

ANTIBIOGRAM PROFILING OF *HELICOBACTER PYLORI* STRAINS AND THE EFFICACY OF *BRASSICA CAPITATA* AGAINST RESISTANT STRAINS ISOLATED FROM THE PATIENTS SUFFERING FROM GASTRODUODENAL DISEASES IN GUWAHATI, ASSAM

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ABSTRACT

Objective: *Helicobacter pylori* resistance toward commonly used antibiotics is increasing leading to the treatment failure; hence, our aim is to determine the antibiogram susceptibility pattern of *H. pylori* strains isolated from Guwahati, Assam (Northeast India) and also to test the efficacy of the *Brassica capitata* against the multi and dual drug-resistant strains of North and Northeast India.

Methods: Minimum inhibitory concentration of different antibiotics was determined by agar dilution method. Disc diffusion method was used to check the efficacy of *B. capitata* against clarithromycin (CLR), metronidazole (MTZ), and levofloxacin (LEV)-resistant *H. pylori* strains.

Results: All the *H. pylori* strains were 100% sensitive to CLR, tetracycline, amoxicillin, and furazolidone. 72.8% of the strains were sensitive toward MTZ and 54.5% were sensitive toward LEV. *B. capitata* showed good efficacy against the resistant strains of *H. pylori* of North and Northeast India.

Conclusion: Most of the *H. pylori* strains from Northeast India were sensitive toward the commonly used antibiotics for the treatment regime. *B. capitata* is effective against *H. pylori* infection, suggesting its potential as an alternative therapy, and opens the way for further studies on identification of novel antimicrobial targets of *B. capitata*.

Keywords: *Helicobacter pylori*, Antibiotic resistance, *Brassica capitata*.

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INTRODUCTION

Helicobacter pylori is a Gram-negative microaerophilic gastric pathogen causing various gastroduodenal (GI) diseases such as gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric cancer. It is classified as Class I carcinogen by IARC in 1994 [1]. Its infection is present in more than half of the population worldwide [1-5] and to almost 80% of the Indian population [6]. Indian population is an amazing combination of diverse races and cultures; thus, its inhabitants also differ in their genetic traits. Till date, it was believed that dietary factors are responsible for high prevalence of such GI diseases in the Northeast region of India because of its geographically, culturally, and ethnically divergent population [7]. *H. pylori*-associated various GI diseases can be cured by proper eradication therapy which includes proton-pump inhibitor-based triple therapy with multiple antibiotics. The commonly used antibiotics are clarithromycin (CLR), amoxicillin (AMX), metronidazole (MTZ), furazolidone (FZ), tetracycline (TET), and levofloxacin (LEV) [8,9]. Use of the antibacterial agents in general population for curing the various respiratory and anaerobic infections has resulted in the emergence of antibiotic-resistant strains of *H. pylori* which is the prime cause of treatment failure. Resistance to various antibiotics such as MTZ (10–90%), CLR (0–15%), FZ (0–13%), TET (1–2%), and AMX (0–1%) has been shown to be variable and dissimilar in various regions within the countries [10–14]. The prevalence of dual resistance and multidrug resistance has increased significantly in many countries and has become a major obstacle in eradicating the *H. pylori* infection [15]. For these reasons, the natural products can be an alternative to the commonly used drugs for the eradication of *H. pylori*.

Nature is a rich source of numerous natural resources which can have the beneficial effects such as anti-inflammatory, antidiabetic, and antioxidative [16]. It was observed and confirmed that diet rich in vegetables and fruits reduced the risk of having several chronic disorders and immune diseases. *Brassica capitata* (cabbage) is considered to be one of the natural gifts that are herbaceous and leafy plant belonging to the family of *Brassicaceae*, having anti-inflammatory property and is generally used in cooking [17,18].

Against this background, the present study has been conducted (1) to study the antimicrobial susceptibility pattern of *H. pylori* strains from Northeast India and (2) to evaluate the efficacy of *B. capitata* as an antimicrobial agent against the multi and dual drug-resistant strains of *H. pylori* isolates from North and Northeast India.

METHODS

Patients and specimens

A total of 98 biopsies were collected from the patients having an average age of 42.6 years and M:F ratio of 1:0.46 visiting Gauhati Medical College, Guwahati, Assam, with clinical symptoms of gastrointestinal disorders and underwent endoscopy. Samples were collected as per the inclusion and exclusion criterion.

An inclusion criterion includes

Patients aged >18 years with symptoms of duodenal or gastric ulcer/gastritis/gastric adenocarcinoma/non-ulcer dyspepsia and no antimicrobial therapy to eradicate *H. pylori* infection.

Exclusion criteria included

Previous gastric surgery, any use of bismuth, antimicrobial agents, H2 receptor antagonists, proton-pump inhibitors within 4 weeks before endoscopic examination, or any of several concomitant medical illnesses including cardiac, respiratory, renal, and liver diseases. The study was approved by the institutional ethical committee. All the patients who were enrolled in the study were not exposed to any prior antibiotics for *H. pylori* infection for the previous 2 weeks.

Isolation and identification of *H. pylori*

Two biopsies were collected from each patient with various GI symptoms, one for rapid urease test and other collected in transport media (Brucella broth + glycerol) were stored in -80°C deep freezer until transportation to Amity University, Noida, for culture. The biopsy samples were transported to Molecular Bacteriology Laboratory at Amity Institute of Biotechnology, Noida, in dry ice. The detail demographic performa of each patient was filled. The biopsies which were in transport media were used for the isolation of *H. pylori* on brain heart infusion agar media (BHIA) Becton Dickinson, Sparks, MD, USA, supplemented with 5% horse serum; 0.4% IsovitaleX (Becton Dickinson, Sparks, MD, USA), amphotericin B (8 $\mu\text{g}/\text{ml}$), trimethoprim (5 $\mu\text{g}/\text{ml}$), and vancomycin (6 $\mu\text{g}/\text{ml}$) and were allowed to incubate at 37°C for 3–6 days under microaerophilic condition (5% O_2 ; 10% CO_2 ; 85% N_2) (double gas incubator, Heracell 150i). Identification of the *H. pylori* was on the basis of colony morphology, Gram staining, and observing the positive results of urease, oxidase, and catalase tests. For further use in future, the *H. pylori* suspension was stored in glycerol stock containing BHI broth and 20% glycerol and were stored at -80°C .

DNA extraction and polymerase chain reaction (PCR) amplification for the confirmation of the *H. pylori*

The bacteria were harvested by colony morphology, and the DNA was extracted by cetyltrimethylammonium bromide method [19] using 24 h grown confluent lawn of bacterial culture on BHI agar (BHIA, Difco Laboratories) plates.

For molecular confirmation of *H. pylori* colonies, the urease gene was amplified using the primers sequence of UreBF5'-CGTCCGGCAATAGCTGCCATAGT3' and UreBR5'-GTAGGTCCTGCTACTGAAGCCTTA3' which gave an amplicon size of 480 bp [20]. The PCR was performed in a final volume of 20 μl containing 10X PCR buffer, 500 nM of each primer, 2 mM MgCl_2 , 200 μM each deoxyribonucleotide triphosphate, 1.5 U Taq DNA polymerase, and 10 ng of DNA sample. PCR was performed in a thermocycler (Eppendorf, Germany) under the following condition; initial denaturation for 2 min at 96°C was followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s. After the final extension of 72°C for 10 min, the PCR products were examined by 1% agarose gel according to the standard procedure. *H. pylori* strain 26695 was used as a control strain.

Antibiotic susceptibility testing

The minimum inhibitory concentrations (MICs) for MTZ, FZ, AMX, TET, LEV (Sigma, St Louis, MO, USA), and CLR (Abbott Laboratories, Abbott Park, IL, USA) were determined by agar dilution method as per European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [21]. The positive isolates were considered to be resistant if the MIC was $>8 \mu\text{g}/\text{ml}$ for MTZ, $>0.12 \mu\text{g}/\text{ml}$ for AMX, $>1 \mu\text{g}/\text{ml}$ for TET, $>0.5 \mu\text{g}/\text{ml}$ for CLR, $>2 \mu\text{g}/\text{ml}$ for FZ, and $>1 \mu\text{g}/\text{ml}$ for LEV [21]. For antibiogram profiling, 2-fold serial dilutions of antibiotics were used: MTZ 0.2–64 $\mu\text{g}/\text{ml}$; FZ 0.2–2 $\mu\text{g}/\text{ml}$; AMX 0.12–2 $\mu\text{g}/\text{ml}$; TET 1–4 $\mu\text{g}/\text{ml}$; CLR 0.125–2 $\mu\text{g}/\text{ml}$; and LEV 0.2–2 $\mu\text{g}/\text{ml}$. *H. pylori* suspensions were prepared equivalent to McFarland 2 (1×10^8 CFU/ml). 3 μl was delivered as a spot on BHIA plates containing different concentrations of antibiotics [22]. Antibiotics free BHIA plates were used as control. All the plates were further incubated under microaerophilic condition at 37°C for 72 h or longer until a visible inhibition ellipse was seen, and the results were interpreted as per EUCAST guidelines [21].

Collection of samples of *B. capitata*

B. capitata was collected from the local Indian market and was further authenticated at the Botany Department of Amity University. The leaves were washed properly and dried in room temperature for 5–10 days. These dried leaves were then grinded into powdered and extracted using methanol, n-hexane, and chloroform.

Preparation of the extract of *B. capitata*

The extract was prepared in methanol, n-hexane, and chloroform. To prepare the extract about 2 g of dried powdered plant sample was extracted with 30 ml of methanol, n-hexane, and chloroform consecutively for 72h under constant stirring. The extracts were filtered and collected separately, and 30 ml of respective solvent was added to it. The extracts were again filtered and collected after 72 h. The filtered extracts were dried under pressure and suspended in the solvent.

Anti-*H. pylori* assay of *B. capitata* by disc diffusion assay

The dual drug-resistant strains of *H. pylori* from Northeast India and multidrug-resistant strains from North India (from our previous study) were included to test the efficacy of n-hexane, methanolic, and chloroform extract of *B. capitata*. The bacterial colonies were taken directly from the BHIA 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 1×10^8 colony-forming units (CFUs)/ml) [23]. Sterile Whatman filter paper disks of 6 mm in diameter were loaded with different concentration of n-hexane, methanol, and chloroform extracts of *B. capitata* and placed on the inoculated plates with 3×10^9 CFU of *H. pylori*. The plates were kept under observation for 2 days at 37°C under microaerophilic conditions (5% O_2 , 10% CO_2 , and 85% N_2). All experiments were performed in triplicates. Pure n-hexane, methanol, and chloroform were used as a negative control and AMX was used as positive control.

Statistical analysis

Differences between groups were statistically evaluated using the Chi-square test. Differences were considered significant at the 5% probability level. Statistical analysis was performed using SPSS ver.20 software.

RESULTS

Isolation of *H. pylori*

A total of 98 biopsies were collected from the patients within the age group of 18–80 years who were suffering from various GI diseases visiting Gauhati Medical College, Guwahati, Assam. Among the 98 biopsies which were transported to Amity University, Noida; in transport media, *H. pylori* was successfully isolated from 11 biopsy sample by culture method under microaerophilic condition. All the positive isolates were within the age of 18–80 years, with M/F ratio of 1:0.46. The clinical diagnosis of all the isolates was antral erosion (n=3), antral ulcer (n=1), conjunctive dystrophy (n=1), duodenal ulcer (n=1), and gastritis (n=5).

Identification of the *H. pylori*

Rapid urease test (RUT)

The detection of the *H. Pylori* was done with RUT. *H. pylori* is urease positive and gives a pink color to the RUT solution (5% urea + 2% phenol red and pH 6.6–6.8) indicating the presence of *H. pylori*.

PCR detection for the confirmation of the *H. pylori* strain

The DNA of all the 11 isolates was amplified for urease gene using UreBF and UreBR primers. Analysis was done on 1% agarose gel confirming the presence of *H. pylori* (Fig. 1).

Antimicrobial susceptibility of the antibiotics

Agar dilution method was used for determining the MIC of all the antibiotics. Resistance toward MTZ was found in three isolates; MIC $>32 \mu\text{g}/\text{ml}$ (n=1), MIC $>16 \mu\text{g}/\text{ml}$ (n=1), and MIC $>8 \mu\text{g}/\text{ml}$ (n=1).

Resistance to LEV founded in 4/11 isolates (MIC >1 µg/ml). Of four-resistant strains, three showed dual resistances to MTZ and LEV. All the (11/11) strains were 100% sensitive toward CLR, TET, AMX, and FZ.

Distribution of antibiotics as per the clinical information

The distribution of MTZ and LEV resistance according to the age, gender, and disease outcome is discussed in Table 1. As per the association of MTZ and LEV within gender, there was no significant association found.

However, the prevalence of MTZ and LEV resistance was observed more in the patients with the age group of 41–60 years than in the patients with age ranging from 18 to 40 and 61 to 80 years. The MTZ and LEV resistance was seen to be higher in the patients suffering from gastritis, but the association was not significant (Table 1).

All the 11/11 (100%) *H. pylori* strains were sensitive toward CLR, TET, AMX, and FZ, and the significant association was found with the gender ($p>0.04$) and the disease diagnosis ($p>0.004$), but no significant association was found with patient's age ($p>0.3$) (Fig. 2).

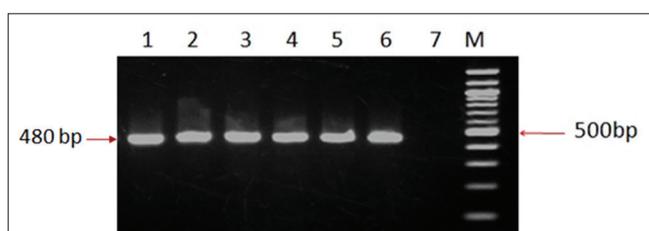


Fig. 1: DNA amplification of urease gene of 480 bp for the confirmation of *Helicobacter pylori* strains. Lane 1- positive control, 26695; Lane 2-6 - *H. pylori* clinical isolates from Lane 7 - negative control, and Lane M - Marker (100 bp)

Table 1: Correlation between MTZ and LEV susceptibility of *H. pylori* isolates with gender, age, and disease diagnosis

Clinical information	MTZ (R)	p	LEV (R)	p
Gender				
Male (n=9)	3 (27%)	0.4	4 (36%)	0.36
Female (n=2)	0 (0%)		0 (0%)	
Age				
18–40 (n=6)	1 (9%)	0.4	1 (9%)	2.9
41–60 (n=3)	2 (18.1%)		3 (27%)	
61–80 (n=2)	0 (0%)		0 (0%)	
Disease diagnosis				
Gastritis (n=9)	3 (27%)	4.6	4 (36%)	0.8
Conjunctive dystrophy (n=1)	0 (0%)		0 (0%)	
Duodenal ulcer (n=1)	0 (0%)		0 (0%)	

MTZ: Metronidazole, LEV: Levofloxacin, *H. pylori*: *Helicobacter pylori*

Effect of *B. capitata* against the resistant *H. pylori* strains

Two clinical isolates, one from North and other one from Northeast India, were included in the study. Of the two strains, one strain isolated from North Indian patient was multidrug resistant showing resistance toward MTZ, CLR, and LEV and the other from Northeast India was dual drug resistant showing resistance toward MTZ and LEV.

Different concentration of n-hexane, methanol, and chloroform extract was loaded on the disc and was air dried. 100 µl of the *H. pylori* strains dissolved in PBS having McFarland 2 (3×10^9 CFU/ml) was spread on the BHI plate. The disc loaded with the extracts was placed upside down on the *H. pylori* plates. After 72 h of incubation under microaerophilic condition, we found that the n-hexane, methanolic, and chloroform extract of *B. capitata* showed anti-*H. pylori* activity on both dual and multidrug-resistant strains (Table 2). The MIC for multidrug-resistant strain of North India was 100 µg for n-hexane, 20 µg for methanol, and 10 µg for chloroform extracts. The diameter of zone of inhibition was found to be 8 mm, 6 mm for n-hexane, 7 mm for methanol, and 6 mm and 8 mm for chloroform extracts of *B. capitata*, respectively, for North and Northeast strains.

The MIC and the diameter of the zone of inhibition for dual drug-resistant strain of Northeast India and multidrug-resistant strains of North India were similar when tested against various extracts of *B. capitata* (Fig. 3).

DISCUSSION

H. pylori infection causes several GI diseases in both developing and industrialized country and has direct impact on health-care system worldwide [24]. Eradication of *H. pylori* with a proton-pump inhibitor-based triple therapy is presently used to treat *H. pylori* infection [21]. Although it has a success rate of 80–90%, problems such as treatment failure and contraindications for some patients are common. Furthermore, rapidly emerging drug resistance in *H. pylori* strains during treatment with various antibiotics is a major obstacle for successful eradication therapies [1]. Due to the prevalence of antibiotic-resistant *H. pylori* strains, there is an increasing search for safe and effective non-antibiotic compounds that inhibit *H. pylori* growth. In the Indian traditional medical system, a number of plants and plant products are known to possess potent medicinal properties, suggesting their usefulness in treatment.

Antibiotics, namely MTZ, CLR, and TET are the major drugs used for the treatment of several different types of infections including the *H. pylori* infection [25] which could be the reason for the high level of antibiotic resistance from mainly MTZ in many parts of India. Northern India such as Delhi and Chandigarh has reported a lower prevalence of resistant strains, i.e. 37.5% and 38.2%, respectively [26]. Eastern India observes high MTZ resistance with 85% [27] along with South India which includes Hyderabad and Chennai having 100% and 88.2%, respectively [28]. The MTZ resistance of *H. pylori* may be because of the extensive use of this inexpensive antibiotic for the treatment of parasitic, genital, and dental infections, especially in developing countries [29].

Table 2: Antimicrobial activity of n-hexane, methanolic, and chloroform extract of *B. capitata* against the dual and multidrug-resistant strains of North Indian (strain1) and Northeast India (strain 2)

Strain nos.	MIC for MTZ, CLR, LEV (agar dilution method)	n-hexane extract of <i>B. capitata</i> (disc diffusion assay)		Methanolic extract of <i>B. capitata</i> (disc diffusion assay)		Chloroform extract of <i>B. capitata</i> (disc diffusion assay)	
		Amount (in µg)	Zone of inhibition (in mm)	Amount (in µg)	Zone of inhibition (in mm)	Amount (in µg)	Zone of inhibition (in mm)
Strain 1	MTZ=64 µg/ml CLR=4 µg/ml LEV=2 µg/ml	100	8	20	7	10	6
Strain 2	MTZ=32 µg/ml LEV=1 µg/ml	100	6	20	7	10	8

B. capitata: *Brassica capitata*, MIC: Minimum inhibitory concentration, MTZ: Metronidazole, LEV: Levofloxacin, CLR: Clarithromycin

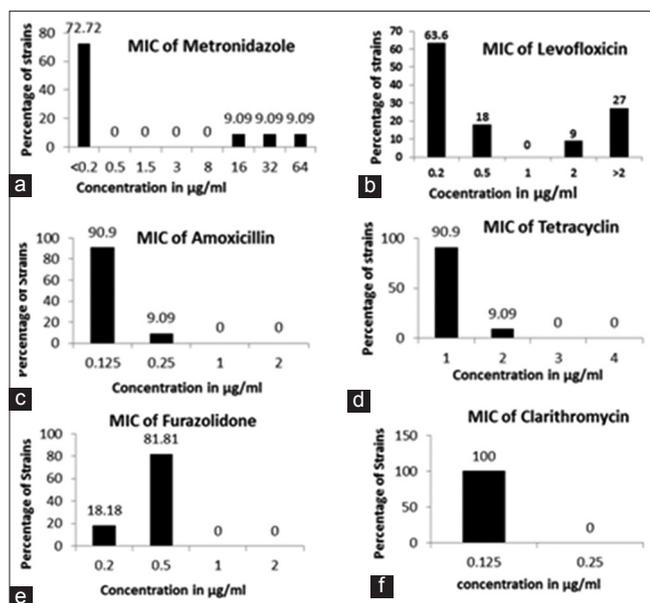


Fig. 2: Distribution of minimum inhibitory concentration of antibiotics among the *Helicobacter pylori* strains isolated from Northeast India. Metronidazole (a), levofloxacin (b), amoxicillin (c), tetracycline (d), furazolidone (e), and clarithromycin (f)



Fig. 3: Disc diffusion method: Antimicrobial activity of *Brassica capitata* against the drug resistance strains of *Helicobacter pylori* (a) n-hexane extract (b) methanolic extract (c) chloroform extract

Studies comprehend that the resistance is due to inactivation of gene *rdxA* and *frx* gene [30,31].

Another drug of choice for the treatment is CLR that is commonly used in the triple therapy for the eradication of *H. pylori* having an efficacy of 90% [32]. The level of resistance for CLR varies in India, namely Lucknow (82%), Mumbai (91%), Hyderabad (96%) [28], Kolkata (0%) [30], Gujarat (58.8%) [33], and Delhi (11.8%) [34]. Studies have shown that the CLR resistance is due to the mutation in the 23S rRNA gene at 2142 (A-G), 2143 (A-G), and 2182 (T-C) positions [34].

LEV can also be an option as a drug for the eradication of *H. pylori* as has been reported in many countries. According to the recommended guidelines, if the LEV resistance in the susceptibility test of *H. pylori* is over 20%, then the antibiotic is not considered as a good option. Its resistance rate in Gujarat is 13.8% [33] and in North India is 73.2% [35].

Our study reports on the antibiotic sensitivity pattern toward AMX, TET, CLR, and FZ of all the 11 Northeast strains isolated from Guwahati, Assam. We found that all the 11 strains were sensitive toward the AMX, TET, CLR, MTZ, LEV and FZ. It has been observed that 27% of the *H. pylori* strains showed resistance toward MTZ, 36.3% of the strains toward LEV and rest 27% toward both MTZ and LEV.

Due to increase use of antibiotics over the last two decades, *H. pylori* has acquired the antibiotic resistance which is one of the main causes of treatment failure. De *et al.* have mentioned the anti-*H. pylori* effect of curcumin and their use in the treatment of the *H. pylori* infection [36]. In our previous study, we have reported the efficacy of Emblica

officinalis [37], *Paedra foetida* [38], and *Parmelia perlata* [39] against the drug-resistant *H. pylori* strains. *B. capitata* which is leafy vegetable and is used in cooking with the nutritive value being rich in calcium and protein, and Vitamin C is utilized for healing process [40,41]. This prompted us to explore its antimicrobial potential against multi and dual drug-resistant strains of North and Northeast India. The n-hexane, methanolic, and chloroform extract of *B. capitata* were tested by disc diffusion method for its efficacy against *H. pylori*. Zone of inhibition with the n-hexane extract for North Indian and Northeast strains at MIC of 100 μg was 8 mm and 6 mm, respectively. The methanol extract showed 7 mm zone for both the strains at MIC of 20 μg and chloroform extract showed the best effect at the MIC of 10 μg with 6 mm of zone of inhibition for North Indian strain and 8 mm zone of inhibition for Northeast strains.

Overall, this study provides novel insights into the therapeutic potential of *B. capitata* against *H. pylori* infections, although further studies are required to extrapolate its effect on humans.

CONCLUSION

Our study highlights the potential antibacterial activity of *B. capitata* against *H. pylori* *in vitro* irrespective of the genetic makeup of the strains. However, its MIC is relatively high which may be due to the poor bioavailability of *B. capitata* extracts. Furthermore, our study for the 1st time in India studied the Northeast Indian strains which were found to be sensitive to most of the antibiotics used for the treatment regime, whereas other parts of India reported high multidrug resistance [29].

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AUTHOR'S CONTRIBUTION

RD and KD have given the concept of antibiogram profiling of *H. pylori* strains of Northeast India. RD and AKM have finalized the manuscript. SM has performed the experiments, analyzed the data, and drafted the manuscript. SB has provided the *B. capitata* sample and finalized the manuscript. VG and MR have performed some of the experiments. SD and AKA have provided the samples and analyzed the clinical data.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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