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Original scientific paper

IN VITRO EVALUATION OF ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF OLEUM HYPERICI: AN ORIGINAL PRODUCT FROM GOČ MOUNTAIN (SERBIA)

Mirjana Ž. Grujović[*1], Katarina G. Mladenović[1], Ljiljana R. Čomić[1] and Aleksandar Glišović[2]

[1]*University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanovića 12, 34000 Kragujevac, Republic of Serbia*
[2]*„Our paradise garden", Kneza Mihajla 278, 34000 Kragujevac, Republic of Serbia*
*****Corresponding author; E-mail: mirjana.grujovic@pmf.kg.ac.rs

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ABSTRACT: This study observed the preliminary investigation of Oleum Hyperici, an original product made from *Hypericum perforatum* flowers, collected from Goč Mountain (Serbia). Some of the chemical properties (pH, the content of metals and metalloids - As, Cr, Pb, Cd, Ni, Hg) and the microbiological safety of the product including the total count of aerobic mesophilic bacteria, coagulase positive staphylococci, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* sp., as well as fungi: molds and yeasts) were evaluated. Antimicrobial activity of *H. perforatum* oil against 18 species of bacteria was determined using the disc diffusion and microdilution methods, by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The antibiofilm activity of Oleum Hyperici was evaluated using the biofilm formation assay. The results indicated that oil did not contain potentially pathogenic microorganisms. Oleum Hyperici product showed the antimicrobial activity on the most of tested bacteria (inhibition zone was in a range from 14 - 24 mm), except on *Klebsiella pneumoniae* ATCC 70063, *K. pneumoniae*, *E. coli* ATCC 25922, *P. mirabilis*, *Bacillus subtilis* ATCC 6633 and *B. subtilis* IP 5832. According to the obtained results, tested bacteria (planktonic and biofilm form) showed sensitivity up to 10^{-2} dilution of Oleum Hyperici. Based on the obtained preliminary results, it could be concluded that Oleum Hyperici can be used in traditional medicine for development control of some potential human pathogens.

Keywords: Oleum Hyperici, original product, food safety, biofilm, antimicrobial activity

INTRODUCTION

Hypericum perforatum (fam. Hypericaceae), also known as St John's wort, is well-known medicinal herb, with its native habitat in Europe and Asia, but it can be found as invasive species in North America and Oceania (NG *et al*., 2017). It was used in folk medicine for treatment of burns, wounds, hematomas, inflammations and muscle pain (ISTIKOGLOU *et al*., 2010; MOFFAT, 2014). An oil macerate of *H. perforatum* flowers (Oleum Hyperici) has been also widely used as a traditional medicine across Balkan countries for a long time,

especially in Bosnia and Herzegovina and Serbia, mostly for healing skin ulcers, burns and wounds (SÜNTAR *et al*., 2010; ŠARIĆ-KUNDALIĆ *et al*., 2010; ŠAVIKIN *et al*., 2013) as an antiseptic, for liver and stomach complaints, as an antiphlogistic agent in the treatment of inflammation of the bronchi and urogenital tract, treatment of biliary disorders, bladder irritation, etc.

According to HPLC analysis, standardized extract of *H. perforatum* contains precisely 0.3% hypericin (250 μg per 300 mg) and twice as much pseudohypericin (0.6% of the total extract) (BROCKMÖLLER *et al*., 1997). TIAN *et al*. (2014) indicated that *H. perforatum* contains hyperforin (phloroglucinol derivative currently thought to be the major psychoactive component of the plant) and adhyperforin. *H. perforatum* flowers contain many derivatives, including the flavonol hyperoside, biflavonols, xanthone derivatives, common phenolic acids (caddeic acid, chlorogenic acid, and ferulic acid), tannins and catechin derivative (DOSTALEK and STARK, 2012; MATEI *et al*. 2015).

The content of bioactive compounds in Oleum Hyperici, especially phenolic derivatives (thymol and carvacrol) depends of some factors, like geographical origin, ecological and agronomic condition and way of picking plants (SÜNTAR *et al*., 2010). It is well-known that many compounds showed sensitivity to light, humidity or heat, so the storage of plant is very important, too (BAKKALI *et al*., 2008, HYLDGAARD *et al*.,2012). SCHEMPP *et al*. (1999) and SADDIQE *et al*. (2010) indicated that hypericin and hyperforin have potential antibiotic properties, especially active against some Gram-positive bacteria. HEYDARIAN *et al*. (2017) indicated that polysaccharides from *H. perforatum* have good antimicrobial potential against some Gram-positive and Gram-negative bacteria. SÜNTAR *et al*. (2016) demonstrated that *H. perforatum* extracts showed good antagonistic potential against several oral bacteria. SARKISIAN *et al*. (2012) indicated that secondary metabolites from *Hypericum* spp. exhibit antibiofilm activities against *Staphylococcus* spp. SCHIAVONE *et al*. (2013) indicated that hyperforin had the ability to inhibit planktonic and biofilm cultures of *S. aureus* and *E. faecalis*.

The aim of this study was to examine some of the chemical properties of the Oleum Hyperici, as original, noncommercial product from Serbia, Goč Mountain and *in vitro* evaluation of microbiological safety of the products. Also, it is shown the possible impact on the planktonic growth and biofilm formation of selected bacteria, in order to evaluate the possible use of the product in commercial proposes.

MATERIAL AND METHODS

Preparation of H. perforatum oil (Oleum Hyperici) original product

The *H. perforatum* plants were collected on the Goč Mountain (1216 m altitude), during the 2017. It is located in central Serbia, in the Kopaonik mountain range. Eastern side of the mountain is covered with mixed forests (*Fagus* sp., *Abies* sp., *Pinus nigra, Quercus petraea*), while the west side is bare and rocky. An endemit of the Balkan, *Acer heldreichii* Orph. subsp. *visianii* (Nym.) K. Maly var. *pancicii* (K. Maly) Hayek), the variety of *P. nigra* (*P. nigra* var. *gocensis* Georgev) as well as 226 medicinal and aromatic plant species, which includes 25 taxa of endemic plants, can be found on Goč. The climate belongs to the type mountain variety of mild continental climate, with an average snow cover of 50 cm. The highest peak of the mountain is Ljukten (1216 m). Goč has more than 250 springs with healthy drinking water and it is considered as a mountain of medical tourism, as it provides conditions for recovery of cardiovascular patients (GAJIĆ, 1984).

The plant material was collected before the sun zenith and after the sun rose (around 10 o'clock in the morning). Only flowers were used for the process of making the Oleum

Hyperici (250 g of flowers was added into 1000 ml of oil). After a 12-hour drying on natural wind, the flowers were macerated in the cold, unrefined olive oil (Olitalia, Olitalia SRI, Forli, Italy). The vessels in which we poured a mixture of olive oil and flowers were transparent, so the sun would be able to warm the mass more easily. These samples were set down at the sunniest point, so that the Sun shines most during the day following the procedure of the traditional practice in that region After 40 days, the vessels were drawn into the darkness and after twelve hours it was filtered through the gauze and the Oleum Hyperici, with familiar red color, was obtained.

Determination of the sensory and chemical properties

The sensory characteristics, as well as the pH and the content of metals and metalloids - As, Cr, Pb, Cd, Ni, Hg were performed according to standard approved methods for testing the food in the Food testing center, Belgrade, Serbia (https://www.cin.co.rs/) (POM-03-116; POM-03-AAS-03; POM-03-AAS-08; POM-03-AAS-09) (The Official Rules of the RS, No. 92/11).

Microbiological safety

Microbiological safety that included the total count of aerobic mesophilic bacteria, coagulase positive staphylococci, *E. coli*, *P. aeruginosa*, *Proteus* sp., molds and yeasts) of Oleum Hyperici was performed according to the accredited methods for testing the food in the Food testing center, Belgrade, Serbia (The Official Rules of the RS, No. 92/11).

Microbial test strains

The overnight cultures of the following 18 bacterial species were used: 8 isolates (*Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium, Salmonella enterica, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus*) originated from human samples, 7 standard strains (*E. coli* ATCC 25923, *K. pneumoniae* ATCC 70063, *P. mirabilis* ATCC 12453, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538) and 3 probiotic strains (*B. subtilis* IP 5832, *Lactobacillus plantarum*, *Bifidobacterium animalis* subsp. *lactis*). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac, Serbia. The other microorganisms (ATCC strains and probiotics) were provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The bacterial strains were kept in glycerol stock at -80°C until use.

The evaluation of antibacterial activity

Disk diffusion method

The concentrated Oleum Hyperici, dissolved in Tween 20 (Fisher Scientific UK, Leicester, UK), was used for testing the antimicrobial activity. For screening of the antimicrobial activity, the disc diffusion method, with some modifications, was used (BAUER *et al.*, 1966). Mueller-Hinton agar (Torlak, Belgrade, Serbia) was spilled into petri dishes and a solid disc of 8 mm diameter were made under sterile conditions. Then, the plates were inoculated with bacteria (suspension density 1.5 x 10^8 CFU/ml), using a sterile swap. After the inoculation of the bacteria, 100 μl of Oleum Hyperici was added into previously made disc, while 100 μl of pure olive oil was used as negative control. The prepared samples were incubated for 24 hours at 37°C. The results were interpreted by measuring the diameter of zone inhibition (the appearance of the inhibitory zone, in mm).

Microdilution method

The bacterial sensitivity on the Oleum Hyperici samples was expressed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), using the microdilution method (SARKER *et al*., 2007). Two-fold serial dilutions of the oil, up to $10^{-7,}$ were made in sterile 96-well microtiter plates containing 100 µl of Mueller-Hinton broth (Torlak, Belgrade, Serbia) per well. From the overnight cultures of the tested strains of bacteria, the suspensions were prepared in a sterile 0.85% saline solution. For the standardization of bacterial suspensions, the Mc Farland standard number 0.5 was used (bacteria density of 1.5×10^8 CFU/ml), and the samples were diluted until the density was 5×10^5 CFU/ml. In each well, it was added 10 µl of the suspension of the tested bacteria. The microtiter plates were incubated at 37°C for 24 hours. Resazurin (Alfa Aesar GmbH & Co., Karlsruhe, Germany), an indicator of cell growth, was added, in order to read the results of the inhibitory effect of the Oleum Hyperici on the growth of bacteria. The extensive reduction of resazurin by metabolically active cells would lead to the color change of the blue-purple in pink, which indicates the emergence of growth and thus an underestimation of cellular activity (O'BRIEN *et al*., 2000). The lowest concentration at which there was no change in the color of the indicator was determined as the minimum inhibitory concentration.

MBCs were determined by screening samples from the wells of the microtiter plates in which no growth was observed after 24 hours on a solid nutrient agar (Torlak, Belgrade, Serbia). After incubation, the lowest concentration at which growth was not observed (without colonies), was considered to be the minimum bactericidal concentration. Each experiment contains growth control, sterility control and positive control (standard antibiotic - ampicillin and tetracycline (Sigma Chemicals Co., USA), dissolved in nutrient liquid medium). Each test was done in duplicate and the results are shown as the mean value.

Antibiofilm activity of Oleum Hypericum

The experiment was performed according to the method described by O'TOOLE and KOLTER (1998). In order to determine the influence of Oleum Hyperici on bacteria which are causers of skin infections, the following bacterial strains were used: *S. aureus*, *S. aureus* ATCC 6538, *P. aeruginosa* and *P. aeruginosa* ATCC 9027. The 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) were used, and in each well (except the first row), a 100 µl of Mueller-Hinton broth was added. A 200 µl of the pure Oleum Hyperici was added to the first row of wells, and twofold serial dilutions were made, up to 10^{-7}. Then 10 µl of bacterial suspension (0.5 McFarland for Gram-negative and 1 McFarland for Gram-positive bacteria) was added to each well. The inoculated microtiter plates were incubated at 37°C for 48 hours (time in which biofilm can be formed). The rest of the experiment was performed according to the modified method described in detail in MURUZOVIĆ *et al*. (2016).

Only broth or broth with Oleum Hyperici served as control to check sterility and nonspecific binding of media. To compensate for background absorbance, OD readings from sterile medium, olive oil, fixative, and dye were averaged and subtracted from all test values. All tests were performed in duplicate and determined with (ELISA) plate reader (T-2100C, Rayto, Shenzhen, China) at 630 nm wavelength.

RESULTS AND DISCUSSION

Sensory, chemical and microbiological properties

The oil tested presents the extract made from flowers of autochthonous plant *H. perforatum*, extracted in the commercial olive oil. Based on the examinations of sensory

characteristics, it was concluded that this is the red, oil liquid with moderately intense scent, which resembles on the olive oil smell. The results of the chemical characteristics of the Oleum Hyperici are presented in Table 1. Based on the results, it could be concluded that the content of metals and metalloids are in accordance with the standards for food samples.

Table 1. The chemical characteristics of the Oleum Hyperici

Parameters	Results	Used method
pH	7.0	POM-03-116
The content of metals and metalloids[1]		
As	< 0.1	POM-03-AAS-09
Cr	< 5	POM-03-AAS-03
Pb	< 5	POM-03-AAS-03
Cd	< 0.5	POM-03-AAS-03
Ni	< 5	POM-03-AAS-03
Hg	< 0.1	POM-03-AAS-08

[1]Values are given in mg/kg

The results of microbiological testing were presented in Table 2. Based on the results, it could be concluded that the oil is safe for using because it does not contain potential pathogenic microorganisms according to the standards for food samples (The Official Rules of the RS, No. 92/11).

Table 2. Microbiological safety of Oleum Hyperici

Parameters	Results	Used method
Total count of aerobic mesophilic bacteria (CFU/g (ml))	< 10	G II/1
Coagulase positive staphylococci (0.1 g (ml) at 37 °C	n.d	GI II/2
***E. coli* 0.1 g (ml)**	n.d	II/4
***P. aeruginosa* 0.1 g (ml)**	n.d	II/3
***Proteus* sp. 0.1 g (ml)**	n.d	II/5
Total count of Molds and yeasts (CFU/g (ml))	< 10	II/1

n.d-not detected in the sample

Antibacterial activity of Oleum Hyperici

The antibacterial activity of the Oleum Hyperici, as the original product from Serbia, for the first time, was investigated in this study, using the two methods: disk diffusion and microdilution method.

According to the results obtained from disk diffusion method (Table 3), the most of tested bacteria (12 strains) showed sensitivity to the Oleum Hyperici reaching the inhibition zone in a range from 14 - 24 mm, but the zones were turbid. None of activity was obtained against *K. pneumoniae* ATCC 70063, *K. pneumoniae*, *E. coli* ATCC 25922, *P. mirabilis* and *B. subtilis* ATCC 6633. Pure olive oil did not show the impact on the bacterial growth. The results of the sensitivity of tested bacteria to antibiotics were showed in Table 4.

According to the results from microdilution method, tested bacterial strains showed sensitivity only to the first and second dilution of Oleum Hyperici, while MBC was only detected in undiluted (pure) Oleum Hyperici (Table 4).

Table 3. Antimicrobial activity of the Oleum Hyperici (disc diffusion method)

Species	Oleum Hyperici	
	ZI[1]	ZA[2]
K. pneumoniae ATCC70063	/	/
K. pneumoniae	/	/
E. coli	18	T
E. coli ATCC 25922	/	/
P. aeruginosa	24	T
P. aeruginosa ATCC 27853	20	T
P. mirabilis	/	/
P. mirabilis ATCC 12543	20	T
S. typhimurium	16	T
S. enterica	18	T
E. faecalis	16	T
E. faecalis ATCC 39212	16	T
S. aureus	20	T
S. aureus ATCC 25923	22	T
B. subtilis ATCC 6633	/	/
L. plantarum	14	T
B. animalis subsp. *Lactis*	16	T
B. subtilis IP 5832	/	/

[1]Zone of inhibition (mm);
[2]Zone appearance (C – clear zone of inhibition;
T - turbid zone of inhibition; / - none zone of inhibition)

Table 4. Antimicrobial activity of the Oleum Hyperici (microdilution method)

Species	Oleum Hyperici		Ampicillin		Tetracycline	
	MIC	MBC	MIC	MBC	MIC	MBC
K. pneumoniae ATCC 70063	10^{-1}	p	n.d	n.d	n.d	n.d
K. pneumoniae	10^{-1}	p	>128	>128	4	32
E. coli	10^{-1}	p	2.1	1.2	2	6
E. coli ATCC 25922	10^{-1}	p	0.37	0.5	4	6
P. aeruginosa	10^{-2}	p	>128	>128	>128	>128
P. aeruginosa ATCC 27853	10^{-2}	p	>128	>128	4	32
P. mirabilis	10^{-1}	p	>128	>128	>128	>128
P. mirabilis ATCC 12543	10^{-1}	p	n.d	n.d	n.d	n.d
S. typhimurium	10^{-1}	p	2	2	2	2
S. enterica	10^{-1}	p	1	1	2	4
E. faecalis	10^{-2}	p	4	6	1	6
E. faecalis ATCC 39212	10^{-1}	p	0.25	0.75	1.5	3
S. aureus	10^{-2}	p	< 0.06	< 0.06	< 0.06	< 0.06
S. aureus ATCC 25923	10^{-2}	p	0.25	0.75	1.5	3
B. subtilis ATCC 6633	10^{-1}	p	3	4	0.25	0.37
L. plantarum	10^{-1}	p	n.d	n.d	n.d	n.d
B. animalis subsp. *Lactis*	10^{-1}	p	< 0.06	0.12	4	8
B. subtilis IP 5832	10^{-1}	p	8	16	< 0.06	< 0.06

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values given in dilutions of *H. perforatum* oil (p-pure *H. perforatum* oil) and for antibiotics as μg/ml; n.d-not determined

Due to the more often appearance of antibiotic resistant bacteria, there is a need for development of natural protection from bacterial infections. According to the KALABA *et al*. (2015), *S. aureus, S. typhimurium* and *P. aeruginosa* are the most common pathogenic bacteria that cause health and economic problems. Oleum Hyperici tested in this study, demonstrated the influence on the mentioned bacteria. HEYDARIAN *et al*. (2017) indicated that polysaccharides from *H. perforatum* have a good antimicrobial potential against *E. coli, S. typhi, B. cereus* and *S. aureus*, according to the disk-diffusion method, which was confirmed in our study. SÜNTAR *et al*. (2016) demonstrated that *H. perforatum* extracts showed antimicrobial potential against *Streptococcus mutans, S. sobrinus, Lactobacillus plantarum* and *Enterococcus faecalis* (oral bacteria). MARČETIĆ *et al.* (2016) indicated that hyperforin and hypericin are responsible for antibacterial activity of *H. perforatum* against *B. subtilis, E. faecalis, Staphylococcus epidermidis* and *Micrococcus luteus.* GIBBONS *et al*. (2002) indicated the activity of oil against Gram-negative bacteria. The results from our study showed that tested Oleum Hyperici, made from flowers of *H. perforatum* from Goč Mountain, had a good antimicrobial potential although detection of compounds should be done in further studies.

Antibiofilm activity

The antibiofilm activity of the Oleum Hyperici was determined using the biofilm formation assay (O'TOOLE and KOLTER (1998). Four strains of tested bacteria (*S. aureus, S. aureus* ATCC 25923, *P. aeruginosa* and *P. aeruginosa* ATCC 27853) were chosen because they are a well-known causative agents of skin infections. The results are presented in Table 5.

The results indicated that tested bacteria showed no ability of biofilm formation in first and second dilution. Up to dilution 10^{-5}, among tested bacteria, only *S. aureus* clinical isolate showed the ability of significant biofilm formation (78%, compared to growth control).

Table 5. Antibiofilm activity of Oleum Hyperici

Dilutions of Oleum Hyperici	***S. aureus***	***S. aureus* ATCC 25923**	***P. aeruginosa***	***P. aeruginosa* ATCC 27853**
10^{-1}	/	/	/	/
10^{-2}	/	/	/	/
10^{-3}	50	28.6	7.8	33.3
10^{-4}	75	35.7	15.7	38.9
10^{-5}	78	50	35.3	55.6

The results are presented as percent of growth of biofilm in different dilutions of *H. perforatum* oil, compared with growth control of biofilm (100%); /- no biofilm formed

Antibiofilm activity of Oleum Hyperici was investigated in a few studies, but the tested original product from Serbia was not investigated so far. SARKISIAN *et al*. (2012) and SCHIAVONE *et al*. (2013) indicated that secondary metabolites from *Hypericum* spp. had antibiofilm activity against *S. aureus, S. epidermidis* and *E. faecalis*. In our study, tested Oleum Hyperici showed antibiofilm activity on all four tested bacteria in first and second dilution. The results of antibiofilm activity were better than results of MBC, which was only detected in pure Oleum Hyperici. This suggests that the traditional Oleum Hyperici formulation, originated plant from specific area (Goč Mountain) may have utility in regulation of bacterial virulence and pathogenesis that are involved in skin infections.

CONCLUSION

H. perforatum oil, Oleum Hyperici, has a positive effect on human health and inhibition of the growth of bacteria. The oil can be used as additional supplement for preventing or a treatment of infections caused by pathogenic microorganisms. The oil tested in this study presents the original product, made from *H. perforatum* flowers, collected from the specific area of Goč Mountain, and olive oil from Italy. Based to the results of this study, it could be concluded that this original product is safe for human use with an influence on the bacteria tested in this study, *in vitro*. Also, the influence on the biofilm formation on some potential skin pathogens, was significant. The results presented in this paper indicate that this folk medical therapy for skin and some tissue infections could represent a safe and efficient therapy.

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