International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 1, 2016

Original Article

THERAPEUTIC POTENTIAL OF LICHEN *PARMELIA PERLATA* AGAINST DUAL DRUG-RESISTANT *HELICOBACTER PYLORI* ISOLATES

VALENTINA GEHLOT[1], SHWETA MAHANT[1], POOJA VIJAYRAGHWAN[1], KUNAL DAS[2,3], SHANU HODA[1], RAJASHREE DAS[1]*

[1]Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh, India, [2]Max Superspeciality Hospital, Patparganj, New Delhi, India, [3]Yasodha Superspeciality Hospital, Ghaziabad, Uttar Pradesh, India
Email: rajashreepatra79@yahoo.co.in

Received: 13 Oct 2015 Revised and Accepted: 18 Nov 2015

ABSTRACT

Objective: *H. pylori* have acquired resistance to the commonly used antibiotics due to their use in many anaerobic and parasitic infections leading to treatment failure of various gastroduodenal diseases associated with *H. pylori* infection. Our aim was to test the efficacy of *Parmelia perlata* which is traditionally used for the treatment of dysentery, diarrhea, and dyspepsia against antibiotic resistant gastric pathogen *H. pylori*.

Methods: The antimicrobial activity of an ethanolic and methanolic extract of *P. perlata* against drug-resistant *H. pylori* isolates from North India *in vitro* was carried out by determining the Minimum inhibition concentration (MIC) using disk diffusion method and microdilution method.

Results: Two *H. pylori* isolates were included in this study. One was resistant to both metronidazole (MIC of 64 µg/ml) and clarithromycin (MIC of 4 µg/ml) and another one was resistant to metronidazole only having a MIC of 64 µg/ml. Methanolic and ethanolic extract of *P. perlata* showed its effectiveness in inhibiting drug resistant *H. pylori* isolates with maximum inhibition at 500 × 10^3 µg/ml concentration of *P. perlata*.

Conclusion: Prevalence of metronidazole resistant ranges between 50–90% in developing countries including India with the emergence of dual-drug resistance was reported in many studies. This study suggests that *P. perlata* used commonly as a spice in food has a potential for the treatment of drug-resistant *H. pylori* infection in a safe and effective manner.

Keywords: *Helicobacter pylori, Parmelia perlata*, Dual-drug resistance.

INTRODUCTION

Helicobacter pylori are a gastric pathogen and are associated with several gastroduodenal diseases such as chronic gastritis, peptic ulcer, and gastric cancer [1-3]. More than one-half of world's population carries the *H. pylori* infection and in India its prevalence exceeds 90% of the population. Eradication of *H. pylori* from infected individuals remains the best choice for an effective treatment of *H. pylori*-associated diseases. Triple therapy, consisting of the combination of two antibiotics and a proton pump inhibitor, gives a high eradication rate [4]. The common antibiotics used against *H. pylori* are Metronidazole, Clarithromycin, Furazolidone, Amoxicillin, and Tetracycline. Misuse of antibacterial agents in general population for various respiratory and anaerobic infections have resulted in the emergence of antibiotic-resistant strains which is the prime cause of treatment failure apart from potential side effects [5, 6]. Metronidazole and clarithromycin are the most commonly used antibiotics in *H. pylori* treatment. Prevalence of resistance against metronidazole has been found to be high in many studies [4, 7, 8].

The emergence of dual and multidrug resistance has also increased significantly in many countries and has become a major obstacle in eradicating the *H. pylori* infection [8-11]. In view of the incomplete cure achieved with a conventional triple therapy comprising metronidazole and clarithromycin, there is an urgent need to develop new treatment strategies for *H. pylori* infection.

Therefore, in such a scenario, there is a crucial need to find new antimicrobial agents. Unlike synthetic drugs, bioactive natural products are beneficial for the humans without causing unwanted side-effects. In this search lichens are the new bioactive preparations of natural origin. Lichens produce secondary metabolites the "lichen substances", which comprise depsides, depsidones, dibenzofurans, xanthones and terpene derivatives. Lichens and their metabolites have multiple biological activities: antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory, anti-herbivore, ecological roles and enzyme inhibitory [12, 13]. *Parmelia perlata,* commonly called as the stone flower is generally used as a spice to enhance the taste and flavor of the foods. *P. perlata* is lichen that is widely distributed in the hilly areas of the Indian subcontinent. In ancient folklore it has been used in cosmetic and has been reported to have medicinal value. It was used for the treatment of dysentery, wound healing, diarrhea, and dyspepsia [14, 15].

Studies have been conducted using different extracts of *P. perlata* against various bacterial and fungal pathogens. It contains many compounds like tridecyl myristate, 3-ketooleanane, icosan-1ol etc. [16]. Recent studies have shown that methanolic extract of Lichen possesses anti-microbial potential [17]. Our aim is to study the antimicrobial activity of the methanolic and ethanolic extracts of the lichen *P. perlata* against dual-drug resistant *H. pylori* in safe and effective manner.

MATERIALS AND METHODS

Collection of sample

P. perlata was collected from the local Indian market and authenticated in the Botany Department of Amity University. The plant was properly washed under running tap water and then rinsed in distilled water. Then it was air dried in shade and grinded into powder using mortar and pestle.

Preparation of extracts

In order to obtain the extract, about 100 gms of *P. perlata* were crushed with mortar and pestle and sieved. The dried powder was then extracted with 400 ml n-Hexane, chloroform, Methanol and Ethanol consecutively for 72 h/solvent under constant stirring. The extract was then filtered and dried under pressure and resuspended in the solvent. Methanolic and ethanolic extract of *P. perlata* were used in the present study.

H. pylori strains and culture

Patients suffering from various gastroduodenal diseases were included in this study. *H. pylori* strains were isolated from antral biopsies collected from patients and were identified on the basis of colony appearance, gram staining, and positive reactions in biochemical tests (catalase, urease, and oxidase). *H. pylori* strain was isolated and cultured on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) supplemented with 5% horse serum (Invitrogen, NY), 0.4% Isovitale X (Becton Dickinson, MD), trimethoprim (5µg/ml), vancomycin (8µg/ml), and polymixin B (10µg/ml). The plates were incubated at 37 °C in a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) (Double gas incubator, Hera cell 150i) for 3 to 6 d. Stock cultures were maintained until use at– 70 °C in Brain heart infusion broth with 20% glycerol.

Suspension preparation

The bacterial suspension was prepared by the direct colony method [18]. The colonies were taken directly from the plate and were suspended in 5 mL of sterile 0.85% phosphate buffer saline (PBS). The turbidity of the initial suspension was adjusted by comparing with McFarland's standard number 2 (3 µl of which contains about 1 $\times 10^8$ colony forming units (CFU)/ml) [19].

Determination of antimicrobial susceptibility and resistance

H. pylori cells growing exponentially on antibiotic free BHI agar were suspended in Phosphate buffered saline (PBS) buffer, a series of 10-fold dilutions of these cell suspensions was prepared, and 10 µl of each dilution was spotted on freshly prepared BHI agar containing various concentration of different antibiotics (µg/ml) viz. Metronidazole (0.2, 0.5, 1.5, 3, 8, 16, 32, 64), Clarithromycin (0.125, 025, 1, 2), Furazolidone (0.2, 0.5, 1, 2), Amoxicillin (0.125, 0.25, 1, 2) and Tetracycline (1, 2, 3, 4).

Minimum inhibitory concentration (MIC)

After 72 h incubation under microaerophilic conditions, the minimal inhibitory concentration was recorded as the lowest concentration that inhibited visible growth of organisms. Minimal inhibitory concentration (MIC) for different antibiotics was defined as Metronidazole (>8 µg/ml) [20], Clarithromycin (>0.5 µg/ml) [20], Amoxicillin (>0.12 µg/ml) [20], Tetracycline (>1 µg/ml) [20] and Furazolidone (>2 µg/ml) [7].

Anti- _H. pylori_ activity assay of _P. perlata_ extracts

(a) Disk diffusion method

Sterile Whatman paper disks (6 mm in diameter) were loaded with different concentration of methanolic and ethanolic extracts of *P. perlata* and placed on the inoculated plates with 3 $\times 10^9$ colony forming unit (CFU) of *H. pylori*. The plates were kept under observation for 2 d at 37° C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). All experiments were performed in triplicates. Pure methanol and ethanol were used as a negative control and amoxicillin were used as positive control.

(b) Micro-dilution method

Minimum inhibitory concentration (MIC) was also determined by microdilution method. The 96-well plate was prepared by dispensing 80 µl of BHI broth into first well. A 20 µl from the stock solution of lichen extract (5×10^4 µg/ml) was added to the first well. Then, five-fold serial dilution was performed till 8th well. The obtained concentration range was from 4000 µg/ml to 0.05 µg/ml. To each well 150 µl of the diluted bacterial cells were added to give final concentration of 3×10^9 CFU/ml. The inoculated plates were incubated at 37 ° C for 2 d at microaerophilic conditions. MIC_{90} was defined as the lowest concentration of extract sample that inhibited the 90 % of *H. pylori* cells when to compare to control i.e. *H. pylori* cells without extract sample. Reading was noted on Elisa reader (Erba Lisa Scan II).

Rapid urease test (RUT) was also done for visualization of microbial growth. The basis of the test is the ability of *H. pylori* to secrete urease enzyme which hydrolyzes the urea to ammonia and carbon dioxide and raises the pH of the medium from yellow (*H. pylori* negative) to pink color (*H. pylori* positive). MIC was defined as the lowest concentration of extract sample that prevented RUT medium color change from yellow to pink (fig. 2).

RESULTS

Two clinical *H. pylori* isolates were included in this study isolated from a patient suffering from Non-erosive reflux disease (NERD) and Gastroesophageal reflux disease (GERD). The MIC of *H. pylori* isolate for metronidazole, clarithromycin, furazolidone, amoxicillin and tetracycline were determined by agar dilution method. Out of the two strains one strain was found resistant to metronidazole only having MIC of 64 µg/ml but sensitive to all drugs namely clarithromycin, furazolidone, amoxicillin, and tetracycline having MIC of 0.125 µg/ml, 0.2 µg/ml, 0.125 µg/ml and 1 µg/ml respectively and another strain was resistant to both clarithromycin and metronidazole having MIC of 4 µg/ml and 64 µg/ml respectively but sensitive to furazolidone, amoxicillin, and tetracycline with MIC of 2 µg/ml, 0.125 µg/ml and 1 µg/ml respectively.

Antimicrobial activity

Disc diffusion method

Different concentration of methanolic and ethanolic extract of *P. perlata* was loaded onto the disc and was air dried. 100 µl of the suspended *H. pylori* strain in PBS having Mac Farland 2 (3×10^9 CFU/ml) was spread plated onto BHI medium. The disc loaded with the extracts was placed upside down in the *H. pylori* plates (fig. 1). After 72 h of incubation, we found that the methanolic and ethanolic extract of *P. perlata* has anti-*H. pylori* activity. MIC of *P. perlata* was < 50×10^3 µg/ml (table 1). Among the different concentration tested for both the extracts 500×10^3 µg/ml displayed maximum inhibition against the drug resistant *H. pylori* strain. The diameter of the zone of inhibition was 15 mm for the methanolic extract of *P. perlata*. (table 1)

(a) **(b)**

Fig. 1: Disk diffusion method: Antimicrobial activity of _P. perlata_ extracts against metronidazole-resistant _H. pylori_ strain (a) Methanolic extract of _P. perlata_. (b) Ethanolic extract of _P. perlata._

Table 1: Antimicrobial activity of methanolic and ethanolic extract of *Parmelia perlata* against drug-resistant *H. pylori* strains

Strain No.	MIC for MTZ* and CLR* (Agar dilution method)	Ethanolic extracts of *P. perlata* (Disk diffusion method)		MIC_{90} for *P. perlata* (micro-dilution method)	Methanolic extracts of *P. perlata* (Disk diffusion method)		MIC_{90} for *P. perlata* (microdilution method)
		Concentration (in µg/ml)	Zone of inhibition (in mm)[a]		Concentration (in µg/ml)	Zone of inhibition (in mm)[a]	
HP 1	MTZ= 64µg/ml CLR= 4 µg/ml	50 × 10^3 250 × 10^3 500 × 10^3	8.0±0.1 10.0±0.5 14.0±0.2	800 µg/ml	50 × 10^3 250 × 10^3 50 × 10^3	10.0±0.0 12.0±0.1 15.0±0.0	32 µg/ml
HP 2	MTZ= 64µg/ml CLR= <0.125µg/ml	50 × 10^3 250 × 10^3 500 × 10^3	8.0±0.0 11.0±0.1 13.0±0.5	800 µg/ml	50 × 10^3 250 × 10^3 500× 10^3	8.0±0.1 13.0±0.0 15.0±0.5	32 µg/ml

[a]= mean±SD (n = 3), MTZ = Metronidazole, CLR = Clarithromycin, GERD = Gastro-esophageal reflux disease, NERD = Non-erosive reflux disease

Microdilution method

The antimicrobial activity of lichen extract was evaluated by microdilution method. The MIC was determined in two drugs resistant clinical *H. pylori* isolates. MIC values ranged between 4000 µg/ml to 0.05 µg/ml (fig. 2). MIC_{90} values of a methanolic and ethanolic extract of *P. perlata* were 0.1 µg/ml and 31.3 µg/ml respectively against *H. pylori* isolate resistant to both metronidazole and clarithromycin.

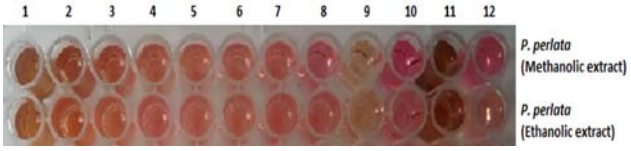

Fig. 2: Micro-dilution method: lane 1 to 8 shows the Five-fold serial dilution of lichen extract from lane 1 to lane 8 with a constant cell count of *H. pylori*. Lane 9 shows media control without cells. Lane 10 shows the cell control without lichen extract. Lane 11 shows extract control without cells. Lane 12 shows the control in which the lichen extract dissolved with cells. MIC_{90} was observed at lane 4 and Lane 2 for methanolic and ethanolic extract of *P. perlata* respectively

DISCUSSION

Due to increasing use of antibiotics over the last two decades, *H. pylori* have acquired antimicrobial resistance and are the main cause of treatment failure. Previous studies have shown that the prevalence of resistance to metronidazole in different parts of India were Delhi (37.5%) [21], Chandigarh (38.2%) [21], Lucknow (68%) [21], Gangetic belt of North India (100%)[22]Hyderabad (100%) [21] Chennai (88.2%) [21], Kolkata (85%) [7] and Gujarat (83.8%) [23]. High prevalence of metronidazole-resistant *H. pylori* has also been reported from other developing countries (China, 77.8%; Bangladesh, 77.8%; Mexico, 76.3%) [24, 25]. In India, the high prevalence of multidrug-resistant strain was observed in (Multicentric study in India, 43.2%; Gujarat, 85%)[21, 23]. Our recent studies have shown the prevalence of Dual and multidrug resistance in 26.5% (18/68) and 8.9% (6/68) of cases in India [8]. The high prevalence of dual drug resistant *H. pylori* isolates has prompted us to look into the natural products which can be effective against *H. pylori*.

Anti- *H. pylori* potential of many plant and plant products and their usefulness for treatment of *H. pylori* infection has been studied. De *et al.* has mentioned the therapeutic potential of curcumin against *H. pylori*-associated gastroduodenal diseases and have shown eradication of *H. pylori* from the infected mouse stomach [26]. Similarly, our previous study has shown that ethanolic extract of *Embilica officinalis* can effectively treat *H. pylori*-associated gastric ulcer [27]. We have also found that methanolic extract of *Paedra foetida* was effective in inhibiting the growth of metronidazole-resistant *H. pylori* strain *in vitro* [28]. In India, *Parmelia* is used in treating a number of diseases and as a food supplement which is mentioned in Indian Materia medica [29]. Previous studies showed its antimicrobial activity against different bacteria namely *Clavibacter michiganensis*, *Pseudomonas solanacearum* and fungi like *Fusarium oxysporum* and *Rhizopus nigricans* [29]. Antimicrobial properties of different lichens have also been tested against different pathogenic bacterial and fungal strains. Tippeswamy *et al.* used hot and cold extracts of the lichen in various solvents and tested them against the variety of bacterial and fungal pathogens [29]. Gulluce *et al.* found that methanolic extract of the lichen *Parmelia saxatilis* has stronger antibacterial property as compared to antifungal activity [30].

We have included dual drug-resistant *H. pylori* strain in this study. Both the methanolic and ethanolic extracts of *P. perlata* were studied. In the present study, we found that methanolic and ethanolic extract of *P. perlata* potentially inhibited the growth of the *H. pylori* isolates *in vitro* that were isolated from the infected patient suffering from NERD and GERD. It is noteworthy that one of the *H. pylori* strain (HP1) was resistant to both clarithromycin and metronidazole and another strain (HP2) was resistant to metronidazole only which is the important component used in the first line therapy for the *H. pylori* treatment. So, our results suggest that methanolic and ethanolic extract of *P. perlata* acts through mechanisms distinctly different from the mode of action of these antibiotics for inhibition of *H. pylori* growth.

Further research needs to be done to determine the compounds that are responsible for antibacterial activity against *H. pylori*. The findings also indicate that scientific studies carried out on medicinal plants having traditional claims of effectiveness [31, 32] can give fruitful results.

ACKNOWLEDGEMENT

We thank Amity University for providing the infrastructure and support to carry out the work.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Correa P. Human gastric carcinogenesis, a multistep and multifactorial process-first American cancer society award lecture on cancer epidemiology and prevention. Cancer Res 1992;52:6735-40.
2. NIH consensus conference. *Helicobacter pylori* in peptic ulcer disease. NIH consensus development panel on *Helicobacter pylori* in peptic ulcer disease. JAMA 1994;272:65-9.
3. Parsonnet J. *Helicobacter* and gastric adenocarcinoma. In: Parsonnet J. editor. Microbes and Malignancy. Infection as a cause of Human cancers. New York, NY: Oxford University Press; 1999.

4. Toracchio S, Cellini L, Di Campli E, Cappello G, Malatesta MG, Ferri A, *et al.* Role of antimicrobial susceptibility testing on efficacy of triple therapy in *Helicobacter pylori* eradication. Aliment Pharmacol Ther 2000;14:1639-43.
5. Bytzer P, O' Morain C. Treatment of *Helicobacter pylori.* Helicobacter 2005;10:40-6.
6. Cameron EA, Powell KU, Baldwin L, Jones P, Bell GD. *Helicobacter pylori*: antibiotic resistance and eradication rates in Suffolk, UK, 1991-2001. J Med Microbiol 2004;53:535-8.
7. Datta S, Chattopadhyay S, Patra R, De R, Ramamurthy T, Hembram J, *et al.* Most *Helicobacter pylori* strains of Kolkatta in India are resistant to metronidazole but susceptible to other drugs commonly used for eradication and ulcer therapy. Aliment Pharmacol Ther 2005;22:51-7.
8. Das R, Gehlot V, Mahant S, Mukhopadhyay AK, Das K. High prevalence of single, dual, and multidrug-resistant *Helicobacter pylori* infection in India. *Helicobacter* 2015;20(Suppl 1):P03.18.
9. Miyazi H, Azuma T, Ito S, Suto H, Ito Y, Yamazaki Y. Susceptibility of *Helicobacter pylori* isolates to metronidazole, clarithromycin, and amoxicillin *in vitro* and in clinical treatment in Japan. Aliment Pharmacol Ther 1997;11:1131-6.
10. Megraud F. Antibiotic resistance in *Helicobacter pylori* infection. Br Med Bull 1998;54:207-16.
11. Xia HX, Keane CT, O' Morain CAA. 5 y survey of metronidazole and clarithromycin resistance in clinical isolates of *Helicobacter pylori*. Gut 1996;39 Suppl 2):A67.
12. Karagoz A, Dogruoz N, Zeybek Z, Aslan A. Antibacterial activity of some lichen extracts. J Med Plants Res 2009;3:1034-9.
13. Kosanić M, Ranković B. Antibacterial and antifungal activity of different lichens extracts and lichen acid. Res J Biotechnol 2011;6:23-6.
14. Kiritikar KR, Basu BD. Indian medicinal plants. Ed 3. Lalit Mohan Basu MB. Allahabad; 1996. p. 2757.
15. Hussain M, Raza SM, Farooq U, Bakhsh H, Majeed A, Aziz A. *In vitro* antimicrobial potential of Lichen (*Parmelia perlata*) against different pathogenic microbes. Int J Pharm Sci 2014;4:666-70.
16. Sharma AK, Sharma MC, Dhobal MP. Phytochemical investigation of therapeutic important lichen: *Parmelia perlata*. J Nat Prod Plant Resour 2012;2:101-6.
17. Mitrović T, Stamenković S, Cvetković V, Tošić S, Stanković M, Radojević I, *et al.* Antioxidant, antimicrobial and antiproliferative activities of five lichen species. Int J Mol Sci 2011;12:5428-8.
18. Andrews JM. BSAC working party on susceptibility testing. BSAC standardized disc susceptibility testing method. J Antimicrob Chemother 2005;56:60-76.
19. Wayne DB, McGaghie WC. Use of simulation-based medical education to improve patient care quality. Resuscitation 2010;81:1455-6.
20. EUCAST. Breakpoint tables for interpretation of MICs and Zone Diameters. Version 3.1; 2013.
21. Thyagarajan SP, Ray P, Das BK. The geographical difference in antimicrobial resistance pattern of *Helicobacter pylori* clinical isolates from Indian patients: a multicentric study. J Gastroenterol Hepatol 2003;18:1373-8.
22. Singh V, Mishra S, Maurya P, Rao G, Jain AK, Dixit VK, *et al.* Drug resistance pattern and clonality in *H. pylori* strains. J Infect Dev Countries 2009;3:130-6.
23. Pandya HB, Agravat HH, Patel JS, Sodagar NRK. Emerging antimicrobial resistance pattern of *Helicobacter pylori* in central Gujarat. Indian J Med Microbiol 2014;32:408-13.
24. Wu H, Shi XD, Wang HT, Liu JX. The resistance of *Helicobacter pylori* to metronidazole, tetracycline and amoxycillin. J Antimicrob Chemother 2000;46:121–3.
25. Nahar S, Mukhopadhyay AK, Khan R, Ahmad MM, Dutta S, Chattopadhyay S, *et al.* Antimicrobial susceptibility of *Helicobacter pylori* strains isolated from Bangladesh. J Clin Microbiol 2004;42:4856-8.
26. De R, Kundu P, Swarnakar S, Ramamurthy T, Chowdhury A, Nair GB, *et al*. Antimicrobial activity of curcumin against *Helicobacter pylori* isolates from India and during infections in mice. Antimicrob Agents Chemother 2009;53:1592-7.
27. Mehrotra S, Jamwal R, Shyam R, Meena DK, Mishra K, Patra R, *et al.* Anti-*Helicobacter pylori* and antioxidant properties of *Emblica officinalis* pulp extract: a potential source for therapeutic use against gastric ulcer. J Med Plants Res 2011;5:2577-83.
28. Chanda S, Gehlot V, Das R, Mahant S, Das K, Singh K, *et al*. Antimicrobial activity of herbal extract against drug-resistant *Helicobacter pylori* isolates from India. World J Pharm Res 2014;3:1234-43.
29. Tippeswamy B, Sushma NR, Naveen Kumar KJ. Evaluation of antimicrobial property of lichen *Parmelia perlata*. Afr J Pharm Pharmacol 2013;7:1242-50.
30. Gulluce M, Aslan A, Sokeman M, Sahin F, Adiguzel A, Agar G, *et al.* Screening the antioxidant and antimicrobial properties of the lichens *Parmelia saxatilis*, *Platismatia glauca*, *Ramalina pollinaria*, *Ramalina polymorpha* and *Umbilica rianylanderiana*. Phytomedicine 2006;13:515-21.
31. Ameenah GF. Medicinal plants: the tradition of yesterday and drugs of tomorrow. Mol Asp Med 2006;27:1-93.
32. Shanmugam S, Rajendran K, Suresh K. Traditional uses of medicinal plants among the rural people in Sivagangai district of Tamil Nadu, Southern India. Asian Pac J Trop Biomed 2012;2:S429-S434.