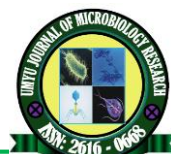




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## In-vitro Antibacterial Activity of Crude Extracts of *Annona senegalensis* Against Selected Bacteria Associated with Urinary Tract Infections

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### Abstract

The treatment of urinary tract infections (UTIs) has become challenging due to antimicrobial resistance exhibited by the causative organisms. Medicinal plants are alternatives to conventional drugs and complement treatment for diverse infections owing to the growing antimicrobial resistance to synthetic drugs. The study assessed the antibacterial activity of aqueous, ethyl acetate, and methanol extracts of leaves, bark, and roots of *A. senegalensis* against selected bacteria associated with urinary tract infections (UTIs). The extracts of *A. senegalensis* were obtained by the soxhlet extraction method. Qualitative phytochemical screening of the extracts was carried out using standard procedures. The bacteria tested were *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from urine samples of UTI patients. Antimicrobial susceptibility testing was carried out using the agar well diffusion method, while minimum inhibitory concentration (MIC) was determined by broth dilution method using two-fold serial dilutions. The qualitative phytochemical analysis showed the presence of phenols, tannins, terpenoids, flavonoids, steroids, saponins, and cardiac glycosides. Zones of inhibition of extracts at 400 mg/mL ranged between 16.33±0.58 - 24.67±0.58 mm (leaf extracts), 14.00±1.00 - 21.33 ±0.58 mm (bark extracts) and 14.67±0.58 - 21.00±1.00 mm (root extracts). The highest zone of inhibition (24.67±0.58 mm) observed was against *Pseudomonas aeruginosa* with aqueous leaf extract, while the least inhibition (14.00±1.00 mm) was with ethyl acetate bark extract, also against *Pseudomonas aeruginosa*. MIC values ranged from 25 to 100 mg/mL, while MBC values ranged from 50 to 200 mg/mL against the test isolates. *A. senegalensis* demonstrated great antibacterial potential and can be recommended for treating UTIs.

**Keywords:** *Annona senegalensis*, Antibacterial Activity, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, Qualitative Phytochemical Screening, Urinary Tract Infection.

### INTRODUCTION

Urinary tract infection (UTI) is among the most common bacterial infections globally. It is defined as the microbial infiltration or colonization of the urinary tract (Barba *et al.*, 2013). Approximately 7 million clinic visits annually result from Urinary tract infections (UTIs) (Fazly Bazzaz *et al.*, 2021).

Treatment of UTIs has become challenging as a result of bacterial pathogens becoming resistant to principal antibiotic classes. Furthermore, many drug regimens prescribed nowadays are very expensive and with serious side effects. This highlights the need for other novel, safe, inexpensive antibacterial agents (Dangarembizi *et al.*, 2013).

Plant medicines have become reputable in preventing and treating various diseases (Khameneh *et al.*, 2019). Vadhana *et al.* (2015) opined that antibiotic-resistant bacteria could

become less common with the help of herbal remedies.

*Annona senegalensis* (Annonaceae), the African Custard Apple or Wild Soursop, is a versatile plant with numerous traditional and therapeutic applications (Babalola *et al.*, 2021). The plant has a widespread distribution in northern Nigeria, particularly in Niger, Benue, Nasarawa, Kano, Kaduna, Plateau States and in the Federal Capital Territory, Abuja (Okhale *et al.*, 2016). The local names are; *Gwándàn dààjii* (Hausa), *dukuu-hi* (Fulani), *Abo* (Yoruba), *ubono-ocha* (Igbo), and *Ahur/Ayam hul* (Tiv) (Ogoli *et al.*, 2011; Babalola *et al.*, 2021).

The stem bark treats gastroenteritis, toothache, malaria, guinea worms, snake bites, diarrhea, and respiratory infections. The leaves are used in treating pneumonia and are also known to boost health. The root treats dizziness, chest colds, stomach aches, and venereal diseases.

Other therapeutic benefits include antioxidant, antimicrobial, anti diarrheal, anti-inflammatory, anticonvulsant, anti-trypanosomal, antsnake venom, antinociceptive, and many other medicinal properties (Awa *et al.*, 2012; Okhale *et al.*, 2016; Djoza *et al.*, 2017).

The plant has found wide applications in traditional medicine among the diverse ethnic groups in Benue State of northern Nigeria. For instance, an infusion of the leaves and stem bark of *A. senegalensis* is used in the treatment of urinary tract infections by herbalists among the Tiv people. The Igedes use the plant with *Ageratum conyzoides* to treat diarrhea infections. It is also combined with *Nauclea latifolia* to treat dysentery. It is thought that *A. senegalensis* produces better efficacy when used synergistically (Ogoli *et al.*, 2011; Babalola *et al.*, 2021). Despite the wide applications of *A. senegalensis* in traditional medicine for curative purposes, there is a dearth of scientific information on the use of the plant against urinary tract pathogens. Thus, the study aimed to assess the antibacterial activity of crude extracts of *A. senegalensis* against bacteria implicated in UTIs.

## MATERIALS AND METHODS

### Plants Collection

Fresh leaves, barks, and roots of *A. senegalensis* were collected from Makurdi Metropolis in Benue State and identified at the Department of Botany, Joseph Sarwuan Tarka University, Makurdi, Benue State, Nigeria.

### Preparation of Plant Samples

The leaves, barks, and roots of the plant were shade-dried (25°C) for three weeks, after which they were pulverized using a mortar and pestle. An electric blender was further used to produce fine powder (Salihu, 2017).

### Extraction of Plant Samples

The dried, powdered plant samples were extracted using a soxhlet apparatus with either water, ethyl acetate (99%), or methanol (99%) as solvents. The soxhlet's thimble was loaded with the plant sample and attached to a round bottom flask with the appropriate solvent. This was fixed on the heating mantle and adjusted to the solvent's boiling temperature. A condenser was attached to the sample holder (thimble) to condense the solvent's vapour. The condensed solvent dripped onto the sample, thus dissolving and extracting the active compounds (Salihu, 2017). Extracts collected in the round bottom flask were transferred to labeled beakers and allowed to evaporate to

dryness, after which they were refrigerated at 4°C prior to use.

### Phytochemical Screening

Qualitative Phytochemical screening of the crude extracts was done to determine the major classes of bioactive components present in the aqueous, ethyl acetate or methanol leaf, bark, and root extracts of *A. senegalensis*.

#### Test for flavonoids

Approximately 2 mL of plant extract was dispensed in the test tube, and 2 mL of 10 % diluted Sodium Hydroxide was added. The formation of a yellow solution that turned colourless with the addition of dilute Hydrochloride indicated that flavonoids were present (Ayoade *et al.*, 2019).

#### Test for tannins

Five (5) mL of plant extract was added to 10 mL of distilled water and mixed with a few drops of 5 % Ferric Chloride (FeCl<sub>3</sub>) solution. The formation of a green precipitate was considered positive for tannins (Fentahun *et al.*, 2017).

#### Test for saponin

One (1) mL of plant extract was added to 5 mL of distilled water in a test tube and vigorously shaken. The presence of persistent frothing indicated the presence of saponins (Fentahun *et al.*, 2017).

#### Test for phenol

Approximately 2 mL of plant extract was treated with 4 drops of 5% Ferric Chloride and observed for deep blue or bluish-black colour formation, indicating that phenol was present (Vijisara and Arumugam, 2013).

#### Test for cardiac glycosides

Two (2) mL of glacial acetic acid with a drop of ferric chloride solution was added to a tube having 5 mL of crude extract. A brown ring interface formed after adding 1 mL of concentrated sulphuric acid indicated a positive result for cardiac glycosides (Ayoola *et al.*, 2008).

#### Test for terpenoids

One (1) mL of plant extract was added to 2 mL of chloroform; then 5 mL of concentrated sulphuric acid was added and observed for the presence of reddish-brown colour at the interface, which gave a positive result (Vijisara and Arumugam, 2013).

#### Test for steroids

A test tube added approximately 2 mL of extract to 2 mL of chloroform. The mixture was shaken and filtered. Ethanoic anhydride and tetraoxosulphate (vi) acid were added to the filtrate. A greenish layer atop the liquid indicated a positive test (Coker and Oaikhena, 2020).

### Preparation of Stock Solution of Extracts

Four (4) grams of aqueous, ethyl acetate, and methanol extracts of leaves, bark, and roots of *A. senegalensis* were weighed and dissolved in 10 mL of dimethylsulfoxide (DMSO). This gave a concentration of 400 mg/mL of the extracts used for the antibacterial activity testing (Salihu, 2017).

### Test Bacteria

Test bacteria, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, isolated from urine samples of patients with UTI were subcultured on ceftrimide and MacConkey agar, incubated at 37 °C for 24 hours (Cheesbrough, 2006). The bacterial isolates were identified based on colonial morphologies, Gram reactions, and biochemical characteristics.

### Standardization of Test Bacteria for Susceptibility Testing

The test organisms were suspended in normal saline and incubated for 6 hours at 37 °C. After incubation, they were standardized according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) by adjusting their turbidity to 0.5 McFarland standard ( $1.5 \times 10^8$  CFU/mL) (Salihu, 2017).

### Preparation of Culture Media for Susceptibility Testing

Muller-Hinton Agar (MHA) (ReadyMED®) was prepared following the manufacturer's guideline, sterilized at 121 °C for 15 minutes, and allowed to cool before dispensing onto plates (Sandhyarani *et al.*, 2014).

### Determination of Antimicrobial Activity

The antibacterial activity of each extract was determined using the agar well diffusion method. Standard culture (0.2 mL) was dispensed aseptically into Petri dishes. Mueller-Hinton agar was dispensed into plates and swirled to allow for even distribution of organisms and left to solidify. On the seeded plates, equidistant wells were drilled using a sterile cork borer with a 6 mm diameter, and 0.2 mL of the appropriate extract dilutions and controls were aseptically distributed into the appropriate wells. All tests were performed in triplicates; the set-up was incubated at 37 °C for 24 hours. Ciprofloxacin was used as the positive control, while DMSO was the negative control. The inhibition zones were measured and recorded in millimeters (mm) after 24 hours of incubation (Cheesbrough, 2006).

### Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the extracts was carried out by broth dilution method using two-fold serial dilutions. The nutrient broth was prepared as directed by the manufacturer. Five milliliter of broth was

poured into seven test tubes, sterilized at 121 °C for 15 mins, and cooled. Five (5) mL of solution of each plant extract at 400 mg/mL was dispensed aseptically into the first test tube containing 5 mL broth and shaken thoroughly to homogenize. Using a fresh pipette, 5mL of the mixture was transferred to the second test tube and mixed by shaking. The same procedure was repeated to test tube six, where 5mL of the mixture was discarded. This gave 200, 100, 50, 25, and 12.5 mg/mL concentrations. Standardized bacteria in normal saline (0.1 mL) were inoculated into the different concentrations of the extracts in test tubes one to five. Test tube six contained the extract but no test organism (negative control), while test tube seven contained the organism in broth without extract (positive control). The tubes were incubated at 37 °C for 24 hours, after which they were observed for turbidity (growth). The lowest concentration in the series that showed no turbidity compared to the control was recorded as the MIC (Dawang *et al.*, 2019).

### Determination of Minimum Bactericidal (MBC)

The extracts' minimum bactericidal concentration (MBC) was determined from the result of the MIC. A loopful from the MIC tubes showing no turbidity (growth) was inoculated on nutrient agar plates, incubated at 37 °C for 24 hours, and observed for growth. The lowest concentrations from MIC tubes without visible growth on agar plates were regarded as MBC (Mourouge *et al.*, 2013).

### Statistical Analysis

The data generated was analyzed using Minitab Version 16.0 Software. Descriptive (mean) and inferential (ANOVA) Statistical analyses were carried out. Mean separation was done using Fisher's method (a modified version of Duncan's multiple-range test). Differences among and between treatments were considered statistically significant at  $P \leq 0.05$ .

## RESULTS

Table 1 shows the qualitative phytochemical composition of *Annona senegalensis* extracts. The seven phytochemicals tested were present in the three organs screened (leaf, bark, and root), although some failed to yield positive results. All extract types (aqueous, methanol, and ethyl acetate) showed the presence of flavonoids, tannins, saponins, and steroids in the leaf, bark, and root. Terpenoids were present in all bark and root of all extract types. Phenol was present in all extracts except the ethyl acetate leaf extract. Cardiac glycosides were present except for ethyl acetate leaf and methanol root extracts.

**Table 1: Results of Phytochemical Analysis of Extracts of *Annona senegalensis***

Phytochemical	Leaf			Bark			Root		
	AE	ME	EAE	AE	ME	EAE	AE	ME	EAE
Flavonoid	+	+	+	+	+	+	+	+	+
Tannin	+	+	+	+	+	+	+	+	+
Saponin	+	+	+	+	+	+	+	+	+
Terpenoid	-	-	+	+	+	+	+	+	+
Phenol	+	+	-	+	+	+	+	+	+
Cardiac Glycoside	+	+	-	+	+	+	+	-	+
Steroid	+	+	+	+	+	+	+	+	+

**Key:** Phytochemical present (+), Phytochemical absent (-), Aqueous extract (AE), Methanol extract (ME), Ethyl acetate extract (EAE).

**Table 2** presents the cultural and biochemical characteristics of test isolates. The isolates were identified as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The isolates were

Gram-negative rods. *P. aeruginosa* was oxidase and citrate positive, indole and urease negative. *K. pneumoniae* was oxidase and indole negative and citrate and urease positive.

**Table 2: Morphology and Biochemical Characteristics of Test Organisms**

Colonial morphology	Gram reaction	Catalase test	Coagulase test	Oxidase test	Citrate test	Indole test	Urease test	Suspected organism
Rod	-	+	-	+	+	-	-	<i>P. aeruginosa</i>
Rod	-	+	-	-	+	-	+	<i>K. pneumoniae</i>

**Key:** positive (+), negative (-)

The zones of inhibition (ZI) of test organisms by leaf, stem bark, and root extracts of *A. senegalensis* at 400 mg/mL are presented in **Table 3**. All extract types showed antibacterial activities. Comparative effects of the leaf extract types on each test organism showed that aqueous leaf extract gave the highest (24.67±0.58 mm) ZI when tested on *P. aeruginosa*. On *K. pneumoniae*, ethyl acetate leaf extract gave the highest (24.00±1.00 mm) ZI. Methanol leaf extracts gave the least inhibition against test isolates. In the stem bark, aqueous bark extract had the highest (21.33±0.58 mm) action on *K.*

*pneumoniae*, while ethyl acetate bark extract had the least (16.33±0.58 mm) activity against *P. aeruginosa*, all at concentrations of 400 mg/mL. In the root extract types, *P. aeruginosa* had its highest (21.33±0.58 mm) inhibition under ethyl acetate extract. Similarly, *K. pneumoniae* was most inhibited (19.67±1.16 mm) by methanol root extract. In all instances, significant inhibition was observed among the treatments for each of the test organisms, as the control (Ciprofloxacin) had higher ZI values than plant extracts (P<0.05).

**Table 3: Zones of Inhibition (mm±SD) of Aqueous, Methanol and Ethyl Acetate Leaf, Bark and Root Extracts of *Annona senegalensis* at 400 mg/mL**

Test Organism	<i>P. aeruginosa</i>			<i>K. pneumoniae</i>		
	Leaf	Stem	Root	Leaf	Stem	Root
AE	24.7±0.6 <sup>a</sup>	15.7±0.6 <sup>b</sup>	14.7±0.6 <sup>c</sup>	19.7±0.6 <sup>c</sup>	21.3±0.6 <sup>b</sup>	16.7±.6 <sup>b</sup>
ME	16.3±0.6 <sup>d</sup>	16.3±7.5 <sup>b</sup>	21.0 ±1 <sup>b</sup>	17.0±1.0 <sup>d</sup>	15.3±0.6 <sup>c</sup>	17.7±1.2 <sup>b</sup>
EAE	19.7±0.6 <sup>c</sup>	14±1.0 <sup>c</sup>	21.3±0.6 <sup>b</sup>	24.0±1.0 <sup>d</sup>	15.3±0.6 <sup>c</sup>	17.3±4.9 <sup>b</sup>
Control(CPX)		28±0.0 <sup>a</sup>			29±1.0 <sup>a</sup>	

\*Superscripts with different letters within a row (for each plant part) are significantly different (P<0.05)

**Key:** AE = aqueous extract, ME = methanol extract, EAE = ethyl acetate extract, CPX =Ciprofloxacin. Values in mm = mean, SD = standard deviation.



The MIC of aqueous, methanol, and ethyl acetate extracts of *A. senegalensis* on the test organisms is presented in Table 4. Five extracts, aqueous leaf, methanol bark and root, and ethyl

acetate leaf and root, gave the lowest (25 mg/mL) MIC. The highest (100 mg/mL) MIC value was observed in aqueous root and methanol leaf extracts tested on *P. aeruginosa*.

**Table 4: Minimum Inhibitory Concentration (MIC) of *Annona senegalensis* Extracts**

Test organisms	Extracts concentration (mg/mL)								
	AL	AB	AR	ML	MB	MR	EAL	EAB	EAR
<i>P. aeruginosa</i>	25	50	100	100	25	25	25	50	25
<i>K. pneumoniae</i>	25	25	50	50	25	25	25	25	25

Key: AL = aqueous leaf, AB = aqueous bark, AR = aqueous root  
 MLE = methanol leaf, MB = methanol bark, MR = methanol root  
 EAL = ethyl acetate leaf, EAB = ethyl acetate bark, EAR = ethyl acetate root.

Table 5 below shows that ethyl acetate bark had an MBC of 50 mg/mL on both *P. aeruginosa* and *K. pneumoniae*. High (200 mg/mL) MBCs were

recorded with aqueous root and ethyl acetate bark extracts on all test organisms.

**Table 5: Minimum Bactericidal Concentration (MBC) of *Annona senegalensis* Extracts**

Test organisms	Extracts concentration (mg/mL)								
	ALE	ABE	ARE	MLE	MBE	MRE	EALE	EABE	EARE
<i>P. aeruginosa</i>	50	200	200	200	200	50	50	200	100
<i>K. pneumoniae</i>	50	100	200	200	200	50	50	200	100

Key: AL = aqueous leaf, AB = aqueous bark, AR = aqueous root  
 MLE = methanol leaf, MB = methanol bark, MR = methanol root  
 EAL = ethyl acetate leaf, EAB = ethyl acetate bark, EAR = ethyl acetate root.

**DISCUSSIONS**

All the seven phytochemicals tested were detected in various extracts of *A. senegalensis*. This study agrees with [Tukur et al. \(2020\)](#) who reported the presence of these phytochemicals in leaf extracts of the plants. Various phytochemicals have also been reported in the leaf and stem bark of *A. senegalensis* ([Jada et al., 2014](#); [Jada et al., 2015](#)). However, the presence of phenols and steroids in the leaf in this study is at variance with the report of [Jada et al. \(2014\)](#). Plants' medicinal qualities result from their phytochemicals, as plants with high phytochemical content have been shown to demonstrate a diverse range of pharmacological effects ([Tunwagun et al., 2020](#)). The presence of these phytochemicals in the plant's leaves, bark, and roots supports its traditional application in the management/treatment of bacterial infections.

All parts of *A. senegalensis* demonstrated great activity against all test organisms as DZI of extracts ranged between 14.0±1.0 to 24.7 ± 0.6 mm. The high antibacterial property of *A. senegalensis* was also reported by [Yandev et al. \(2022\)](#) in their study of the antibacterial activity of the root and stem of the plant. However, their study was only based on the aqueous and methanol crude extracts.

[Dawang et al. \(2019\)](#) reported the antibacterial activity of the methanol leaf extract of *A.*

*senegalensis* against *P. aeruginosa*. However, higher inhibition zones were observed in this study against the organism. Similarly, the aqueous stem bark extract showed higher activity against *K. pneumoniae*. However, methanol and ethyl acetate root extracts showed better activity against the test isolates than the stem bark extracts. The leaves and roots of *A. senegalensis* could be better antibacterial agents than the plant's stem bark. This could be that the biologically active ingredients, even though found in all parts tested, may have been present at higher concentrations in the leaves and roots than in the plant's stem bark.

The test organisms were more susceptible to methanol and ethyl acetate root extracts than the aqueous root extracts, even though the aqueous root extract had all the phytochemicals. [Das et al. \(2010\)](#) noted that flavonoids and phenolics that are water soluble have no antimicrobial significance but are only important as antioxidant compounds. Also, the optimal solvent for extraction of phytochemical components is dependent on the plant materials and the phytochemical of interest, this is because different phytochemicals have varying solubility properties in different solvents ([Mahdi-Pour et al., 2012](#)).

The control drug, ciprofloxacin, showed higher inhibition zones at a lower concentration than the crude extracts. According to Tyokusa *et al.* (2019), this could be attributed to the fact that crude extracts may contain less active ingredients, whereas ciprofloxacin, being highly purified, contains higher concentrations of the active ingredients. Also, though crude extracts contain many phytochemicals, a few may have potent antimicrobial properties. Moreover, the non-active components may interfere with the activity/actions of the potent phytochemical. The MIC of 50 mg/mL and MBC of 200 mg/mL exerted by methanol leaf extract against *P. aeruginosa* in the current study is at variance with the study of Dawang *et al.* (2019), which reported MIC and MBC of 9.37 mg/mL and 300 mg/mL respectively against the same organism. However, in both studies, the MBC values observed were two or more-fold the MIC values,

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indicating that higher concentrations of the plant may be required to kill bacteria. This may not be good, as it is generally preferred that low concentrations of drugs be taken to eliminate or minimize side effects, adverse drug reactions, or toxicity to host tissues.

## CONCLUSION

The study's findings demonstrated that the leaves, bark, and roots of *A. senegalensis* are rich in phytochemical compounds capable of exerting bacteria, as demonstrated by their effectiveness against test bacteria, thus supporting the traditional claim for its application in treating urinary tract infections. However, greater refinement would lead to higher potency, and stop consumption of components that are non-active against pathogens.

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