Phytochemical Studies and Antibiofilm Activity of Annona Senegalensis Plant Extracts against Three Diarrhoeagenic Bacteria

Yandev D [1,2]***, Ogbonna IO*** [2,3]***, Orhii CT*** [2] ***and Adie A*** [2]***,***

[1]*Department of microbiology university of Nigeria Nsuka.* [2]*Department of Microbiology University of Mkar, Mkar, Benue State, Nigeria.* [3]*Department of Microbiology, Joseph Sarwuan Tarka University, Makurdi, Benue State, Nigeria.*

ABSTRACT

The aim of this study was to evaluate the stem and root extracts of *Annona senegalensis* for phytochemical analysis and determine the antibiofilm activity against *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli.* Collection of plant and bacterial stock samples followed standard practices. Plant stem and roots extracts were prepared using aqueous and methanolic methods of cold maceration and tested for the presence of various phytochemicals. Quantitative assessment of active ingredients was carried out using spectrophotometric method. Stock samples of bacteria were sub cultured on a nutrient agar and the isolates were subjected to confirmatory tests using cultural, microscopic and biochemical characteristics. Qualitative and quantitative biofilm assays were carried out using the tube method. Data were analyzed using descriptive and inferential methods on the Minitab software. While confidence limit was set at 95% level. Results of phytochemical screening revealed the presence of all phytochemicals tested in the methanolic root and stem extracts. Methanolic root had the highest amount of saponin (1.57%), terpenoid (3.39%) and flavonoid (3.44%). Aqueous stem had the highest amount of phenol (4.30%), steroid (4.71%) and cardiac glycoside (1.9%) while aqueous root had the highest amount of tannin (1.29%) and alkaloid (1.12%). The phytochemicals had significantly varied quantity in the plant ($F = 6.60$, $P<0.05$) but the extract types used had similar effect. The root had the highest percentage distribution of phytochemicals (26%) regardless of the extraction method used followed by the aqueous stem (25%) and methanolic stem (23%). Among the phytochemicals, the mean quantity of phenol was the highest (3.845) followed by steroid (3.1%) and terpenoid (2.58%). Biofilm formation was moderate in *S. typhimurium* (++) but weak the other two (+). Result of biofilm inhabitation assay of *A. senegalensis* plant showed that methanolic extracts (stem and root) performed better than the aqueous method, as low as 12.5mg/ml in *S, flexneri*. Results have proven the potentials of the plant stem and root as a possible remedy for gastro-intestinal diseases and diarrhoea due to the presence of all basic active ingredients that were earlier reported to have antibacterial effects and due to the antibiofilm activities of the methanolic extracts..

Keywords: *Annona senegalensis*, Phytochemicals, Bacteria, Biofilm.

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****Correspondence:***
Yandev, D, 1. Microbiology Department University of Mkar, Mkar, Benue State, Nigeria.
Email:*yandevdoowuese@gmail.com*
Tel: *+2348036911514*

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INTRODUCTION

Plants have been used since time immemorial for their medicinal properties, mainly due to the presence of their natural active ingredients known as phytochemicals. These phytochemicals include tannins, saponins, phenols, flavonoids, alkaloids, steroids, terpenoids, cardiac glycosides etc. which posses therapeutic and antimicrobial effects on different classes of organisms [1]. One of such plants possessing abundant phytochemicals is *Anonna senegalensis,* a plant that is native to the African continent and known to have various medicinal properties and therapeutic effects, including antibiotic activity [2]. The plant was reported to have the potentials of curing diseases as a medicinal fruit bearing plant [3,4,5]. Various ethnomedicinal characters have been attributed to all the parts of *A. senegalensis*. The fruit of the plant is commonly used as a source of food, and also contains essential oils like car-3-ene, which possess various antibacterial and anti-inflammatory characteristics as well head and body ache relieving factors [2]. The root extracts of the plant has been said to possess effects against convulsion, inflammation, snake venom, trypanosome parasites and is also investigated for analgesic properties [6,7]. The leaves are said to contain essential oils like linalool, which are also known to be a potent antibacterial effects [3]. In folk medicine, the plants root bark is used in the treatment of tuberculosis, guinea worm, respiratory infections, malaria parasite and sleeping sickness in northern Nigeria [8, 9]. Among the Tiv people of Benue State, North Central Nigeria, the phytochemicals and essential oils contained in the plants roots, bark and stems are extracted with boiling hot water, then orally administered to a person suffering from diarrhoea, dysentery, vomiting or convulsion, which are all diseases known to be caused majorly by enteric bacterial pathogens, hence *A. senegalensis* is said to be a potent phytomedicinal plant, used unconventionally against various enteric diseases [10]. There is a link between the phytochemical constituents in plants and their antibiofilm activities against disease causing agents. Biofilm is defined as microbial cells immobilized in a matrix of extracellular polymers acting as an independent functioning ecosystem, homeostatically regulated [11]. It has been seen that as a mode of survival, bacteria have the natural ability to cohere and colonize solid surfaces in a wide range of environments, by producing a sessile aggregate of cells encased within an extracellular polymeric substance (EPS) known as a biofilm [12]. The biofilm structures produced by bacteria have a wide range of effects to human and environmental health, as they have the ability to form on a wide range of natural and man-made surfaces including aquatic systems, living tissue, medical devices and industrial piping systems [13]. Many chronic diseases as well as acute infections and several reoccurring diseases become more prevalent due to the formation of a biofilm by pathogenic bacteria, since a biofilm is known to promote bacteria resistance and pathogenicity by producing harmful toxins that become encased in the biofilm matrix. The use of antibiotics against biofilm bacteria is an emerging problem to modern medicine, as conventional medications may do well to eliminate planktonic cells, but are readily defeated by the presence of a biofilm due to the reduced ability of the agents to access the microbes found within the biofilm matrix [14].

Hence, bacterial biofilms pose an enormous health risk in clinical environments, food processing industries and water systems [15,16]. Therefore, eradicating biofilm formation is one effective approach in checking bacterial virulence and resistance. Bacteria found inside a biofilm are known to have a high tolerance to antibacterial agents as well as hosts natural immune defenses and disinfectants

on abiotic surfaces, the biofilm matrix is composed of sessile bacterial cells encased with extracellular polymeric secretions which protect the cells and prevent permeability. For an antibacterial agent to succeed within a biofilm, it must be able to withstand and overcome several biofilm activities such as a variety of resistant mutants, high cell density, efflux pumps, persistence cells, substance delivery and molecular exchanges. All these characteristics make it increasingly difficult, if not impossible for antibacterial agents to be efficient against biofilm forming bacteria. Antibiotic agents must be able to penetrate through the biofilm coat before it can affect the cells within, but with the extracellular polymeric matrix covering the cells, this significantly reduces the entry of antibiotics. The minute amounts of antibiotic agents that do gain entry into the matrix interacts various biofilm properties which produce an anti-spread barrier for the antibiotic agent, hence it is rendered useless within the matrix [17]. The aim of this study was to evaluate the stem and root extracts of *Annona senegalensis* for qualitative and quantitative phytochemical constituents and also to determine the antibiofilm activity of the extracts against three diarrhoeagenic bacterial species, namely: *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli.*

MATERIALS AND METHODS

Sample Collections

The plant samples were collected within Makurdi metropolis, Benue State. Plants were authenticated by technologists in the Benue State Ministry of Agriculture and Natural Resources, Makurdi. Three stock samples of test bacteria (*Salmonella, Shigella* and *E.coli*) were collected aseptically from the Microbiology and Parasitology Laboratory at Federal Medical Centre, Makurdi.

Preparation of Plant Extracts

Stem and root parts were sundried and pounded using wooden mortar and pestle to obtain separate powdered forms. The extracts were prepared were using aqueous and methanolic methods of cold maceration as adopted by [18]. This was done by adding 100g of each plant powder into 946ml glass jars filled with 850ml the extraction solvent for 24 hours and it was followed by appropriate separation technique including filtration and evaporation at 80^{o}C.

Phytochemical Analysis of Extracts

The various extracts were tested for the presence of various phytochemicals through procedures identified by [18] and recorded as + (present) and (absent). Saponin was tested using the Frothing test where 2ml of extract was vigorously shaken with distilled water for about 2 minutes in a test tube, the appearance of stable foaming indicated the presence of saponins. Tannins and phenols were tested by adding a few drops of $FeCl_3$ to 2ml of extract, the development of a dark blue or dark green color indicated the presence of tannins and phenols. A few drops of concentrated H_2SO_4 was added to 2ml of extract, the appearance of a creamy or light yellow coloration was an indication for the presence of flavonoids. Few drops of Meyers reagent was added to 2ml of extract, the appearance of a blue black turbidity was a indication for the presence of alkaloids. About 1g of extract was dissolved in 2ml of acetic anhydride, 1ml of conc. H_2SO_4 was slowly added and a color change to blue or green meant the presence of steroids. Extracts were mixed with 2ml of chloroform then 2ml of conc. H_2SO_4. The appearance of a reddish brown coloration at the interface indicated the presence of terpenoids. Legal's test of cardiac glycosides was conducted where 1ml of pyridine and a few drops of sodium nitroprusside along with a few drops of NaOH solution were put into the extract solution. A deep red colouration

indicated a positive test for cardiac glycosides. Spectrophotometric method was used in the quantitative analysis of active ingredients from each plant extracts using standard procedures.

Cultural and Biochemical Characterization of Isolates

Stock samples sub cultured on a nutrient agar.in the Biochemistry Laboratory of Federal University of Agriculture, Makurdi, Nigeria. They isolates were subjected to confirmatory tests using cultural, microscopic and biochemical characteristics according to the procedures described by [18 19]. The biochemical tests carried out included Grams stain, catalase, citrate, urease, hydrogen sulphide and indole tests. Motility test was also carried out.

Qualitative Biofilm Forming Ability

The biofilm assay was carried out using standard methods [15,16]. Exactly, 6.27g of trypticase soy broth was weighed and dissolved in 200ml of distilled water and 1% glucose, 9ml were dispensed into 18 test tubes then sterilized in an autoclave at 121°c for 15 minutes. After cooling, the tubes were inoculated with 1.00µl of test organism and incubated at 37°C for 24hrs. After incubation the content was decanted and washed out three times with distilled water to get rid of excess planktonic cells then dried at an inverted position. The test tubes were then stained with crystal violet stain and left for 15mins, then rinsed out three times to remove excess stain then inverted and left to dry. The biofilm formation of the isolates was considered positive with the appearance of a visible film line around the test tube. The qualitative biofilm formation was recorded as + (weak), ++ (moderate) and +++ (high).

Antibiofilm Assay

Antibiofilm assay was done using the tube method [15, 16]. Exactly 4.5ml of each extract was added to 9ml of trypticase soy broth with 1% glucose and serially diluted into 36 test tubes, divided into 3 groups arranged in 4 rolls and 3 columns, giving an extract concentration of 50.00 mg/ml, 25.00 mg/ml and 12.50 mg/ml in each tube. Then each group inoculated with 1.00µl of test organism and incubated at 37°C for 24hrs. After incubation, the extracts were considered to have an antibiofilm property with the disappearance of the film line surrounding the tube. The concentration which inhibited the formation of a biofilm was recorded as the antibiofilm concentration. Afterwards, tubes were washed out with 99% acetone to remove all adhered stain.

Data Analysis

The Minitab 16.0 statistical software was used. Data were analysed using descriptive and inferential methods. Confidence level was set at 95% level (5% level of significance)

RESULTS

Results of phytochemical screening of *Annona senegalensis* (Table 1) revealed the presence of all phytochemicals tested in the methanolic root and stem extracts. Thus, Saponin, Tannin, Phenol, Terpenoid, Flavonoid, Steroid, Cardiac Glycoside and Alkaloid were confirmed in the two parts of the plant. However, the aqueous method failed to reveal tannin and phenol in the stem extract as well as alkaloid in the root extract. Methanolic root had the highest amount of saponin (1.57%), terpenoid (3.39%) and flavonoid (3.44%). Aqueous stem had the highest amount of phenol (4.30%), steroid (4.71%) and cardiac glycoside (1.9%) while aqueous root had the highest amount of tannin (1.29%) and alkaloid (1.12%). The phytochemicals had significantly varied quantity in the plant ($F = 6.60$, ($P<0.05$) but the extract types used had similar effect. The root had the highest percentage distribution of phytochemicals (26%) regardless of the extraction method used followed by the aqueous stem (25%) and

methanolic stem (23%) (Figure 1). Among the phytochemicals, the mean quantity of phenol was the highest (3.845) followed by steroid (3.1%) and terpenoid (2.58%) while alkaloid was the lowest (0.69%) (Figure 2). From the results (Table 3), three species of bacteria were identified from the stock bacteria based on morphological and biochemical characteristics. They were *Salmonella typhimurium, Shigella flexneri* and *Escherichia coli*. Among these bacteria, biofilm formation was moderate in *S. typhimurium* (++) but weak the other two (+) as given in Table 4.

Table 1: Qualitative Phytochemical Analysis of *Annona senegalensis* Extracts.

Phytochemicals	Methanoic root	Methanoic stem	Aqueous stem	Aqueous root
Saponin	+	+	+	+
Tannin	+	+	-	+
Phenol	+	+	-	+
Terpenoid	+	+	+	+
Flavonoid	+	+	+	+
Steroid	+	+	+	+
Cardiac Glycoside	+	+	+	+
Alkaloid	+	+	+	-

Table 2: Quantitative Phytochemical Analysis of *Annona senegalensis* Extracts

Phytochemicals	Methanoic root	Methanoic stem	Aqueous stem	Aqueous root
Saponin (%)	1.57	1.43	1.10	1.51
Tanin (%)	1.13	0.54	0.17	1.29
Phenol (%)	3.01	4.14	4.30	3.92
Terpenoid (%)	3.39	2.22	1.72	3.00
Flavonoid (%)	3.44	1.00	0.97	1.94
Steroid (%)	1.42	4.18	4.71	2.09
Cardiac Glycoside (%)	1.24	0.54	1.90	1.40
Alkaloid (%)	0.96	0.33	0.33	1.12

F-test (Extract type) = 0.13, P=0.943 (P>0.05) No significant difference; F-test (Phytochemical type) = 6.60, P=0.000 (P<0.05) Significant

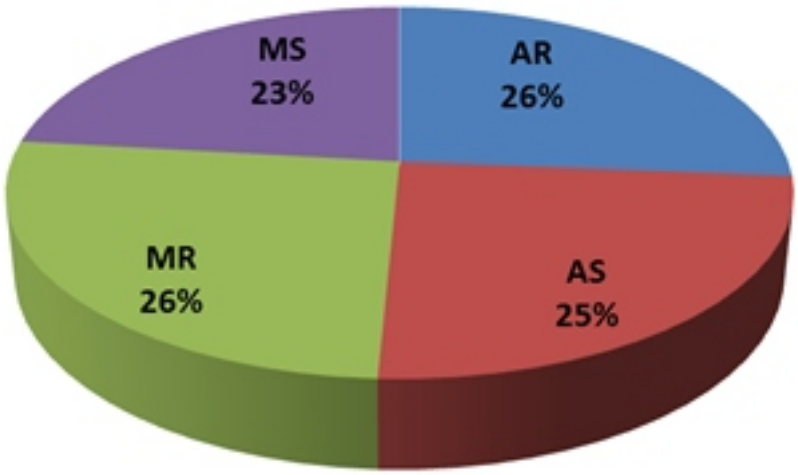

Figure 1: Percentage distribution of quantity of phytochemicals according to extracts types

Key: M.R = methanoic root, M.S = methanoic stem, A.S = aqueous stem, A.R = aqueous root.

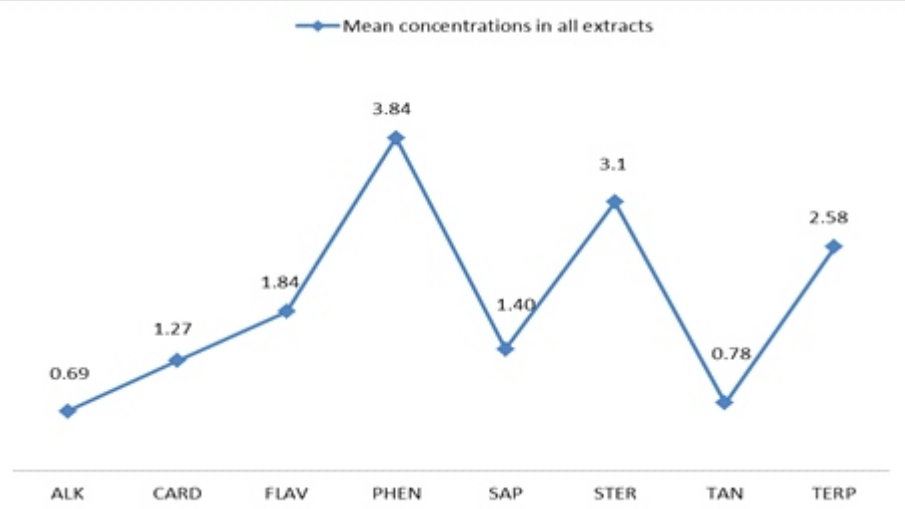

Figure 2: Mean concentration of phytochemicals in *Annona senegalensis*

Alk = Alkaloids, Card = Cardiac Glycosides, Flav = Flavonoids, Phen = Phenol, Sap = Saponin, Ster = Steroid, Tan = Tanin, Terp = Terpenoid.

Table 3: Morphological and Biochemical Characteristics of Bacterial Isolates

Morphological description	Biochemical Characteristics	Bacteria
Pale colony, circular in shape and raised elevation. Rod like in shape	Positive reactions to catalase, citrate, motility and hydrogen sulphide tests. Negative reaction to Gram stain, urease and indole tests	*Salmonella typhimurium*
Pale colony, circular in shape and raised elevation. Rod like in shape	Positive reaction to catalase test only. Negative reaction to Gram stain and other tests	*Shigella flexneri*
Pink colony, circular in shape and raised elevation. Rod like in shape	Positive reaction to catalase, indole and motility tests. Negative reaction to Gram stain and other tests	*Escherichia coli*

Table 4: Qualitative Biofilm Formation of Bacterial Isolates

Isolate	Biofilm formation
Salmonella typhimurium	++
Shigella flexneri	+
Escherichia coli	+

Key: (+++) = strong biofilm producer; (++) = moderate biofilm producer; (+) = weak biofilm producer; (-) = negative biofilm producer

Table 5: Quantitative Biofilm Inhibition Assay of *Annona senegalensis* Extracts on Bacterial Biofilms (mg/ml).

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

T- test (root/stem methanoic extract on isolates) = 0.00, P = 1.000 (P>0.05) No significant difference

Result of biofilm inhabitation assay of *A. senegalensis* plant (Table 5) showed that methanolic extracts (stem and root) had the same effect on the isolates where it was 25mg/l in *S. typhimurium* and *E.coli* but as low as 12.5mg/ml in *S, flexneri*. Effect produced by the root and stem using methanoic extraction was the same (P>0.05). Aqueous stem extract had the highest quantity of 50mg/ml on biofilms produced by *S. typhimurium* and *E.coli.* Aqueous root had the same effect on the three test organisms with values of 25mg/ml.

DISCUSSION

The results of the phytochemical screening of the methanol and aqueous root and stem extracts of *Anonna senegalensis* shows the presence of saponins, tannins, phenols, flavonoids, alkaloids, steroids, terpenoids and cardiac glycosides as previously reported [8, 10]. The methanol extracts possessed the most abundance of these phytochemicals, while the aqueous extracts showed a moderate to high abundance. Results have proven the potentials of the plant stem and root as a possible remedy for gastro-intestinal diseases and diarrhea due to the presence of all basic active ingredients that were earlier reported to have antibacterial effects [3]. The results for the biofilm formation ability reveals that *S. typhimurium* isolate produced a moderate biofilm in the tube, whereas *S. flexneri* and *E. coli* isolates both produced a weak biofilm. This is in agreement with previous studies [15, 16]. Also, methanoic extracts were more potent against the isolates biofilms than the aqueous extracts.

CONCLUSION

This study suggested that the methanoic and aqueous root and stem extracts of *A. senegalensis* are a good potential source for antibiofilm agents, and may hold pharmacological significance in antibiotic therapy as well as combating bacterial resistance associated with gastroenteric infections and diarrhoea. Further studies are needed on the plant to ascertain its toxicity, dosage and other aspects of pharmacological importance. It is however recommended in herbal therapy against the test organisms.

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