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 this 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) [11-15]. 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 linked gastric pathogenic progressions.

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 × 1 L). The extracts containing solvent was 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. 

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

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 PAL 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.
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-antiancancer 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 antioxidant property which has potent gastric healing properties [22]. Few other reports showed an effective hepato protective 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 µg/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 possesses potent-antimicrobial activity, antitoxic, 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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