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In-vitro antibacterial activity of *Annona senegalensis* root and stem extracts against *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*

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Abstract
The aim of the preset study was to determine the efficacy of *Annona Senegalensis* stem and root extracts against *Salmonella typhimurium Shigella flexneri* and *Escherichia coli* through the evaluation of bacterial sensitivity and determination of the minimum inhibitory and bactericidal concentration of the extracts against the test isolates. Plant materials were collected and duly authenticated. The methanoic and aqueous extracts prepared from the powdered forms were tested on the bacterial after cultural and biochemical identification of the isolates. The antibiotic sensitivity test was carried out using the Kirby Bauer disk diffusion method while chloramphenicol was used as the standard control. The Minimum Inhibitory Concentration (MIC) of the plant extracts were determined by broth dilution method while the Minimum Bactericidal Concentration (MBC) was determined by a method described using standard protocols. The ratio of MBC:MIC was computed to determine the bactericidal or bacteriostatic effects of the extracts. Data were analyzed using the Minitab 16 statistical package. Descriptive statistics (mean and standard error) and analysis of variance tools were applied while mean separation was done Fischer's method at 5% level of significance. Antibacterial sensitivity test showed that the control test (Chloramphenicol) had significantly higher antibacterial sensitivity ($P<0.05$) than any of the plant extract. Minimum Inhibitory Concentration (MIC) of the plant extract ranged from 6.25 mg/ml to 25.0 mg/ml. The lowest MIC of 6.25mg/ml was observed in *Salmonella typhimurium* among all extract types. Root and stem had similar effects on the test organisms ($P>0.05$) but, methanoic root extract had the lowest MBC of 6.25mg/ml against *S.typhimurium* and *S. flexneri*. Based on the MBC/MIC ratio, all extract types had bactericidal effects on *Salmonella typhimurium* and *Shigella flexneri* except aqueous root extract that showed bacteriostatic effect. Only the methanoic root and stem extracts exhibited bactericidal effects on *Escherichia coli. Annona Senegalensis* root and stem could possibly be explored commercially as an antibacterial agent against species of *Salmonella, Shigellia* and *Escherichia.*

Keywords: *Annona Senegalensis*, Antibacteria, *Salmonella*, *Shigella flexneri*, *Escherichia coli*

Introduction
Microbial infections are the cause of numerous endemic, current and reoccurring diseases worldwide, which can be transmitted effectively through physical contact, air, water, food or living vectors (Doron and Gorbach, 2008). Throughout history, infectious microbial diseases have been great threat to health worldwide, as factors like immigration and emigration could bring a diseases from an endemic region to a region where it was previously unknown (Fernandez *et al.*, 2020). Increased spread and transmission of infectious bacterial diseases has led to an overall increase in the use of antibiotics, which has subsequently resulted to an increase the mortality rate due to antibiotic resistance (Almagor *et al.*, 2018).

A number of human lives have been saved since the discovery of antibiotics, due to the significant cutback in the mortality rate caused by bacterial infection, which was achieved through antibiotic therapy. Although in recent times, the efficacy of antibiotics has been threatened by the increase in resistance of bacteria species. Antibiotic resistance is seen to be the ability of some bacteria species to protect themselves and withstand being inhibited or killed by particular antibiotics, even after the normal approved antibiotic dosage has been attained. Bacterial infections caused by resistant species are more likely extend hospitalization and increase the likelihood of mortality (Kaigongi, 2014). Resistance of bacteria to antibiotic agents can be achieved through a natural means; by possessing a gene that is resistant to the antibiotic they produce e.g. *Streptomycete* has the gene of resistance to its own antibiotic. Natural resistance can also be seen in organisms that may lack an effective transport system to carry the antibiotic, or in other organisms that lack a target site to be destroyed by an antibiotic. Also, Gram-negative bacteria have a cell wall barrier against antibiotics, or may completely lack a cell wall e.g. *Mycoplasma*. Bacteria can also gain resistance through the process of natural selection, where the resistant strains are left unharmed and reproduce other organisms, thereby transferring the resistant gene to the next generation and so on (Afolami and Onifade, 2018).

Increasing antibiotic resistance along with emerging bacterial infections have become a worldwide concern, hence highlighting the need to develop alternative antibiotic drugs that will effectively combat not only the resistant bacterial strains, but al so the emerging and persistent microbial pathogens at a global scale (Karahutova *et al.*, 2021). The effect of conventional antibiotics on organisms is played out in several ways which prevent the organism from reaching its full potential, such as; (i). Inhibition of cell membrane functions, such as protection of the cytoplasm (glycopeptides and β-lactam agents). (ii). Inhibition of nucleic acid synthesis (rifampin and fluoroquinolones). (iii). Inhibition of protein synthesis (tetracyclines and macrolides). (iv). Interference with cell wall formation (daptomycin and polymyxin). (v). Interruption of metabolic pathways and (vi). Disruption of bacterial membrane structure. Antimicrobial resistance to conventional medication has been an emerging and reoccurring problem to modern medicine, as many bacterial strains are impervious to conventional antibiotics by way of various mechanisms, as well as having the ability to transfer their resistance properties to other bacteria (Ebrahim, 2010; Sanchez *et al.*, 2016).

Historically, plant parts have been used for therapeutic purposes since the dawn of time and till date serve as a source of revolutionary antibiotic compounds (Saga and Yamaguchi, 2009; Sarita *et al.*, 2019). An estimated 50% of Western medicines today are of plant origin or have plant materials embedded in them. Antibiotic compounds of plant origins typically affect bacteria growth in ways different from conventional antibiotics, as several plant extracts have been reported to have phytochemical constituents that exhibit biological activity. The medical advantage of these plant extracts on pathogens mainly comes about due to the combination of these phytochemicals, which are produced and stored in specific or all part of the plant. *Annona senegalensis* of the family Annonaceae, known majorly as the wild custard apple is a perennial shrub or small fruit tree of about 2-7m tall, found in the savanna and also parts of the tropical rain forest regions of Africa. The plant has been reported to possess antimicrobial properties but limited work exists to ascertain this claim (Adzu *et al.*, 2005; Ajaiyeoba *et al.*, 2006). It is possible to be a complete cure for gastrointestinal diseases where the causative agents are known for have antimicrobial resistance genes. The aim of the preset study was to determine the efficacy of *Annona Senegalensis* stem and root extracts against *Salmonella typhimurium Shigella flexneri* and *Escherichia coli* through the evaluation of bacterial sensitivity and determination of the minimum inhibitory and bactericidal concentration of the extracts against the test isolates.

Methodology

Study Area

The study was carried out in Makurdi local government area of Benue State, Nigeria, which is located on Latitude 7.7322° N and Longitude 8.5391° E. Stock samples of the bacterial isolates were collected from Federal Medical Center, Makurdi at the Microbiology & Parasitology Unit under the Department of Diagnostics.

Collection and Authentication of Plant Samples

The plant samples were collected from Demekpe at Agboughol village, located in Wadata behind the Makurdi modern market of Modern Market Council ward. The roots and stems of *Annona senegalensis* were obtained from the study area and identified by Mr. Guda Agudu, an agriculturist with the Benue State Ministry of Agriculture and Natural Resources, Makurdi. All materials were transported to the Biochemistry Lab of the Federal University of Agriculture, Makurdi for analysis and experimentation.

Preparation of Plant Extracts

Methanol and distilled water were used as the solvents of choice. After collection, the plant parts were thoroughly washed with clean water, then the root and stem barks of the plant were removed with a pointed knife and shade dried at room temperature for four weeks. After they had dried, the part parts were pounded separately using a wooden mortar and pestle, and the coarse power was subjected to extraction using the cold maceration method. The dry extracts were weighed using a digital weighing balance, methanol root 7.00g, methanol stem 6.50g, aqueous stem 10.50g and aqueous root 7.00g and each dry extract dissolved in 70ml, 70ml, 100ml and 70ml of Dimethyl sulfoxide (DMSO) respectively, giving an initial concentration of 100mg/ml for each extract.

Collection and Identification of Bacterial Isolates

The stock samples of *Salmonella*, *Shigella* and *E.coli* were collected aseptically from the microbiology and parasitology laboratory at Federal Medical Centre, Makurdi and sub cultured on a nutrient agar. After which the isolates were subjected to various confirmatory biochemical, morphological and cultural tests as identified by Shoaib *et al.* (2020). The biochemical tests carried out included Grams stain, catalase test, citrate test, urease test, H_2S test, motility test, microscopy and indole test. Stock solutions were sub cultured into nutrient broth and kept for later use.

Grams stain and Microscopy

A loopful of water was placed on a clean glass slide and inoculated with a wire loop of bacteria, then smeared and heat

fixed by passing it through a flame. A primary stain (crystal violet) was poured and left for about 30-60 seconds then rinsed off with water. Grams iodine was then added and kept for abour 30-60 seconds and washed off. The slide was then washed with 99% acetone to remove excess stain and rinsed off. The secondary stain (safranin) as added and left for 30-60 seconds then washed off. The slide was then left to dry and afterwards viewed under a microscope for the morphological features. Gram-positive bacteria appear purple while Gram-negative bacteria appear pink.

Media Preparation for Antibacterial Effects

Media were prepared aseptically in readiness for antibacterial activities using standard protocols (Hussain *et al.*, 2012). Exactly 500ml of distilled water was added to 11g of Mueller Hinton broth weighed on a digital weighing balance for the determination of Minimum Inhibitory Concentration (MIC). Exactly 250ml of distilled water was added to 7g of Mueller Hinton agar for the determination of Minimum Bactericidal Concentration (MBC)

Sensitivity Test

The antibiotic sensitivity test was carried out using the Kirby Bauer disk diffusion method. Briefly, 9.5g of nutrient agar was dissolved in 250ml of distilled water and sterilized, then poured out into 6 petri dishes, 2 for each organism and allowed to gel. Exactly 0.1µl of test organism was collected in a micropipette and placed on the agar, then uniformly spread across the dish with a spreader. Using the rear end of a micropipette tip, 5 wells were aseptically made in the agar and labeled A,B,C,D,E. After which 0.1µl of each extract was collected using a micropipette and put into each corresponding well, A=methanoic root extract, B=methanoic stem extract, C=aqueous stem extract, D=aqueous root extract and E=chloramphenical (standard control) and incubated at 37ºC for 24 hrs. After incubation, the various zones of inhibition around each well was measured with a transparent metre rule and recorded as the zone of inhibition. This procedure was done in duplicate.

Minimum Inhibitory Concentration (MIC) Test

The MIC of the plant extracts were determined by broth dilution method as described by Mogana *et al.* (2020) with modifications. Briefly, 5mls of each extract was collected and poured into the first tube of each roll in a group, then 5mls drawn and serially diluted into the other tubes, giving an extract concentration of 50.00 mg/ml, 25.00 mg/ml, 12.50 mg/ml, 6.25 and 3.12 mg/ml in each tube. The roles in each group were labeled according to the extract present and each group labelled according to test organism used and then inoculated with 1.00µl of test organism from a micropipette. The tubes were then incubated at 37ºc for 24hrs. After incubation, the tube with the lowest concentration of extract that showed no visible growth or turbidity was recorded as the MIC.

Minimum Bactericidal Concentration (MBC) Test

The MBC was determined by a method described by Varley *et al.* (2021) with modifications. Briefly, Mueller Hinton agar was poured into 3 Petri dishes and allowed to cool and gel, then with a black marker divided into four compartments labeled ABCD. A wire loop from each test tube that showed no visible growth or turbidity was picked and smeared on each corresponding segment on the agar plate, then incubated at 37ºC for 24hrs. After incubation, the segment from the least concentration that showed no visible growth on the Petri dish was recorded as the MBC.

Determination of MBC: MIC Ratio

The ratio of MBC:MIC was done to determine antibacterial activity as identified by Spellberg, (2017) and Mogana *et al.*, (2020), if this ratio fell between 1-4, the effect of the extract on the bacterial isolate was considered bactericidal, but if it was above 4 its effect was seen as bacteriostatic. The ratio was obtained by the formula: MBC value divided by MIC value.

Statistical Analysis

Data were analyzed using the Minitab 16 statistical package. Descriptive statistics (mean and standard error) and analysis of variance tools were applied while mean separation was done Fischer's method at 5% level of significance

Result

Table 1 and 2 provide information on the identity of the test isolate. All isolates formed negative reactions under gram staining and urease test, pale or pink colonial characteristics with circular shape, raised elevation and rod like. They were identified as *Salmonella typhimurium, Shigella flexneri* and *Escherichia coli*. Antibacterial sensitivity test (Table 3) showed that the control test (Chloramphenicol) had significantly higher antibacterial sensitivity ($P<0.05$) than any of the plant extract. *Salmonella typhimurium* was sensitive to the control (51.0mm) followed by the methanoic stem (17.5mm). *Shigella flexneri* was sensitive to the control test (39.0 mm) followed by the methanolic stem and root (23.0mm). *Escherichia coli* was sensitive to the control test (39.0mm) followed by methanoic root (15.0mm). Minimum Inhibitory Concentration (MIC) of the plant extract (Table 4) ranged from 6.25 mg/ml to 25.0 mg/ml. The lowest MIC of 6.25mg/ml was observed in *Salmonella typhimurium* among all extract types and *Shigella flexneri* except the 12.5mg/ml recorded in aqueous stem extract. *Escherichia coli* had the highest MIC value ranging from 12.5mg/l to 25.0mg/ml (Figure 1). Root and stem had similar effects on the test organisms ($P>0.05$). As given in Table 5, methanoic root extract had the lowest Minimum Bactericidal Concentration (MBC) of 6.25mg/ml against *Salmonella typhimurium* and *Shigella flexneri* while aqueous and methanoic root extract had the highest MBC of 50.0mg/ml against *Shigella flexneri* and *Escherichia coli* respectively (Figure 2).

Table 1: Cultural and Morphological Characterization of Bacteria Isolates

Colony colour	Colony shape	Elevation	Morphology	Suspected bacteria
Pale	Circular	Raised	Rod	*Salmonella typhimurium*
Pale	Circular	Raised	Rod	*Shigella flexneri*
Pink	Circular	Raised	Rod	*Escherichia coli*

Table 2: Biochemical Characterization of Bacterial Isolates

Grams reaction	Catalase test	Citrate test	Urease test	Indole test	Motility test	H_2S test	Suspected bacteria
-	+	+	-	-	+	+	*Salmonella typhimurium*
-	+	-	-	-	-	-	*Shigella flexneri*
-	+	-	-	+	+	-	*Escherichia coli*

Table 3: Antibacterial Sensitivity Test of Extracts on Bacterial Isolates (mm).

		Extracts			
Isolates	**Control**	**M.R**	**M.S**	**A.S**	**A.R**
Salmonella typhimurium	51.0 ± 1.0^{a}	15.5 ± 0.5^{bc}	17.5 ± 0.5^{bc}	14.0 ± 0.0^{c}	17.0 ± 1.0^{b}
Shigella flexneri	39.0 ± 3.0^{a}	23.0 ± 3.0^{b}	23.0 ± 6.0^{b}	22.0 ± 0.0^{b}	18.0 ± 0.0^{b}
Escherichia coli	39.0 ± 0.0^{a}	15.0 ± 1.0^{b}	11.5 ± 0.5^{c}	12.0 ± 0.0^{c}	11.0 ± 0.0^{c}

Key: control = Chloramphenicol, M.R = methanoic root, M.S = methanoic stem, A.S = aqueous stem, A.R = aqueous root.
F (*Salmonella typhimurium*)= 506.35, P= 0.000 ($P<0.05$)
F (*Shigella flexineri*) = 6.06, P= 0.037 ($P<0.05$)
F (*Escherichia coli*)= 576.80, P=0.000 ($P<0.05$)
Means that do not share a letter within a row are significantly differen

Table 4: Minimum Inhibitory Concentration (MIC) of *Annona senegalensis* Extracts on Bacterial Isolates (mg/ml).

Isolate	Methanoic root (mg/ml).	Methanoic stem (mg/ml).	Aqueous root (mg/ml).	Aqueous stem (mg/ml).
Salmonella typhimurium	6.25	12.5	6.25	6.25
Shigella flexneri	6.25	6.25	6.25	12.5
Escherichia coli	12.5	12.5	12.5	25.0

T- test (root/stem methanoic extract on isolates) = 0.71, P = 0.519 ($P>0.05$) No significant difference
T- test (root/stem aqueous extract on isolates) = 1.06, P = 0.349 ($P>0.05$) No significant difference

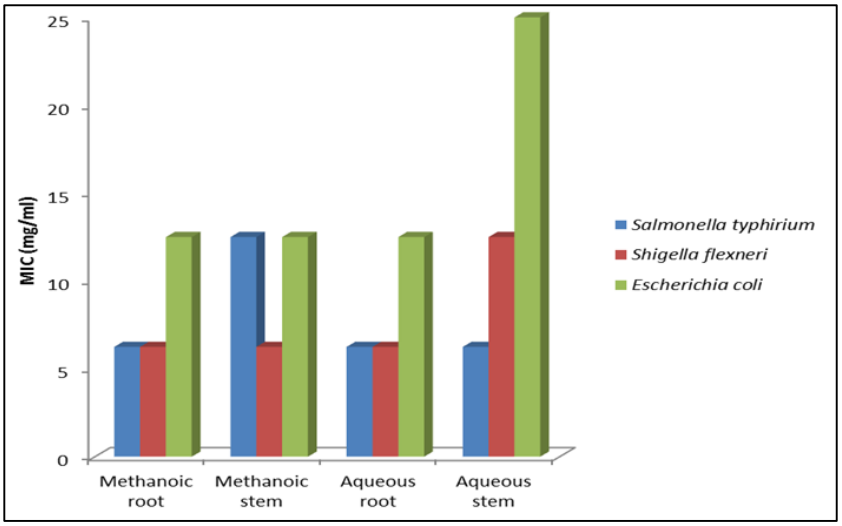

Fig 1: Comparative MIC of different extracts on isolates

Table 5: Minimum Bactericidal Concentration (MBC) of *Annona senegalensis* Extracts on Bacterial Isolates (mg/ml)

Isolate	Methanoic root (mg/ml).	Methanoic stem (mg/ml).	Aqueous root (mg/ml).	Aqueous stem (mg/ml).
Salmonella typhimurium	6.25	12.5	-	12.5
Shigella flexneri	6.25	25.0	50.0	25.0
Escherichia coli	50.0	25.0	-	-

Key: (-) = no activity
T- test (root/stem methanoic extract on isolates) = 0.00, P = 1.000 ($P>0.05$) No significant difference
T- test (root/stem aqueous extract on isolates) = 0.23, P = 0.830 ($P>0.05$) No significant difference

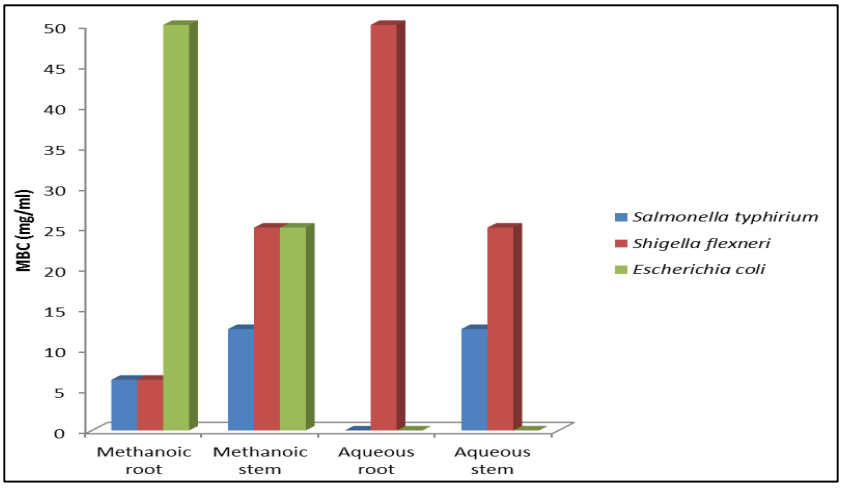

Fig 2: Comparative MBC of different extracts on isolates

All extract types had bactericidal effects on *Salmonella typhimurium* except aqueous root extract. The MBC/MIC ratio was in equal proportion in methanoic root and stem extracts (1:1) while MBC doubled MIC values in aqueous stem extract (Table 6). All extract types had bactericidal effects on *Shigella flexneri* except aqueous root extract that showed bacteriostatic effect. The value of MBC to MIC was the same in methanoic root (1:1) but 4 times more than MIC in methanoic stem. It was doubled in aqueous stem and 8 times more than MIC in aqueous root (Table 7). Only methanoic root and stem extracts exhibited bactericidal effects on *Escherichia coli*. The value of MBC to MIC doubled in methanolic stem and 4 times more than MIC in methanoic root (Table 8). The results for the sensitivity testing reveals that *Annona senegalensis* had a reduced potency on *E.coli*, and a higher effect on *S. typhimurium*, as previously shown the in research of Yaro (2017). The results for the minimum bactericidal concentration of the extracts show the methanoic extracts having significant bactericidal effects at various concentrations while the aqueous extract shows no effects on *E.coli*. According to Spellberg, (2017) and Mogana *et al*. (2020), an antibiotic that achieves an above 1000 fold reduction in bacterial density, but does so at a concentration over 4 fold of the MIC is considered bacteriostatic even though it clearly kills bacteria. By this, it is seen that the aqueous roots extracts possess a bacteriostatic effect on *S. flexneri*, the effect of the aqueous stem extract on *E. coli* as well as the aqueous root extracts on *S. typhimurium* and *E. coli* could not be determined as a result no MBC, while all other extracts possessed bactericidal effects against the isolates.

MIC is defined as the lowest concentration of an antimicrobial agent that inhibited the visible growth of a microorganism after overnight incubation (Cheruiyot *et al.*, 2009). The least concentration where no bacteria growth is recorded is the MBC. In situations where low MIC and MBC values were recorded, it is an indication of the potency of that particular plant against the bacteria tested. The plant investigated has demonstrated strong antimicrobial activities against the three gram-negative bacteria which are associated with different types of diseases such as wound infections, typhoid fever and shigellosis though under specific MIC's and MBC's (Kala, 2005). It is also an indication that the plants are potential sources for production of drugs with broad spectrum activity.

Table 6: MBC: MIC Ratio Showing Effect of Extracts on *Salmonella typhimurium*

Isolate	MIC	MBC	MBC:MIC	Effect
Methanoic root	6.25	6.25	1:1	Bactericidal
Methanoic stem	12.5	12.5	1:1	Bactericidal
Aqueous stem	6.25	12.5	2:1	Bactericidal
Aqueous root	6.25	-	-	ND

ND = not determined, (-) = no activity.

Table 7: MBC: MIC Ratio Showing Effect of Extracts on *Shigella flexneri*

Isolate	MIC	MBC	MBC:MIC	Effect
Methanoic root	6.25	6.25	1:1	Bactericidal
Methanoic stem	6.25	25.0	4:1	Bactericidal
Aqueous stem	12.5	12.5	2:1	Bactericidal
Aqueous root	6.25	50.0	8.1	Bacteriostatic

Table 8: MBC: MIC Ratio Showing Effect of Extracts on *Escherichia coli*

Isolate	MIC	MBC	MBC:MIC	Effect
Methanoic root	12.5	50.0	4:1	Bactericidal
Methanoic stem	12.5	25.0	2:1	Bactericidal
Aqueous stem	25.0	-	-	ND
Aqueous root	12.5	-	-	ND

ND = not determined, (-) = no activity.

Conclusion

The three test bacteria were sensitive to *Annona Senegalensis* stem and root extracts. All antibacterial parameters tested showed that the methanoic stem and root extracts had bactericidal and bacteriostatic effects on the test isolates The plant could possibly be explored commercially as a source of antibacterial agent subject to further pharmacological evaluations.

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