Clinical Microbiology: Open Access
ISSN: 2327-5073

Olorunnipa et al., Clin Microbial 2013, 2:7
DOI: 10.4172/2327-5073.1000132

Research Article Open Access

Anti-*Helicobacter pylori* Activity of *Abelmoschus esculentus* L. Moench (okra): An *in vitro* Study

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Abstract

The anti-*Helicobacter pylori* activity of the methanol and hexane extracts of *Abelmoschus esculentus* L. Moench (okra) dried fruits were evaluated on forty-one clinical isolates and a standard ATCC 43504 strain by the use of agar well diffusion technique. The methanol extract of *A. esculentus* showed *A. esculentus* L. Moench (okra) dried fruit had inhibitory effects against *Helicobacter* strains; with diameters zone of inhibition between 13 and 28 mm on 32 out of the 42 isolates tested. No noticeable zone of inhibition was observed from the hexane extract of the tested plant on all the *H. pylori* strains tested. The bioactive methanol extract of *A. esculentus* demonstrated *A. esculentus* L. Moench (okra) dried fruit had Minimum Inhibitory Concentration (MIC) values of 70 to 85 mg mL^{-1} on selected susceptible strains except *H. pylori* AT CC 43504 which had MIC value of 250 mg mL^{-1}. The time-kill study of the methanol extract of *A. esculentus* on *H. pylori* BAA009, *H. pylori* BAA026 and *H. pylori* ATCC 43504, revealed a decline in the surviving population of the organisms after 8 h of exposure to the methanol extracts of *A. esculentus* L. Moench dried fruit at doses equivalent to MIC2 × MIC and 4 × MIC, and a total kill of the population at 24 h. Therefore, alternative antimicrobial agents may be isolated from further bioassay-guided fractionation of edibles such as *A. esculentus* L. Moench for the treatment of *H. pylori* infections, especially as they are readily available.

Keywords: Anti-*Helicobacter pylori; Abelmoschus esculentus*; Fruit; Kill kinetics; Methanol; Hexane

Introduction

Helicobacter pylori is a Gram-negative spiral-shaped, fastidious, microaerophilic bacillus [1] hum an pathogen currently being investigated worldwide due to its prevalence in almost 50% of the world's population and has been implicated as a major etiologic agent of chronic gastritis, peptic ulcer disease (PUD), gastric adenocarcinoma, and lymphoma [2,3]. Since its first acceptance by the international guidelines in 1996, the standard first-line treatment options for *H. pylori* eradication involves triple therapies which utilize an antisecretory agent (usually a Proton-Pump Inhibitor (PPI)) and two antim icrobial agents most of the ten selected from amoxicillin, clarithromycin, and metronidazole [3]. In the last decade however, a progressive decline in cure rates below the acceptable level of 80% has been reported [4] with increasing antimicrobial resistance of *H. pylori* in m any countries leading to difficulty in the successful treatment of *H. pylori* infections [5,6]. Estimates suggest that ~80% of people living in dev eloping countries depend primarily on traditional medicine [7] with the use of herbs from plants as major source for treating diseases [8]. One of such common plant readily available in dev eloping countries like Nigeria is *Abelmoschus esculentus* L. Moench. Also known as lady's finger or okra, *A. esculentus* is edible and well known for its nutritional value and healing properties such as anticancer, reduced heart attack, lower blood cholesterol, relieve intestinal disorder, relieve inflammation of the colon, relieve diverticulitis, relieve stomach ulcer, neutralize acid, lubricate large intestine, treatment of lung inflammation, treatm ent of irritable bowel, keep joints limber, as well as the treatm ent of sore throats, burns, reducing poisonings and psoriasis [9-12]. *A. esculentus* has also been shown to possess antibacterial properties against infectious disease causing bacterial pathogens such as *Bacillus subtilis, Streptococcus pyogens, Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Proteus mirabillis and Pseudomonas aeruginosa* [13], *Rhodococcus opacus, Mycobacterium* sp. and *M. aurum, Staphylococcus aureus, Escherichia coli,* and *Xanthobacter py2* [14], inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa [15] and inhibits the adhesion of *Campylobacter jejuni* to mucosa isolated from poultry *in vitro* but not *in vivo* [16]. In Nigeria and m ost dev eloping countries, *H. pylori* infection is a public-health issue [17]. The aim of this study is to evaluate the *in vitro* anti-*Helicobacter pylori* activity of *A. esculentus*: specifically to determine its zone of inhibition, Minimum Inhibitory Concentration (MIC) and kill rate with time on the organism.

Materials and Methods

Plant collection, extraction, and preparation of extracts

Dried fruits of *A. esculentus* L. Moench (okra) were purchased from Bodija Market, Ibadan, Oyo State, Nigeria; between the months of December 2010 and March 2011; and then identified and authenticated at the Department of Botany and Microbiology, University of Ibadan, and Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State. Voucher specimen was deposited at FRIN with herbarium number FHI 109558. The fruits were dusted and air dried at room temperature for 4 to 5 weeks and then grounded to coarse powder using a dry electric mill (Moulinex). The pulverized plant material (8.6 kg) was extracted (in smaller portions) by subjecting it to exhaustive Soxhlet extraction with *n*-hexane and methanol in

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Received September 06, 2013; **Accepted** October 17, 2013; **Published** October 24, 2013

Citation: Olorunnipa TA, Igbokwe CC, Lawal TO, Adeniyi BA, Mahady GB (2013) Anti-*Helicobacter pylori* activity of *Abelmoschus esculentus* L. Moench (okra): An *in vitro* study. Clin Microbial 2: 132. doi: 10.4172/2327-5073.1000132

succession. Extracts were collected, dried under reduced pressure, weighed, and stored at −20°C for 24 h before use. Stock solutions of lyophilized extracts were reconstituted in 20% DMSO with final concentrations of 100 to 400 mg/ml prepared for the initial screening. Lower concentrations in the range 20 to 300 mg/ml were also prepared to determine the Minimum Inhibitory Concentrations (MICs) of the bioactive crude extracts.

Antimicrobial agents

The chemotherapeutic agents used in the test as positive control were Gentamicin 100 µg/mL (Nichol as Laboratories Limited, England), Ofloxac in 100 µg/mL and Metronidazole 100 µg/mL, while the negative control was 20% DMSO.

Phytochemical screening

Phytochemical screening was carried out t o detect the pres enc e of secondary metabolites such as anthraquinones, tannins, saponins, alkaloids, and carbenolides using methods described by Harborne [18].

Strains of *Helicobacter pylori* and culture methods

Fort y-one clinical isolates and a standard strain ATCC 43504 were used for this investigation. All the clinic al strains were isolated, characterized and identified at The Department of Pharmaceutical Microbiology, University of Ibadan, Ibadan, Nigeria; while the ATCC strain was from College of Pharmacy, University of Illinois, Chicago, USA.

Susceptibility testing

Susceptibility was determined using the agar well diffusion technique. A 0.1 ml aliquot of logarithmic phase broth culture of each bacterium (optical density equivalent to 10^7-10^8 cf µ/ml) was used to seed sterile molt en Mueller-Hint on agar (O XOID) medium with 5% sterile horse blood maintained at 45°C. The seeded plates were allowed t o dry in the incubator at 37°C for 20 min. A standard cork borer (8 mm diameter) was used to cut uniform wells on the surface of the agar, into which was added increasing concentrations of the test extract dissolved in 20% DMSO. A pre-incubation diffusion of the extracts into the seeded medium was allowed for 1 h. Plates were incubated at 37°C in an automatic CO_2-O_2 incubator under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2) for 2-3 days after which diameters of zones of inhibition (mm) were measured. Since each of the extracts was reconstituted in 20% DMSO, these diluents were included in each plate as a solvent control besides the chemotherapeutic agents included as positive controls. This method has been adopted from previous published procedures [19].

Determination of minimum inhibitory concentrations

Minimum Inhibitory Concentrations (MICs) were performed by a modification of standard agar dilution method procedures as previously described [20]. Extracts were tested at various concentrations. The positive control antibiotic included was of loxacin. The MICs were determined after 3 to 5 days of incubation at 37°C under microaerophilic conditions. The MIC was regarded as the lowest concentration that showed no visible growth from a duplicate experiment.

Time-kill Assay

Determination of bactericidal activity of the methanol extract of *A. esculentus*

The viable counting technique was employed for this assay as previously described [21]. An overnight broth culture in 4.5 ml of Trypticsoy broth inoculated in a static growth condition of each organism was made. Two of the *H. pylori* strains coded BAA009 and *H. pylori* BAA026 and a standard strain ATCC 43504 were used for this experiment. A 0.5 ml of each culture was subculture into a warm (37°C) 4.5 ml Tryptic Soy broth and incubated for 90 min using a Gallenkamp orbit al incubator to give a logarithmic phase culture. A 0.1 ml of the logarithmic phase culture was then inoculated into a warm 4.9 ml of Tryptic Soy broth containing the test extract to give 1 in 50 dilution of the culture (equivalent to approximately 1×10^7 colony forming units) and the required concentration of the extract. A loopful of the test sample (extract-culture mixture) was withdrawn immediately, diluted out in Tryptic Soy broth and 0.2 ml of 1:1000 dilution plated on an oven dried Mueller-Hint on agar to give control time 0 min count. Samples were taken at 30 min, 1, 2, 4, 6 and 24 h. The procedure was carried out in duplicate. Plates were incubated at 37°C for 24 h before counting the colonies. Control plates for negative and positive controls were also incubated. The number of colony forming unit were counted after the period of incubation. The numbers of surviving bacterial c ells per ml were calculated by taking into consideration the dilution factor and the volume of the inoculum. All the procedure was repeated for 2 × MIC and 4 × MIC. A graph of percentage viable count against time in hour (h) was plotted to show the rate of k ill of the test organisms after duplicate experiments.

Results

Bactericidal effects against *Helicobacter strains*; with diameters zone of inhibition of the ex tract between 11 and 28 mm, in 31 out of the 42 isolates tested. No noticeable zone of inhibition was observed by the hexane extract of the tested plant on all the *Helicobacter* strains tested.

The Phytochemical screening of the m ethanol and hexane extracts of *A. esulentus* (data shown in Table 1) showed the presence of alkaloids, saponins, cardenolides, anthraquinones and tannis. These various plant metabolites have earlier been reported to possess medicinal, antimicrobial and physiological activities [22,23]. The presence of these secondary metabolites could be the reason for the observed antimicrobial activities of this plant [24]. Many phytomedicines exert their effects through the additive or synergistic action of several com pounds acting at a single or multiple target sites associated with physiological process [25]. It is noteworthy to state that a large concentration of alkaloids were observed in this study, with all the fractions obtained from the m ethanol extract possessing different degrees of antimicrobial activities on *H. pylori* strains.

The MICs of methanol extract of *A. esulentus* on the entire test *H. pylori strains* in Table 1 were observed to be generally high. This is similar to previous works on crude extracts of plants by other researchers, who reported high MIC values against their test microorganisms [26-28]. However, the MIC values confirmed the existence of inhibitory effects of *A. esulentus* dried fruit with MIC

Name Of Extract Plant/Solvent	Alkaloids	Saponins	Cardenolides	Anthraquinones	Tannis	Flavonoid
A. Esculentus (L)/Hexane	+	+	-	-	+	N.D
A. Esculentus (L)/Methanol	+++	+	++	+	+	N.D

Note: +=Low (trace amount), ++=Medium concentration, +++=High concentration, N.D=Not done

Table 1: Phytochemical Analysis of the Crude Extracts of *A. esulentus*.

values of 70 to 85 m g mL^{-1} for both extracts on selected susceptible strains except *H. pylori* ATCC 43504 which had MIC^{-1}

The Phytochemical screening of the methanol and hexane value of 250 mm ethanol extract of *A. esulentus* is shown in Table 1. Anti-*Helicobacter pylori* activity was demonstrated by the crude m ethanol extract of *A. esculentus* at concentration ≤ 400 mg/ml as shown in Table 2. No activity was demonstrated by the hexane extract against *H. pylori* strains. The MIC of the crude m ethanol extract of *A. esculentus* against *H. pylori* strains with susceptibility of 14 mm and above ranged between 70 to 85 mg/ml. The time-kill study of the m ethanol extract of the plant on *H. pylori* BAA009, *H. pylori* BAA026 and *H. pylori* ATCC 43504 are shown in Figures 1-3.

Discussion

In this study, the anti-*H. pylori* activity of the m ethanol and hexane extracts of *A. esulentus* dried fruits was evaluated. The antimicrobial screening results of the anti-*Helicobacter* activity of the extracts by the use of agar well diffusion technique were presented in Table 2. The MICs of 13 out of the 42 isolates of *H. pylori* using m ethanol extracts of *A. esulentus* was determined, while two of the *H. pylori* strains coded BAA009 and *H. pylori* BAA026 and a standard strain ATCC 43504 were used for bactericidal (kill) studies. The studies showed that the m ethanol extracts of *A. esulentus* dried fruit had esculentus. Chaichanawongsaroj et al. [29] has reported similar MIC (>512 µg/mL) result of anti-*H. pylori* activity of while

H. pylori	Methanol extract (mg/ml)		Hexane extract (mg/ml)		MIC (mg/ml)	Ofloxacin (µg/ml	Gentamicin (µg/ml)	Metronidazole (µg/ml)	20% DMSO
	100	400	100	400		100	100	100	0
BAA003	-	-	-	-	N.E	20 ± 0.0	23 ± 0.0	-	0
BAA003	-	-	-	-	N.E	22 ± 0.0	-	-	0
BAA003	-	-	-	-	N.E	18 ± 0.5	20 ± 0.5	-	0
BAA003	-	-	-	-	N.E	22 ± 0.0	16 ± 0.0	-	0
BAA003	16 ± 0.0	N.E	-	-	85	26 ± 0.5	25 ± 0.5	-	0
BAA003	17 ± 0.5	N.E	-	-	N.E	24 ± 0.0	26 ± 0.5	-	0
BAA003	17 ± 0.0	N.E	-	-	80	26 ± 0.0	22 ± 0.5	-	0
BAA003	-	-	-	-	N.E	-	24 ± 0.0	-	0
BAA003	-	-	-	-	N.E	30 ± 0.5	22 ± 0.0	-	0
BAA003	-	-	-	-	N.E	22 ± 0.0	12 ± 0.5	-	0
BAA003	-	-	-	-	N.E	22 ± 0.0	-	-	0
BAA003	-	-	-	-	N.E	24 ± 0.5	30 ± 0.0	-	0
BAA003	15 ± 0.0	N.E	-	-	N.E	22 ± 0.0	26 ± 0.0	-	0
BAA003	16 ± 0.0	N.E	-	-	80	22 ± 0.0	22 ± 0.5	-	0
BAA003	16 ± 0.5	N.E	-	-	80	30 ± 0.5	26 ± 0.0	-	0
BAA003	13 ± 0.5	N.E	-	-	N.E	-	-	-	0
BAA003	17 ± 0.0	N.E	-	-	85	16 ± 0.0	20 ± 0.0	-	0
BAA003	-	-	-	-	N.E	26 ± 0.0	22 ± 0.5	-	0
BAA003	-	-	-	-	N.E	22 ± 0.0	28 ± 0.0	-	0
BAA003	21 ± 0.5	-	-	-	70	28 ± 0.0	22 ± 0.0	-	0
BAA003	17 ± 0.0	-	-	-	85	22 ± 0.5	20 ± 0.0	-	0
BAA003	19 ± 0.5	N.E	-	-	N.E	18 ± 0.0	23 ± 0.0	-	0
BAA003	28 ± 0.5	N.E	-	-	70	25 ± 0.0	-	-	0
BAA003	18 ± 0.0	N.E	-	-	N.E	28 ± 0.0	22 ± 0.5	-	0
BAA003	23 ± 0.0	N.E	-	-	80	22 ± 0.0	16 ± 0.0	-	0
BAA003	15 ± 0.0	N.E	-	-	N.E	17 ± 0.0	16 ± 0.5	-	0
BAA003	19 ± 0.0	N.E	-	-	N.E	16 ± 0.0	18 ± 0.5	-	0
BAA003	17 ± 0.0	N.E	-	-	N.E	18 ± 0.0	16 ± 0.0	-	0
BAA003	20 ± 0.5	N.E	-	-	N.E	24 ± 0.0	44 ± 0.0	-	0
BAA003	16 ± 0.0	N.E	-	-	N.E	25 ± 0.0	24 ± 0.5	-	0
BAA003	18 ± 0.5	N.E	-	-	N.E	36 ± 0.0	28 ± 0.0	-	0
BAA003	22 ± 0.5	N.E	-	-	N.E	34 ± 0.0	26 ± 0.5	-	0
BAA003	19 ± 0.0	N.E	-	-	N.E	27 ± 0.0	23 ± 0.0	-	0
BAA003	21 ± 0.0	N.E	-	-	80	34 ± 0.0	24 ± 0.0	-	0
BAA003	22 ± 0.0	N.E	-	-	N.E	37 ± 0.0	24 ± 0.0	-	0
BAA003	17 ± 0.5	N.E	-	-	N.E	40 ± 0.0	21 ± 0.0	-	0
BAA003	17 ± 0.0	N.E	-	-	N.E	35 ± 0.0	24 ± 0.0	-	0
BAA003	22 ± 0.0	N.E	-	-	N.E	37 ± 0.0	24 ± 0.0	-	0
BAA003	21 ± 0.0	N.E	-	-	N.E	32 ± 0.0	23 ± 0.0	-	0
BAA003	23 ± 0.5	N.E	-	-	80	28 ± 0.0	-	-	0
BAA003	20 ± 0.0	N.E	-	-	80	22 ± 0.0	18 ± 0.0	-	0
ATCC43504	-	26 ± 0.0	-	-	250	21 ± 0.0	15 ± 0.0	-	0

*Result is average of duplicate experiment. -=No activity, N.E=Not evaluated. Diameter of cork borer=8 mm. Note: The MICs of Ofloxacin=40 µg/ml, Gentamicin=80 µg/ml, Metronidazole=N.E on all the *H. pylori* strains

Table 2: Antimicrobial susceptibility of *Helicobacter pylori* t o methanol extracts of *A. esulentus*. Diameter of zones of inhibition (mm) and MICs.

investigating the anti-*H. pylori* and anti-internalization activities of thirteen Thai plant extracts used for gastric ailments in traditional medicine. The time-kill study of the m ethanol extracts on *H. pylori* BAA009, *H. pylori* BAA026 and *H. pylori* ATCC 43504 as shown in Figures 1-3, revealed a dose dependent decline in population after 8 h of exposure to the m ethanol extracts at doses equivalent to MIC, 2 × MIC and 4 × MIC, followed by a total kill of the population at 24 h. A higher kill rate by the extract at higher concentration (4 × MIC) was generally observed, suggesting resistance of the *H. pylori* strains to lower concentrations. The bactericidal activity was observed to be dependent on time and dose/concentration as the percentage reduction in viable count of surviving population increased with increase in exposure time and concentration of the extracts. This is similar to previous kinetics study [30].

H. pylori infection is associated with chronic gastritis, gastric and duodenal ulcers and gastric cancer in humans [31]. Several treatment regimens have been dev eloped and proved to eradicate *H. pylori* with a cure rate of up to 90% [32]. However, these regimens may have side

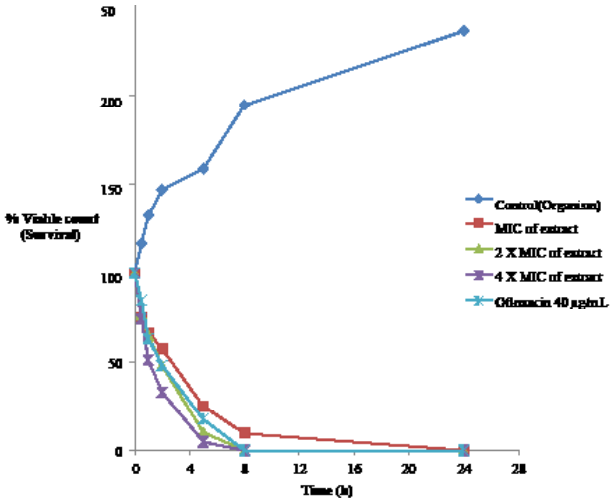

Figure 1: Plot of viable count (survival) vs. time (h) of methanol extract of *A. esculentus* L. Moench (okra) on *H. pylori* BAA009.

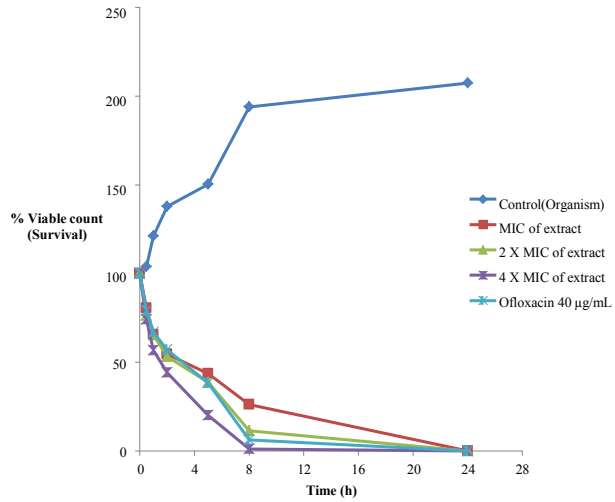

Figure 2: Plot of viable count (survival) vs. time (h) of methanol extract of *A. esculentus* L. Moench (okra) on *H. pylori* BAA026.

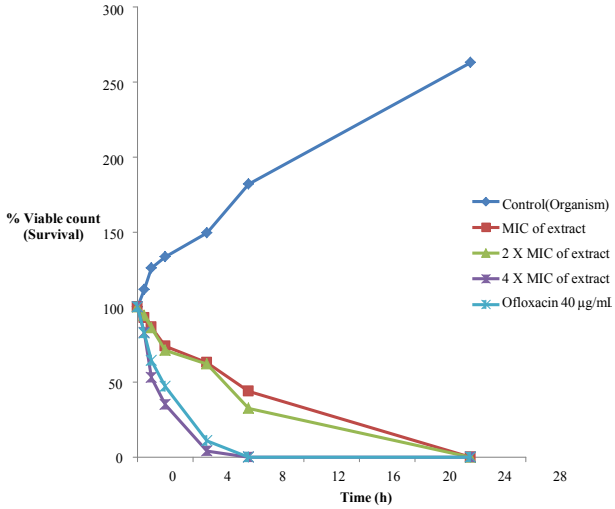

Figure 3: Plot of viable count (survival) vs. time (h) of methanol extract of *A. esculentus* L. Moench (okra) on *H. pylori* ATCC 43504.

effects, poor compliance, and antibiotic resistance [33]. Therefore, alternative antimicrobial agents such as *A. esculentus* L. Moench with fewer side effects are necessary for the treatment of *H. pylori* infection in dev eloping countries, especially as they are edible and readily available.

Conclusion

The anti-*H. pylori* activities exhibited by *A. esculentus* L. Moench suggests its local use in the treatment of gastro-intestinal diseases associated with the *H. pylori* species. Our result show the MIC value does not show potent activity to focus on isolation. However, isolation for phytochemical characterization of active components can be done. Moreover, since this plant is edible it can be safely taken in copious amounts regularly. Thus, it is a potential health food source.

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Citation: Olorunnipa TA, Igbokwe CC, Lawal TO, Adeniyi BA, Mahady GB (2013) Anti-*Helicobacter pylori* activity of *Abelmoschus esculentus* L. Moench (okra): An *in vitro* study. Clin Microbial 2: 132. doi: 10.4172/2327-5073.1000132