

ANTIMICROBIAL ACTIVITY OF ANISOCHILUS CARNOSUS (L.F.) WALL AGAINST THE HUMAN GASTRIC PATHOGEN *HELICOBACTER PYLORI***VIGNESH SHETTY¹, RICHARD LOBO², NIMMY KUMAR², RAMACHANDRA LINGADAKAI³, GANESH C PAI⁴, GIRISH BALARAJU⁴, MAMATHA BALLAL^{1*}**

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Received: 29 May 2017, Revised and Accepted: 01 July 2017

ABSTRACT

Objective: The prevalence of *Helicobacter pylori* in India is high, and majority leads to severe gastrointestinal infections. Existing treatment regimens for *H. pylori* infections have increased failure rates and adverse side effects that desire the search for an effective substitute therapy. *Anisochilus carnosus* (L.f.) wall (Lamiaceae), a herb which grows once in a year at high elevation is used widely in traditional treatment for the complaints of gastric ulcer and skin diseases. The present study was performed to assess the antibacterial activity of *A. carnosus* (L.f.) wall, against clinical isolates of *H. pylori* *in vitro*.

Methods: *A. carnosus* leaves were collected-dried and extracted with water and ethanol by cold maceration with ethanol by Soxhlet method. The minimum inhibitory concentration (MIC) of extracts was made and tested against 32 clinical and 1 reference strains of *H. pylori*.

Results: *A. carnosus* (L.f.) wall inhibited the growth of most of the clinical *H. pylori* strains. The MIC of *A. carnosus* (L.f.) wall extracted by cold maceration (aqueous and ethanol) and Soxhlet apparatus (ethanol) ranged from 500 to 62.5 µg/ml, and the majority of the clinical *H. pylori* strains were inhibited at the MIC of 500 µg/ml of aqueous, ethanol, and Soxhlet ethanol extraction were 63.63%, 43.75%, and 71.87%, respectively.

Conclusion: *A. carnosus* (L.f.) wall is an efficient inhibitor of *H. pylori* growth *in vitro*. *A. carnosus* (L.f.) wall revealed enormous therapeutic potential to *H. pylori* infection as it was extremely active in the suppression of *H. pylori*. Hence, it can be taken as a potential agent against several *H. pylori* linked gastric pathogenic progressions.

Keywords: *Anisochilus carnosus*, *Helicobacter pylori*, Minimum inhibitory concentration, Antimicrobial activity.

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INTRODUCTION

Helicobacter pylori is a unipolar flagellated, microaerophilic bacterium which has the capability to survive in human stomach and setup infections that may be able to last for years or decades. One-half or more people worldwide carry these bacteria, as well as its prevalence in developing countries surpasses 90%. The role of *H. pylori* in causing peptic ulcer disease had attracted greater attention. Indeed, this bacterium is categorized as a carcinogen Group I by the International Agency for Research on Cancer [1-3]. Significant differences among *H. pylori* strains geographically, by molecular characterization and disease outcome has shown strains from India are genetically different from those from East Asia to Western strains [4,5]. There are numerous recognized virulence-related factors including cag pathogenicity Island (cag PAI), that enhance the pathogenesis and risk of more severe infections like gastric adenocarcinoma [4,6]. However, these *H. pylori* virulence markers are not always linked with disease, eradication from diseased persons will be the promising/excellent option for an active treatment of *H. pylori*-related infections. A number of combination therapies have been developed for *H. pylori* eradication to treat or prevent infections. However, treatment with the triple therapy is not successful always, due to the acquisition of drug resistance including metronidazole, and clarithromycin which leads to a serious problem for treatment failure [7]. Several studies have demonstrated that the rate of resistance to antibiotics differs geographically, reaching 30-80% for metronidazole and from 5% to 30% in clarithromycin. Understanding

the reduced cure rate by conventional therapy due to increasingly resistant strains, adverse side effects, refusal among the patients, the antibiotic regimens cost, and some added factors leading to less effective therapy. Therefore, there is an essential need to progress/develop novel treatment approaches for *H. pylori* infection [8-10].

A. carnosus (L.f.) wall, an annual herbaceous plant which is a member of the family Lamiaceae, breeds on high elevation amid small rocks. Distribution in India is mainly seen in Karnataka, Maharashtra, Rajasthan, and Tamil Nadu and practiced traditionally in tribal communities for the treatment of ulcer, stomach ache, cough, and eczema. This plant's phytochemical study has revealed it to be rich in active compounds such as saponins, tannins, flavonoids (apigenin and luteolin), phytosterols, triterpenoids, and essential oil components (carvacrol, β-selinene, camphor, α-cis-bergamotene, and caryophyllene) [11-13].

Considering the advantageous background of *A. carnosus*, the current work has been directed to assess the effectiveness of the *A. carnosus* (L.f.) wall as an antimicrobial means against *H. pylori* clinical strains obtained from patients in South India.

METHODS**Collection of plant materials**

A. carnosus leaves were collected during September 2014 from rock crevices at Hayagreeva Nagar, Udupi, and it was authenticated by

Dr. Richard Lobo, Pharmacognosist, Manipal College of Pharmaceutical Sciences (MCOPS), Manipal, Karnataka. A receipt sample with accession number (PP 573) has been placed in the Department of Pharmacognosy, MCOPS, Manipal, India.

Extraction of *A. carnosus* leaves

The *A. carnosus* leaves (500 g) were dried, coarsely powdered, and extracted with Soxhlet apparatus using ethanol (3 L×1 L). The extracts containing solvent were dried by evaporation on a rotary evaporator kept under low pressure. Dried leaves were crushed (500 g) and kept for cold maceration for 4 days in chloroform:water (1:99) and ethanol at room temperature. The solvent-containing extracts were then filtered and filtrates gained were concentrated to obtain crude aqueous extract and ethanol extract, respectively, on a water bath (Table 1).

Culture and strains of *H. pylori*

Archived 32 *H. pylori* strains which were isolated from the body and antral mucosal biopsy specimens obtained from patients at the Kasturba Medical College and Hospital, India, who had symptoms of the upper gastrointestinal disorders. Along with the clinical strains, one reference *H. pylori* strain American Type Culture Collection (ATCC 26695) was used for this study. The identification of the strains was based on colony morphology, gram staining, and biochemical reactions positive for catalase, urease, and oxidase. Revived *H. pylori* strains were obtained by culturing on brain heart infusion (BHI) agar (Difco Laboratories) supplemented with 7% horse serum (GIBCO), 0.4% IsoVitalX (Becton Dickinson), trimethoprim (5 g/ml), vancomycin (8 g/ml), and polymyxin B (10 g/ml). The culture plates were incubated in the microaerophilic atmosphere (5% O₂, 10% CO₂, and 85% N₂) (double gas incubator; Thermo Fisher Scientific) at 37°C. The stock cultures were preserved until use at -80°C.

Minimum inhibitory concentrations (MIC)

Stock cultures which were frozen were then cultured on BHI agar supplemented with 7% horse serum and incubated at microaerophilic conditions for 3 days as noted earlier. Isolates were cultured again on fresh BHI agar plate and incubated for 24 hr at 37°C. Growing cells of *H. pylori* were put in sterile phosphate-buffered saline and turbidity adjusted to McFarland Standard 2. Three microliters of the adjusted inoculum were spot inoculated to BHI agar comprising several concentrations of *A. carnosus*. Dimethyl sulfoxide (Merck Chemical) was used as a solvent for *A. carnosus* extract to dissolve and made a stock concentration of 50 mg/ml. Working concentrations were prepared and are 500 µg/ml, 250 µg/ml, 125 µg/ml, and 62.5 µg/ml for aqueous, ethanol maceration, and Soxhlet ethanol extraction. For each experiment, growth control plates were included containing only BHI agar. Incorporating the solvent dimethyl sulfoxide in BHI agar was also considered as growth control to confirm that the viability of the microorganism was not interfered by the solvent used for dissolving extract. 1 reference strain (ATCC 26695) and 32 clinical strains were used for antimicrobial susceptibility testing. Later incubated in the microaerophilic atmosphere at 37°C for 5 days. The MIC was well-defined as the lowest concentration of the test compound at which there was no visible growth. Antibiotics clarithromycin (Sigma Chemical Co.) and amoxicillin (Sigma Chemical Co.) were tested for each batch which was considered for quality control and comparative analyses.

RESULTS AND DISCUSSION

In vitro differential inhibition of *H. pylori* growth by *A. carnosus*

Among 32 *H. pylori* strains which were isolated from patients with gastritis, peptic ulcer, respectively, was tested against *A. carnosus* extract. Out of these 36 strains, 24 strains, including ATCC 26695, showed/tested positive for *cagA* amplicon and the other 8 showed positive for empty site amplicon, indicative of a lack of the entire *cag* PAI. Agar dilution method was used to illustrate MICs.

Cold macerations (crude aqueous extract and ethanol extract) of *A. carnosus* against *H. pylori* showed activity with increasing concentration of MIC. The MIC of *A. carnosus* leaf extract ranged from

62.5 µg/ml to 500 µg/ml, and the majority of the strains inhibited (63.63% and 43.75%) in both aqueous and ethanol extract showed a MIC of 500 µg/ml (Figs. 1 and 2). Soxhlet Ethanol extract of *A. carnosus* at the MIC 500 µg/ml inhibited the growth of 23 (71.87%) clinical isolates (Fig. 3) (Table 2). The reference strain ATCC 26695 showed activity at MIC 500 µg/ml by all the three extracts.

These findings noticeably confirm that *A. carnosus* can be an effective inhibitor of Indian *H. pylori* strains growth regardless of the disease

Table 1: Final product obtained after extraction of 500 g *Anisochilus carnosus* leaves

Method	Yield (%w/w)
Aqueous maceration	0.54
Alcohol (ethanol) maceration	0.27
Ethanol extract-Soxhlet	0.34

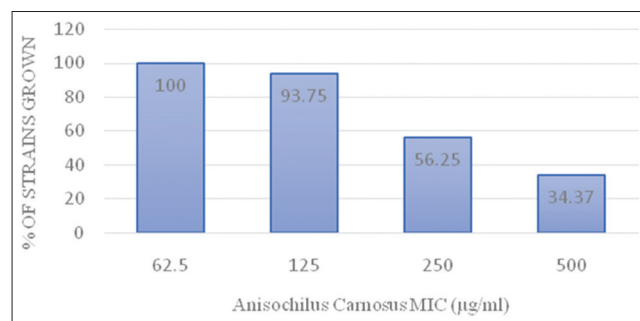


Fig. 1: *Helicobacter pylori* strains grown in minimum inhibitory concentration of aqueous maceration of *Anisochilus carnosus* leaves extract

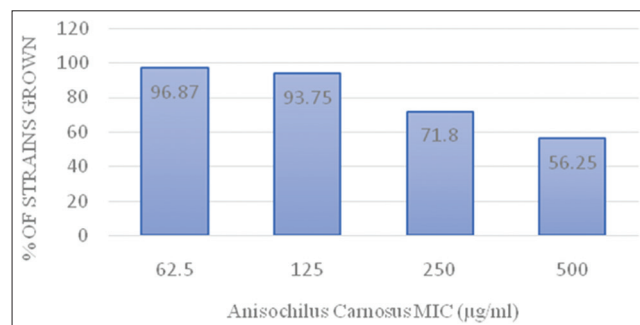


Fig. 2: *Helicobacter pylori* strains grown in minimum inhibitory concentration of ethanol maceration of *Anisochilus carnosus* leaves extract

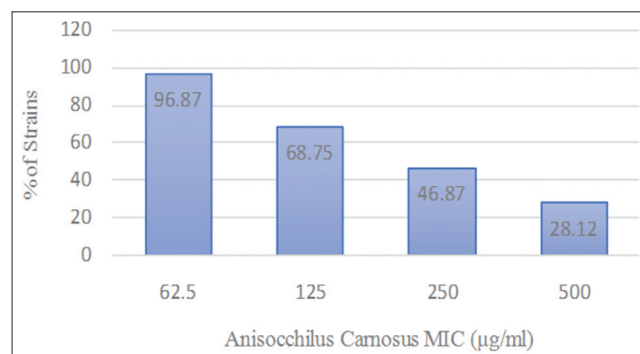


Fig. 3: *Helicobacter pylori* strains grown in minimum inhibitory concentration of Soxhlet ethanol extract of *Anisochilus carnosus* leaves

Table 2: Rate of *H. pylori* clinical strains inhibited against the MIC of *A. carnosus* extract

MIC conc. ($\mu\text{g/ml}$)	Aqueous maceration		Ethanol maceration		Soxhlet-ethanol extraction	
	Inhibited N (%)	Grown N (%)	Inhibited N (%)	Grown N (%)	Inhibited N (%)	Grown N (%)
62.5	0 (100)	32 (100)	1 (3.12)	31 (96.88)	1 (3.12)	31 (96.88)
125	2 (6.25)	30 (93.75)	2 (6.25)	30 (93.75)	10 (31.25)	22 (68.75)
250	14 (43.75)	18 (56.25)	9 (28.12)	23 (71.88)	17 (53.12)	15 (46.88)
500	21 (65.62)	11 (34.38)	14 (43.75)	18 (56.25)	23 (71.87)	9 (28.13)

H. pylori: *Helicobacter pylori*, MIC: Minimum inhibitory concentration

status. Among the three methods followed for extraction of *A. carnosus*, more promising results were obtained from Soxhlet ethanol extraction. However, further study is needed to observe the strain-specific MIC differences and polymorphism in gene targets.

Several strategies for eradication of *H. pylori* are followed; among them currently, proton pump inhibitor-based triple therapy, sequential triple therapy is followed for treating *H. pylori* infection. Despite it having a good rate of success, difficulties such as contraindications and failure in treatment are common in few patients [14-16]. Moreover, quickly developing resistance to the drugs commonly used for treating infections caused by *H. pylori* is yet another hurdle toward achieving an effective eradication therapy [9]. Because of the increasing development of drug resistance by *H. pylori* strains, there is an increasing need to develop an effective, safe and cost-effective therapy which inhibits *H. pylori*. Traditional system in Indian medicine makes use of treatment based on medicinal plants and their phytochemicals which are effective and are also said to have lesser side effects [17,18]. Previous studies on *A. carnosus* have shown that it has potent-anticancer activity and is also traditionally used as a treatment for gastric ulcer and skin diseases. This encouraged us to evaluate its potential antimicrobial activity against *H. pylori* strains isolated from India which has diverse population, i.e., hpAsia2 and hpEurope strains [19]. Moreover, there is a higher prevalence of *H. pylori* in Indian Population with a good number of patients suffering from gastrointestinal disorders [20].

A. carnosus, an annual herb plant which grows more commonly in higher elevation such as rock crevices have shown immense benefits in traditional medicine. Some of the previous studies have specified nanoparticles synthesized from an extract of *A. carnosus* to show effective antimicrobial action against microorganisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* [21]. Another study, showed *A. carnosus* to exhibit antiulcer property which has potent gastric healing properties [22]. Few other reports showed an effective hepatoprotective activity by ethanol extract of *A. carnosus* [23]. In addition, leaves of *A. carnosus* presented good antipyretic and analgesic activity [24]. A recent study in India studied the cytotoxic potential of *A. carnosus* [12]. This suggests that *A. carnosus* contain strong pharmacological properties, which could probably be attributed to the phytochemicals present in it, making it an important medicinal plant.

CONCLUSION

In this study, we have examined the *A. carnosus* activity against 32 clinical strains which were isolated from patients suffering from gastrointestinal infections. *A. carnosus* extract inhibited *H. pylori* strains at MIC 500 $\mu\text{g/ml}$. The anti-*H. pylori* activity is irrespective of the virulence genotypes harbored by strains observed by checking cagPAI-positive and cagPAI-negative strains. It is noteworthy to be considered as a potential antimicrobial agent for the eradication of *H. pylori* especially due to the development of increasing drug resistance by *H. pylori* strains. Our findings prompt for further studies to elucidate the mechanism by which *A. carnosus* inhibits *H. pylori* growth. All these observations along with the previous study suggest *A. carnosus* possess potent-antimicrobial activity, antiulcer, and anticancer activity. All these important data can be taken into consideration for developing an

effective alternative therapy for *H. pylori*. However, further studies are needed to extrapolate its effect *in vivo* on animal and humans.

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