	diff=1.089 *p>0.001*	diff=4.844 *p<0.001*	diff=9.589 *p<0.001*	diff=4.800 *p<0.001*		
F	diff=-4.911 *p<0.001*	diff=-4.533 *p<0.001*	NA	diff=-3.900 *p<0.001*	diff=-7.444 *p<0.001*		F	diff=-7.878 *p<0.001*	diff=-4.122 *p<0.001*	diff=0.622 *p<0.001*	diff=-4.167 *p<0.001*	diff=-8.967 *p<0.001*	
G	diff=-2.722 *p<0.001*	diff=-2.344 *p<0.001*	NA	diff=-1.711 *p<0.001*	diff=-5.256 *p<0.001*	diff=2.189 *p<0.001*	G	diff=-3.800 *p<0.001*	**diff=-0.044** ***p=0.999***	diff=4.700 *p<0.001*	**diff=-0.089** ***p=0.967***	diff=-4.889 *p<0.001*	diff=4.078 *p<0.001*

The diff values designate mean differences between responses of line and column species; A = S. aureus, B = S. typhimurium, C = P. aeruginosa, D = E. coli, E = K. pneumoniae, F = E. faecalis, G = C. albicans (significance value á < 0.05)

Conclusions

The analyzed oil can represent an inexpensive natural source of antiseptic compounds, an alternative to synthetic preservatives.

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