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In vitro susceptibility of *Helicobacter pylori* to extracts of *Eucalyptus camaldulensis* and *Eucalyptus torelliana*

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Abstract

The *in vitro* susceptibility of *Helicobacter pylori* to extracts of *Eucalyptus camaldulensis* Dehnh. and *Eucalyptus torelliana* F. Muell. (Myrtaceae), Nigerian medicinal plants, was investigated in six strains of *H. pylori*, namely, ATCC 4504, ATCC 47619, A2, TI8984, 019A, and A6. The susceptibility of these strains was determined using a standardized agar dilution method (National Committee for Clinical Laboratory Standards guidelines) with Mueller–Hinton agar, supplemented with defibrinated horse blood. The minimum inhibitory concentrations of the crude extracts against all the tested strains ranged from 12.5 to 400 μg/mL. Phytochemical screening of the plant extracts revealed the presence of tannins, saponins, and cardenolides. The anti-*H. pylori* activities demonstrated by these plants may be attributed to their chemical constituents, and explain their reported traditional uses, as well as their gastroprotective properties as demonstrated previously in experimental animals. The results of this work suggest that, in accordance with their traditional medical use in Nigeria, *E. camaldalensis* and *E. torelliana* have some therapeutic potential against *H. pylori*, and thus are of interest for the treatment of *H. pylori* infections.

Keywords

Eucalyptus camaldalensis; *Eucalyptus torelliana*; *Helicobacter pylori*; anti-*H. pylori* activity

Introduction

Helicobacter pylori, a Gram-negative, spiral-shaped microaerophillic bacillus, is the leading cause of peptic ulcer disease, a gastrointestinal disorder that affects about 10% of the world's population (Kurata, 1993; Rosenstock & Jorgensen, 1995). The bacterium is associated with up to 95% of duodenal and up to 75% of gastric ulcerations (Alarcon et al., 1999). It has also been associated with gastric carcinoma and, more recently, colorectal cancer (Fujimori et al., 2005). *H. pylori* invades the stomach and is able to survive the extreme acidity of the stomach by excreting urease, an enzyme that hydrolyzes urea to ammonia thus creating an alkaline microenvironment in which the bacillus can survive (Mobley et al., 1988; David, 1996).

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Infection with *H. pylori* causes both acute and chronic gastric inflammation, which may eventually lead to the development of peptic ulcer disease. In fact, Kuipers et al. (1995) reported a lifetime prevalence of peptic ulcer disease of 10–20% in *H. pylori*-positive individuals. Treatment and complete eradication of *H. pylori* results in ulcer healing and reduces the recurrence of infection. Eradication of *H. pylori* usually involves the administration of a combined treatment with two or more antibiotics and a proton-pump inhibitor. However, these drug regimens are complicated, have significant adverse effects, and suffer compliance problems, leading to relapse since complete cure is not always achieved and possible antibiotic resistance.

Since drugs for the eradication of *H. pylori* are not always effective, and antibiotic resistance is becoming a problem worldwide, there is a need to investigate potential new sources of drugs that can eradicate *H. pylori*, treat existing cases, and prevent recurrence and the development of complications.

In Nigeria, plants of the genus *Eucalyptus* (Myrtaceae), including *Eucalyptus camaldulensis* Dehnh. and *Eucalytus torelliana* F. Muell., are used to treat gastrointestinal disorders (Adeniyi et al., 2006). In addition, a decoction of the leaves is reported to be a remedy for sore throat and other bacterial infections of the respiratory and urinary tracts (Bruneton, 1999). The poultice of the leaves is applied over wounds and ulcers (Gill, 1992). The essential oils of the leaves have been used in the treatment of lung diseases and were stated to have anti-tubercular effect (Oyedeji et al., 1999). In animal models, extracts of the leaves of *E. camaldulensis* and *E. torelliana* are reported to decrease gastric acid production and thus appear useful for the treatment of gastric ulcers (Adeniyi et al., 2006). However, their effects against *H. pylori* had not been investigated.

As a follow-up to the gastroprotective activity of these two plants, the current investigation measured the *in vitro* susceptibility of six *H. pylori* strains to extracts of *E. camaldulensis* and *E. torelliana* leaves.

Materials and Methods

Plant collection, extraction, and preparation of extracts

The leaves and stem bark of *E. camaldulensis* and *E. torelliana were* collected and authenticated at the University of Ibadan Herbarium, Ibadan, Nigeria. The plant samples were air-dried and then ground to a fine powder prior to extraction. The pulverized plant material (2.5 kg) of each sample was successively subjected to exhaustive Soxhlet extraction with *n*-hexane, chloroform, and methanol. Extracts were collected, dried under reduced pressure, weighed, and stored at −20°C before use. Stock solutions of lyophilized extracts were reconstituted in 50% DMSO, with final concentrations of 100 to 400 μg/mL prepared for the initial screening. Lower concentrations in the range 12.5 to 100 μg/mL were also prepared to determine the minimum inhibitory concentrations (MICs) of the bioactive crude extracts.

H. pylori strains and culture conditions

Six clinical isolates of *H. pylori* were used for the study. They were ATCC 43504, ATCC 47619 (ATCC, Rockville, MA, USA), A2, T18984, 019A, and A6. The clinical strains were encoded to protect the identity of the patient from whom they were obtained. Some of the isolates were obtained from the Microbiology Laboratory, University of Illinois Medical Center (Chicago, IL, USA), Abbott Laboratories (Abbott Park, IL, USA), and Dr. D. Y. Graham (Houston, TX, USA). The isolates obtained from Abbott Laboratories included organisms obtained from patients in Richmond, VA; Charlottesville, VA; Nashville, TN (USA); and Southampton, UK. Clinical isolates were obtained from different geographic

regions to ensure that the organisms were genetically distinct. Gram stain appearance and a positive urease test confirmed the identification of each organism. The organisms were stored frozen at −70°C in skimmed milk plus 17% glycerol. The following media were used for the study: Tryptic soy broth, blood agar (Remel, Lexana, KS), and Mueller-Hinton agar (Difco, Detroit, MI) supplemented with 10% defibrinated horse blood (Remel). Clarithromycin was used as the positive control, at a concentration of 0.5 μg/mL.

Determination of antimicrobial activity

Susceptibility testing was performed using the agar dilution procedure according to the guidelines described by the National Committee for Clinical Laboratory Standards (NCCLS, 2008). The extracts were dissolved in methanol and sterile distilled water was used for further serial dilutions of the dissolved plant extracts. The medium used was Mueller-Hinton agar supplemented with 10% defibrinated horse blood. McFarland 2 suspensions of *H. pylori* strains were prepared in Tryptic soy broth from 4–5-day-old *H. pylori* on blood agar. Six hundred μl of each suspension of the organism was placed into replicator wells on the already set agar places containing the crude extracts at final concentrations of 100 to 400 μg/mL. When all of the suspension drops had dried, the plates were inverted and incubated at 37°C with 10% CO_2 for 4–5 days, after which the plates were read. All procedures were performed in duplicate. The MIC, defined as the lowest concentration of the compound at which there was no visible growth or only a faint haze, was determined for each plant extract and pure compound. The concentration of the crude extracts ranged from 12.5 to 100 μg/mL. The plates were incubated appropriately and read after 4–5 days of incubation.

Phytochemical screening

Phytochemical screening was carried out to detect the presence of secondary metabolites such as anthraquinones, tannins, saponins, alkaloids, and cardiac glycosides using methods described by Harborne (1991).

Results

The crude extracts were concentrated under reduced pressure and subjected to phytochemical analysis. Screening for secondary metabolites showed the presence of tannins and saponins in the stem bark of *E. camadulensis* and *E. torelliana*, and in the leaf of *E. camaldulesis*. The leaf of *E. torelliana* was found to contain anthraquinone and glycosides in addition to the other metabolites. Alkaloids were absent in all samples tested.

Antimicrobial screening of crude extracts at 100, 200 and 400 μg/mL showed that all of the extracts, except the methanol extracts of *E. camaldulensis* leaf and stem bark as well as *E. torelliana* leaf, demonstrated good activity against *H. pylori*. The MIC values for the active crude extracts ranged from 12.5 to 400 μg/mL for the *H. pylori* T18984 strain (Table 1).

Discussion

The eradication of *H. pylori* has proved difficult in parts of Africa, including Nigeria, especially since effective drugs are not available or resistance has developed. Treatment of *H. pylori* infection usually involves the combination of two or more antibiotics and a proton-pump inhibitor. However, the organism has been found to develop resistance to these antibiotics, leading to relapse and the development of complications from infections. Resistance to metronidazole, the most commonly used antimicrobial agent, has been reported worldwide. It is higher in developing countries and could reach 80–90% in Africa (Alarcon et al., 1999). The eradication of *H. pylori* in patients with pre-existing ulcer cures the ulcer disease and can prevent recurrence (Marshall, 1986; Alarcon et al., 1999).

The activity demonstrated by crude extracts of Nigerian medicinal plants justifies their use in folklore medicine in the treatment of wounds and ulcers (Gill, 1992). This indicates that the plants may be used in the treatment of symptomatic and asymptomatic forms of *H. pylori* infections.

The anti-*H. pylori* properties of these plants may be attributed to the presence of tannins and saponins, which are known to possess antimicrobial potential and offer protection against ulcers. *Eucalyptus* species have been reported to contain a large variety of compounds such as triterpenoid saponins (Glasby, 1999) and tannins (Vaghasiya et al., 1997) that are effective in the treatment of peptic ulcers. Essential oils obtained from these plants have been reported to have antimicrobial activity (Oyedeji et al., 1999).

The results of the present study indicate that *E. camaldulensis* and *E. torelliana* may present new therapeutic alternative for the treatment of gastrointestinal diseases associated with *H. pylori* infections, such as gastric and duodenal ulcers. The data further support the use of these two plants in Nigerian traditional medicine. Further phytochemical studies are in progress to isolate specific compounds in the plants responsible for the anti-*H. pylori* activity.

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Table 1

Minimum inhibitory concentrations[a] of the crude extracts of *Eucalyptus camaldulensis* (Ec) and *Eucalyptus torelliana* (Et) against six strains of *Helicobacter pylori* (Hp).

	Hp ATCC43504			Hp ATCC47619			Hp A2			Hp T18984			Hp 019A			Hp A6		
Extract[b]	**Stock**	**−2**	**−4**	**Stock**	**−2**	**−4**	**Stock**	**−2**	**−4**	**Stock**	**−2**	**−4**	**Stock**	**−2**	**−4**	**Stock**	**−2**	**−4**
H, Ec, L	<25	<25	<25	<25	<25	<25	<25	<25	<25	50	50	<25	<25	<25	<25	25	<25	<25
C, Ec, L	<25	<25	<25	50	<25	<25	50	<25	<25	50	<25	<25	<25	<25	<25	<25	<25	<25
H, Ec, S	<12.5	<12.5	<12.5	50	<12.5	<12.5	<12.5	<12.5	<12.5	200	50	25	<12.5	<12.5	<12.5	25	<12.5	<12.5
C, Ec, S	<25	<25	<25	<25	<25	<25	<25	<25	<25	100	50	25	25	25	25	25	25	25
H, Et, L	25	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	50	25	25	25	<12.5	<12.5	<12.5	<12.5	<12.5
C, Et, L	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	400	100	25	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5
H, Et, S	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	200	200	<12.5	25	<12.5	<12.5	25	<12.5	<12.5
C, Et, S	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	100	100	50	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5
M, Et, S	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5	200	100	12.5	<12.5	<12.5	<12.5	<12.5	<12.5	<12.5

[a] MICs are in μg/mL, stock dilution as well as 2- and 4-fold dilutions (−2 and −4, respectively) of the Hp strains in broth.

[b] H = n-hexane, C = chloroform, M = methanol, L = leaves, S = stem bark