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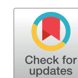
A colorimetric broth microdilution method for assessment of *Helicobacter pylori* sensitivity to antimicrobial agents



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ABSTRACT

Helicobacter pylori is a major infective etiological agent of the upper gastrointestinal tract diseases. The bacterium exhibits resistance to various conventional antibiotics, being usually challenging for eradication. Since there is an urge to consider alternative therapeutic strategies, the aim of the study was to examine selected essential oils of plants belonging to families Cupressaceae (*Juniperus communis*) and Lamiaceae (*Hyssopus officinalis, Salvia officinalis, Melissa officinalis, Lavandula angustifolia, Ocimum basilicum* and *Thymus serpyllum*) against *H. pylori*, using an improved microdilution broth method. The oils were examined in concentration range from 0.03 to 4 μL/mL. The method comprises Brain-heart infusion broth supplemented with yeast extract, horse serum and IsoVitaleX. After 3 day incubation, an equal volume of double strengthen Christensen's urea was added into each well and incubated for additional 4 h. In wells with present *H. pylori*, the medium changed color from yellow to purple, allowing MIC determination even without a microtitre plate reader. The microtitre format method is convenient as it is less expensive, easier to perform and requires less amount of an anti-*H. pylori* agent. The improved method enhances specificity to *H. pylori*, as fast urease activity is almost an exclusive property of this bacterium. The application of the second step incubation with Christensen's urea decreases the possibility of false positive/negative results due to contaminant growth or commonly poor *H. pylori* growth. Among the examined oils, *J. communis, H. officinalis* and *O. basilicum* were not active with the highest applied concentrations, while the most active was *T. serpyllum*, with MIC 2.0–4.0 μL/mL. This is the first report on essential oils activity of *T. serpyllum* and *H. officinalis* against *H. pylori*.

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1. Introduction

Helicobacter pylori is a Gram negative spiral bacterium isolated for the first time by Warren and Marshall more than three decades ago [1]. It is a fastidious, microaerophilic, motile bacterium which is oxidase, catalase and urease positive [2]. *H. pylori* is an etiological agent of duodenal/gastric ulcer, chronic gastritis, gastric adenocarcinoma and gastric mucosa–associated lymphoid tissue lymphoma (MALToma). In addition, it has been recognized by the International Agency for Research on Cancer (IARC) and the World Health Organization as a class I cancerogen [3,4].

H. pylori eradication is necessary in case of ulcer or gastritis, it is important as an efficient method for prevention of gastric cancer, and improves prognosis of gastric MALToma patients by inducing remission [5]. The bacterium is generally sensitive to penicillin, ampicillin, cephalothin, kanamycin, gentamicin, rifampin, and tetracycline, variably sensitive to metronidazole and clarithromycin and resistant to nalidixic acid, trimethoprim, sulfonamides, amphotericin B, cefsulodin, cefoperazone, polymyxin B and vancomycin [2,6]. The most frequently used therapy is a triple therapy, which comprises a proton pump inhibitor (PPI) and two antibiotics: amoxicillin-clarithromycin or clarithromycin-metronidazole. There is also a quadruple therapy (PPI with metronidazole, tetracycline and bismuth) and a sequential therapy (PPI with amoxicillin for 5 days, then next 5 days PPI + metronidazole + clarithromycin) [7]. The levofloxacin is used for triple therapy as a second choice while as a third choice furazolidone in quadruple and rifabutin in triple therapy are used [8,9]. Beside the sequential therapy, there are also a concomitant and a hybrid therapy comprising the above mentioned antibiotics, but

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they still need further validation and clinical assessment [10]. However, the eradication treatment failure is an increasing issue, due to antibiotic resistance of *H. pylori*. For instance, the first-line therapy should eradicate *H. pylori* in at least 80% or more patients, but failure of the treatment is more frequent than expected [11]. According to a literature review from 2009 to 2014, the overall *H. pylori* antibiotic resistance was 47.22% for metronidazole, 19.74% for clarithromycin, 18.94% for levofloxacin, 14.67% for amoxicillin, 11.70% for tetracycline, 11.5% for furazolidone and 6.75% for rifabutin [12]. The emergence of *H. pylori* resistance to common antimicrobial agents is evident, and there is an urge to consider alternative therapeutic strategies.

Various alternative anti-*H. pylori* treatments have been examined so far, including agents based on natural resources such as plants, probiotics and nutraceuticals, as well as novel alternatives based on microorganisms, peptides, polysaccharides, and intragastric violet light irradiation [13–17]. Among these agents, plant products are of particular interest, due to continuous advances in their composition analysis [18] and promising *in vitro* antibacterial activity. For instance, up to date, anti-*H. pylori* activity has been confirmed for EOs obtained from *Cymbopogon citratus* and *Lippia citriodora* in concentration of approximately 0,01% [19], *Myrtus communis* 2.5–0.01% [20], *Thymus caramanicus* 0.001–0.005% [21], *Thymus vulgaris* and *Eucalyptus globulus* 0.001–0.005% [22], *Origanum minutiflorum* 0.005% [23], *Satureja bachtiarica* in concentration of 0,0035% [24], *Pistacia lenticus* approx. 0.02% [25], a mixture of *Satureja hortensis* and *Origanum vulgare* subsp. *hirtum* EOs in ratio 2:1 and final concentration of 0.05% [26], etc. The most extensively examined phytotherapies *in vivo* comprise plant based diet or *per os* use of herbal extracts, but scarcely plant EOs. The general results of these studies based on phytotherapy are improvement of some symptoms, but lack of *H. pylori* eradication [15]. The activity of some EOs was confirmed *in vivo*: for instance, the feeding needle administration of 43.75 μL of *Satureja hortensis* and *Origanum vulgare* subsp. *hirtum* EOs 2:1 mixture per kilogram of mice body weight during 5 days resulted in *H. pylori* eradication in 70% of the infected animals [16]. On the contrary, a blind non-randomized trial with infected patients to whom 400 mg of garlic essential oil was administered orally 4 times per day for 14 days showed neither significant change of symptoms presentations nor *H. pylori* gastric density [17]. These data indicate necessity for further examination of alternative agents and methods for anti-*H. pylori* assessment.

The essential oils of many plants have not been examined against *H. pylori* and previously used *in vitro* methods vary from study to study, potentially being one of the reasons of discrepancy between the *in vitro* and *in vivo* results. As there is no standardized method available to test the anti-*H. pylori* activity in particular of natural substances, Weseler et al. [27] have developed a colorimetric microdilution assay with p-iodonitrophenyltetrazolium violet (INT) as an indicator of bacterial cell viability. Bacteria containing dehydrogenase transform the substrate to a formazane. However, as INT can be metabolized by many bacteria, including potential contaminants of media [28], the method is not specific for *H. pylori*. In the present study we aimed at the improvement of microtitre plate method enhancing selectivity to *H. pylori* and the use of the method for testing anti-*H. pylori* activity of selected plant essential oils.

2. Material and methods

2.1. Plant materials and extraction of essential oil

In the study six plants from family Lamiaceae, originating from an organic farm (Organic farm "Farago", Orom, Serbia) were used to obtain EOs: *Hyssopus officinalis, Salvia officinalis, Melisa officinalis, Lavandula angustifolia, Ocimum basilicum* and *Thymus serpyllum*. In addition, the EOs were extracted from needles and cones of *Juniperus communis* (family Cupressaceae) and plant material was collected from two different localities (Romania mountain, Bosnia and Hercegovina and Fruska Gora mountain, Serbia; BUNS Vouchers 2-1649 and 2-1648, respectively). The plant origin and other relevant details are listed in Table 1.

The plant material was air-dried and finely grounded. The essential oils were isolated by hydrodistillation according to the recommended procedure by Ph. Eur. IV [29]. The obtained essential oils were stored at −20 °C prior to analysis.

2.2. Gas chromatography–mass spectrometry (GC–MS) analysis of essential oils

Qualitative and semi-quantitative chemical composition of essential oils was determined by GC–MS, using Agilent Technologies 6890N gas chromatograph coupled with Agilent Technologies 5975 B electron ionization mass-selective detector. Essential oil was dissolved in hexane (10 μL/mL) and 1 μL of solution was injected into a split/splitless inlet at 250 °C, with a split ratio 1:10. Helium (purity 5.0) was used as a carrier, with a constant flow of 1 mL/min. A non-polar Agilent Technologies HP–5 ms column (30 m × 0.25 mm, 0.25 μm) was used for separation of components, at temperature programme starting at 50 °C, increasing 8 °C/min–120 °C, then 15 °C/min–230 °C, and finally 20 °C/min–270 °C, and holding at 270 °C for 16.9 min (total run time 35 min). Effluent was delivered to the mass spectrometer via a transfer line held at 280 °C. Ion source temperature was 230 °C, electron energy 70 eV and quadrupole temperature 150 °C. To achieve better correlation between experimental and library spectra, standard spectra tune was used. Data were acquired in scan mode (*m/z* range 35–400), with solvent delay of 2.30 min. Agilent Technologies MSD ChemStation software (revision E01.01.335) combined with AMDIS (ver. 2.64) and NIST MS Search (ver. 2.0d) were used for processing of data. AMDIS was used for deconvolution, i.e. co-eluting compounds peak area determination and pure spectra extraction, and NIST MS Search provided search algorithm complementary to ChemStation PBM algorithm. The compounds were identified by comparison of mass spectra with data libraries (Wiley Registry of Mass Spectral Data, 7th ed. and NIST/EPA/NIH Mass Spectral Library 05) and confirmed by comparison of linear retention indices with literature data [30]. Diesel oil, containing C8–C28 n-alkanes, was used as a standard for determination of retention indices. Relative amounts of components, expressed in percentages, were calculated by normalization procedure according to peak area in total ion chromatogram.

2.3. Bacterial strains

In this study, two strains of *H. pylori* were used: SS1, a metronidazole resistant mouse-adapted strain originally isolated from a patient with peptic ulcer [31] and a reference strain *H. pylori* ATCC 26695 (ATCC 700392). The bacteria were grown on Mueller Hinton agar amended with 5% horse blood (bioMérieux, France) for three days at 37 °C under microaerobic conditions and in moist atmosphere. The conditions were obtained using an anaerobic jar with a carbon dioxide generator for microaerophilic bacteria (GENbox microaer, Biomerieux, France). The bacterial suspensions were prepared in 0.9% physiological saline and adjusted to an optical density equivalent to 2×10^8 CFU/mL [32].

In addition, the following urease producing strains from the American Type Culture Collection (ATCC) were used to confirm method specificity: *Klebsiella variicola* ATCC 31488, *Bordetella bronchiseptica* ATCC10508, *Proteus mirabilis* ATCC 12453, *Staphylococcus*

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