ANTIMICROBIAL ACTIVITY OF OREGANO (*Origanum vulgare* L.) AND BASIL (*Ocimum basilicum* L.) EXTRACTS

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The commercial samples of aerial parts of *Origanum vulgare* L. and *Ocimum basilicum* L. (Lamiaceae) were tested for antimicrobial activity. The activity of the extracts with different polarity was tested against a panel of microorganisms, including laboratory strain *Helicobacter pylori* NCTC 12868. The tested extracts showed a moderate activity. The extracts of *O. vulgare* were more active against bacteria, especially against Gram positive bacteria with the minimal inhibitory concentration (MIC) between 62.5 and 125 µg/mL, than the extracts of *O. basilicum* which were active against *Candida albicans* (MIC 125 µg/mL). Cyclohexane extract of *O. vulgare* did not show any activity against tested *H. pylori*, while all other tested extracts were active with MICs between 250 µg/mL and 500 µg/mL. Identified and quantified rosmarinic acid and other polar compounds could be active antibacterial compounds in these spices.

Introduction

Helicobacter pylori is Gram negative bacterium, one of the most common pathogens in the human stomach with the potential to cause chronic gastritis and peptic ulcer disease. In addition, persistent infection by *H. pylori* favours the development of gastric cancer [1,2]. Today a strong association between *H. pylori* infection and both cancer development and progression is known, and this is the first bacterium recognized as a class I carcinogen [3]. Eradication of *H. pylori* is successful by using the antibiotic therapy, but the resistance to antibiotics is emerging, especially to metronidazole and clarithromycin [4,5,6,7]. Natural products could be very useful in the treatment of *H. pylori* infection, but also as an adjutant to antibiotic therapy. The compounds isolated from plants could be safe and effective against both antibiotic-resistant and susceptible *H. pylori* strains, and this is the reason why the research in the field of natural products is essential.

The traditional use of plants as medicine provide the basis for suggesting that essential oils and plant extracts may be useful for specific medical conditions. The antimicrobial activity of natural compounds is well documented, including a few reports on the effects of essential oils against *H. pylori* [8,9,10].

The herbs of two aromatic species *O. vulgare* L. and *O. basilicum* L. (Lamiaceae) are used as spices in everyday life, as well as medicine for treating different conditions, including respiratory and digestive disorders [11,12]. In Serbian traditional medicine, except for culinary use, *O. basilicum* was used as mild sedative, antihelmintic, and also against flatulence or urinary infections, while *O. vulgare* has a long tradition in the treatment of respiratory infections, gastrointestinal diseases (particularly diarrhoea) and against inflammation of skin and mucous membranes [13].

The chemistry of essential oil of *O. vulgare* and *O. basilicum* and its antimicrobial activity is well studied [14,15], as well as the chemistry and activity of different herb extracts [16]. Phenolic compounds, phenolic acids and flavonoids are major compounds of oregano and basil herb according to the literature [11,17-22]. Recent reports have shown a very good antimicrobial activity of the essential oils of *O. vulgare* subsp. *glandulosum* rich in carvacrol, while some phenolics isolated from *O. vulgare* possessed weak to moderate anti-viral activity [11,23]. Several reports presented the results of weak or moderate anti-*H. pylori* activity of the essential oil or different extracts of Oregano species [8,20,24]. Also, some reports suggested synergistic effects against *H. pylori in vitro* of phenolics from oregano and cranberry water soluble extracts (0.1 mg of phenolics/disc) through urease inhibition and disruption of the energy production by inhibition of proline dehydrogenase at the plasma membrane [25].

O. basilicum is also an aromatic plant rich in essential oil and phenolic compounds (flavonoids, phenolic acids) with known antimicrobial activity. Nakhae et al. (2006) studied anti-*H. pylori* activity of *O. basilicum* extracts using the agar-diffusion method, while Castillo-Juárez et al. (2009)

obtained significant minimal inhibitory concentrations (MICs) of *O. basilicum* extracts of different polarity [26,27].

The aim of this study was to investigate the antimicrobial activity of selected commercial samples of oregano and basil, especially against *H. pylori*, and to quantify the most abundant component of the extracts, rosmarinic acid.

Experimental ────────────────────────────

Chemicals and reagents

Rosmarinic acid (RA), and standard antibiotics (ampicillin, amikacin, metronidazol, tetracycline, amoxicillin) were purchased from Sigma-Aldrich (St. Louis, MO). All organic solvents were HPLC grade and were purchased from J.T.Baker (Deventer, The Netherlands). All other reagents for antimicrobial activity testing were: Mueller-Hinton broth, antibiotic supplement, Columbia Blood Agar Base and CampyGen™ (Oxoid, Thermo Scientific, UK), glycerol (Merck, USA), yeast extract and horse blood (Torlak, Belgrade, Serbia), casitone (Difco, USA), RPMI-1640 medium (Sigma, St. Louis, MO, USA), 10% heat inactivated foetal bovine serum FBS (GIBCO, Thermo Fisher Scientific, Life Technologies). All other reagents were from Sigma (St. Louis, MO).

Plant extract preparation

The commercial samples of aerial parts of *O. vulgare* L. and *O. basilicum* L. (Lamiaceae) were purchased (Institute for Medicinal Plant Research "Josif Pančić", Belgrade) and used for the antimicrobial test. The air-dried, powdered aerial parts (10 g-basil; 10 g-oregano) were extracted with cyclohexane during three days on room temperature (1:5, two times, successively). After filtration, the plant material was dried and extracted with dichloromethane and methanol using the same procedure. The solvent was evaporated under low pressure and dried to obtain C_6H_{12} (0.0817 g), CH_2Cl_2 (0.1153 g) and MeOH extracts (0.3958 g) of *O. vulgare* and C_6H_{12} (0.0946 g), CH_2Cl_2 (0.1760 g) and MeOH extracts of *O. basilicum* (0.3422 g). The methanol extract was used for quantification of rosmarinic acid (RA) and all three extracts for antimicrobial analysis.

Quantification of rosmarinic acid in the plant extract

HPLC separation was performed using a Agilent 1100 Series system equipped with a G-1312A binary pump, a G-1328B injector (20 µL loop) and G1315B DAD detector. The column used was a ZORBAX Eclipse XDB-C18 (4.6 × 250 nm, 5 µm) and operated at the temperature of 25 °C. A gradient elution was performed with solvent A (H_2O and H_3PO_4, pH=2.8) and B (solvent A: acetonitrile) as follows: 10-25% B (5 min), 25% B isocratic (10 min), 25-30% B (5 min), 30-50% B (5 min), 50-70% B (5 min), 70-10% B (5 min) at a flow rate of 0.8 mL/min. The injection volume was 20 µL. The presence of RA was identified on the bases of its retention time and UV spectra, as well as by direct comparison with the standard. Quantification of RA was performed under the same condition; detection wavelength was 320 nm. All chromatographic operations were carried out at room temperature. The calibration curve was constructed with five concentrations of standard solutions of RA. Within the concentration range of 0.100-1 mg/mL, the relationship between the peak area of RA was linear with a regression equation y = 27071x - 457.5 (x- concentration of RA; y – peak area of RA). The retention time of standard substance RA was 18.8 min. The linearity of the calibration curve was verified by the correlation coefficient (r2 = 0.9998). Each measurement was performed in triplicates. The concentration of 5 mg/mL of the methanol extract of *O. vulgare* and *O. basilicum* in acetonitrile and water (40:60, V/V) was used to calculate the amount of RA.

Antimicrobial activity

For antimicrobial testing, methanol, dichlormethane and cyclohexane extracts were used. The screening of antimicrobial activity of the tested extracts samples was evaluated using nine different laboratory strains of bacteria - Gram positive: *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), M*icrococcus luteus* (ATCC 9341), *Bacillus subtilis* (ATCC 6633), *Enterococcus feacalis* (ATCC 29212) and Gram negative: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (NCIMB 9111), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella abony* (ATCC 13076) and one strain of the yeast *Candida albicans* (ATCC 10231). The broth microdilution method was used to determine minimal inhibitory concentrations (MICs) of the tested extracts according to Clinical and Laboratory Standards Institute [28]. These tests were performed in Müller-Hinton broth for bacteria and in Sabouraud dextrose broth for Candida. Test strains were suspended in medium to give a final density of 2.0×10^6 cfu/mL. The samples of the extracts were dissolved in dimethylsulfoxide (DMSO) in concentrations of 1.0 mg/mL. Serial dilutions of the stock solutions in broth medium were prepared in a microtitre plate (96 wells). The MIC was defined as the lowest concentration of the extract at which the microorganism does not demonstrate a visible growth. All antimicrobial tests were performed in duplicate and two positive growth controls were included. The MICs of ampicillin and amikacin were determined in parallel experiments.

Anti-*Helicobacter pylori* activity

Laboratory strain of *H. pylori* (NCTC 12868) was used as an indicator for the detection of antimicrobial activity of plant extracts. *H. pylori* was maintained at -80 °C in Mueller-Hinton Broth supplemented with 10% horse blood and 20% (v/v) glycerol until required for experiments. Before being used, the strain was subcultured twice on Columbia Blood Agar Base supplemented with 0.5% (w/V) yeast extract, 1% (w/V) casitone, 10% (v/v) horse blood and antibiotic supplement. Plates were incubated for 72 h at 37 °C under microaerophilic conditions in a jar with CampyGenTM. Colonies were suspended in RPMI-1640 medium supplemented with 10% heat inactivated foetal bovine serum and 0.5% yeast extract to achieve the turbidity requested for the antimicrobial test. Metronidazol, tetracycline and amoxicillin were used as standard antibiotics.

Determination of MIC by Agar Dilution Method

Antimicrobial activity of the plant extracts was followed on plates prepared as described above. Two-fold dilutions of each plant extract were prepared in RPMI-1640 medium. The strains were plated on agar plates and 10 µl drops of twofold dilutions of the plant extracts were spotted on the plates in triplicates. The plates were incubated at 37 °C under microaerophilic conditions (CampyGenTM). The plates were examined visually after 72 h of incubation. The minimal inhibitory concentration was defined as the lowest concentration of the extract inhibiting the visible bacterial growth. Metronidazol, tetracycline and amoxicillin showed ten-fold lower activity than the extracts with MIC values ≤ 0.625 mg/ml.

Results and discussion ————————————

Determination of rosmarinic acid content

Rosmarinic acid was previously isolated from oregano extracts by different authors [11,29,30], and concerned previously as an important antioxidant and antimicrobial agent. Park et al. (2011) determined the content of RA in herb extracts of different Lamiaceae species and found that the percentage was between 0.22-0.97%, while Shekarchi et al. (2012) showed the content of 25.0 ± 0.1 mg/g of dried *O. vulgare* [29,31]. Austrian plants of *O. vulgare* contained from 0.6-37.2 mg/g dry mass, while Lithuanian plants possessed the maximum value of 9.7 mg/g dry mass in the flower extract [32,33].

The quantity of RA in different tissues of *O. basilicum* grown *in vitro* or in hydroponic culture was different. RA ranged approximately from 4 to 63 mg/g DW [21] or in phenolics rich extracts 3.2 mg/g extract [19]. In commercial samples of methanolic extracts of oregano and basil, different concentrations of RA and other phenolics were determined after (basil: 14.59-86.01 mg/100 g; oregano: 2.04-575.80 mg/100 g) [3]. Our results have shown that the content of RA in oregano was 14 mg/g and basil 2.4 mg/g. Concerning the wide range of previously obtained results, commercial samples of oregano and basil were in accordance with literature data.

Antimicrobial activity

Tested extracts showed a moderate antimicrobial activity. Their MIC values are presented in Table 1. Antimicrobial activity of rosmarinic acid was published before by Stanojković et al. (2013) and MIC values for antibacterial activity were 0.0125-0.1 mg/mL [34]. Antifungal activity was stronger with MICs 0.0125-0.05 mg/mL. Antibiotics were more active than the tested extracts. The MIC values of antibiotics are presented in Table 2.

The obtained MIC values for bacteria, except *H. pylori* were between 62.5 and 125 µg/mL for *O. vulgare* extracts, and 125 and 250 µg/mL for *O. basilicum* extracts. The extracts of *O. vulgare* were more active against bacteria, especially against Gram positive bacteria (except *B. subtilis*) than the extracts of *O. basilicum* which were active against *C. albicans* (MIC 125 µg/mL). Dichlormethane and metha-

nol extracts of *O. vulgare* were more active against *S. typhimurium* (MIC 62.5 µg/mL) than corresponding extracts of *O. basilicum* (MIC 250 µg/mL).

Table 1. Antimicrobial activity of tested *O. vulgare* and *O. basilicum* extracts (MIC µg/mL)

Bacteria	MIC µg/mL					
	OVC*	OVD*	OVM*	OBC	OBD	OBM
Staphylococcus aureus ATCC 6538	125	125	62.5	125	125	125
S. epidermidis ATCC 12228	62.5	125	62.5	125	125	125
Micrococcus luteus ATCC 9431	62.5	125	62.5	125	125	125
Bacillus subtilis ATCC 6633	125	125	125	250	250	125
Escherichia coli ATCC 25922	125	125	125	250	250	250
Klebsiella pneumoniae NCIMB 9111	125	125	125	125	125	125
Pseudomonas aeruginosa ATCC 9027	125	125	125	250	125	125
S. typhimurium ATCC 14028	125	62.5	62.5	250	250	250
Helicobacter pylori NCTC 12868	>1000	500	250	500	250	500
Candida albicans ATCC 10231	>500	>500	125	125	125	125

*OV- *O. vulgare* extracts; OB – *O. basilicum* extracts (C-cyclohexane, D- dichloromethane, M-methanol)

Previous studies were focused on the antimicrobial activity of essential oils of oregano and basil. A strong antimicrobial activity attributed to present phenolic compounds *i.e.* carvacrol, thymol and linalool [14,15,16,35]. There were less researches concerning antimicrobial activity of oregano or basil extracts. Because of toxic effects of carvacrol and thymol (oregano), and estragol (basil) that are concentrated in the essential oils, testing of these extracts can have practical application [36].

The recent paper of Martins et al. (2014) showed a moderate antibacterial activity of the ethanol extract of oregano herb in the concentration of 20 mg/mL against Gram negative bacteria *E. coli, P. aeruginosa*, *E. sakazakii* and *P. vulgaris* [37]. The other authors showed that methanol extract of *O. basilicum* did not have any antibacterial activity even in the concentrations which were higher than 2640 mg/mL [38].

Cyclohexane extract of *O. vulgare* did not show any activity against tested *H. pylori*, while all other tested extracts were active with MICs between 250 µg/mL (methanol extract of *O. vulgare* and dichlormethane extract of *O. basilicum*) and 500 µg/mL (cyclohexane and methanol extract of *O. basilicum*; dichlomethane extract of *O. vulgare*). *O. basilicum* and *O. vulgare* extracts were moderate active, with the strongest activity of dichlormethane and methanol extracts suggesting that polar compounds were the most active compounds. Ethnopharmacological survey of anti-*H. pylori pylori* activity of different aromatic plants showed that *O. majorana* ethanol extract

possessed activity against seven clinical isolates of *H. pylori* in the concentration of 250-500 µg/ml [20]. Ohno et al. (2003) showed in vitro inhibitory activity against *H. pylori* growth at the concentration of 0.1% (V/V) of *O. majorana* and *O. basilicum* album essential oils [8].

Table 2. Antimicrobial activity of tested antibiotics (MIC µg/mL)

Bacteria	MIC µg/mL				
	Ampicillin	Amikacin	Amoxicillin	Tetracycline	Metronidazole
Staphylococcus aureus ATCC 6538	0.5	n.t.*	-	-	-
S. epidermidis ATCC 12228	0.5	n.t.	-	-	-
Micrococcus luteus ATCC 9431	1.5	n.t.	-	-	-
Bacillus subtilis ATCC 6633	1.8	n.t.	-	-	-
Escherichia coli ATCC 25922	2.0	1.5	-	-	-
Klebsiella pneumoniae NCIMB 9111	2.8	2.0	-	-	-
Pseudomonas aeruginosa ATCC 9027	n.t.	2.5	-	-	-
S. typhimurium ATCC 14028	n.t.	n.t.	-	-	-
Helicobacter pylori NCTC 12868	-	-	<62.5	<62.5	<62.5
Candida albicans ATCC 10231	-	-	-	-	-

*n.t. – not tested

The relatedness between antimicrobial activity and phenolic compounds present in some plants was already observed when using microorganisms other than *H. pylori*. The results of Chun et al. (2005) showed that aqueous (50 mg phenolics per disk) and ethanol extracts of cloned *O. vulgare* against *H. pylori* ATCC 43504 than commercial samples (100 mg of phenolics) [39]. The authors concluded that a higher antioxidant activity as well as higher phenolics content could be the reason for anti-*H. pylori* activity due to different mechanisms. Methanol extracts of *O. dictamnus*, *O. majorana* and *O. vulgare* from Greece exhibited a moderate antimicrobial activity against 15 clinical isolates of *H. pylori* with MICs 0.625-5 mg/mL [24]. Similar results were obtained with *O. basilicum* methanol extract (MIC 2.5-5 mg/mL). Our results for *O. vulgare* extracts could be comparable with the results of Mahady et al. (2005) who showed very low MICs *in vitro* of methanol extracts of *O. majorana* herb and *O. vulgare* against 14 clinical isolates and 1 ATCC strains (50-100 µg/mL) [40], because all other authors obtained much higher MIC values.

Different *Ocimum* species were tested against *H. pylori* and for gastroprotective activity [41,42,43]. MICs of the extracts against 4 most sensitive clinical isolates of *H. pylori* were 677 and 729 µg/mL. Very low MICs (31.2 µg/ml) were obtained for the methanol extract of *O. basilicum* from Mexico [27], and for methanol, n-hexane and butanol fraction of *O. basilicum* methanol extract from Iran (39.1, 41 and 117.2 µg/ml), while aqueous fraction did not have any activity against tested clinical isolates

of H. pylori [26]. Our results for tested basil extracts were between 250 and 500 µg/mL against standard *H. pylori* strain. It was obvious from the literature that clinical isolates of *H. pylori* were more sensitive to tested *O. basilicum* and *O. vulgare* extracts.

Rosmarinic acid was the major compound in tested methanol extracts. A previous study of Lin et al. (2005) considered the synergistic effects against *H. pylori in vitro* of phenolics from oregano and cranberry water soluble extracts (0.1 mg of phenolics/disc) through urease inhibition from one side and disruption of the energy production on the other. The inhibition of proline dehydrogenase at the plasma membrane was linked with higher-molecular-weight phenolics from oregano which operated at the plasma membrane level and subsequently, upon urease inhibition [25]. Also, partial hydrophobicity of phenolics of oregano may allow the attachment and inhibition at the plasma membrane level.

As a major phenolic in oregano methanol extract, rosmarinic acid could be involved in exhibited activity. Even in the content of RA in oregano 14 mg/g and basil 2.4 mg/g for commercial samples and with very high anti-*Helicobacter pylori* activity, we can conclude that rosmarinic acid was not the only active compound in the tested methanol extracts. Some other compounds were probably involved in anti-*H. pylori* activity.

Conclusion

Origano and basil are very common spices in every day diet and could be important in both prevention and treatment of many diseases, including bacterial infection. As a rich source of phenolic compounds and other secondary metabolites with antimicrobial activity, they could be subjected for further preventive role in H. pylori infection in vivo, and to chemical structure determination of active compounds.

Acknowledgments

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Izvod ——————

ANTIMIKROBNA AKTIVNOST EKSTRAKATA ORIGANA (*Origanum vulgare* L.) I BOSILJKA (*Ocimum basilicum* L.)

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Komercijalni uzorci nadzemnih delova *Origanum vulgare* L. i *Ocimum basilicum* L. (Lamiaceae) su korišćeni za određivanje antimikrobne aktivnosti. Aktivnost ekstrakata različite polarnosti je testirana protiv izabranih mikroorganizama, uključujući laboratorijski soj *Helicobacter pilori* NCTC 12868. Testirani ekstrakti su imali umerenu aktivnost. Ekstrakti *O. vulgare* su bili aktivniji protiv bakterija, posebno Gram pozitivnih bakterija sa minimalnim inhibitornim koncentracijama (MIK) između 62,5 i 125 µg/mL, od ekstrakata *O. basilicum* koji su pokazali aktivnost protiv *Candida albicans* (MIK 125 µg/mL). Cikloheksanski ekstrakt *O. vulgare* nije pokazao antimikrobnu aktivnost na testirani *H. pylori*, dok su ostali testirani ekstrakti bili aktivni sa MIK vrednostima između 250 µg/mL i 500 µg/mL. Identifikovana i kvantifikovana ruzmarinska kiselina i druga polarna jedinjenja mogu predstavljati aktivna jedinjenja sa antibakterijskom aktivnošću u ovim začinima.

Ključne reči: *Origanum vulgare* L.; *Ocimum basilicum* L.; antimikrobna aktivnost; *Helicobacter pylori*