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Chemical composition and antibacterial activity of the essential oil and extract from *Mentha longifolia* against *Helicobacter pylori*

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Helicobacter pylori infection is prevalent, affecting almost half of the world's population. Eradicating *H. pylori* with antibiotics is a global challenge due to medication resistance. Developing new treatment regimens is one of the most important tactics for combating medication resistance. Herbal treatments have attracted a lot of interest recently among the many *H. pylori* infection adjunct therapies. *M. longifolia* is an edible plant found in northern Iran; its leaves have traditionally been used to cure gastrointestinal ailments. This study aims to determine the chemical composition of the essential oil (EO) derived from *M. longifolia* leaves using GC-MS and assess the anti-*H. pylori* activity of the EO and methanolic extract using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods. Results showed that the main components of *M. longifolia* EO were cis-Piperitenone oxide (67.064%), piperitenone oxide (9.135%), L-Menthone (5.745%), Trans Caryophyllene (5.271%) and Eucalyptol (3.524%). *M. longifolia* EO exhibited the greatest anti-*H. pylori* activity (MIC=187 µg/mL), while the methanolic extract was (MIC=1500 µg/mL). The phytochemicals studied in this study have the potential to be utilized as adjuvant therapy with standard antibiotics against *H. pylori*.

Keywords: Antimicrobial activity, Chemical composition, Essential oil and extract, *Helicobacter pylori*, *Mentha longifolia*

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Introduction

Helicobacter pylori is a Gram-negative bacillus, without spores, microaerophiles, spirals, and inhabits the gastric mucosa in more than half of the world's population and is distributed worldwide[1,2]. This bacterium was previously known as *Campylobacter pylori*. It has been identified with high urease activity and as a cause of stomach inflammation and stomach ulcer. Also, the infection of this bacterium has a strong connection with peptic ulcers and stomach cancer[3,4]. In developing countries, 70 to 90% of people before the age of 10 are infected with this bacterium. The clinical causes of bacterial infection are influenced by several factors, including differences in the response of different hosts to bacterial stimulation, bacterial virulence factors such as Cag A, Vac A, social factors, individual behaviors, and environmental factors[5]. Antibiotics are one of the most common drugs used inappropriately. The result of the widespread use of antibiotics is the emergence of resistant pathogens, which has created the need to constantly produce newer types of antibiotics[6]. The attention of researchers has been drawn to finding ways to prevent the onset of resistance and to find appropriate drugs with fewer side effects[7,8]. For this purpose, medicinal plants are of particular interest.

Herbal medicines are used widely for the prevention of bacterial as well as infectious diseases. On the other hand, antimicrobials used to treat multiple drug resistant (MDR) and total drug resistant (TDR) infections are becoming more popular in the healthcare community and worldwide, new treatments to treat these infections are being pursued[9–11]. Traditional Iranian medicine is also one of the richest and most famous places in the world[12,13]. *Lamiaceae* is one of the largest plant families with a global distribution *M. longifolia* is a stimulant, antispasmodic, anti-flatulence, gallbladder shrinkage, antibacterial, sedative and laxative[14,15]. In clinical studies, *M. longifolia* significantly improves fever, cough and fatigue in COVID-19 and other suspected

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cases and reduces the duration and proportion of cases progressing to a more serious condition[16-19]. Recent research has shown that the most antibacterial and anti-infective effects of *M. longifolia* are due to its various and important compounds, such as Menthone, Pulegone, Piperitenone oxide and Thymol[20-22]. Due to the resistance of these bacteria to antibiotics and the prevalence of infection, the effect of medicinal plants on the prevention of the growth of bacteria due to the presence of secondary metabolites, this study was to identify the chemical compounds of essential oil using GC-MS and to evaluate and compare the antimicrobial effect of methanolic extract and essential oil of *M. longifolia* against *H. pylori.*

Materials and Methods

Plant material

The *M. longifolia* was freshly harvested around the mountains of Babol in July 2022. A voucher specimen (accession no. Mubabol 234) was deposited in the Department of Pharmacology at the Babol University of Medical Sciences. The voucher samples were authenticated by Professor S. A. Mozaffarpour, Head of the Traditional Medicine Research Center of Babol University of Medical Sciences.

Preparation of essential oil

After washing with sterile water, it was dried in a dark place and powdered with a mill. Essential oils were obtained by hydro-distillation of 100 g of dried aerial parts using a Clevenger-type apparatus for 4 h. The essential oil was extracted from the lower shaft and stored in 5 cc tubes in a dark place at 4°C.

Preparation of extracts

The maceration method was used to prepare the extract. For this purpose, 50 g of powdered plant was mixed with 500 cc of methanol solution, then placed at room temperature and 100 rpm in an incubator shaker for 72 h, and then strained through a sterilized filter cartridge and concentrated by rotary evaporation. The concentrated extract was poured into a glass plate, stored in an oven for drying, and then stored at 4°C.

Identification of essential oil chemical compounds

The GC–MS analysis of bioactive compounds from the *M. longifolia* EO was done using Agilent Technologies GC systems with a model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.15 µm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high-energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with a flow rate of 0.8 mL/min. The initial temperature was set at 50–250°C with an increasing rate of 5°C/min and a holding time of about 10 min. Finally, the temperature was increased to 300°C at 10°C/min. One microliter of the prepared 1% of the essential oil with respective solvents was injected in a split less mode. The relative amount of chemical compounds in each *M. longifolia* EO is expressed as a percentage based on calculating the area under the curve and with a link to the Wiley library in the chromatogram.

Agar dilution method for *H. pylori* bacteria

Method of preparing the culture medium

Clinical *H. pylori* strain isolates from biopsy specimens were collected from Rouhani Hospital (Babol, Iran). Test strains were cultured on Brucella agar (Merck, Germany) containing defibrinated blood (7%), and 0.005, 0.01, and 0.025 g of trimethoprim, vancomycin and polymyxin B antibiotics were added to plates[23]. The plates were incubated at 37ºC, under microaerophilic conditions (5% O2, 10% CO2 and 85% N2), in an anaerobic jar for 3–7 days. All *H. pylori* strains were sub-cultured on selective media containing Brucella agar (Merck, Germany), 7% defibrinated sheep blood and trimethoprim. The plates were then incubated in microaerobic conditions at 37ºC. Bacterial suspension in normal saline adjusted to McFarland standard[24].

Minimum inhibitory concentration and minimum bactericidal concentration method

The Anti-*H. pylori* activities of EO and methanolic extract of *M. longifolia* were tested with the agar dilution method. Different concentrations of methanolic extract of *M. longifolia* (6000, 3000, 1500, 750, 375, 187, 93 µg/mL) and *M. longifolia* EO (1500, 750, 375, 187, 93 µg/mL) were determined. Amoxicillin (160 µg/mL) and tetracycline (80 µg/mL) were used as a positive control, and 1% of DMSO was used as a reagent control. Different concentrations of extract and essential oil were added to each medium plate containing 20 mL of *H. pylori`s* selective culture medium described above. It was inoculated with a suspension of the fresh *H. pylori* (Approximately 1×108, turbidity compared with 0.5 McFarland). All plates were incubated at 37°C with specific conditions (5% O2, 10% CO2 and 85% N2) in moist atmosphere for 3–7 days. MIC was determined as the lowest concentration that growth of *H.*

pylori could be visible. Overall, it was compared with bacterial growth in the control plate's sample. The concentration of samples which killed and inhibited the growth of *H. pylori* completely is known as MBC)[22].

Result and Discussion

GC-MS of *M. longifolia* essential oils

Fig. 1 shows the total ion current of EO, the analysis of which by GC–MS revealed 42 peaks; 12 identified peaks represent 99.3% of the oil component (Table 1). It can be seen that the composition of the *M. longifolia* EO is dominated by monoterpenes and sesquiterpenes. The major compounds were cis-Piperitenone oxide (67.064%), piperitenone oxide (9.135%), L-Menthone

Table 1 — GC-MS compound name, retention time and peak value obtained of *M. longifolia* EO.

No	Retention time	Compounds	% Of total oil
1	7.602	Eucalyptol	3.524
2	9.954	L-Menthone	5.745
3	10.114	L-Menthone	0.859
4	10.274	DL-Menthol	0.967
5	11.745	Pulegone	2.130
6	12.380	cis-Piperitenone oxide	67.064
7	12.849	Thymol acetate	2.278
8	13.112	Cyclohexen	1.137
9	14.623	Piperitenone oxide	9.135
10	15.756	trans-Caryophyllene	5.271
11	16.814	Benzenethiol	0.918
12	16.992	Germacrene D	0.971

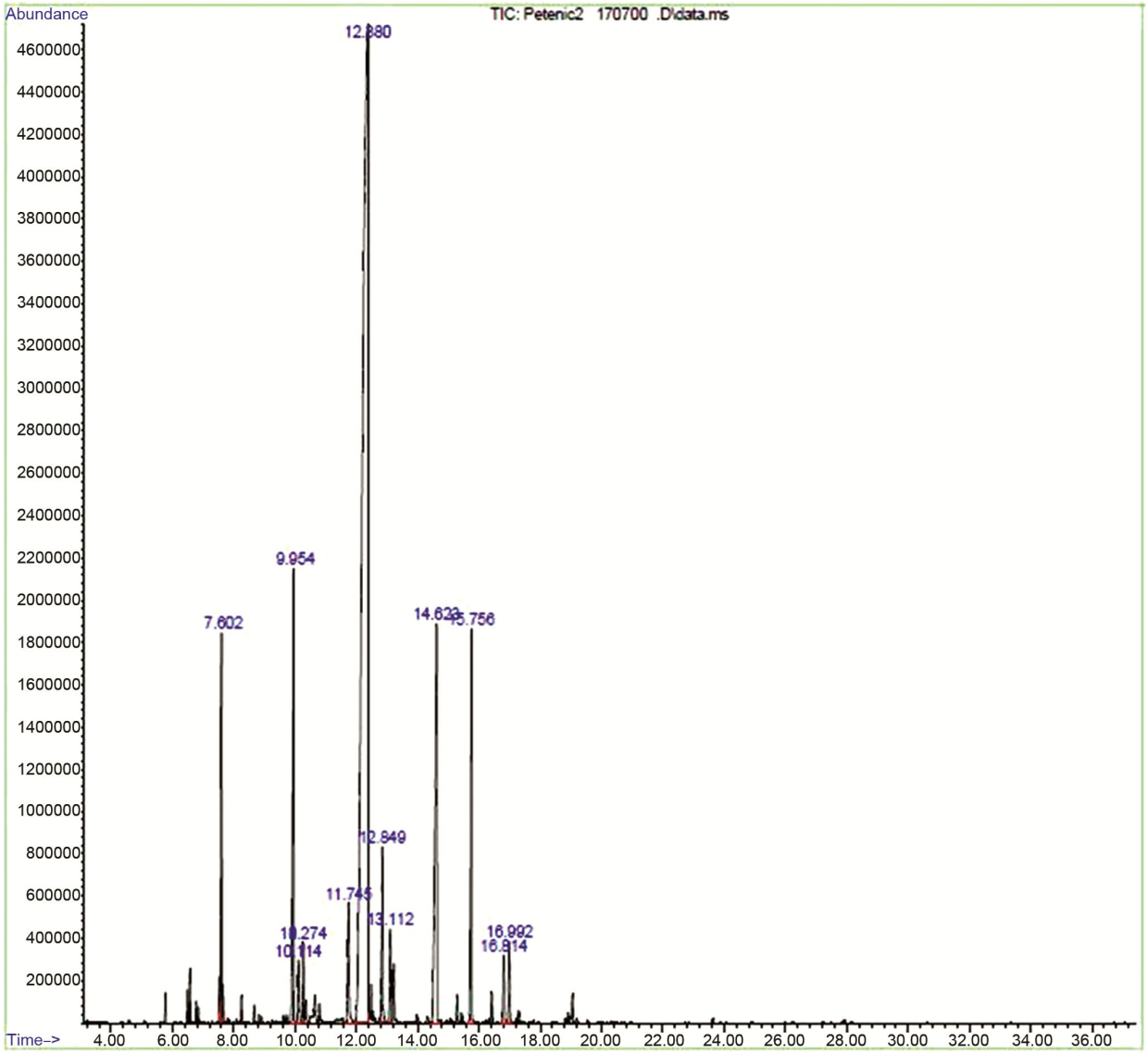

Fig. 1 — GC-MS analysis of the respective compounds of *M. longifolia* E.

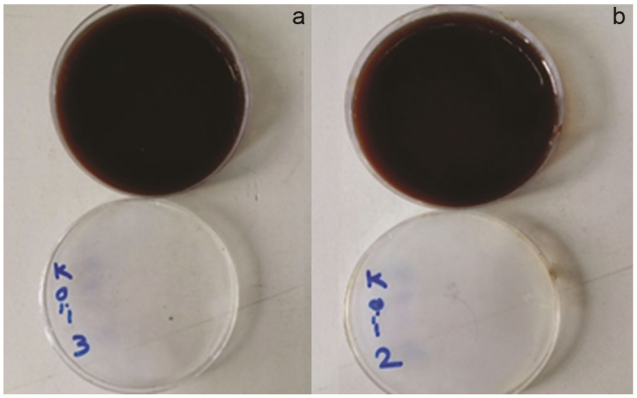

Fig. 2 — Experimental setup to determine MIC and MBC. a) MIC of *M. longifolia* EO 187 µg/mL; and b) MBC of *M. longifolia* EO 375 µg/mL.

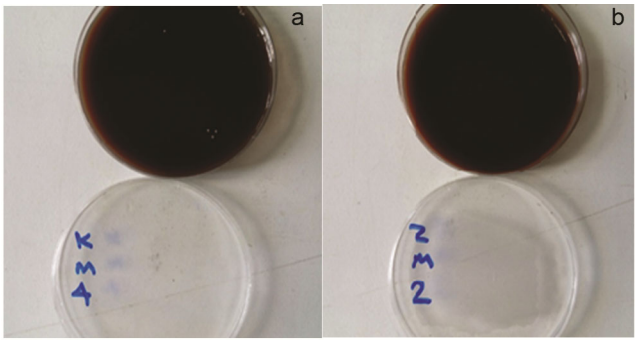

Fig. 3 — Experimental setup to determine MIC and MBC. a) MIC of methanolic extract of *M. longifolia* 1500 µg/mL; and b) MBC of methanolic extract of *M. longifolia* 3000 µg/mL.

(5.745%), Trans Caryophyllene (5.271%) and Eucalyptol (3.524%). The present study recognized piperitenone as the highest amount of the analysed compound. In contrast, in other studies, Pulegone was the main component of *M. longifolia* EO, which was not even recognized in the present study[25-27]. Carvone, pulegone, and piperitenone oxide have been found in substantial quantities in the *M. longifolia* EO ecotypes[27]. The current study's results are generally consistent with prior research, except that the main components and percentages of other components change. For example, the main components of *M. longifolia* EO from Iran were pulegone (26%), L-menthone (13.4%) and cis-para-menthan-3,8-diol (10.2%)[28], and China was carvone (47.39%) and limonene (12.48%)[29], while pulegone (26.07%), piperitone oxide (19.72%), and piperitone (11.88%) were found from the oil of India[26]. Piperitenone oxide is a very important compound since it is related to some of the beneficial properties displayed by the plant and its essential oils: Anti-nociceptive activity[30,31]; cardiovascular beneficial

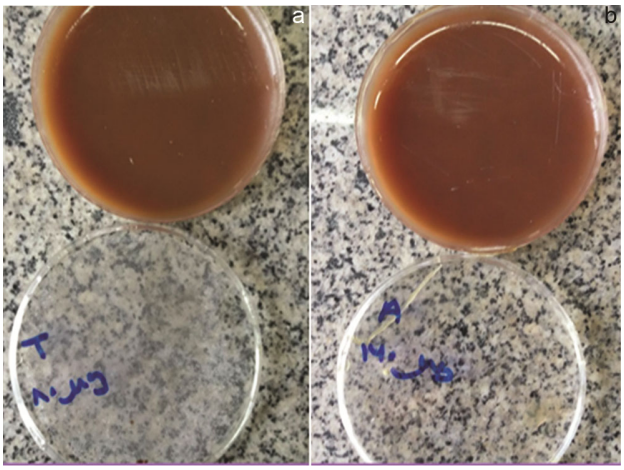

Fig. 4 — Experimental setup to determine positive control. a) Tetracycline on *H. pylori* strains; and b) Amoxicillin on *H. pylori* strains.

effects[32,33], antimicrobial potential and antifungal activity[21,25,27,29].

MIC and MBC of *M. longifolia* essential oil

The MIC and MBC of the extract and essential oil against *H. pylori* ranged from 93 to 3000 µg*l*mL. The results of the present study showed that the *M. longifolia* EO had a high antimicrobial effect on the *H. pylori* strains isolated from clinical cases of gastrointestinal disorders. The MIC and MBC for the essential oil were 187 µg/mL (Fig. 2a) and 375 µg/mL (Fig. 2b), respectively, while for methanolic extracts were 1500 µg/mL (Fig. 3a) and 3000 µg/mL (Fig. 3b). Also, the effects of amoxicillin and tetracycline antibiotics on *H. pylori* were investigated as control samples. Fig. 4 shows that the presence of the antibiotic's tetracycline Fig. 4a and amoxicillin Fig. 4b at concentrations of 80 and 160 µg/mL, respectively, inhibited the presence and proliferation of *H. pylori.* Antimicrobial substances generated from plants kill bacteria using methods distinct from those utilised by antibiotics, and this distinction is therapeutically significant in the management of diseases spurred on by resistant microbial strains[21]. *M. longifolia* exhibits antibacterial activity against various bacteria, yeasts, insects, and other species. A previous study has reported that *M. longifolia* EO has more antimicrobial activity than hydroalcoholic extract[21]. Several studies have approved the anti-*H. pylori* effects of some medicinal plants. Piasecki *et al*.[22] evaluated the effects of *M. longifolia* EO on *H. pylori,* and found that the concentration of *M. longifolia* had a minimum inhibitory range (15.6–31.3 mg/L). Another study by Bakr *et al*.[34] showed anti-*H. pylori* potential of methanolic extract of *M. longifolia*

with minimum inhibitory concentrations and minimum bactericidal concentrations of 6.5 and 50 mg/mL, respectively. Also, earlier studies reported the effects of *M. longifolia* EO and extract on *H. pylori*[35,36].

Conclusion

The results of the present study showed that the MIC for the essential oil was 187 µg/mL, and for methanolic extracts was 1500 µg/mL. The highest composition of *M. longifolia* EO is cis-Piperitenone oxide (67.064%) piperitenone oxide (9.135%). The present investigation reveals a real solution for anti-*H. pylori* resistance by using a natural herbal extract and essential oil may be used as food additives and food preservatives to control such microbial population and conserve human health. Medicinal herbs might also provide successful approach to decrease stomach cancer. However, potential cytotoxicity and side effects might present from those herbs. Therefore, further cytotoxicity investigation will be required.

Conflicts of interest

All the authors of this manuscript declare that there is no conflict of interest.

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