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

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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 mgml^{-1} on selected susceptible strains except *H. pylori* ATCC 43504 which had MIC value of 250 mgml^{-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 MIC, 2 × 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.

Key words: Anti-*Helicobacter pylori, Abelmoschus esculentus*, fruit, kill kinetics, methanol, hexane.

INTRODUCTION

Helicobacter pylori is a Gram-negative spiral-shaped, fastidious, microaerophilic bacillus (Marshall and Warren, 1984; Goodwin et al., 1986) human 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 (IARC, 1994; Malfertheiner et al., 2007; Egan et al., 2008; Asaka et al., 2009). Since their 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 antimicrobial agents most often selected from amoxicillin, clarithromycin, and metronidazole (European *H. pylori* Study Group, 1997; Malfertheiner et al., 2002; Malfertheiner et al., 2007; Asaka et al., 2009). In the last decade however, a progressive decline in cure

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rates below the acceptable level of 80% has been reported (Graham et al., 2007; Chey and Wong, 2007) with increasing antimicrobial resistance of *H. pylori* in many countries leading to difficulty in the successful treatment of *H. pylori* infections (Osato et al., 2001; Nahar et al.,2004; Megraud, 2004; Rimbara et al., 2005).

Estimates shows that 80% of people living in developing countries depend mostly on traditional medicine (Sullivan and Shealy, 1997; Singh, 2002) with the use of herbs from plants as major source of treating diseases (Gbile and Adesina, 1986; Ranabir et al., 2006). One of such common plant readily available in developing 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, treatment of irritable bowel, keep joints limber, treatment of sore throats, heal burn soothe poison, soothe psoriasis (Bakhru, 2000; Balch, 2003; Oyelade et al., 2003; Mars, 2004; Arapitsas, 2008; Adelakun et al., 2009). *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* (Yogesh et al., 2011), *Rhodococcus opacus, Mycobacterium* sp. and *M. aurum, Staphylococcus aureus, Escherichia coli*, and *Xanthobacter py2* (Carla et al., 2011), inhibit adhesion of *Helicobacter pylori* to human gastric mucosa (Lengsfeld et al., 2004) and inhibit adhesion of *Campylobacter jejuni* to mucosa isolated from poultry *in vitro* but not *in vivo* (Lengsfeld et al., 2007). In Nigeria and most developing countries, *H. pylori* infection is a public-health issue (Hunt et al., 2010). 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 by a botanist at the department of Botany and Microbiology, University of Ibadan, Oyo State. 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 succession. Extracts were collected, dried under reduced pressure, weighed, and stored at –20°C 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 (Nicholas Laboratories Limited, England), Ofloxacin 100 µg/ml and Metronidazole 100 µg/ml, while the negative control was 20% DMSO.

Phytochemical screening

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

Strains of Helicobacter pylori and culture methods

Forty-one clinical isolates and a standard strain ATCC 43504 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; while the ATCC strain was from College of Pharmacy, University of Illinois, Chicago, USA.

Susceptibility testing

Susceptibility was determined using the agar cup diffusion technique. A 0.1 ml aliquot of logarithmic phase broth culture of each bacterium (optical density equivalent to 10^7-10^8 cfu/ml) was used to seed sterile molten Mueller-Hinton agar (OXOID) medium with 5% sterile horse blood maintained at 45°C. The seeded plates were allowed to 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% N2, 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, this diluent was 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 (Adeniyi et al., 1996; Annuk et al., 1999).

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were performed by a modification of standard agar dillution method procedures as previously described by Adeniyi et al. (2009 a and b). Extracts were tested at various concentrations. The positive control antibiotic included was ofloxacin. 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 described by Lajubutu et al. (1995). An overnight broth culture in 4.5 ml of Tryptic Soy 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 orbital 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-Hinton 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 cells 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 was plotted to show the rate of kill of the test organisms after duplicate experiments.

RESULTS

Anti-*Helicobacter pylori* activity was demonstrated by the crude methanol extract of *A. esculentus* at concentration ≤400 mg/ml as shown in Table 1. No activity was demonstrated by the hexane extract against *H. pylori* strains. The MIC of the crude methanol 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 methanol extract of the plant on *H. pylori* BAA009, *H. pylori* BAA026 and *H. pylori* ATCC 43504 are shown in Figures 1 to 3.

DISCUSSION

In this study, the anti-*H. pylori* activity of the methanol and hexane extracts of *A. esulentus* dried fruits were evaluated. The antimicrobial screening results of the anti-*Helicobacter* activity of the extracts by the use of agar well diffusion technique were presented in Tables 1. The MICs of 13 out of the 42 isolates of *H. pylori* using methanol 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 (kil) studies. The studies showed that the methanol extracts of *A. esulentus* dried fruit had bactericidal effects against *Helicobacter* strains; with diameters zone of inhibition of the extract between 11 and 28 mm, on 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 methanol and hexane extracts of *A. esulentus* (data not shown) 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 (Iwu et al., 1999; Sofowora, 1993). The presence of these secondary metabolites could be the reasons for the observed antimicrobial activities of this plant (Rotimi et al., 1988). Many phytomedicines exert their effects through the additive or synergistic action of several compounds acting at a single or multiple target sites associated with physiological process (Tyler, 1999). It is noteworthy to state that large percentage of alkaloid was observed in this study, with all the fractions obtained from the methanol 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 various researchers who reported high MIC values against their test microorganisms (Mansouri et al., 2001; Akinyemi et al., 2005; Mbata et al., 2006; Ndukwe et al., 2007; Junaid et al., 2008; Chenielle et al., 2009; Adnan and Ayaz-Khan, 2011).However, the MIC values confirmed the existence of inhibitory effects of *A. esulentus* dried fruit with MIC values of 70 to 85 $mgml^{-1}$ for both extracts on selected susceptible strains except *H. pylori* ATCC 43504 which had MIC value of 250 $mgml^{-1}$ on methanol extract of *A. esculentus.* Chaichanawongsaroj et al. (2012) has reported similar MIC(>512 µg/ml) result of anti-*H. pylori* activity of while 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 methanol extracts on *H. pylori* BA009, *H. pylori* BA026 and *H. pylori* ATCC 43504 as shown in Figures 1 to 3, revealed a dose dependent decline in population after 8 h of exposure to the methanol 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 (Funatogawa et al., 2004). It was also observed that a more kill rate was achieved by methanol extract of *A. esculentus* (3 to 4%) across the various

Table 1. Antimicrobial susceptibility of *Helicobacter pylori* to methanol extracts of *A. esulentus*. Diameter of zones of inhibition (mm) and MICs.

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	
BAA003	-	-	-	-	N.E	20±0.0	23±0.0	-	0
BAA004	-	-	-	-	N.E	22±0.0	-	-	0
BAA005	-	-	-	-	N.E	18±0.5	20±0.5	-	0
BAA006	-	-	-	-	N.E	22±0.0	16±0.0	-	0
BAA007	16±0.0	N.E	-	-	85	26±0.5	25±0.5	-	0
BAA008	17±0.5	N.E	-	-	N.E	24±0.0	26±0.5	-	0
BAA009	17±0.0	N.E	-	-	80	26±0.0	22±0.5	-	0
BAA010	-	-	-	-	N.E	-	24±0.0	-	0
BAA011	-	-	-	-	N.E	30±0.5	22±0.0	-	0
BAA012	-	-	-	-	N.E	22±0.0	12±0.5	-	0
BAA013	-	-	-	-	N.E	22±0.0	-	-	0
BAA015	-	-	-	-	N.E	24±0.5	30±0.0	-	0
BAA016	15±0.0	N.E	-	-	N.E	22±0.0	26±0.0	-	0
BAA018	16±0.0	N.E	-	-	80	22±0.0	22±0.5	-	0
BAA019	16±0.5	N.E	-	-	80	30±0.5	26±0.0	-	0
BAA021	13±0.5	N.E	-	-	N.E	-	-	-	0
BAA022	17±0.0	N.E	-		85	16±0.0	20±0.0	-	0
BAA024	-	-	-	-	N.E	26±0.0	22±0.5	-	0
BA025	-	-	-	-	N.E	22±0.0	28±0.0	-	0
BA026	21±0.5	-	-	-	70	28±0.0	22±0.0	-	0
BA027	17±0.0	-	-	-	85	22±0.5	20±0.0	-	0
BA028	19±0.5	N.E	-	-	N.E	18±0.0	23±0.0	-	0
BA029	28±0.5	N.E	-	-	70	25±0.0	-	-	0
BA030	18±0.0	N.E	-	-	N.E	28±0.0	22±0.5	-	0
BA032	23±0.0	N.E	-	-	80	22±0.0	16±0.0	-	0
BA033	15±0.0	N.E	-	-	N.E	17±0.0	16±0.5	-	0
BA034	19±0.0	N.E	-	-	N.E	16±0.0	18±0.5	-	0
BA036	17±0.0	N.E	-	-	N.E	18±0.0	16±0.0	-	0
BA037	20±0.5	N.E	-	-	N.E	24±0.0	44±0.0	14±0.0	0
BA038	16±0.0	N.E	-	-	N.E	25±0.0	24±0.5	-	0
BA039	18±0.5	N.E	-	-	N.E	36±0.0	28±0.0	-	0
BA040	22±0.5	N.E	-	-	N.E	34±0.0	26±0.5	-	0
BA042	19±0.0	N.E	-	-	N.E	27±0.0	23±0.0	-	0
BA043	21±0.0	N.E	-	-	80	34±0.0	24±0.0	-	0
BA044	22±0.0	N.E	-	-	N.E	37±0.0	24±0.0	-	0
BA046	17±0.5	N.E	-	-	N.E	40±0.0	21±0.0	-	0
BA047	17±0.0	N.E	-	-	N.E	35±0.0	24±0.0	-	0
BA048	22±0.0	N.E	-	-	N.E	37±0.0	24±0.0	-	0
BA049	21±0.0	N.E	-	-	N.E	32±0.0	23±0.0	-	0
BA050	23±0.5	N.E	-	-	80	28±0.0	-	-	0
BA052	20±0.0	N.E	-	-	80	22±0.0	18±0.0	-	0
ATCC 43504	-	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.

dose concentrations (MIC, 2 × MIC and 4 × MIC) on the *H. pylori* strains.

H. pylori infection is associated with chronic gastritis, gastric and duodenal ulcers and gastric cancer in humans (Ferreira et al., 2008). Several treatment regimens have been developed and proved to eradicate *H. pylori* with a cure rate of up to 90% (O'connor et al., 2010). However, these regimens may have side effects, poor compliance, and antibiotic resistance (Ki et al., 2011). Therefore, alternative antimicrobial agents such as

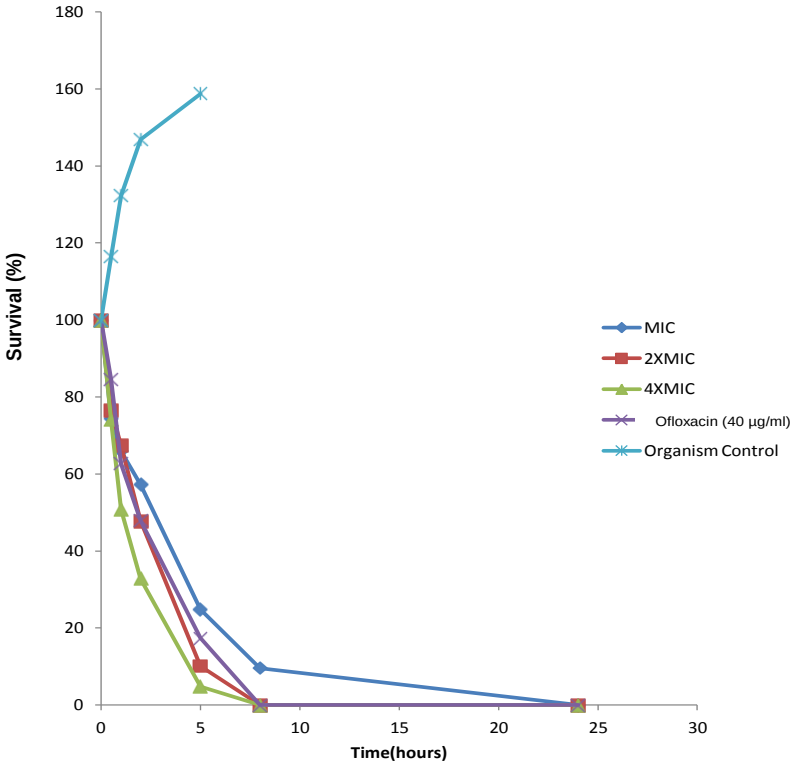

Figure 1. Plot of viable count (survival) vs time (hr) of methanol extract of *A. esculentus* L. Moench (okra) on *H. pylori* BAA009.

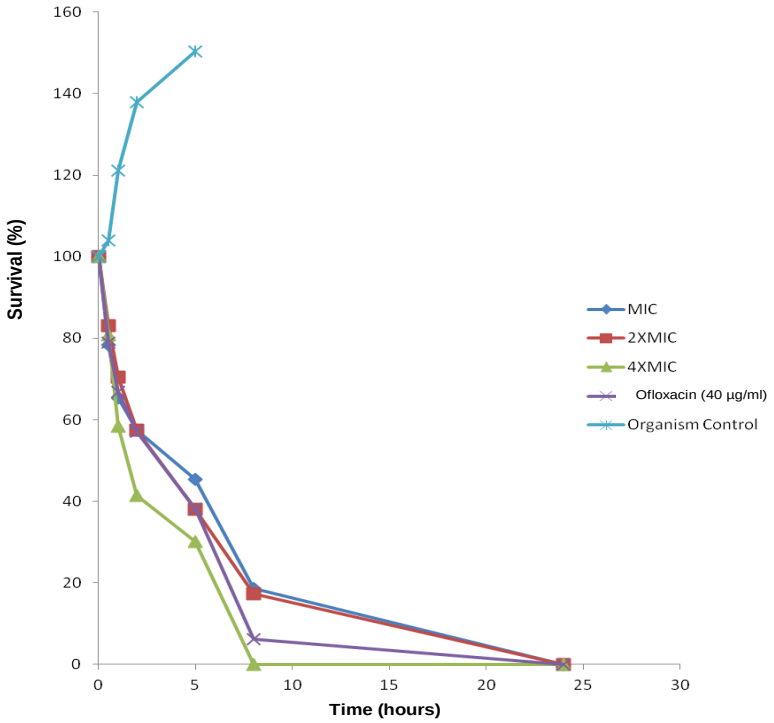

Figure 2. Plot of viable count (survival) vs time (hours) of methanol extract of *A. esculentus* L. Moench (okra) on *H. pylori* BAA026.

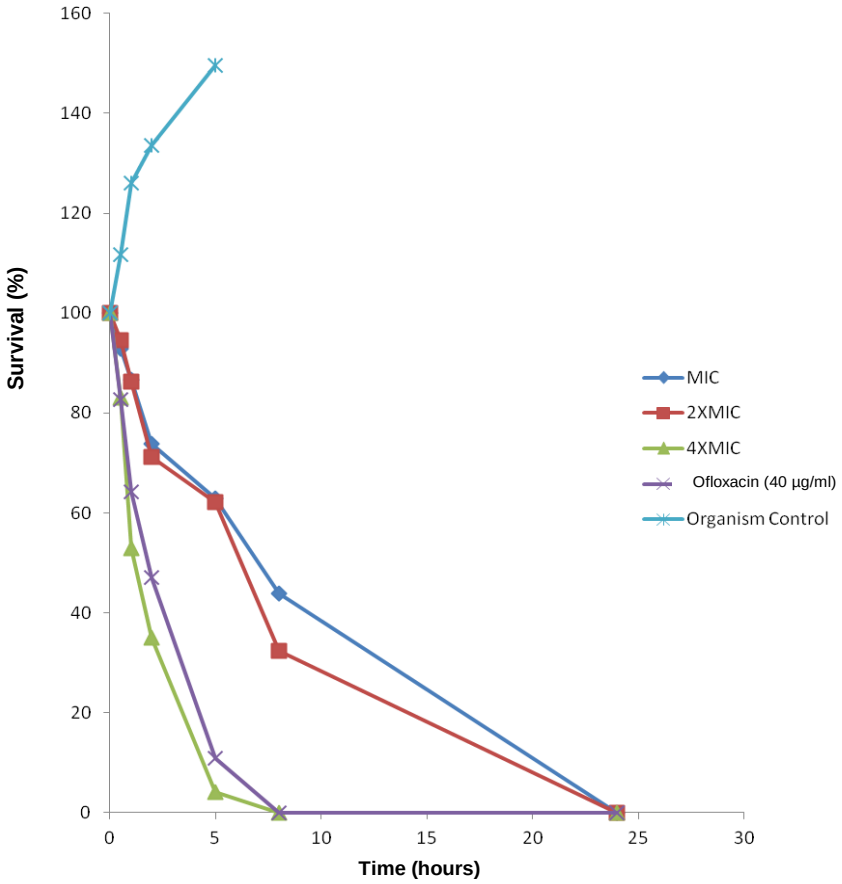

Figure 3. Plot of viable count (survival) vs time (hours) of methanol extract of *A. esculentus* L. Moench (okra) on *H. pylori* ATCC 43504.

A. esculentus L. Moench with fewer side effects are necessary for the treatment of *H. pylori* infection in developing 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 at higher doses. Thus, it is a potential health food source.

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