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Effect of *Myristica fragrans* Houtt. Seed (Nutmeg) on *Helicobacter pylori*-induced gastritis in albino rats: *in vitro* and *in vivo* studies

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ABSTRACT

The anti-*Helicobacter pylori* (*H. pylori*) activities of dichloromethane and methanol extracts of *Myristica fragrans* Houtt. seed (nutmeg) was studied to authenticate traditional use in gastrointestinal disorder. Anti-*H. pylori* activities using the agar dilution method was investigated in 39 strains of *H. pylori* comprising 38 clinical isolates and a standard strain. Bactericidal studies were done by the viable counting technique. The effect of 500 mg/kg and 250 mg/kg body weight of the methanol extract of nutmeg on *H. pylori*-induced gastritis and colonization was investigated in albino rats. The minimum inhibitory concentration (MIC) was 6.25 mg/mL while the minimum bactericidal concentration (MBC) ranged from 6.25 mg/mL to 100 mg/mL. Bacterial density score of the gastric mucosa reduced from $5.0 \pm 7.07 \times 10^8$ to $1.6 \pm 1.4 \times 10^4$ and $3.45 \pm 1.4 \times 10^4$ CFU/mL (mean ± SD, $p < 0.05$) after treatment with 500 mg/kg body weight and Ofloxacin 400 mg/kg respectively. Analysis of variance (ANOVA) tested the effect of the groups on the treatment days and revealed a significant difference between the treatments at $p< 0.05$. The results of these studies have proven the activities of *Myristica fragrans* Houtt. seed on *H. pylori* - induced gastritis in albino rats.

Keywords: Crude extracts, susceptibility testings, bactericidal studies, bacterial inoculation, histopathological examinations.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is an important etiologic impetus usually leading to chronic gastritis, gastroduodenal ulcer and low grade gastric mucosa associated lymphoid tissue lymphoma. Epidemiological data shows that a high *H. pylori* infection rate is related to the high incidence of gastric cancer and gastric adenocarcinoma (Forman and Burley, 2006). The World Health Organization through the International Agency for Research on Cancer in 1994 recognized *H. pylori* as a class 1 carcinogen and it is now recognized as a primary bacterial gastric pathogen in humans (Blaser and Atherton, 2004). The incidence of

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infection by *Helicobacter pylori* in human varies with age and socioeconomic condition, however, symptomatic *H. pylori* gastritis occurs with high frequency in the Western World (Perez-Perez et al., 2005). *H. pylori* infection is common worldwide with prevalence rates ranging from 30 to 40% in the United States, 80 to 90% in South America and 70 to 90% in Africa (Ndububa et al., 2001; Genta, 2002; Perez-Perez et al., 2005; Tsai et al., 2005). A very high prevalence of *H. pylori* (88.9%) in South West, Nigeria has been reported (Adeniyi et al., 2012). A recent trend in therapeutic regimens for *H. pylori* eradication is adoption of a triple therapy with proton pump inhibitor and two antimicrobials-amoxicillin and clarithromycin. However, the occurrence of strains resistant to clarithromycin has given rise to concern, and this problem might be of particular importance in areas where many people are infected with *H. pylori* (Wang et al., 2005). Therefore, it is important to search for non-antibiotic substances, which are highly safe in terms of gastrointestinal protection from *H. pylori*-associated diseases.

Myristica fragrans Houtt. (nutmeg) is used as a cure for headache and as a gastrointestinal drug in the Indian ancient Ayurveda; and has been used for dyspepsia, bellyache, diarrhoea and vomiting in the traditional Chinese medicine. *Myristica fragrans* has reportedly been used as a fruit paste and applied to teeth. Extracts of *M. fragrans* have been reported to have antimicrobial activity against *Escherichia coli, Salmonella*, and other bacteria not typically found in the mouth, and not known to have any implication in causing plaque or gingivitis (Indu, 2006). In traditional medicine, the seed kernel (nutmeg) is widely used as carminative, astringent, hypolipidaemic, antithrombotic, antiplatelet aggregation, antifungal and aphrodisiac (Sonavane et al., 2002). It is also used in the treatment of flatulence, nausea, and dyspepsia (Zaidi et al., 2009). In Nigeria, nutmeg is used as culinary spices and preservatives in snacks production. While some researchers have reported the *in vitro* anti- *H. pylori* activity of nutmeg (Mahady et al., 2005; O'Mahony et al., 2005), the medicinal purpose most especially for gastrointestinal disorder has not been investigated in Nigeria, hence the importance of this study. In addition *H. pylori*-induced gastritis in albino rat model has not also been investigated in Nigeria.

MATERIALS AND METHODS

Plant collection, extraction, and preparation of extracts

The seed of *Myristica fragrans* Houtt. (nutmeg) was obtained at Bodija Market, Ibadan, Nigeria and authenticated at the Forest Research Institute of Nigeria (FRIN), Ibadan and at the Department of Botany, University of Ibadan, Ibadan, Nigeria. The plant sample was air-dried and then meshed prior to extraction. The plant material (3 kg) was subjected to exhaustive Soxhlet extraction with methanol. Partitioning of the extracts with *n*-hexane and dichloromethane based on their polarity was done to have three different fractions: *n*-hexane, dichloromethane and methanol fractions. The resulting extracts were concentrated to dryness under reduced pressure, weighed, lyophilized and stored at 4 ^{o}C. Ten grams (10 g) of each extracts reconstituted with 20% ethanol to obtain the stock solutions which were further diluted to final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml were used for the *in vitro* susceptibility testing against the various *H. pylori* strains. For the animal studies, 50 grams of the extracts were prepared to final concentrations of 500 and 250 mg/kg body weight.

Phytochemical screening of extracts

Phytochemical screening was carried out to detect the presence of secondary metabolites such as anthraquinones, tannins, saponins, alkaloids, and phenols using methods described by (Harborne, 1998).

In vitro susceptibility testing

The susceptibility and minimum inhibitory concentration (MIC) testing were

performed using the agar dilution procedure guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008). The medium used was Mueller-Hinton agar supplemented with 5% defibrinated horse blood. The final concentrations of the extracts tested were 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL for each sample. A total of 38 clinical isolates and a standard strain ATCC 43504 of *H. pylori* were used in the susceptibility testing. The identification of each organism has been confirmed by Gram stain appearance and a positive urease test. Ofloxacin at a concentration of 10 µg/mL was used as positive control. MacFarland 2 suspensions of *H. pylori* strains were prepared in Tryptic soy broth from 4–5 days old *H. pylori* on blood agar (Adeniyi et al., 2009). The organisms were inoculated onto agar plates containing the plant extracts via a graduated inoculating device which delivers two hundred microlitre (200 µL) per spot. The spots were air-dried before incubating the plates at 37 ^{o}C under a microaerophilic gas mixture composed of 10% CO_2, 5% O_2, and 85% N_2 (Campygen, Oxoid, UK) at 100% humidity and examined for growth after 5 days. All procedures were repeated for accuracy. The least concentration that gave no visible growth was taken as the minimum inhibitory concentration (MIC) of the extract. The minimum bactericidal concentration (MBC) testing was performed as previously described by (Adeniyi et al., 2009; Aibinu et al., 2007). The lowest concentration that prevented bacterial growth after days of incubation was recorded as the minimum bactericidal concentration (MBC). The bactericidal activity was performed using viable counting techniques previously described (Ogudo et al., 2014).

In vivo studies: albino rat model

Animal

Specific pathogen-free male albino rats (Central animal house, College of Medicine, University of Ibadan, Nigeria) 6-8 weeks old, were housed in plastic cages with hard wood chips in an air-conditioned biohazard room for infection with a 12 hours light-12 hours dark cycle. They were given food (Ladokun & Sons pelletized rat feeds, Ibadan, Oyo State, Nigeria) and water in bottles *ad libitum*.

Bacterial inoculation

H. pylori BAA 037 susceptible to methanol extract of *M. fragrans in vitro* was grown in Tryptic soy broth (Oxoid, UK) and cultured on Mueller Hinton agar (Oxoid, UK) containing 5% ^{V}/v horse blood at 37^{o}C in a microaerophilic gas mixture composed of 10% CO_2, 5% O_2, and 85% N_2 (Campygen, Oxoid, UK) at 100% humidity. After each rat had fasted for 24 hours, 0.8 mL sample containing 1×10^9 colony-forming units (CFU) per milliliter (1×10^9 CFU/mL) of *H. pylori* was used as the inoculum and delivered via the oral canular.

Extract preparation

Lyophilized methanol extract of *M. fragrans* Houtt. (nutmeg), was reconstituted in 20% ethanol to final concentrations equivalent to 250 mg/kg body weight and 500 mg/kg body weight of rats.

Experimental protocol

Thirty-three (33) albino rats were divided into five groups. Group A and B were the treatment groups and were given 500 mg/kg (Group A, n= 9) and 250 mg/kg (Group B, n= 9) body weight of methanolic extract of *Myristica fragrans* 12 hourly. Group C (n=5) served as positive (drug) control and received *H. pylori* challenge and given 400 mg/kg body weight of Ofloxacin 24 hourly. Group D (n=5), the negative control group received *H. pylori* - challenge but no treatment. Group E (n=5) did not receive the treatment nor *H. pylori* challenge. At 8-10 weeks of age, all animals except Group E were fasted for 24 hours and then inoculated with *H. pylori* BAA 037 by gavage (0.8 mL, 1.0×10^9 CFU/animal). The infected groups (A - D) were given standard diet and water alone for a week to allow colonization and establishment of infection in the gastric mucosa. From day one till the end of the experimental period, body weights of the rats were measured once a week and animals were monitored daily for their general health. After a week of infection, rats from groups A, B and C were

sacrificed and the stomach excised. The excised stomachs were cut open along the greater curvature and then were divided in half along the lesser curvature. One half was used for a culture study while the other was used for histopathological analyses to ascertain the establishment of infection. This procedure was carried out one week and two weeks after treatment to monitor the effect of the extract on *Helicobacter pylori* and gastritis. The excised stomach (half) was homogenized with 5 mL of Tryptic soy broth with the help of vortex mixer and then diluted serially from 1: 10 (10^{-1}) to 1: 1000000 (10^{-6}). Aliquots (100 µL) of the dilution was inoculated onto Columbia agar (Oxoid, UK) plates supplemented with 5% horse blood and incubated under microaerophilic condition and high humidity for 3 to 5 days. After 5 days the colonies were counted to determine the level of *H. pylori* colonization and effect of treatment. Colonies were identified as *H. pylori* based on their characteristic morphology. The density of *H. pylori* was assessed as CFU per whole stomach. This was carried out at a week after infection, week 1 of treatment and 2 weeks after treatment. The other half was fixed in 10% neutral buffered formalin for histopathological examination. The effect of treatment was evaluated using the Updated Sydney System which was reported as severe, moderate and mild.

Statistical analysis

Analysis of variance (ANOVA) tested the effect of the extract in animal groups on the treatment days and it revealed a significant difference between the treatments at $p< 0.05$. The possibility of the methanol extract of *Myristica fragrans* reducing *H. pylori* load and thus effecting healing of the gastritis was compared with effect of the control drug-Ofloxacin in *H. pylori*-induced gastritis groups.

RESULTS

***In vitro* susceptibility**

The crude methanol extract of nutmeg was phytochemically screened for the presence of secondary metabolites; the result revealed the presence of alkaloids, anthraquinones, flavonoids, tannins and phenols. The dichloromethane extract inhibited the growth of four out of the thirty-nine *H. pylori* isolates tested *in vitro* with MIC value of 6.25 mg/mL and MBC values of 6.25 mg/mL- 12.5 mg/mL (Table 1). *H. pylori* BAA 037 was the only strain susceptible to methanol extract of *M. fragrans* with MIC and MBC values of 25 mg/mL and 100 mg/mL respectively. Bactericidal studies (kill kinetics) of the extracts on the *H. pylori* isolates revealed a drastic dose dependent decline in the surviving population after 8 hours of exposure to the extract at doses equivalent to MIC, 2 x MIC and 4 x MIC, accompanied with a total kill of the population at 24 hours (Figures 1-3).

Effects in Albino rats

The *in vivo* study investigated the possibility of *H. pylori* colonizing the gastric mucosa of albino rats and eliciting infection (gastritis) and also if the treatment with different concentrations of methanol extract of nutmeg seed would have effect on the *H. pylori*-induced gastritis. It was noted that *H. pylori* was able to colonize and elicit infection in rat model (gastritis); and that the extract reduced the microbial load and had effect on the induced gastritis. The results of this experiment are shown in Table 2 and Figures 4-8. In Groups A, B, C and D all the animals inoculated with *H. pylori* were infected and developed severe gastritis. The submucosal layer was very prominent, appears severely congested and haemorrhagic after 7 days of infection (Figure 4). In Group A (infected animals treated with 500 mg/kg body weight of methanol extract of *Myristica fragrans*) there was a moderate cellular infiltration and congestion at the lamina propria/submucosal 7 days post-treatment, followed by a mild cellular infiltration after 14 days of treatment (Figure 5). In group B (infected animals treated with 250 mg/kg body weight of methanol extract of *Myristica fragrans*); there was a large extensive focus of severe submucosal and epithelial necrosis as well as cellular infiltration by mononuclear cells after

7 days of treatment. There was no visible lesion seen after 14 days of treatment. Group C (infected animals treated with 400 mg/kg body weight of Ofloxacin) showed a moderate to severe cellular infiltration at the laminar propria with few extending to the mucosal glands after 7 days of treatment and moderate cellular infiltration and congestion at the laminar propria/submucosal after 14 days of treatment (Figure 6). Group D had severe congestion, dispersal of the submucosal connective tissue, with severe cellular infiltration by mononuclear cells. The basal region of the epithelial glands was also congested; there was a focus of epithelial erosion after 21 days of infection without treatment (Figure 7). Group E was the control, normal stomach in which there was no visible lesion seen (Figure 8). The *H. pylori* load in Group A animals (treated with 500 mg/kg body weight of methanol extract of *Myristica fragrans*) was significantly reduced from 5.0 ± 7.07 × 10^8 CFU/mL to 2.7 ± 1.4 × 10^4 CFU/mL and 1.6 ± 1.4 × 10^4 CFU/mL on days 7 and 14 respectively compared to Group C animals (infected animals treated with 400 mg/kg body weight of Ofloxacin) which had a microbial load reduction from 5.0 ± 7.07 × 10^8 CFU/mL to 4.65 ± 1.4 × 10^4 CFU/mL and 3.45 ± 1.4 × 10^4 CFU/mL on days 7 and 14 respectively (Table 2). Importantly, the extract did not increase morbidity or mortality of the animals nor had effect on other organs of the rats.

Table 1: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of lyophilized extracts of *Myristica fragrans* Houtt. (nutmeg) seed on susceptible *H. pylori* isolates.

H. pylori	**Methanol extract (mg/mL)**		**Dichloromethane extract (mg/mL)**		**Ofloxacin (µg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
BAA 025	R	R	6.25	12.5	10	40
BAA 037	25	100	R	R	10	40
BAA 044	R	R	6.25	6.25	120	120
BAA 049	R	R	6.25	12.5	7.5	30
BAA 050	R	R	6.25	12.5	30	30

Note: R = Resistance (i. e. no activity of the extracts)

Table 2: Effect of methanol extract of *Myristica fragrans* Houtt. (nutmeg) on *Helicobacter pylori* colonization in the gastric mucosa of albino rats at day 7 and 14 of treatment.

Group	**Density of *H. pylori* in the gastric mucosa before treatment (CFU/mL)**	**Density of *H. pylori* in the gastric mucosa after treatment- Day 7 (CFU/mL)**	**Density of *H. pylori* in the gastric mucosa after treatment- Day 14 (CFU/mL)**
A	$5.0 \pm 7.07 \times 10^{8}{}_{a}$	$2.7 \pm 1.4 \times 10^{4}{}_{b}$	$1.6 \pm 1.4 \times 10^{4}{}_{c}$
B	$5.0 \pm 7.07 \times 10^{8}{}_{a}$	$5.75 \pm 1.4 \times 10^{4}{}_{b}$	$5.65 \pm 1.4 \times 10^{4}{}_{c}$
C	$5.0 \pm 7.07 \times 10^{8}{}_{a}$	$4.65 \pm 1.4 \times 10^{4}{}_{b}$	$3.45 \pm 1.4 \times 10^{4}{}_{c}$

Means with different subscripts along the same row are significantly ($p < 0.05$) different; Note: The result is mean ± S.D ($p < 0.05$). Group A- *H. pylori* challenged treated with 500 mg/kg body weight of nutmeg extract; Group B- *H. pylori* challenged treated with 250 mg/kg body weight of nutmeg extract; Group C- *H. pylori* challenged treated with 400 mg/kg body weight of Ofloxacin (positive control).

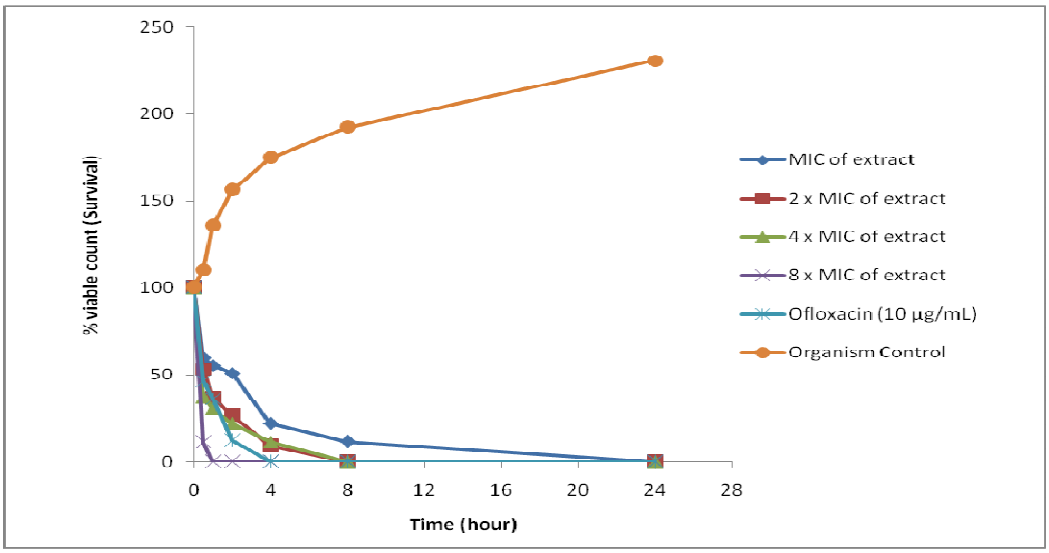

Figure 1: Percentage viable count (Survival) vs Time (hour) of dichloromethane extract of *Myristica fragrans* Houtt. (nutmeg) on *Helicobacter pylori* BAA025 showing the rate of kill at different concentrations.

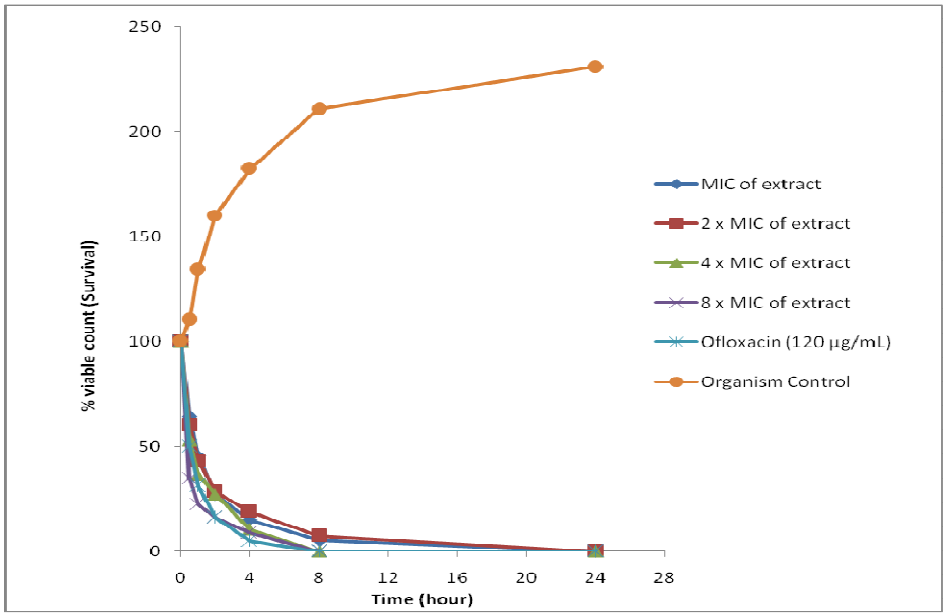

Figure 2: Percentage viable count (Survival) vs Time (hour) of dichloromethane extract of *Myristica fragrans* Houtt. (nutmeg) on *Helicobacter pylori* BAA044 showing the rate of kill at different concentrations.

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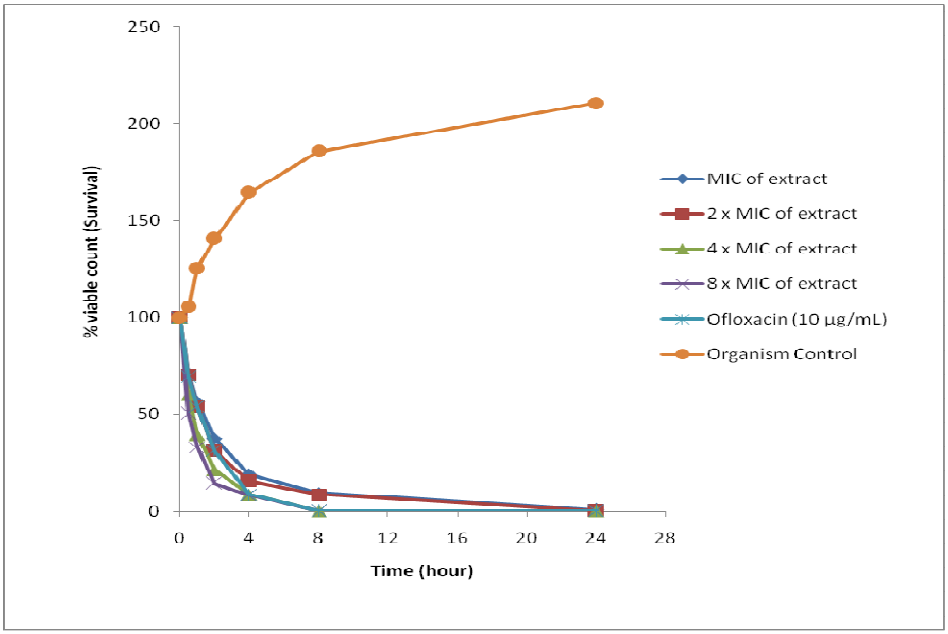


Figure 3: Percentage viable count (Survival) vs Time (hour) of methanol extract of *Myristica fragrans* Houtt. (nutmeg) on *Helicobacter pylori* BAA037 showing the rate of kill at different concentrations.

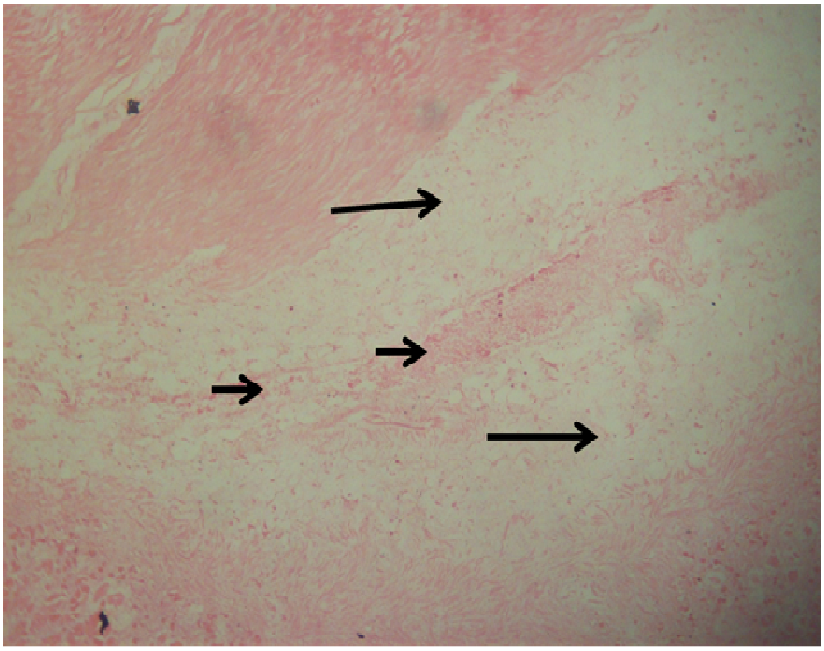
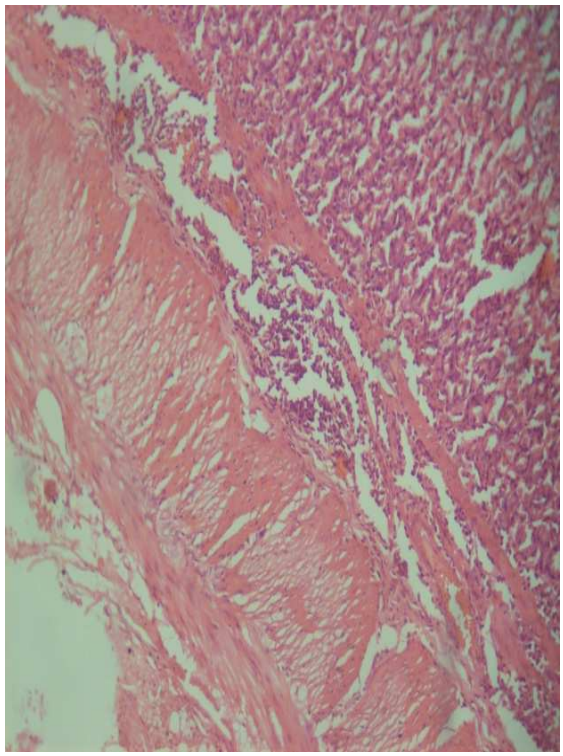


Figure 4: Groups A-D; *H. pylori*- infected stomach before treatment- The submucosal layer is very prominent (longer arrows), and appears severely congested and haemorrhagic (shorter arrows). M x 100, Hematoxylin & Eosin.

A



B

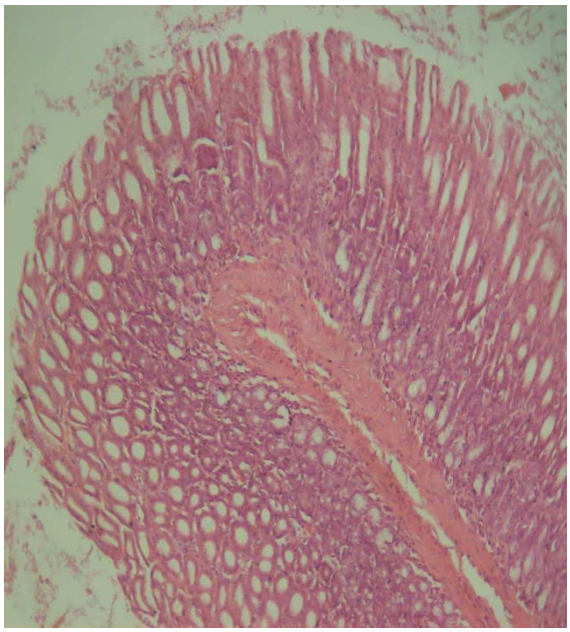
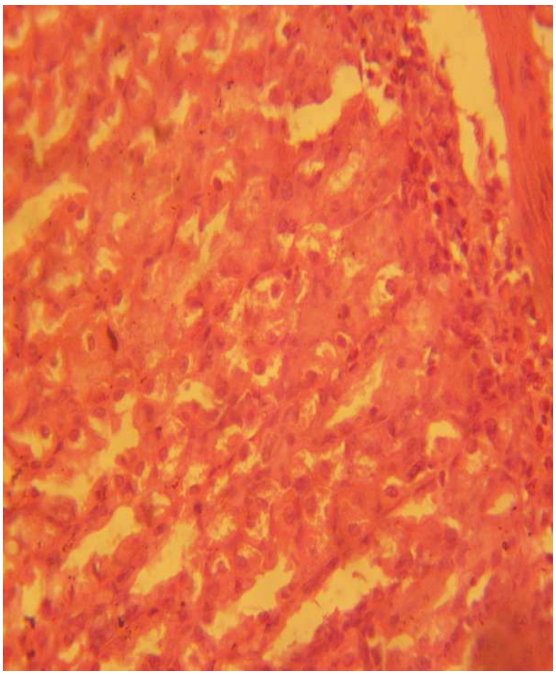
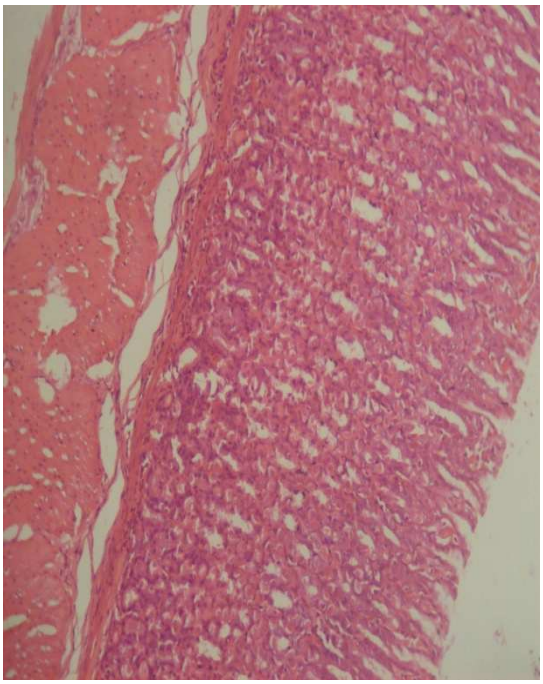


Figure 5: Group A (*H. pylori* challenged treated with 500 mg/kg body weight of nutmeg extract) **A**. Day 7 of treatment- There is a moderate cellular infiltration and congestion at the laminar propria/submucosal. **B**: Day 14 of treatment- There is a very mild cellular infiltration at the lamina propria/submucosal. M x 100, Hematoxylin & Eosin.



A



B

Figure 6: Group C (*H. pylori* challenged treated with 400 mg/kg of Ofloxacin) - **A.** Day 7 of treatment - There is no visible lesion seen. **B:** Day 14 of treatment- There is no visible lesion seen. M x 100, Hematoxylin & Eosin.

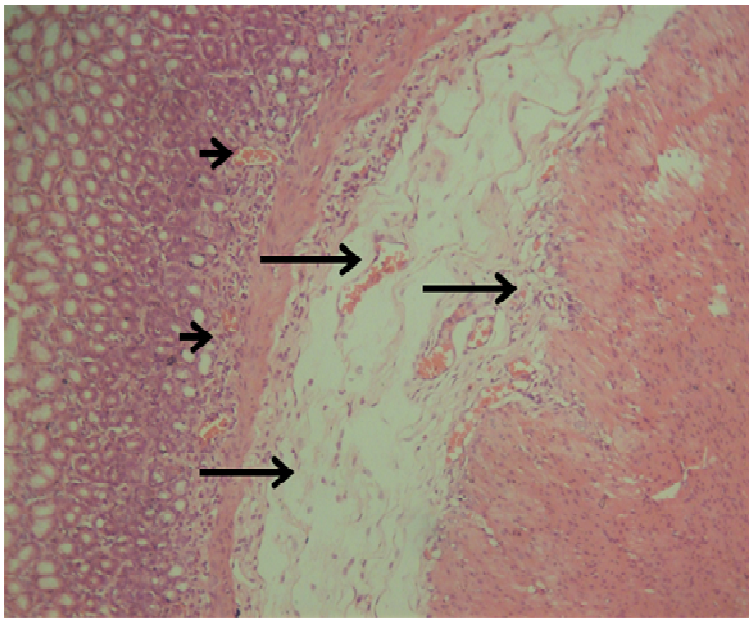


Figure 7: Group D (negative control group received *H. pylori* - challenge but no treatment) - There is severe congestion, dispersal of the submucosal connective tissue, with severe cellular infiltration by mononuclear cells (longer arrows). The basal regions of the epithelial glands are also congested (shorter arrows).

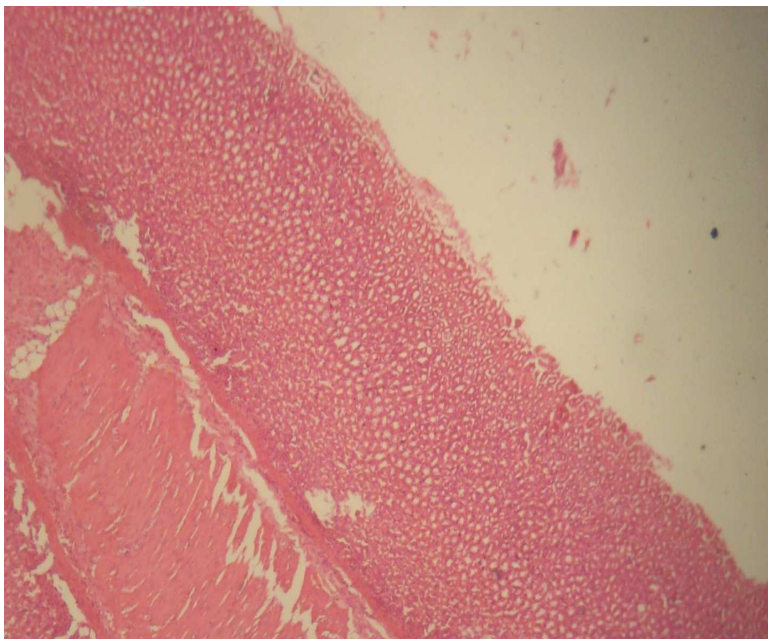


Figure 8: Group E- Normal stomach (receive neither treatment nor *H. pylori* challenge). There is no visible lesion seen. M x 100, Hematoxylin & Eosin.

DISCUSSION

In Nigeria *Myristica fragrans* Houtt. (nutmeg) is used for culinary purpose and as preservatives in snacks production. It is known as a cure for headache and as a gastrointestinal drug in the Indian ancient Ayurveda, and has been used for dyspepsia, bellyache, diarrhoea and vomiting in the traditional Chinese medicine (Jaiswal, 2011). *In* vitro susceptibility testing of *M. fragrans* on *Helicobacter pylori* (*H. pylori*) has been reported (Mahady et al., 2005; O'Mahony et al., 2005). This study investigated the anti-*H. pylori* activity and gastroprotective ability of *M. fragrans* in Nigeria and also to create a rat model to understand the pathogenesis of *H. pylori*-induced gastritis. The present study showed the *in vitro* and *in vivo* effects of nutmeg extracts on *H. pylori*-induced gastritis in albino rat model. Both culture and histopathological examinations revealed a clear reduction in the *H. pylori* colonization as well as reduced mucosal inflammation and epithelial proliferation in the stomach of *H. pylori*-infected rats (Table 2 and Figure 5). The activity of *Myristica fragrans* in reducing *H. pylori* load and thus effecting healing of the gastritis compared favourably well with the control drug (Ofloxacin) which would have been expected to have better activity being a pure drug. There are several animal models available to help understand the pathogenesis of *H. pylori* infection, including gnotobiotic piglets, athymic mice and monkeys (Poutahidis et al., 2001; Raghavan et al., 2003; Kodama et al., 2005). Other studies have reported animal model of *H. pylori* such as induction of ulceration and severe gastritis in *Mongolian gerbil* by *H. pylori* infection (Ohkusa et al., 2003), suppression of *H. pylori*-induced gastritis by green tea extract in *Mongolian gerbil* (Ishizone et al., 2007), among others. However, there are no reports found on rat model *H. pylori*-induced gastritis as at the time this research was carried out. It is therefore interesting to report that rat model could also be used to understand the pathogenesis of *H. pylori* and that rat model could as well mimic human body in addition to previously reported animal models. Although nutmeg has been reported to demonstrate anti-*H. pylori* activity *in vitro* (Mahady et al., 2005; O'Mahony et al., 2005), there are no reports on anti- *H. pylori* activity of nutmeg *in vivo*.

In addition, the *in vitro* experiment revealed a broad antibacterial spectrum of nutmeg extracts including activity against some restricted organisms such as *H. pylori*. However, it did not demonstrate any antibacterial activity against *Escherichia coli* and *Klebsiella pneumoniae* tested in our preliminary study. *Escherichia coli* and *Klebsiella pneumoniae* are well known human intestinal flora. These safety characteristics of nutmeg may therefore be appropriate for its use in prevention of *H. pylori* infection. Chronic inflammation and increased cell proliferation are features common to the pathogenesis of many human cancers, and as these features seem to play a central role in the initiation and promotion of carcinogenesis (Ishizone et al., 2007); the reduction of inflammation and cell proliferation by nutmeg may thus be an effective modality for preventing *H. pylori*-induced carcinogenesis in the stomach.

Conclusion

The *in vitro* and *in vivo* studies have proven the anti-*H. pylori* activity of nutmeg and the ability of *H. pylori* to induce gastritis in albino rats. The development of the albino rat model for *in vivo* studies will enhance researches focused on the discovery and development of new and effective therapeutic agents from medicinal plants for the treatment of *H. pylori* infections especially in the developing world where the majority of the population depends on herbal remedies for cure. They also proved the anti-*H. pylori* and anti-inflammatory potential of *M. fragrans* (nutmeg). Hence nutmeg could serve as a good lead in the development of therapeutic agent for the treatment of *H. pylori*-associated infections. Since nutmeg serves culinary purposes, intake as food supplements could help alleviate or prevent *H. pylori*-associated

diseases. Further researches will focus on bioassay-guided fractionation of active extract.

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