ABSTRACT

*Helicobacter pylori* can cause chronic gastritis, peptic ulcer, and gastric carcinoma. This study compares chemical composition and anti-*H. pylori* activity of mandarin leaves and marjoram herb essential oils, and their combined oil. GC/MS analysis of mandarin oil revealed six compounds (100% identified), mainly methyl-N-methyl anthranilate (89.93%), and 13 compounds (93.52% identified) of marjoram oil, mainly trans-sabinene hydrate (36.11%), terpinen-4-ol (17.97%), linalyl acetate (9.18%), and Caryophyllene oxide (8.25%). Marjoram oil (MIC = 11.40 μg/mL) demonstrated higher activity than mandarin oil (MIC = 31.25 μg/mL). The combined oil showed a synergistic effect at MIC of 1.95 μg/mL (same as clarithromycin).

**In-silico** molecular docking on *H. pylori* urease, CagA, pharmacokinetic and toxicity studies were performed on major compounds from both oils. The best scores were for Caryophyllene oxide then linalyl acetate and methyl-N-methyl anthranilate. Compounds revealed high safety and desirable properties. The combined oil can be an excellent candidate to manage *H. pylori*.

GRAPHIC ABSTRACT

1. Introduction

Despite enormous progress in medicinal strategies for the treatment of many human health problems, infectious diseases continue to pose a significant threat to public health. *Helicobacter pylori* is an extracellular gram-negative spiral bacterium, that is now recognised as a major cause of gastroduodenal diseases such as chronic gastritis, which affects nearly everyone and leads to peptic ulcers or gastric adenocarcinoma, the second most common cause of cancer death worldwide. Standard medications can cure the infection in more than 80% of *H. pylori*-infected patients. Patient compliance, antibiotic resistance, and recurring infections, on the other hand, are all the major concerns that limit the use of antibiotics in the treatment of *H. pylori* infection that needs to be addressed.

Natural products are reported to demonstrate various biological activities and as promising antimicrobials and clarithromycin.
Essential oils derived from aromatic and medicinal plants have recently gained popularity and great scientific interest as they are a part of traditional medicine predominating all over the globe for the alleviation of various health problems. Essential oils have been shown to possess potential antibacterial, antifungal, antiviral, anticancer, and antioxidant properties such as cinnamon, orange, lemon, pepper, thyme, and Schinus. Besides, they act as an important milestone in alternative medicine as well as natural therapies. Therefore, it is reasonable to expect that a variety of plant compounds in these oils have antimicrobial effects.

Among several essential oils that may be useful as antimicrobial agents, marjoram oil (Origanum majorana L., Lamiaceae) is an aromatic medicinal plant with the greatest potential for industrial applications because it shows different biological activities, including antibacterial, antifungal, antihypertensive, anti-inflammatory, and antioxidant properties. Origanum majorana leaves and essential oil have been claimed to be useful for the treatment of respiratory and gastrointestinal problems. It is one of the most popular spices used in cooking, arousing interest not only in the use of its leaves but also in its essential oil for therapeutic purposes.

On the other hand, the genus Citrus (Rutaceae) has been one of the most popular and commercially important crops for thousands of years. Citrus fruits are known for their nutritional values as an excellent source of vitamin C, their unique flavour, and their medicinal properties. Interestingly, essential oil (EO) is the most vital by-product of citrus processing. Petigrain mandarin essential oil is extracted from Citrus reticulata leaves. It could relieve stress and digestive problems while helping with flatulence, diarrhoea, and constipation. It is mostly used to increase circulation to the skin, reducing fluid retention and helping prevent stretch marks. Mandarin oil is used to calm the nervous system and has a tonic effect. Moreover, it showed broad-spectrum antibacterial and antifungal agents. It inhibited the growth of several bacterial and fungal strains. Furthermore, petitgrain mandarin essential oil showed potential antioxidant, anticancer (HL-60 and NB4), and radical scavenging activities.

The increasing emergence of H. pylori infections worldwide as well as the emerging tolerance against most currently available antibiotics has necessitated the urgent need to discover novel therapies available to control H. pylori infections. Meanwhile, it has been noticed that a few plants have been investigated recently for their H. pylori bactericidal activity. Antibacterial drug interactions can change the efficacy and either synergistic or antagonistic action, interaction between different compounds can lead to the reduction of the inhibitory activity.

This has driven our interest to assess the constituents of essential oils of marjoram (Origanum majorana L.) and mandarin leaves by using GC-MS, as well as evaluate the synergistic anti-H. pylori activity in-vitro of these oils, as compared to clarithromycin. An in-silico study was performed, where molecular docking was carried out on the major compounds identified from both oils on H. pylori virulent factors domains such as urease and CagA. Further in-silico pharmacokinetic and toxicity studies were performed on these major components to determine their safety margins and properties.

2. Materials and methods

2.1. Essential oils

The whole herb of Origanum majorana was subjected to steam distillation for 5 h. The oil produced has a pale yellow colour and herbaceous sweet odour. Citrus reticulata oil was prepared by water distillation of the leaves using the Clevenger apparatus for 5 h. Its colour is pale yellow and of intensely sweet and fresh scent. Both oils were purchased from Somitt Aromatic Company that were kept in dark bottles.

2.2. Analysis of essential oils by gas chromatography

2.2.1. GC/FID analysis

The GC/FID analyses were carried out on a Varian 3400 apparatus (Varian GmbH, Darmstadt, Germany) equipped with an FID detector and an RtX-SMS fused-bonded silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; Ohio Valley, Ohio, USA); the operating conditions were: The initial column temperature was kept at 45 °C for 2 min (isothermal), and then programmed rising at a rate of 5 °C/min to 300 °C and held for 5 min. Detector and injector temperatures were 300 °C and 250 °C, respectively. The sample volume was 0.03 μl. Helium carrier gas flow rate was 2 ml/min. Peak Simple 2000 chromatography data system (SRI Instruments, Torrance, USA) was used for recording and integrating the chromatograms.

2.2.2. GC/MS analysis

The analyses were carried out on a Hewlett Packard gas chromatograph (GC HP 5890 II; Hewlett Packard GmbH, Bad Homburg, Germany) equipped with the same column and conditions as for the GC/FID. The capillary column was directly coupled to a quadrupole mass spectrometer (SSQ 7000; Thermo-Finnigan, Bremen, Germany). The injector temperature was 250 °C. Helium carrier gas flow rate was 2 ml/min. All the mass spectra were recorded with the following analytical conditions: filament emission current, 60 mA; electron energy, 70 eV; ion source temperature, 200 °C; and scan range was from 40 to 400 Amu. The diluted samples (0.5% v/v n-hexane used as solvent) were injected with split mode (split ratio, 1:15). Compounds were identified by comparison of their mass spectral data and retention indices. The identified compounds were matched with Wiley Registry of Mass Spectral Data 8th edition and NIST Mass Spectral Library (December 2005). The identification was further confirmed by the calculation of the retention indices (RI) relative to a homologous series of n-alkanes (C6 - C22), under identical experimental conditions, as well as matching with the literature.

2.3. Assessment of the anti-Helicobacter pylori activity

2.3.1. Determination of the minimal inhibitory concentration (MIC)

The micro-well dilution method was used to evaluate the antibacterial activity of the Marjoram and Mandarin oils against Helicobacter pylori (ATCC 43504, the reference strain being obtained from the American Type Culture Collection) adopting the NCCLS guidelines (1998) and as previously described by Cerda et al. 100 μg of the tested samples were combined with 100 μL of 20% (v/v) bacterial suspensions (OD at 600 nm = 1.0) in a flat bottomed 96-well microplate. Serial two-fold dilutions of the oils and the standard were prepared directly in a sterile 96-well microtiter plate. Deionised water was used as a negative control meanwhile clarithromycin was used as a positive control. The reaction mixture was incubated using Mueller-Hinton broth. After incubation at 37 °C for 3 days under microaerophilic conditions (10% CO2 and 80% humidity). 25 μL of 10 mM 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) freshly prepared in water were added into the mixture (final volume was 225 μL) to each well and incubate for 30 min. The developed purple colour was measured at 550 nm using a microplate reader. All tests were performed in triplicate.
triplicate. Inhibition (%) was calculated as follows: \[
\frac{([\text{Initial control absorbance}]-[\text{Final absorbance}])}{([\text{Initial control absorbance}])} \times 100.
\]

The Agar dilution checkerboard method is used to evaluate the synergistic action of both essential oils. The MIC90, the concentration of samples with 90% inhibition was calculated from dose-response curves. The MIC values were assessed in triplicate, using an automatic ELISA microplate reader.

### 2.4. In-silico studies

#### 2.4.1. Molecular docking

The X-ray 3D structures of human *H. pylori* Urease and Cag A oncogenic proteins were downloaded from the protein data bank using the following IDs: 1e9y and 4dvy, respectively. All the docking studies were conducted using MOE 2019 which was also used to generate the 2D and 3D interaction diagrams between the docked ligands and their potential targets. In the beginning, the two enzymes and the seven isolated major compounds were prepared using the default parameters. The active site of each target was determined and the seven isolated compounds were saved into a single file with MDB extension. Finally, the docking was finalised by docking the mdb file containing the seven compounds into the active site of both the enzymes.

#### 2.4.2. In-silico toxicity and ADME prediction

Both the Toxicity and pharmacokinetic properties of the seven compounds were predicted by the online servers ProTox II (https://tox-new.charite.de/protox_II/index.php?site=compound_input) and Swiss adme (http://www.swissadme.ch/index.php), respectively.

### 3. Results

#### 3.1. Determination of the volatile oil compositions using GC/MS

Six compounds were identified from the oil of mandarin leaves (Figure 1, Table 1). The predominant constituents of the oil were methyl-\(N\)-methyl anthranilate (89.93%) and \(\gamma\)-terpinene (6.25%), respectively. While 93.52% of marjoram oil was identified (Figure 2, Table 2), the major peaks were for *trans*-sabinene hydrate (36.11%), terpinen-4-ol (17.97%), linalyl acetate (9.18%), carophyllene oxide (8.25%), and \(\alpha\)-terpineol (6.17%), respectively.

#### 3.2. Determination of anti-Helicobacter pylori activity

This study’s focal objective is to compare the antimicrobial activities of essential oils of mandarin leaves and marjoram against *Helicobacter pylori*. The MIC results for the two tested oils separately and combined with clarithromycin as a positive control are shown in Figure 3(A–D), respectively.

The results of this study revealed that marjoram oil showed higher antibacterial activity against *H. pylori* at a MIC of 11.4 \(\mu\)g/mL, (Figure 3B) relative to mandarin essential oil, which exhibited a MIC value of 31.25 \(\mu\)g/mL (Figure 3A). This may be attributed to the presence of high content of oxygenated compounds in marjoram oil that were identified as shown in (Table 2), including *trans*-sabinene hydrate (36.11%), terpinen-4-ol (17.97%), linalyl acetate (9.18%), carophyllene oxide (8.25%), and \(\alpha\)-terpineol (6.17%).

While methyl-\(N\)-methyl anthranilate (89.93%) has been identified as the major constituent of mandarin leaf oil, followed by \(\gamma\)-terpine (6.25%) as shown in (Table 1). A combined mixture of both oils exhibited a potentially synergetic inhibitory effect against *H. pylori* at a MIC of 1.95 \(\mu\)g/ml, yielding higher inhibition (Figure 3C) relative to marjoram and mandarin oils separately. Furthermore, clarithromycin demonstrated the same MIC value (1.95 \(\mu\)g/ml).

#### 3.3. Docking study

A molecular docking study was carried out on the major compounds identified from both oils on *H. pylori* virulent factors domains such as urease and CagA. Results showed that carophyllene oxide showed the best fitting scores followed by linalyl acetate and methyl-\(N\)-methyl anthranilate, as demonstrated in Table 3. The binding affinities of carophyllene oxide, linalyl acetate and methyl-\(N\)-methyl anthranilate to urease and CagA are further demonstrated in Figures 4, 5, respectively. Carophyllene oxide showed the best binding affinities to the urease enzyme by 4 hydrogen bonds (H-bonds) and solvent interaction, while it revealed an interaction with only 2 H-bonds with cagA. Linalyl acetate exhibited 5 H-bonds and 2 metal (Nickel) co-ordinates with urease and 3 H-bonds with cagA. Regarding, methyl-\(N\)-methyl anthranilate, it showed 3 H-bonds and solvent interaction with urease, while it demonstrated 2 H-bonds and 2 hydrophobic interactions with the active sites of CagA. Figures 4G, 5G revealed concomitant interactions of seven major compounds identified in both oils with the active sites of urease and cagA, respectively, to reveal more of the synergistic effect of these components as anti-*H. pylori*.

#### 3.4. In-silico toxicity study

As demonstrated in Table 4 all the compounds have high margins of safety and they were predicted to have no potential toxicity.
3.5. Pharmacokinetics study

It is important for therapeutic candidates to have both acceptable pharmacokinetics and pharmacodynamics profiles. Accordingly, the pharmacokinetic profiles of the seven major compounds were computed using the online server of swiss adme. As depicted in Table 2. All the compounds were predicted to have high GIT absorption making them excellent oral candidates against H. pylori.

This high bioavailability of the seven compounds is attributed to their desired physicochemical properties including FLEX (Flexibility), LIPO (Lipophilicity), INSATU (Saturation), INSOLU (Solubility), SIZE and POLAR (Polarity) as demonstrated in Table 5 and Figure 6. In addition, all compounds showed no or minimal interaction with microsomal cytochromes and then could be taken concurrently with other medications. Most importantly, no compound was found to be a substrate for the p-glycoprotein known to be one of the resistance mechanisms of H. pylori for existing antibiotics.40 A worthy note, the seven compounds were aligned with all of Lipinski’s rules, besides none of them had any reported Pan Assay Interference (PAINS).

4. Discussion

The continuous evolution of many drawbacks with the current therapies for H. pylori, such as the prevalence of antibiotic-resistant, drug interventions, side effects, and poor satisfaction, all highlight the
search for safe and effective non-antibiotic alternative medicines. This has driven our interest in evaluating the bactericidal activity of marjoram and mandarin oils against H. pylori.

At present, interest in essential oils has increased because of their bactericidal activity against several bacteria without the marked toxic effects of synthetic drugs. Bactericidal activity is a well-known property of volatile oils, particularly those of marjoram and mandarin. Numerous studies have confirmed the antimicrobial activity of marjoram essential oils and mandarin leaf essential oils. This study investigates the bactericidal activity of the hydro-distilled essential oils of the leaves of mandarin and marjoram. The results of this study revealed that marjoram oil showed a higher effect against H. pylori than mandarin essential oil. This may be attributed to the presence of high content of oxygenated compounds identified in marjoram oil, including trans-sabinene hydrate (36.11%), terpinen-4-ol (17.97%), linalyl acetate (9.18%), caryophyllene oxide (8.25%), and α-terpineol (6.17%). While methyl-N-methyl anthranilate (89.93%) was identified as the major constituent of mandarin leaf oil, followed by c-terpinene (6.25%).

Many studies of in vitro antimicrobial activity of marjoram and mandarin oils in the literature may be probably due to the action of the major compounds which have been previously tested for their bactericidal activity, such as terpinen-4-ol, α-terpineol, and γ-terpinene were found as the predominant components of the essential oils obtained from the aerial parts of Origanum scabrum and Origanum microphyllum, both endemic species in Greece, exhibited a very interesting antimicrobial profile after they were tested against six Gram-negative and Gram-positive bacteria and three pathogenic fungi. Furthermore, the acetone crude extract of the stem bark of Sclerocarya bijerea is a promising source for anti-H pylori compounds.

### Table 3. Docking scores of marjoram and mandarin major oil compounds on H. pylori virulent factors domains (urease and CagA).

| Compound name                  | Urease (1e9y) | Cag A (4dv) |
|-------------------------------|--------------|------------|
| Caryophyllene oxide           | -11.4        | -9.5       |
| Linalyl acetate               | -9.1         | -8.3       |
| Methyl-N-methyl anthranilate  | -8.9         | -8.2       |
| α- Terpineol                  | -7.6         | -7.4       |
| trans-Sabinene hydrate        | -7.4         | -6.9       |
| Terpine-4-ol                  | -7.2         | -7.1       |
| γ-Terpine                     | -6.6         | -5.4       |

Figure 3. The MIC90 & MIC graphs of Anti-Helicobacter pylori activity of mandarin oil leaves (A), marjoram oil (B), equal amounts of both oils(C), and Clarithromycin (D) as standard. All determinations were carried out in a triplicate manner and values are expressed as the mean ± SD.
with terpinen-4-ol, an essential oxygenated monoterpene oil, being the most abundant agent (35.83%), and it was reported as a major mediator of the anti-H. pylori activity. The inhibitory activity of terpinen-4-ol in this study was similar to that of amoxicillin, one of the most effective drugs used in the eradication of H. pylori infections worldwide. Additionally, trans-sabinene hydrate, terpinen-4-ol, α-terpineol, and γ-terpinene have been reported as major components of marjoram oil, which exhibited antibacterial activity against food-related bacteria like E. coli, Salmonella cholraesius, and S. aureus in fresh sausage. Because of their antimicrobial activity against foodborne bacteria EOs could be added to food products to extend their shelf life, but changes in the taste, as well as formulation problems, could represent a problem there.

Linalool (8.5%), α-terpineol (4.4%), and linalyl acetate (4.2%) are considered the most important components of Myrtle oil that showed significant antimicrobial activities against Salmonella typhimurium, Lactobacillus spp., Yersinia enterocolitica, Helicobacter pylori, and significant antifungal activity when combined with amphotericin B. Moreover, the antimicrobial activity of the essential oil of Thymus capitatus was tested using the broth dilution method. γ-Terpinene in Thymus capitatus essential oil (10%) induced strong bactericidal activity against H. pylori strains.

β-Caryophyllene, is a natural bicyclic sesquiterpene, which is extracted from clove and tested for the eradication of H. pylori in a mouse model, and its effects on the inflammation of the gastric mucosa. Interestingly, β-caryophyllene showed potent antimicrobial activity against H. pylori by direct killing action. In addition, it improved the inflammation of the gastric mucosa by decreasing H. pylori number. The use of compounds with natural origin has gained popularity in scientific research focussed on drug innovation against H. pylori because of their broad flexibility and low toxicity. For example, monoterpines limonene and β-pinene resulted in MICs against H. pylori of 75 µg/mL and 500 µg/mL respectively.

Regarding the biological properties, we know that essential oils are complex mixtures of numerous constituents. As a result, their biological effects can be the result of the synergism of all their constituents, thus components are working together. In this case, the effect of the mixture would be greater than the pure sum of its single parts. The essential oils could be used on their own, as well as in combination with other oils or synthetic active agents since synergy was observed by combining these substances. Various studies showed that the extent of antimicrobial activity and the mode of action is dependent on the additive, synergistic, or even antagonistic effects of the individual constituents.

Results of this study clearly showed the synergistic effect of both marjoram and petitgrain mandarin oils on their anti-H. pylori activity. The combined oil sample showed the highest inhibitory effect against H. pylori at MIC 1.95 µg/mL. Clarithromycin, the used
reference drug, demonstrated the same MIC value as the combined oil, at the same concentration used. Thus, it should be noted that the combined oils’ effects are comparable to clarithromycin. An in-silico study was carried out to further verify the observed results. Docking studies are performed to reveal the binding affinity of the major components to the target enzymes, where caryophyllene oxide showed the best fitting scores followed by linalyl acetate and methyl-N-methyl anthranilate. Furthermore, all the tested compounds showed high margins of safety in-silico, they were predicted to have no potential toxicity and were aligned with all Lipinski’s rules. Additionally, all the tested compounds showed no or minimal interaction with microsomal cytochromes, thus, they could be taken concomitantly with other medications. Moreover, neither of the tested compounds was found to be a substrate for the p-glycoprotein (one of the resistance mechanisms of H. pylori for existing antibiotics) or had any reported Pan Assay Interference (PAINS). From this perspective, the two oil extracts are considered promising inhibitors for both sensitive and resistant strains of H. pylori with a notable safety margin and good desirable pharmacokinetic properties.

5. Conclusion
Marjoram and mandarin oils are the most widely available and highly consumed by humans due to their nutritional and medicinal values and very low toxic effects. The current study revealed the promising synergistic effects of the volatile constituents from marjoram and mandarin leaves against Helicobacter pylori, offering a
natural remedy for pharmaceutical industries to combat this bacterial infection. Furthermore, the major compounds of both oils were evaluated in-silico, and demonstrated superior biological activities, excellent safety margins and desired pharmacokinetic properties making them excellent candidates to manage *H. pylori*.

**Disclosure statement**

The authors declare that they do not have any conflict of financial interests or personal relationships that could influence the reported work.

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**Table 5.** The predicted pharmacokinetics of marjoram and mandarin major oil compounds.

| Parameter          | *trans*-Sapinene hydrate | Terpine-4-ol | Linalyl acetate | Caryophyllene oxide | *α*-Terpineol | Methyl-N methyl anthranilate | *γ*-Terpineol |
|--------------------|--------------------------|--------------|-----------------|--------------------|---------------|----------------------------|---------------|
| GIA                | High                     | High         | High            | High               | High          | High                       | High          |
| BBB                | Yes                      | Yes          | Yes             | Yes                | Yes           | Yes                        | Yes           |
| P-gP substrate     | No                       | No           | No              | No                 | No            | No                         | No            |
| CYP1A2 inhibitor   | No                       | No           | No              | No                 | No            | Yes                        | No            |
| CYP2C19 inhibitor  | No                       | No           | No              | Yes                | No            | No                         | No            |
| CYP2C9 inhibitor   | No                       | No           | No              | Yes                | No            | No                         | No            |
| CYP2D6 inhibitor   | No                       | No           | No              | No                 | No            | No                         | No            |
| CYP3A4 inhibitor   | No                       | No           | No              | No                 | No            | No                         | No            |
| Lipinski Violations| No                       | No           | No              | No                 | No            | No                         | No            |
| PAINS              | No                       | No           | No              | No                 | No            | No                         | No            |

GIA (gastrointestinal absorption), BBB (Blood Brain Barrier), P-gP (P-glycoprotein transporter), CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 are the five forms of cytochromes P450 (CYP). PAINS (Pan Assay Interference).

**Figure 6.** The predicted physicochemical properties for selected compounds, such as linalyl acetate (A) and *trans*-sabinene hydrate (B).
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