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Original Research Article

Susceptibility testing and bactericidal activities of *Theobroma cacao* Linn. (cocoa) on *Helicobacter pylori* in an *in vitro* study

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ABSTRACT

The *in vitro* anti-*Helicobacter pylori* properties of *n*-hexane and methanol extracts of the dried seeds of *Theobroma cacao* Linn. were investigated in forty-one (41) *Helicobacter pylori* (*H. pylori*) clinical isolates collected from a cross-section of residents living in Oyo State, Nigeria and standard *H. pylori* ATCC 43504 strains. Phytochemical screening was carried out on the extracts to detect the presence of secondary metabolites. The extracts were tested against the *H. pylori* strains using the agar well diffusion method at varying concentrations (≤100 mg/mL). The minimum inhibitory concentration (MIC) of the crude extracts was determined against the susceptible strains by the agar dilution method. Bactericidal (kill-kinetics) studies were further carried out on two *H. pylori* clinical isolates. The methanol extracts of *T. cacao* L. (cocoa) seed had an inhibitory effect against *H. pylori* strains to a varying degree; with diameter zones of inhibition between 12 mm and 17 mm on 31 out of the 41 clinical isolates tested with MIC values of 80 to 90 mg/mL. The time–kill study of the methanol extract on *H. pylori* BAA009 and *H. pylori* BAA026, revealed a decline in the surviving population after 8 h of exposure at doses equivalent to MIC, 2× MIC and 4× MIC, accompanied with a total kill of the population at 24 h.

From this study it may be concluded that regular consumption of *T. cacao* L. (cocoa) seed or its products in Nigeria and other developing countries where the plant is cultivated could greatly reduce the incidence of peptic ulcer, as well as suppress acute and chronic gastrointestinal inflammation caused by *H. pylori* a common gastric pathogen.

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1. Introduction

Helicobacter pylori is a Gram-negative, spiral-shaped microaerophilic bacterium often implicated in duodenal and gastric ulcerations, gastritis, gastric carcinoma, colorectal cancer, peptic ulcer disease, nonulcer dyspepsia and gastroeosophageal reflux disease (GERD) (Fujimori et al., 2005; Bekir et al., 2009). However, many who are infected by *H. pylori* do not show any symptoms of disease (Bekir et al., 2009). Having been classified as a Group 1 carcinogen in man by the International Agency on Cancer (IARC, 1994), *H. pylori* has a high global prevalence, especially in developing countries (Bekir et al., 2009). Currently, treatment and eradication of *H. pylori* involves the use of new triple therapies composed of two antibiotics and a proton pump inhibitor; but the increasing antibiotic resistance by the organism, coupled

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with increasing side effects arising from the drug usage is creating a general health problem especially in the developing countries like Nigeria. As a result a great need has arisen to discover and develop new anti-*H. pylori* remedies especially from herbs that will not only eradicate and possibly prevent the organism, but would have minimal side effects, be easily accessible and be affordable even by the poor.

Theobroma cacao Linn. (cocoa) is a common edible plant and cash crop which can grow in most parts of the world. Along with its by-products, it is one of the most widely consumed plants as food globally (Addai, 2010). Developing countries are among the world's leading producers of the crop (Addai, 2010). A large body of research has shown that *T. cacao* contains polyphenols and other antioxidants leading to its use in the treatment of diseases such as cardiovascular disease, diabetes mellitus and immunomodulatory diseases (Vinson et al., 2006). *T. cacao* has also been shown to possess antimicrobial activity against cariogenic microorganisms such as *Streptococcus mutans* and *S. sanguinis* (Ferrazzano et al., 2009), enterohaemorrhagic *Escherichia coli* (Takahashi et al., 1999); with an 18th century claim of its effects in the treatment of microbial mediated diseases such as tuberculosis, yellow fever, cholera and smallpox (Deanna and Louis, 2008).

Due to the increasing number of cases of *H. pylori* infection in developing countries such as Nigeria; coupled with the large production, consumption and availability of *T. cacao* in this country; this study was carried out not only to validate its ethnomedicinal value but to access the anti-*H. pylori* potential of this plant in developing countries.

2. Materials and methods

2.1. Plant collection, extraction, and preparation of extracts

The fresh seeds of *T. cacao* Linn. (cocoa) were purchased between the months of November 2010 and February 2011. They were identified and authenticated by a botanist at the Department of Botany and Microbiology, University of Ibadan, Ibadan, and Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria. A voucher specimen was deposited at FRIN with herbarium number FHI 109559. The seeds were dusted and air dried at room temperature for 4–6 weeks, then ground to coarse powder using a dry electric mill (Moulinex, France). The pulverized plant material (5.8 kg) in small portions was subjected in succession to exhaustive Soxhlet extraction with *n*-hexane and then methanol. For each extraction cycle about 800 g of the pulverized plant material was loaded into the Soxhlet thimble and extracted with *n*-hexane until the solvent became clear (usually between 18 and 24 h). The marc from the *n*-hexane extraction was then further subjected to extraction with methanol until this solvent became clear. The extracts from each solvent were combined, concentrated and dried under reduced pressure using rotary evaporator (Xian Ltd., China) at 45 °C, weighed, and stored at 4 °C before use. The extracts were then lyophilized (i.e. freeze-dried to remove all the solvent) at the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria. Stock solutions of the freeze dried extracts were prepared for the initial screening by reconstituting in 20% DMSO, with a final concentration of 50–100 mg/mL. Lower concentrations in the range 20–90 mg/mL were also prepared to determine the minimum inhibitory concentrations (MICs) of the bioactive crude extracts.

2.2. Antimicrobial agents

The chemotherapeutic agents used in the test as positive controls were Gentamicin (Nicholas Laboratories Limited, England) and Ofloxacin (Sanofi-Aventis, Switzerland), while the negative control was 20% DMSO.

2.3. Phytochemical screening

Phytochemical screening was carried out on the *n*-hexane and methanol extracts to detect the presence of secondary metabolites such as anthraquinones, tannins, saponins, alkaloids, and cardenolides using methods described by Harborne (1998).

2.4. Strains of H. pylori and culture methods

Forty-one (41) clinical isolates of *H. pylori* and *H. pylori* ATCC 43504 strains were used for this investigation. All the clinical strains were isolated, characterized and identified at the Department of Pharmaceutical Microbiology, University of Ibadan, Ibadan, Nigeria using standard methods (Nedrud and Blanchard, 2000); while the standard *H. pylori* ATCC 43504 strains were supplied by the College of Pharmacy, University of Illinois, Chicago, USA.

2.5. Susceptibility testing

Susceptibility of *H. pylori* to *n*-hexane and methanol extracts was determined by the agar cup diffusion technique as previously described (Lawal et al., 2014). Inoculated plates were incubated at 37 °C in an incubator under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2) for 2–3 days after which the diameters of the zones of inhibition (mm) were measured. Twenty percent of DMSO was included in each plate as a solvent (negative) control while Gentamycin and Ofloxacin were included as positive controls.

2.6. Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of the methanol extract, the only extract to show any activity in the susceptibility testing, was determined for susceptible *H. pylori* strains with a diameter zone of inhibition of 14 mm and above. The test was performed by a modification of standard agar dilution method procedures as previously described (Adeniyi et al., 2009). The extract was tested at various concentrations ranging from 20 to 90 mg/mL. The positive control antibiotic included was Ofloxacin. The MICs were determined after 3–5 days of incubation at 37 °C under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2). The MIC was regarded as the lowest concentration that prevented visible growth from a duplicate experiment.

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