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COMPARISON OF IN-VITRO ANTI-*HELICOBACTER PYLORI* ACTIVITIES OF *ORIGANUM MAJORANA* AND *ORIGANUM VULGARE*

Efe Sert[*1]

[*1]Research Intern, Kocaeli University Faculty of Medicine and TEV Inanc High School, Kocaeli, Turkey.

ABSTRACT

Through this study, the potential *Origanum vulgare* and *Origanum majorana* usage for the eradication of *Helicobacter pylori* was investigated by determining anti-*H. pylori* activities, minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC). *H. pylori* was supplied from the ATCC and cultivated according to guidelines. *H. pylori's* susceptibility to different types of extracts (aqueous, ethanol, and essential oil) of these two species was determined by the disc diffusion assay. Furthermore, the MIC and MBC values of the essential oils, which showed the highest anti-*H. pylori* activities, were determined by the agar dilution method.

Most extracts of *Origanum vulgare* and *Origanum majorana* exhibited antibacterial activity. Although, extracts of *Origanum vulgare* always showed a greater activity than the *Origanum majorana* extracts. The strongest comparable inhibitory activity was demonstrated by the *Origanum vulgare* essential oil at 1% v/v, followed by the essential oil of *Origanum majorana* at the same concentration. The MIC and MBC concentrations of *Origanum vulgare* essential oil were 0.372 g/mL and 1.471 g/mL respectively. The anti-*H. Pylori* potential of both *Origanum vulgare* and *Origanum majorana* essential oils were highly comparable to commonly used antibiotics (the positive control) such as clarithromycin, metronidazole, amoxicillin.

Keywords: *Helicobacter pylori*; anti-microbial; *Origanum*; MIC and MBC.

I. INTRODUCTION

Helicobacter pylori, which has a gram-negative spiral rod, is a microaerophilic bacterium that possesses a high morbidity and mortality rate. Causing many burdens to health care services around the world, *H. pylori* infection in the stomach is correlated with diseases such as peptic ulcer disease, chronic gastritis, and many stomach related diseases in both adults and children. [1-3]

Although most diseases are asymptomatic, around 50 percent of the world's population is diagnosed with *H. pylori* infection, with the highest occurrence in developing countries. The standard triple medication combination [4], which is the combination of clarithromycin and amoxicillin or metronidazole and gastric acid suppressants or ranitidine bismuth citrate, has been the prescribed therapy for the *H. pylori* infection for the last two decades. However, owing to the recent low susceptibility rates of *H. pylori* to key antibiotics, particularly clarithromycin, but also metronidazole and levofloxacin, the effectiveness of these triple regimens has recently decreased to levels below 70 percent and resulted in various resistant strains. [4] Increasing resistance has recently made it less efficient to use antibiotics. Therefore, to control *Helicobacter pylori* infection, the development of a new antimicrobial agent is needed. Herbs are one of the most used and powerful agents of these potential antimicrobial agents.

Herbal extracts of thyme, oregano, rosemary, and Aloe Vera have been used for centuries and demonstrate high antimicrobial activities both within in-vitro and in-vivo studies. [5] While the antimicrobial activity mostly depends on the composition of the species, the extraction method that is used is also an important factor. The anti-*H. pylori* property of thyme essential oil, being primarily composed of thymol and carvacrol, has been confirmed with previous studies. [6]

Furthermore, *Origanum vulgare*, which possesses these phenols in its essential oil composition propose the possible *anti-Helicobacter pylori* characteristic. [7] On the other hand, the *anti-Helicobacter pylori* characteristic of *Origanum majorana* which is closely related to *Origanum vulgare* but different in essential oil composition cannot be inferred without further investigation.

The aim of this investigation is to measure the anti-Helicobacter activity of different types of extracts of the two species and compare these findings.

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II. METHODOLOGY

Helicobacter pylori strain

The reference strain of this study, *H. pylori* (ATCC 700392), was obtained from the American Tissue Type Culture Collection (ATCC). While Brucella broth is used to cultivate the frozen *H. pylori* culture with 5% fetal bovine serum, Brain Heart Infusion agar containing 15% sheep blood is used to incubate the stain under microaerophilic conditions for 72 h at 38◦C. [8] Afterward, gram staining biochemical tests were carried out in order to identify the strain.

Plant extraction

Freshly harvested leaves and roots of *Origanum vulgare* and *Origanum majorana* were air-dried and grinded with pestle and mortar to obtain a powder form. Through incubating 30 g of dry plant material for 10 minutes in 800 mL of hot water (80 °C), preparation of the aqueous extract was carried out briefly. Afterward, centrifuge process is carried out at 4000 x g for 15 minutes. The extract was then filtered by using a filter paper, refrigerated at 4 °C in a freezer and kept at 4 °C before experimental stage.

Through the Soxhlet extractor, ethanol extracts of *Origanum vulgare* and *Origanum majorana* were obtained. In short, 30 g of the crushed dried plant sample was transferred to separate filter papers that were cone-shaped and then inserted into a Soxhlet extractor. In round bottom flasks, plant materials were positioned and extracted with 400 ml of ethanol. For 6 h, the Soxhlet extraction procedure was performed. By using a rotary evaporator, the solvent ethanol was evaporated at a decreased pressure at 38 °C after extraction until only about 15 mL of ethanol remained. The extracts were freeze-dried at −85 °C for 40 minutes and stored at −4 °C until the experimental stage was carried out.

The essential oil extraction process was carried out according to the hydro-distillation process by utilizing a Clevenger apparatus for both *Origanum vulgare* and *Origanum majorana.* In order to extract any remaining water from it, the essential oils were then dried through anhydrous sodium sulfate.

Determination of anti-*H. pylori* activity

In order to examine the anti-*H. pylori* properties of the different types of extracts the disc diffusion method was carried out. Brain Heart Infusion agar containing 15 % sheep blood was used to carry out the disc diffusion method. To inoculate plant extracts, sterile discs with diameters of 6 mm were used. Essential oils, ethanol and aqueous extracts of *Origanum vulgare* and *Origanum majorana* were impregnated with the discs at various concentrations. Following the method Koneman *et al*. suggested [9], a 0.5 McFarland standard was prepared and 10mL of sample was placed into a sterile centrifuge tube. *H. pylori* inoculum from the bacterial suspension subculture was prepared. In essence, the method was done follows as: 4-5 *Helicobacter pylori* colonies were emulsified in distilled water and approximately 1.5 x 10^8 CFU/mL turbidity was set (according to 0.5 McFarland standards). A sterile cotton swab was used to evenly inoculate the BHI agar plates. About 10 minutes, the plates were allowed to dry. Subsequently, all discs were positioned on the agar plates and pushed softly to achieve maximum contact with the inoculated bacteria. A distance of at least 20 mm from the edges of the plates was preserved in order to prevent interference of the inhibition areas. A clarithromycin disc, a metronidazole disc, and an amoxicillin disc were used as the positive control.

Fifteen minutes after the discs were set, the plates were incubated for 3-6 days at 37 °C. Then, plates were examined and the inhibition zones occurred around discs were checked. The diameters of different inhibition zones were measured. The experiment has been replicated three times and the average was noted.

Evaluation of Minimum Inhibitory Concentration (MIC)

To further confirm and evaluate the anti-*H. pylori* activities of tested extracts, the minimum inhibitory concentrations (MICs) were measured by the process of broth microdilution. [10] *H. pylori* was grown in Müller-Hinton broth in order to perform MIC tests. After suspending *H. pylori* in a medium to acquire 2.0 x 106 CFU/mL as the final density, extract samples were prepared by dissolving extracts in dimethyl sulfoxide (DMSO) with concentrations of 1.0 mg/mL. In a microtiter plate, serial dilutions of stock solutions were performed. After the process, the lowest concentration in which a visible growth of the microorganism is not present is determined as the MIC value.

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Evaluation of Minimum Bactericidal Concentration (MBC)

MBC was measured according to the procedures of Clinical and Laboratory Standards Institute. [11] An amount of 0.5 mL of content was taken from each MIC tube and diluted for 10 time in sterile distilled water. The prepared sample was plated on Brain Heart Infusion agar plates containing 15% sheep blood and incubated for 48 hours at 37 °C under microaerophilic conditions. After the analysis of the plates, the lowest concentration of an extract at which a *H. pylori* colony was not present was determined as the MBC value.

III. RESULTS AND DISCUSSION

Table-1: Anti-*H. pylori* activities of aqueous, ethanol and essential oil extracts of *O. vulgare* and *O. majorana* in the context of growth inhibition evaluated by the disc diffusion method. [8]

Tested Sample	Inhibition Zone (mm)*
100% aqueous *O. majorana* extract	0
100% ethanol *O. majorana* extract	10.2 ± 4.01
100% *O. majorana* EO	≥ 90.0
10% *O. majorana* EO	≥ 90.0
1% *O. majorana* EO	19.3 ± 2.61
100% aqueous *O. vulgare* extract	5.6 ± 5.62
100% ethanol *O. vulgare* extract	15.6 ± 398
100% *O. vulgare* EO	≥ 90.0
10% *O. vulgare* EO	≥ 90.0
1% *O. vulgare* EO	35.7 ± 3.17
CLR	59.2 ± 6.05
MTZ	72.8 ± 1.45
AMX	62.3 ± 6.63
DMSO	0

The positive controls were CLR, clarithromycin; MTZ, metronidazole; and AMX, amoxicillin. As a negative control, DMSO (0.5 percent) was used.

* The mean standard deviations are presented as ±.

Anti-*Helicobacter pylori* activities of *O. vulgare* and *O. majorana* extracts

The anti-*H. pylori* efficacy of the ethanol, aqueous, and essential oil extracts of *Origanum vulgare* and *Origanum majorana* tested for *Helicobacter pylori* (ATCC 700392) by the agar disc diffusion assay are presented in Table 1. Although no antimicrobial activity against *Helicobacter pylori* (ATCC 700392) at a concentration of 3 mg/mL was observed in the aqueous extracts of *Origanum majorana*, the aqueous extracts of *Origanum vulgare* showed antimicrobial activity. Ethanol extracts of both species demonstrated poor inhibitory effects with respect to their anti-*H. pylori* activity at the same concentration. However, the ethanol extract of *O. vulgare* had a larger zone of inhibition and a significant difference was again observed between two species. Related findings have previously been observed with *Origanum minutiflorum* against *Shigella sonnei*, *Listeria monocytogenes*, and *Staphylococcus aureus*. [8] The difference of the solvents used to extract various components explains the variations in anti-*H. pylori* activity, with different polarities and inherent bioactivity of the solvents. [12]

100 % and 10% essential oil extracts of both O. vulgare and O. majorana showed a strong anti-*H. pylori* activity with corresponding concentrations of 920 mg/mL and 92mg/mL. Demonstrating inhibition zones greater than 90 mm (Table 1), these concentrations of essential oil extracts showed higher anti-*H. pylori* activity than the

control antibiotics clarithromycin, metronidazole, and amoxicillin with zones of inhibition: 59mm, 62mm, 71mm, respectively. Although when we look at the inhibition zone values of 1% essential oil extracts of these two species, it is observed that *O. vulgare* showed a greater anti-*H. pylori* activity than *O. majorana* extract.
Essential oil compositions were compared according to previous research to see in order to find possible variance that can be attributed to the difference in anti-*H. pylori* behaviors between the two species. [13] Main differences were between Carvacrol and Thymol amounts. While it's been stated that *O. vulgare* essential oil was comprised of 33.92% Thymol and 6.90% Carvacrol, *O. majorana* essential oil was comprised of 0.33% Thymol and an unknown value of Carvacrol. As previous studies suggested the antimicrobial activities of these phenols, the significant differences in the essential oil compositions of *O. majorana* and *O. vulgare* resulted in different anti-*Helicobacter pylori* activities.

The anti-*Helicobacter pylori* effects of essential oils and their constituents are thought to weaken the structure of the bacterial membrane. Essential oils' lipophilic structure helps them to move across the phospholipid membrane by disturbing its function and structure which is rich in polysaccharide, fatty acid, and phospholipid.[14]

Table 2: The agar dilution procedure was used to assess the MIC and MBC concentrations of *O. vulgare* and *O. majorana* essential oil extracts against *H. pylori*.

Tested Sample	MIC (g/mL)	MBC (g/mL)
O. majorana EO	0.612	3.418
O. vulgare EO	0.372	1.471

MIC and MBC values

Showing the highest anti-*H. pylori* activities, essential oils of *O. vulgare* and *O. Majorana* were further investigated by the evaluation of the MIC and MBC values. The MIC values for the *O. vulgare* and *O. majorana* essential oils were found as 0.372 g/mL and 0.612 g/mL respectively. The MBC values for *O. vulgare* and *O. majorana* essential oils were measured as 1.471 g/mL and 3.418 g/mL, respectively. This findings correlate with the results of disk diffusion assay and confirm that *O. vulgare* essential oil possesses a greater anti-*H. pylori* activity than *O. majorana*.

IV. CONCLUSION

The anti-*Helicobacter pylori* effects of *Origanum vulgare* and *Origanum majorana* extracts on *H. pylori* (ATCC 700392) were demonstrated in this research. For the purpose of validating the potential usage of *O. vulgare* essential oil to treat *H. pylori* infections, further experiments need to be carried out.

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