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Full Length Research paper

Antibacterial effects of crude extract of *Azadirachta indica* against *Escherichia coli, Salmonella* spp and *Staphylococcus aureus*

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Most agents used by humans in the treatment of diseases are of plant origin. *Azadirachta indica* (neem) is a tree which has been found to possess antibacterial, antifungal, anti-inflammatory, anti-tumour properties and is also used as a pesticide. In this work, antibacterial effect of methanolic and aqueous extracts of the stem bark of *A. indica* was determined using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and Kill-time of extracts as indices. Clinical bacterial isolates such as *Escherichia coli*, *Salmonella* spp and *Staphylococcus aureus* were used as test organisms. For the aqueous extracts, a minimum concentration of 43.75 mg/ml was found to inhibit the growth of *E. coli* in nutrient broth. *S. aureus* was inhibited by 87.50 mg/ml and *Salmonella* spp by 175 mg/ml. *Salmonella* spp and *S. aureus* were killed by a minimum concentration of 175 mg/ml but at 1200 and 130 s respectively; *while E. coli* was killed at 87.50 mg/ml at 600 s. For methanolic extract, 43.75, 87.50 and 175 mg/ml concentrations inhibited the growth of *E. coli, Salmonella* spp *and S. aureus* respectively in nutrient broth. Both *Salmonella* spp *and S. aureus* were killed by 175 mg/ml at 600 and 60 s respectively in nutrient broth, while *E. coli* was killed by 250 mg/ml at 1200 s. A comparison of the effects of commonly used antibiotics and the extracts of *A. indica* at 1, 3 and 5 mg per disc on the bacterial isolates by disc diffusion method, showed that the extracts had similar effects on the bacteria as the fluoroquinolones. The crude extracts of *A. indica* were able to inhibit the growth of bacterial isolates *in vitro,* it therefore means that the plant has antibacterial properties. It is recommended that further work be done to identify the specific ingredient(s) responsible for the effect, purify it and standardize same as a drug against bacteria.

Key words: *Azadirachta indica, Escherichia coli*, *Salmonella* spp, *Staphylococcus aureus*, fluoroquinolones.

INTRODUCTION

Medicinal plants have a long history of use and their use is widespread in both developing and developed countries. According to reports of the World Health Organization, 80% of the world's population relies mainly on traditional therapies which involve the use of plant extracts or their active substances (WHO, 1993). Microorganisms have developed resistance against many antibiotics due to the indiscriminate use of antimicrobial drugs (Ahmad et al., 1998). Furthermore, antibiotics are sometimes associated with side effects (Cunha, 2001), whereas there are some advantages of using antimicrobial compounds of medicinal plants, such as fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature (Vermani and Garg, 2002). It is

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known that more than 400, 000 spp. of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern medicine (Odugbemi, 2006). Some plant decoctions are of great value in the treatment of diarrhoea or gastrointestinal disorder, urinary tract infections, skin infections, infertility, wound and cutaneous abscesses (Ergene et al., 2006). The tree*, Azadirachta indica* of the family Maliaceae*;* popularly known as neem tree or dogon yaro (Hausa) is an evergreen tree, native to the Southeast Asia and found in most tropical countries. It has been in use since ancient times, to treat a number of human ailments and also as household pesticide (Chattopadhyay et al., 1993; 1996; Chattopadhyay and Bandyopadhyay, 2005). Extracts from the bark, leaves, fruits and roots have been used to control leprosy, intestinal helminthosis and respiratory disorders (Ketkar and Ketkar, 1995). Every part of the neem tree has been used as traditional medicine for house-hold remedy against various human ailments from antiquity. The tree is still regarded as 'Village dispensary'. It is a plant known over 2000 years as one of the most versatile medicinal plants having a wide spectrum of activity (Larkshmanan and Subramanian, 1996). *Enterobacteriaceae*, the enteric bacteria are facultative anaerobic Gram-negative rods that live in the intestinal tract of animals in health and diseases. The *Enterobacteriaceae* are among the most important bacteria medically. A number of genera within the family are human and animal intestinal pathogens (for example, *Salmonella, Shigella* and *Yersinia*). Several others are normal colonist of the human gastrointestinal tract (for example, *Escherichia coli*, *Enterobacter*, *Klebsiella*), but these bacteria, as well, may occasionally be associated with diseases in humans and animals (Todar, 2008). This study was therefore carried out to determine the antibacterial activity of crudely extracted *A. indica* on clinical isolates of *E. coli, Salmonella* spp. and *Staphylococcus* spp.

MATERIALS AND METHODS

Isolation and characterization of microbes

The test organisms: *E. coli*, *Salmonella* species and *Staphylococcus* species were clinical isolates obtained from the Microbiology Diagnostic Laboratory of Ahmadu Bello University Veterinary Teaching Hospital, Zaria. *E. coli* and *Salmonella* species were subcultured on Brilliant Green Agar (BGA) and MacConkey agar, while *Staphylococcus* species was on sheep blood agar and incubated at 37°C for 24 h. Colonial morphology was observed and Gram's staining was carried out.

Herbal extraction

Source of stem bark and leaves for extraction

The stem bark and leaves were harvested from a neem tree in Ahmadu Bello University environment, close to the Ahmadu Bello University Biological Garden and indentified at the Department of Biological Sciences, Ahmadu Bello University, Zaria. These were properly dried at room temperature, grinded and taken to the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria for extraction.

Extraction by the maceration method

The powdered form of the stem bark and leaves were dissolved in Methanol. The mixture was properly agitated and allowed to stand for 24 h. This was then filtered and the filtrate was poured into an evaporating dish and concentrated to dryness over a water bath at 40°C. A knife was used to scrape the extracts into containers and preserved. The sediments after methanolic extraction were soaked in water for 24 h and same procedure as of the methanolic extraction was followed.

Antibacterial screening test

10 and 50% solutions of methanolic and aqueous extracts of both stem bark and leaves were prepared by dissolving 1 and 5 g in 10 ml each of distilled water. 10 ml each of the prepared concentrations were pipetted into sterile test tubes. Bacterial aliquots of the test organisms were made by scooping 2 colonies each of a 24 h growth of the bacteria into 4 ml of sterile distilled water. 0.2 ml of each of the aliquots containing approximately 5×10^4 bacterial cells or colony forming units was transfered into both of the extract concentrations and allowed to stand for an hour for reaction to take place between the extracts and the bacterial organisms. The mixtures were then inoculated on separate nutrient agar plates and incubated at 37°C for 24 h. Methanolic and aqueous extracts of the leaves at 10 and 50%, and 10% methanolic and aqueous extracts of stem bark had a suppressive antibacterial effect. 50% concentration of both methanolic and aqueous stem bark extracts had inhibitory effect, hence chosen as the working concentration.

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and kill-time of crude extracts on Isolates

Working concentration

50% of methanolic and aqueous stem bark extracts were subjected to double fold serial dilution in 3 sets of 7 test tubes each containing 5 ml of double strength nutrient broth. The nutrient broth served as diluent and media. 0.1 ml of each of the test organisms' aliquot was added to each of the serially diluted extracts and incubated at 37°C for 24 h. MIC was read as the concentration equivalent to the last test tube showing visibly complete clearance. Contents of 3 consecutively retrospective test tubes from the MIC test tubes, were plated on nutrient agar and incubated at 37°C for 24 h. The concentrations of extract in the last plates that showed no growth were taken as the MBC. To obtain the Kill-time, a dilution of the extract using normal saline was made to the MBC equivalents obtained. 0.1 ml aliquot of each of the test organisms were added to the respective extract concentration and plated on nutrient agar at time intervals of 10 s, 20 s, 30 s, 1 min, 2 min, 5 min, 10 min, 20 min, 40 min and 60 min. This was to determine the contact time needed by the crude extract to kill the bacteria. The plates were incubated at 37°C for 24 h. The first plates that yielded no growth were recorded against their corresponding time.

Sensitivity test using the disc diffusion method

Paper discs were made by cutting filter papers into various suitable

sizes and weighed. The discs were immersed into the solution of the extracts, dried and weighed. The amount of extract adsorbed by the discs were determined by subtracting the initial weights of the discs from the final weights of the discs. The discs of different concentrations were then placed on nutrient agar whose surfaces were evenly smeared with the isolates and incubated at 37°C for 24 h and results read by measuring the diameter of clearance around the discs.

RESULTS

Table 1 showed that discs containing extracts concentrations of 1 mg, 3 mg and 5 mg of *A. indica* had no inhibitory effect on *E .coli.*

Table 2 showed discs containing 1, 3 and 5 mg of extracts having 10 to 20 mm zone of inhibition. Table 3 showed that the disc containing 5 mg of extracts produced the largest zone of inhibition of 10 to 20 mm, while discs containing 1 and 3 mg produced less than 10 mm each.

Table 4 showed that *E. coli* required the highest concentration (250 mg/ml) of the plant extract to be able to cause a bactericidal effect and also took the longest time of 20 min before the effect could be seen. *Salmonella and Staphylococcus* needed an equal concentration of 175 mg/ml for bactericidal effect but *Salmonella* took more time (10 min) as opposed to *Staphylococcus* (1 min)

From Table 5 it could be seen that all the bacteria were resistant to enrofloxacin at 5 µg and septrin at 25 µg. All the bacteria were susceptible, at different degrees, to Nitrofurantoin. The highest susceptibility (32 mm diameter) was recorded by chloramphenicol on *Staphylococcus aureus* (plate 3) and Erythromycin on *Salmonella* spp.

Table 1. Inhibitory effect of methanolic leave extracts of *Azadirachta indica* on *Escherichia coli* by disc diffusion method.

Concentration of extract (mg/disc)	Zone of Inhibition (mm)	Interpretation
1	0	Resistant
3	0	Resistant
5	0	Resistant

Table 2. Inhibitory effect of methanolic extracts of the stem-bark of *Azadirachta indica* on *Staphylococcus aureus* by disc diffusion method.

Concentration of Extract (mg/disc)	Zone of Inhibition (mm)	Interpretation
1	10-20	++
3	10-20	++
5	10-20	++

Table 3. Inhibitory effect of methanolic extracts of the stem-bark of *Azadirachta indica* on *Salmonella spp* by disc diffusion method.

Concentration of extract (mg/disc)	Zone of Inhibition (mm)	Interpretation
1	<10	+
3	<10	+
5	10-20	++

Table 4. MIC, MBC and kill-time of methanolic stem-bark extract of *A. indica* on bacterial isolates.

Isolate	MIC (mg/ml)	MBC (mg/ml)	Kill-time (sec)
Salmonella spp	87.50	175.00	600
S. aureus	175.00	175.00	60
E. coli	143.75	250.00	1200

DISCUSSION

From the antibacterial screening tests of the crude extracts of *A. indica* carried out on the selected bacterial isolates (*E. coli*, *Salmonella* spp and *S. aureus*), the methanolic and aqueous extracts of the leaves, up to the concentration of 500 mg/ml were not able to inhibit the bacterial growth on nutrient agar (Figure 1 and plate 1). However, the stem bark extracts, even at 100 mg/ml had inhibitory effects on the bacterial isolates (Tables 2, 3 and Figure 2). This implied that the stem-bark extracts were more effective than the leave extract in terms of antibacterial activity. This may also explain why the stem bark is bitterer than the leaves, meaning it contains more of Nibidin which Biswas et al. (2002) showed in their work to be the main active anti-bacterial ingredient of *A. indica*. The zero zone of inhibition shown in Figure 1 on *E. coli* at concentrations of 1, 3 and 5 mg also agreed with reports by Yagoub et al. (2007) who in their preliminary screening for anti-microbial activity of different plants against different organisms, methanolic extracts of *A. indica* produced zero zone of inhibition against *E. coli.* It is important to state here that the Clinical and Laboratory Standard Institute (CLSI 2010) method for interpreting MIC was not applied here owing to the fact that we were dealing with crude extracts here while the CLSI system deals in purified active ingredients. So because there are quite a number of other ingredients in these crude extracts we could not determine the concentration of the active ingredients. Therefore our measurements here were in mg instead of µg.

Both the aqueous and methanolic stem-bark extracts of *A. indica* had anti-bacterial effects. The growth of *E. coli* in nutrient broth was inhibited by the least concentration of 43.75 mg/ml of the stem-bark extracts of *A. indica*; This was followed by *S. aureus* at 87.50 mg/ml and highest concentration of 175 mg/ml was needed by *Salmonella* spp. *Salmonella* spp. had the highest MIC of the aqueous stem bark extract, and its MBC was at the

Table 5. Susceptibility of bacteria to commonly used antimicrobials.

Antimicrobial	Conc. (µg)	*Salmonella* spp	*E. coli*	*S. aureus*
Septrin (SXT)	25	R	R	R
Chloramphenicol (C)	30	R	S	S
Nitrofurantoin (F)	50	S	I	S
Ciprofloxacin (CIP)	5	I	R	R
Enrofloxacin (ENR)	5	R	R	R
Augmentin (AMC)	30	R	R	I
Erythromycin (E)	10	S	I	R
Tetracycline (TE)	30	S	I	R

Key: S=sensitive (zone diameter of bacterial inhibition of ≥ 15 mm for TE; ≥ 16 mm for SXT, E & F ; ≥18 mm for C & AMC; ≥ 21 mm for CIP & ENR). I = intermediate (zone diameter of bacterial inhibition of 12 to 14 mm for TE, 11 to 15 mm for SXT, E & F: 13 to 17 mm for C & AMC; 16 to 20 mm for CIP & ENR). R = resistant (zone diameter of bacterial inhibition ≤ 11 mm for TE, ≤ 10 mm SXT, E & F; ≤ 12 mm C & AMC; ≤ 15 mm CIP & ENR) (CLSI, 2010).

Figure 1. Plate 1; Inhibitory effect of methanolic leaf extracts of *A. indica* on *E. coli* by disc diffusion method. It showed no zone of inhibition i.e. *E. coli* was resistant. Plate 2; Inhibitory effect of methanolic extracts of the stem bark of *A. indica* on *S. aureus* by disc diffusion method. It showed zones of inhibition i.e. *S. aureus* was moderately susceptible.

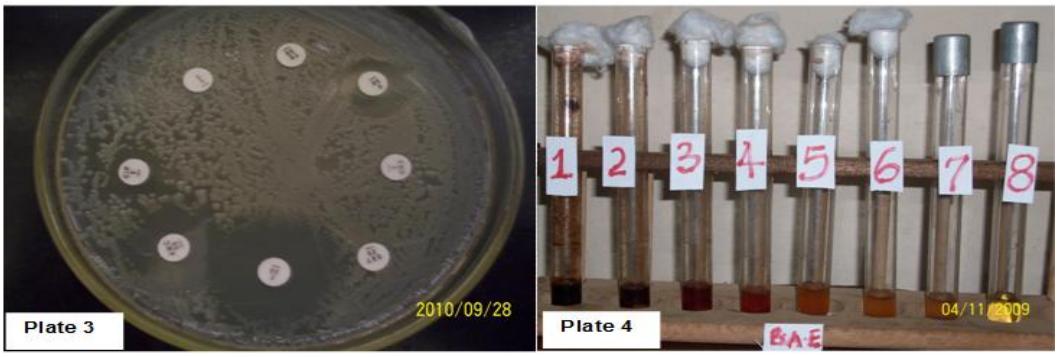

Figure 2. Plate 3; Susceptibility of *S. aureus* to commonly used antimicrobial agents. It showed the highest susceptibility by chloramphenicol. Plate 4; MIC of the stem-bark extracts of *A. indica* on *E. coli* MIC was at tube no. 3, i.e. the tube with least concentration showing clearance.

interval of 130 s. *E. coli* was killed at the least minimum concentration of 87.5 mg/ml within 600 s. The same concentration but it needed the longest time of 1200 s taken to have the killing effect. The MBC of *S. aureus* was 175 mg/ml but it required the shortest time consistency in the effects of these extracts on the test

organisms, implied that neither of the solvents of extraction had a negative effect on the active ingredient. The difference in the effect of this plant extracts within the organisms suggested that there are different antibacterial compounds in the plant extracts and that the compound that acted on one may not be the same as the one that acted on the others since antibacterial agents have different modes of action (Aliu, 2007). This phenomenon of varied susceptibility was also observed by Ergene et al. (2006). The kill-time of both the methanol and aqueous extracts of *A. indica* on Gram-negative organisms was much longer than on Gram-positive organisms. This might be due to the more complex nature of the cell wall of Gram negative organisms as compared with Gram positive organisms. The cell wall of Gram-positive organisms is single-layered; while that of Gram-negative bacteria is multilayered and also bound by an outer cell membrane (Yoa and Moellering, 1995).

In the antibiotic sensitivity tests, all the isolates were resistant to septrin and enrofloxacin. The varying susceptibility of the various bacterial isolates to commonly used antibiotics could be attributed to indiscriminate and irrational use of these drugs in the animals which usually results in resistance developed by the microbes.

CONCLUSION AND RECOMMENDATION

The present work has shown that *Salmonella* spp., *S aureus* and *E .coli* were susceptible to crude extracts of *A. indica in vitro* which means the plant has antibacterial property. It is hereby recommended that further research be done towards isolating, purifying and standardizing the active antibacterial ingredients in *A. indica*. Also more work should be carried out to determine the pharmacokinetics, pharmacodynamics and possible toxicity of the pharmacoactive ingredient(s).

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