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Antibacterial Activity of Extracts and Essential Oils of Two Iranian Medicinal Plants, *Salvia mirzayanii* and *Zhumeria majdae*, Against *Helicobacter pylori*

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Abstract: *Helicobacter pylori* is the major etiological agent of chronic active gastritis, peptic ulcer and gastric carcinoma. Therapeutic protocols used in eradication of this organism fail in many cases, mainly due to emerging antibiotic resistance; hence developing better therapeutic regimens is a high demanded necessity. In an effort to discover natural anti-*Helicobacter pylori* compounds, we investigated the anti-*Helicobacter pylori* effects of both extracts and essential oils of *Salvia mirzayanii* and *Zhumeria majdae* that are used for treatment of gastritis and peptic ulcer diseases in folk medicine. Antibacterial activity of these materials against *Helicobacter pylori* isolated from biopsy samples was evaluated *in vitro* using disc diffusion method. Our results revealed that the essential oils (1/10 and 1/20) and the extracts (20, 40, 50 mg/ml) of both plants have strong antibacterial effects against *H.pylori*, although the strongest activity was seen for *Salvia mirzayanii* essential oils. The essential oils of two plants; *Salvia mirzayanii* and *Zhumeria majdae,* contain components that could be potential industrial sources for preparing effective drugs against *H. pylori* strains.

Key words: *Helicobacter pylori* · *Salvia mirzayanii* · *Zhumeria majdae* · Extracts · Essential Oils

INTRODUCTION

Helicobacter pylori, a Gram-negative bacterium, is one of the most widespread infections in human worldwide that persistently infects up to 50% of the world's population. Infections has been reported to be higher in the developing than in the developed countries with up to almost 100% of the population being infected in some developing nations. The infection is associated with atrophic gastritis, duodenal ulcer and gastric adenocarcinoma at a later stage [1]. Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high risk population [1].

The current and most effective treatment for peptic ulcer disease is a triple therapy regimen consisting of a proton pump inhibitor, such as omeprazole and two antibiotics, clarithromycin and either amoxicillin or metronidazole. However, antibiotics consumption causes an increase in prevalence of antibiotic resistance, which is remarkable in some areas of the world [2].

The need of new anti-infective agents due to the emergence of multiple antibiotic resistances has led to the search of new sources of potential antimicrobials by researchers. Among them, the plant kingdom or medicinal plants offers a wide range of biodiversity of great value for the pharmaceutical industry [3].

Plants provide a multitude of flavors and fragrances which have found their way into everyday life. Essential oils and some of their constituents are used not only in

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pharmaceutical products for their therapeutic activities but also in agriculture, as food preservers and additives for human or animal use, in cosmetics and perfumes and other industrial fields [4].The complex composition of the essential oils and the variety of chemical structures of their constituents are responsible of a wide range of biological activities many of which are of increasing interest in the fields of human and animal health. Particularly, many essential oils and their constituents have traditionally been used for their antimicrobial activity which has long been recognized [3]. The extracts of some plants are being used in Iranian folk medicine as the basis of anti-ulcer medicines for treatment of peptic and duodenal ulcers and dyspepsia. Some of them have been known to aid the body's fight against infections. Several medicinal plants, particularly from the mint (Lamiaceae) family have been reported to exhibit a broad spectrum antibiotic activity against both Gram-negative and Gram-positive bacteria [5].

In an effort to discover natural anti-*Helicobacter pylori* compounds, we screened two Iranian medicinal plants of Lamiaceae family to detect their relevant biological activities.

Zhumeria majdae Rech. F. & Wendelbo known locally by the name of "Mohrekhosh" as a monotypic genus of Zhumeria occurring in the southern parts of Iran, have been credited with a high potential medicinal uses as antinociceptive, anti-inflammatory, anti-leishmanial and anti-plasmodial. Its leaves have been used for many years as a curative for stomachaches, flatulence, diarrhea, indigestion, cold, headache, wound healing and as antiseptic and treatment of painful menstruation [6].

Salvia mirzayanii Rech. F. & Esfand, is an endemic plant growing in Iran [7] that known locally by the name of "Mohretalkh" and belongs to the genus Salvia. Several species of salvia are used in folk medicine as antiseptics, astringents and spasmolytics. The leaves of *Salvia mirzayanii* have been used for many years in folk remedies for gastritis relief or as an antiseptic agent [7]. The GC-mass analysis of *Salvia mirzayanii* essential oil had been shown that Linalyl acetate (7.6%), 1, 8-Cineole (8.0%), Linalool (9.0%) and 8-acetoxy linalool (11%) are the major compounds in this plant [8]. Moreover, some literatures are shown the antimicrobial [9] and immunomodulatory activities of *Salvia mirzayanii* on cell-mediated and humoral antibody-mediated response [10].Many studies indicated antioxidant, antimicrobial and antiviral activities of some salvia species [8].

In the present study, we aimed to investigate the effects of both extracts and essential oils of *Salvia mirzayanii* and *Zhumeria majdae* on *Helicobacter pylori* strains isolated from biopsy samples.

MATERIALS AND METHODS

Plant Materials: The aerial parts of *Salvia mirzayanii* and *Zhumeria majdae*were collected from south of Iran, Hormozgan province, in April 2012.

Preparation of the Essential Oils: Dry aerial materials of plants (100g) were pulverized; essential oil of plants was extracted by Clevenger-type apparatus for 4h. The essential oils were prepared in Fars Center of Agricultural Research, Shiraz, Iran.

Preparation of Plants Extracts: Air-dried and powdered aerial parts of each plant (250 g) were macerated with 1000ml methanol (methanolic extract) and chloroform (chloroformic extract) for 24h separately. Then they were shaken, filtered and evaporated in a rotary evaporator under reduced pressure at low temperature until dryness. The extracts were prepared in Shiraz University of Medical Sciences, Shiraz, Iran.

Isolation and Identification of *H. pylori*: Five biopsy samples from patients attending the endoscopy ward of Namazee Hospital of Shiraz were collected during September 2012. Samples were gently homogenized and cultured on rapid ureas-test media and colombia agar base (Merck, Germany), supplemented with 10% lysed horse blood and 7% fetal calf serum and the antibiotics amphotericin B (5µg/L), trimethoprim (5µg/L) and vancomycin (10µg/L). The cultures were kept in a microaerophilic atmosphere (7% O_2, 7.1% H_2, 79.8% N_2), provided by Anoxomate (Merck II, Mart Microbiology BV, Netherlans) at 37°C for 2-4 days. The isolates were then confirmed as *H. pylori* by positive oxides, catalase and rapid urease-test[11]. If any of the two tests were positive simultaneously, the sample was considered *H. pylori* positive. These *H. pylori* strains where then used as microbial tests in our antibacterial assays.

Disc Diffusion Antibacterial Activity Assay Method: To eliminate the contamination from the extracts they were filtered by 0.22milipore filters. Then 30µl of dilutions of 1/10, 1/20 and 1/30 solutions of essential oils and 10, 20,

40 and 50 mg/ml solutions of extracts in ethanol were applied to sterile paper discs (6mm in diameter). The discs were allowed to be dried in an open sterile Petri dish in a biological safety vertical laminar flow cabinet. The antimicrobial activity of the extracts was determined based on disc diffusion method using *H. pylori* cell suspension whose concentrations were equilibrated to 3 McFarland standard tubes [11].The bacterial suspension was inoculated on a Muller-Hinton agar plate and then the prepared discs were placed on the surface of the inoculated plates and incubated at 37°C under microaerophilic condition and then the diameter (mm) of inhibition zones were determined after 24hours incubation. In all the tests the discs containing ethanol as solvent were used for negative control and clarithromycin, metronidazole and Levofloxacin antibiotics discs were used as positive controls. The experiment was performed in duplicate and the results were expressed as average values.

Statistical Analysis: All extraction and determination were conducted in triplicate. Data were expressed as means. The means were compared by using the one-way and multivariate analysis of variances (ANOVA) followed by Duncan's multiple range tests. The difference between individual means were deemed to be significant at $P<0.05$.

RESULTS

Methanolic and chloroformic extracts of two different plants species of Lamiaceae family used in Iranian traditional medicine to treat gastrointestinal disorders were investigated for their anti-*Helicobacter pylori* activity. The results were shown in Tables 1 and 2. The extracts from *Salvia mirzayanii* and *Zhumeria majdae* possessed a wide range of antibacterial spectrum, because they inhibited the growth of bacteria and the diameter of inhibition zone varied from 10-26.8mm, depending on the concentration of extracts that applied in this study. Methanolic and chloroformic extracts of *Salvia mirzayanii* and *Zhumeria majdae* showed the highest inhibitory effect in 50mg/ml concentration. In contrast these two extracts of plants had little action against *H.pylori* in concentration of 10mg/ml. For the methanolic and chloroformic extracts, the most active plant was *Salvia mirzayanii*. It's worth noticing that we tested

Table 1: Inhibition zones of methanolic extracts at different concentrations

	Inhibition zone diameter (mm)*						
					Antibiotics (µg)		
	Methanolic extract (mg/ml)				Lev	Cla	Met
Plant species	10	20	40	50	5	15	Met
Salvia mirzayanii	16.3^{a}	19.8 ^{ab}	23 ^{c}	26.8^{d}	> 12 (S)	>30 (S)	<16 (R)
Zhumeria majdae	10^{a}	20.6 ^{b}	20.8 ^{b}	21 ^{b}	> 12 (S)	>30 (S)	<16 (R)

(*): Data are the mean values of three replicates (diameter of inhibition zone mm), lev: Levofloxacin, Cla: Clarithromycin, Met: metronidazole, R: Resistance, S:Sensitive

Values (means of three replicates) of each parameter followed by at least one same letter are not significantly different at $P<0.05$

Table 2: Inhibition zones of chloroformic extracts at different concentrations

	Inhibition zone diameter (mm)*						
					Antibiotics (µg)		
	Chloroformic extract (mg/ml)				Lev	Cla	Met
Plant species	10	20	40	50	5	15	5
Salvia mirzayanii	14.25 ^{a}	18^{bc}	22 ^{bc}	26.3 ^{c}	> 12 (S)	>30 (S)	<16 (R)
Zhumeria majdae	16.4 ^{a}	16.6 ^{a}	20 ^{c}	21.6 ^{c}	> 12 (S)	>30 (S)	<16 (R)

(*): Data are the mean values of three replicates (diameter of inhibition zone mm), lev: Levofloxacin, Cla: Clarithromycin, Met: metronidazole, R: Resistance, S:Sensitive

Values (means of three replicates) of each parameter followed by at least one same letter are not significantly different at $P<0.05$

Table 3: Inhibition zones of essential oils at different concentrations

	Inhibition zone diameter (mm)*					
				Antibiotics (µg)		
	Essential oil concentration			Lev	Cla	Met
Plant species	1/10	1/20	1/30	5	15	5
Salvia mirzayanii	>35 [b]	>30 [a]	28 [a]	> 12 (S)	>30 (S)	<16 (R)
Zhumeria majdae	>30 [c]	26 [b]	22 [a]	> 12 (S)	>30 (S)	<16 (R)

(*): Data are the mean values of three replicates (diameter of inhibition zone mm), lev: Levofloxacin, Cla: Clarithromycin, Met: metronidazole, R: Resistance, S:Sensitive

Values (means of three replicates) of each parameter followed by at least one same letter are not significantly different at P<0.05

the extracts but not the fractionated compounds, hence the fractionating compounds from the most active extracts could be encouraging.

Results on the antimicrobial activity of the essential oils of *Salvia mirzayanii* and *Zhumeria majdae* were summarized in Table 3. The essential oils of both plants showed very strong antibacterial effects against *H.pylori* in both 1/10 and 1/20 concentrations, although the strongest activity have gained for *Salvia mirzayanii* in three concentrations. It was seen that *Salvia mirzayanii* and *Zhumeria majdae* affected *H.pylori* as much as Levofloxacin, Clarithromycin and metronidazole antibiotics. It is interesting to highlight that the two plants essential oils were more effective than their extracts.

DISCUSSION

Increasing antibiotic resistance among some pathogens causing infectious diseases as well as the appearance of undesirable side effects of antibiotics had increased the interests of producing new nontoxic and more effective antimicrobial agents from herbal plants. Among the medicinal or aromatic plants, members of Lamiaceae family have a wide range of compound such as terpenoids, iridiods and phenolic [12]. The large amount of these substances in plant extracts and essential oils of herbal belong to this family have shown antibacterial and antifungal powerful effects against harmful microorganisms in the world [13,14]. For example, the ethanol extract of *Ocimum basilicum* had shown antimicrobial activities against nine strains in the genera Acinetobacter, Bacillus, Escherichia and Staphylococcus [15]. On the other hand, many of the most active anti *Helicobacter pylori* plants have a long history of traditional use as water-based remedies for gastrointestinal afflictions and many of them are found among lamiaceae plants [16].

Before the recognition of *Helicobacter pylori* as the main etiological agent of chronic gastritis and peptic ulcer disease, the research studies elucidating the mechanisms by which traditional medicinal plants exert their actions, were focused on their gastroprotective and/or anti-acid and/or anti-inflammatory effects [17].But in the recent years a great deal of research has been under taken to determine a direct action of plants upon *Helicobacter pylori.*

The leaves of these two plants have been used for many years in folk remedies for gastritis relief or as an antiseptic agent [7]. Our research revealed the strong effects of essential oils of two plants against *Helicobacter pylori*. All these activities can be related to the major constituents, mainly linalool [18,19] or other compounds whose antimicrobial properties have been previously reported. GC and GC-MS analysis of the essential oils of both plants have shown 26 compounds in *Zhumeria majdae* and 34 compounds in *Salvia mirzayanii* essential oils. Among them, linalool and camphor were the main components in *Zhumeria majdae* essential oil [20] and linalyl acetate, 1,8-cineol, linalool and 8-acetoxy linalool were reported as the main components for *Salvia mirzayanii*[8]. Accordingly, more investigation on the anti- *Helicobacter pylori* activity of these compounds could be recommended.

It has been reported that most Salvia species which have terpens, pinen, lorneol and cineol are recommended for use as a diuretic and an antiseptic agents [21]. In addition, camphor and 1,8-cineol are well-known chemicals with their pronounced antimicrobial potential [18].

However, the potent antimicrobial activity of *Salvia mirzayanii* and *Zhumeria majdae* extracts may be attributed to the various phytochemical constituents presented in the crude extract. Some researchers have proved the presence of some inhibitory compounds

(secondary metabolite) or factor in the plant extracts that could have powerful antimicrobial effect on pathogens [22]. In the effort to discover natural anti-*Helicobacter pylori* compounds, Ghannadi and coworker [5] screened seven Iranian members of mint (Lamiaceae) family to detect their relevant biological activities. They showed thatmethanolic extract and polar fractions of *Zataria multiflora*, *Dracocephalum moldavica*, *Lavandula angustifolia* and *Teucrium polium* had strong bactericidal effects against *H.pylori*[5].

Our results provide voluble information about new plants with high anti-*Helicobacter pylori* activity, which will become the starting materials for bioassay guided fractionation to determine the active constituents of the plant extracts and essential oils. The essential oils of these two plants, *Salvia mirzayanii* and *Zhumeria majdae,* contain components that could be potential industrial sources for preparing effective drugs against *H.pylori* strains.

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