doi.org/10.36721/PJPS.2020.33.6.SUP.2809-2814.1

In vitro and *in vivo* anti-*Helicobacter pylori* activity of selected medicinal plants employed for the management of gastrointestinal disorders

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Abstract: Five medicinal plants *Mentha piperita* L., *Trachyspermum ammi* L., *Viola odorata* Linn., *Matricaria chamomilla* L. and *Foeniculum vulgare* Mill. were selected for their *in vitro* and *in vivo* evaluation of anti-*Helicobacter pylori* activity. *In vitro* evaluation was performed by using disk diffusion method and minimum inhibitory concentrations were noted while rat models were selected for *in vivo* activity against four *Helicobacter pylori* strains isolated form gastric mucosa. *Mentha piperita* showed largest zone of inhibition with 9 mm diameter among all other extracts. All the plants showed promising anti-*Helicobacter pylori* activity against four isolates and a reference strain at concentrations of 125, 250, 500 and 1000 µg/ml in comparison with Amoxicillin 1 µg/ml but least MIC was exhibited by *Mentha piperita* followed by *in vivo* testing where it competed Amoxicillin at 1000 mg/kg by achieving 80% eradication of *Helicobacter pylori* in mucosa of infected rats justified by histological examination of stomach. It was concluded that medicinal plants possess strong anti-*Helicobacter pylori* activity and can be considered a potential source of safe and effective alternative regimens for the eradication of *Helicobacter pylori*.

Keywords: *Helicobacter pylori*,*in vitro*, *in vivo,* medicinal plants, gastric disorders.

INTRODUCTION

Helicobacter pylori, a gram negative, microaerophilic bacteria which is considered the most common pathogen of gastrointestinal tract since its discovery. Barry Marshall in 1982 and Robin Warren in 1984 assumed special significance in scientific world for the first isolation of *Helicobacter pylori* from the stomach of human (Asif & Akram, 2014; Benktander *et al*., 2018). The organism infects about half of the world population i.e. 50%. *Helicobacter pylori* infection is tremendously prevalent in developing countries and runs over 90% of their populations, on the other hand high living standards of developed countries (apart from Japan) has suppressed the infection rate below 40% and are trying to be further more (Holsonback, 2018). In Pakistan, non-invasive techniques revealed that 72.3% healthy children possess *Helicobacter pylori* in their stomach with negligible gender difference as 70.3% and 74.0% in girls and boys respectively. Recent data revealed that in 1-15 and 15-65 years of age, 47% and 92% of population in Pakistan is *Helicobacter pylori* positive, respectively (Mansori *et al*., 2020). The re-infection rate of *Helicobacter pylori* is less than 2% and found mostly in lower socioeconomic conditions (Stefano *et al*., 2018).

Conventional medicines have been used for times as eradication therapy for *Helicobacter pylori* with dual, triple or quadruple regimens by combining antibiotics, bismuth compounds, proton pump inhibitors and H_2 receptor antagonists. In spite of such effective therapies, the conventional medicine has to face 5-20% failure rate (Nguyen *et al*., 2019) with usual relapses most often due to incompliance of patient to the therapeutic scheme, life style and diet but the development of drug resistance and side effects of antibiotics remained the main stay (Fallone *et al*., 2019). Thus, there is a great need to look for alternative treatment options particularly traditional herbs commonly utilized for the management of gastrointestinal ailments. In this scenario studies has performed to evaluate medicinal herbs possessing excellent anti-*Helicobacter pylori* activity including *Mallotus philippinensis* Muell., *Curcuma longa* L., *Glycyrrhiza glabra* L., *Zingiber officinale* Rosc. and *Matricaria*

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chamomilla L. (Zaidi *et al.*, 2009; Asif & Akram, 2014).

The aim of this research work was to prove scientifically the efficacy of traditional medicinal plants such as *Matricaria chamomilla* L., *Foeniculum vulgare* Mill., *Trachesprmum ammi* L., *Mentha piperita* L., *Violo odorata* Linn., for the eradication of *Helicobacter pylori* as these medicinal plants are commonly prescribed for the management of gastrointestinal problems (Ahmed, 2016; Atinafu, 2016). Moreover, the study was conducted to provide safe, effective and rational alternative therapy with least side effects and having fine patient compliance against *Helicobacter pylori* infection.

MATERIALS AND METHODS

Plant material

The medicinal plants employed in this study *Mentha piperita* L., *Trachesprmum ammi* L., *Violo odorata* Linn., *Matricaria chamomilla* L., *Foeniculum vulgare* Mill. were purchased from local market of Rawalakot and identified by Dr. Tassaduq Hussain, Department of Botany, The University of the Poonch Rawalakot Azad Jammu & Kashmir, Pakistan. Voucher specimens were deposited for each plant.

Extract preparation

The dried plants were crushed to get fine powders. Powdered plants material 100g were sonicated twice with 200ml of aqueous ethanol for 48h at room temperature. The sample solutions were then filtered using Whatman No. 1 filter paper and followed by evaporation with the help of rotary evaporator under reduced pressure to get final volume of 100ml. Stock solutions were prepared to be used in disk diffusion test at 100mg/ml with sterile distilled water and for minimum inhibitory concentration (MIC) with Dimethylsulphoxide (DMSO). In culture media the concentration of DMSO remained below 1% (Hu *et al*., 2020).

Qualitative phytochemical analysis of extracts

The selected plants were analyzed qualitatively for carbohydrates, proteins, saponins, tannins, alkaloids, flavonoids, glycosides, phenols and steroids using the standard procedures for phytochemical screening (Mahmood *et al*., 2019).

Bacterial culture and suspension preparation

For *in vitro* study, aqueous ethanol plant extracts were tested against four clinical isolates of *Helicobacter pylori* i.e. FR515, PIMS519, BMT526 and UBT528. These *Helicobacter pylori* isolates were cultured from antral gastric biopsy specimens using Columbia blood agar (CM 0331B, Oxoid, UK) containing 7% laked horse blood (SR 0048, Oxoid, UK) in a moist microaerophilic atmosphere (Campylobacter gas generating kit, BR 0056A, Oxoid, UK) and *Helicobacter pylori* selective supplement dent (SR 0147E, Oxoid, UK). Bacterial suspensions were prepared by transferring fresh colonies of *Helicobacter pylori* isolates in tubes consisting of sterile physiological saline solution and turbidity was adjusted to 0.5 Mac-Farland's standard (1.5×10^8 CFU/ml).

In vitro anti-Helicobacter pylori activity

Disk diffusion test (Helicobacter pylori sensitivity test)

Freshly prepared pre-incubated blood agar plates were spread over by bacterial suspensions of each isolate. Disks of filter paper (6 mm) soaked with 50µl of stock solutions of each extract was placed over agar plates and incubated at 37°C in microaerophilic condition for 3-7 days. The antimicrobial activity was estimated by measuring inhibitory zone diameters (mm) around the disks (disk + zone) using digital patchy meter. To verify Amoxicillin (positive control) sensitivity of isolates concentration of 1µg/ml was prepared where phosphate buffer saline (PBS) was used as solvent.

Evaluation of minimum inhibitory concentration (MIC)

MIC was determined by using agar dilution method (Cai *et al*., 2014) for the extracts that showed minimum 6 mm inhibition zone. DMSO based stock solution of each extract was further diluted with distilled sterile water to make a volume of 900µl to get final concentrations of each extract in the mixture as 125, 250, 500 and 1000 µg/ml respectively and was added with 100µl of bacterial suspension with turbidity corresponding to 0.5 Mc-Farland's standard ($1.5x10^8$ CFU/ml). After incubation of 1h 100µl of this mixture was spreaded onto freshly prepared preincubated blood agar plates and incubated at 37°C under microaerophilic conditions for 3 days and the colonies formed were subsequently enumerated. To determine MIC which is defined as minimum concentration of plant extract that can completely inhibit the growth of bacteria, plants that showed complete inhibition at 125µg/ml were further subjected to the same procedure with two folds dilution i.e. 62 µg/ml and more if required (Lee *et al*., 2019). DMSO (100µl) was used as negative control. Amoxicillin (100µl) with a concentration of 1µg/ml contributed as standard drug or positive control for comparison. All the experiments were performed in duplicate.

In vivo anti-Helicobacter pylori activity

For *in vivo* study, male Wistar albino rats (160-210g) were used. The animals were kept in propylene cage in maintained environmental conditions at suitable temperature (21-25°C) and relative humidity (45-65%) with light and dark cycle of 12 h. Pellet feed (2 h/day) and purified water (free access) *ad libitum* was supplied except fasting period. Standard operation procedure (SOP) was used for all experiments performed on animals and approved by Ethical Committee and Directorate of Advanced Studies and Research, The University of the Poonch, Rawalakot, Azad Jammu & Kashmir.

Experimental design and dosage determination

Animals were divided in to seven groups each consist of five rats (n=5) for one plant extract that showed significant MICs against most sensitive strain by *in vitro* study. All the groups were administered by bacterial strain (1ml/mouse) (6×10^8 CFU/ml) except group-I i.e. normal control having no administration only distilled water (10ml/kg) was given to this group. Group-II was negative control inoculated with bacteria and ingested sterile distilled water (10ml/kg), group-III was positive control given standard drug i.e. Amoxicillin (50 mg/kg), group-IV, V, VI and VII contained animals that have delivered variable doses (1000, 500, 250 and 125 mg/kg) of plant extract, respectively. *Helicobacter pylori* strain at 1 ml dose (2-Mc-Farland) was inoculated intragastric using orogastric cannula two times per day at two days interval, simultaneously 1000, 500, 250 and 125 mg/kg of extracts were given orally twice a day for seven days.

Detection of active infection induced by Helicobacter pylori

To determine *Helicobacter pylori* infection animals were slaughtered under deep anesthesia induced by ether after 03hrs of last administration. The incision was made from xiphoid process on median abdominal line and biopsy samples (3x3cm) were collected from gastric mucosa which were minced, applied to recently prepared rapid urease test (RUT) vile and incubated at 37ºC for 24h to examine urease activity for confirmation of active infection. The color change determined urease activity i.e. negative for bright yellow, false (partially) positive for thick yellow, r positive for red or pink (Skrebinska *et al*., 2018).

Histopathological analysis

After removing the stomach of animals, half of each stomach was fixated in 10% buffered formalin. 5µm sections were made for histopathology block preparation and were stained by a modified Giemsa stain for *Helicobacter pylori* detection. All tissues were microscopically examined to analyze parameters such as score of *Helicobacter pylori* presence in tissues of gastric mucosa.

STATISTICAL ANALYSIS

All results are expressed as Mean±SEM, (n=6). Statistical analyses were performed by using SPSS version 18, with one way analysis of variance (ANOVA) followed by Tukey-Kramer test. Fisher's exact test was used for frequency comparison and $P< 0.05$ was considered to be statistically significant.

RESULTS

Qualitative Phytochemical Analysis

Phytochemical analysis revealed the presence of phytoconstituent given in table 1.

Results of in vitro anti-Helicobacter pylori testing

Disk diffusion test

The *Mentha piperita* L. exhibited larger inhibition zone compared to other four extracts but isolate PIMS519 found most sensitive i.e. 9 mm. (table 2)

In vitro MIC evaluation

All the plants showed variable MICs for different isolates. The strongest *in vitro* anti-*Helicobacter pylori* activity was depicted by *Mentha piperita* L. which absolutely arrested the growth of bacteria at 125, 250, 500 µg/ml (table 3, fig. 1).

In vivo anti-Helicobacter pylori activity

Most significant *in vitro* anti-*Helicobacter pylori* activity was reported for aqueous ethanol extract of *Mentha piperita* L. with highest sensitivity displayed by *Helicobacter pylori* isolate PIMS519. Therefore, for further evaluation *in vivo,* study was performed. Seven groups of orally inoculated rats with *Helicobacter pylori* isolate PIMS519 were examined histologically for presence of bacteria in mucosal epithelial cells of gastrum after intra-gastric administration of aqueous ethanol extract of *Mentha piperita* L. at concentrations of 1000, 500, 250 and 125 mg/kg (body weight). Rapid urease test performed for detection of active infection induced by isolate was negative in group-I animals because they were deprived of isolate inoculation and working as normal control whereas group-II to VII showed positive results indicated by reddish pink discoloration in RUT after incubation of biopsy samples for 24h at 37 ºC (Figure 2).

DISCUSSION

The phytochemical analysis performed in current study revealed the presence of many active chemical compounds in plants where sufficient amount of phenolic compounds are reported in all plants extracts that are suspected to be responsible for bactericidal activity of these plants. Such activities of phenolic phytochemicals are evident from a study executed by Zhang *et al*. (2019). The mechanism of action of phenols and polyphenols is to inhibit the bacterial enzymes activity most probably via reaction sulfhydryl or nn-specific interaction with proteins by oxidized compounds. Other constituents are also proved to have definite mechanisms for bactericidal actions by various studies such as flavonoids has the capability to form complex with proteins found in bacterial cell (Oteiza *et al*., 2018). More over tannins are reported to disable the bacterial proteins i.e. adhesins, transport proteins of cellular envelop and down regulate the enzymes efficiency (De Lima *et al*., 2018). Tannins are also found in large amount in plants analyzed by current study where *Violo odorata* Linn. and *Trachesprmum ammi* L. are rich in flavonoids although present in all studied plants. The plants under study are part of our food and commonly used as flavouring and

aromatic constituents that make their use easy and also prevent the infection by discouraging the growth and acquisition of *Helicobacter pylori*.

After initial screening, *Helicobacter pylori* exhibited no resistance for plants extracts. By disk diffusion method all *Helicobacter pylori* isolates showed sensitivity for plant extracts by variable diameters of clear zone around each disk. There was no isolate found to be resistant for antibiotic Amoxicillin, all the extracts inhibited bacterial growth at a concentration and were active against *Helicobacter pylori* isolates.

In agar dilution method, results showed different values of MIC for same plant extracts. The variation in MICs of individual herb for different isolates is assumed to be due

Table 1: Qualitative phytochemical analysis of aqueous ethanol extracts

	Violo odorata Linn.	*Mentha piperita* L.	*Matricaria chamomilla* L.	*Trachesprmum ammi* L.	*Foeniculum Vulgare* Mill.
Carbohydrates	-	+	-	-	-
	-	+	-	-	-
	+	+	+	+	+
Flavonoids	+	+	+	+	+
	++	+	+	++	+
Protein	++	++	++	++	++
Glycosides	+	+	+	+	++
	++	++	++	+	+
Alkaloids	+	+	-	+	+
Saponins	+	-	+	-	+
Steroid	++	-	+	-	+
Phenols and Tannins	++	++	++	++	++

(-) absent; (+) moderately present; (++) significantly present

Table 2: Sensitivity zone diameter (mm) of plant extracts against *Helicobacter pylori* isolates

Plant extracts (50µl)	FR515	PIMS519	BMT526	UBT528
Mentha piperita L.	8	9	7	8
Foeniculum vulgare Mill.	8	6	7	6
Trachesprmum ammi L.	8	7	6	7
Matricaria chamomilla L.	6	7	6	6
Violo odorata Linn.	7	7	6	6
Amoxicillin	10	8	7	9

Table 3: *In vitro* MIC (µg/ml) of aqueous ethanol extracts of five medicinal plants against *Helicobacter pylori* isolates

Medicinal Plants	*Helicobacter pylori* isolates			
	FR515	PIMS519	BMT526	UBT528
Mentha piperita L.	250	125	500	250
Trachesprmum ammi L.	125	250	1000	250
Foeniculum vulgare Mill.	125	500	250	1000
Violo odorata Linn.	250	250	>1000	500
Matricaria chamomilla L.	>1000	125	250	500
Amoxicillin	01	01	01	01

Table 4: *In vivo* histological identification of *Helicobacter pylori* after ingested isolate PIMS519 followed by treatment with *Mentha piperita* L. and Amoxicillin

Group	Experimental groups	Dosage (mg/kg)	Positive ratio (Bacterial presence)	Percentage (%)
I	Normal control (Distilled water only)	10ml/kg	0/5	0
II	Negative control (Distilled water)	10ml/kg	5/5	100
III	Positive control (Amoxicillin)	50mg/kg	1/5	20
IV	*Mentha piperita* L.	1000mg/kg	1/5	20
V	*Mentha piperita* L.	500mg/kg	2/5	40
VI	*Mentha piperita* L.	250mg/kg	2/5	40
VII	*Mentha piperita* L.	125mg/kg	3/5	60

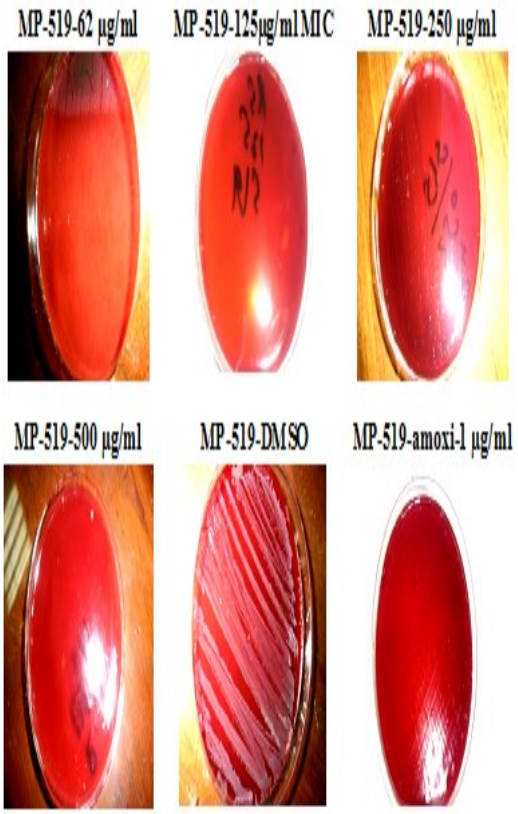

Fig. 1: Agar plates showing MIC for *Mentha piperita* L. (MP) against *Helicobacter pylori* isolate PIMS519

to variable virulence and genetic factors of bacteria as well as host from which the *Helicobacter pylori* were isolated. *Helicobacter pylori* isolate PIMS519 showed most significant MICs for plant extracts.

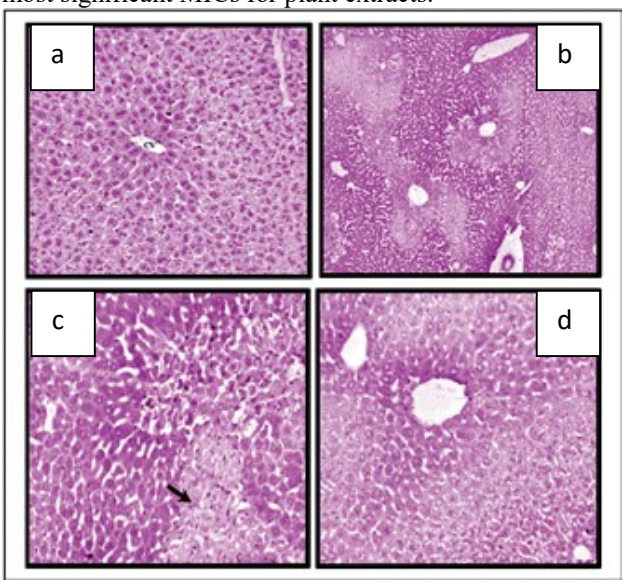

Fig. 2. a: Tissue changes in control group, b: tissue changes in standard (Amoxicillin treated) group, c: tissue changes in test group (*Mentha piperita* pre-treated), d: recovery phase of test group. The findings reported for *Violo odorata* Linn. in current study were much significant than previous study where agar well dilution method is used by Gautam *et al.* (2012) to evaluate antibacterial activity of methanol extract of Sweet violet against respiratory tract pathogens *Haemophilus influenzae, Streptococcus pneumonia, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus*, MIC remained at 6.25, 12.5 and 3.25mg/ml respectively.

The current study revealed significant inhibitory zone of 7-9mm and excellent anti-*Helicobacter pylori* activity at concentration of 125µg/ml that is significant MIC among all plants extracts for different isolates. *Helicobacter pylori* exhibited no resistance for extracts as well as tested in present study. Whereas, the resistance is reported in previous studies for Amoxicillin and Clarithromycin (Bluemel *et al*., 2020). The extracts showed improved results (125µg/ml) than earlier study conducted on garlic powder that inhibited growth at 250µg/ml (Loolaie *et al*., 2017). Furthermore, it is expected that the MICs recorded in present study can be reduced to further lower values by applying particular isolated compounds detected and obtained by compound isolation of plants.

CONCLUSION

The study justified the presence of chemical constituents possessing anti-*Helicobacter pylori* activities through phytochemical analysis. These plants yield fabulous anti-*Helicobacter pylori* activities that provided an alternative way towards eradication and safe and cost effective treatment of associated diseases of *Helicobacter pylori*.

ACKNOWLEDGEMENT

The authors are thankful to Dean, Faculty of Medical & Health Sciences, The University of Poonch, Rawalakot, AJ&K for providing assistance to carry out this research work.

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