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Phytochemical and Antibacterial Activities of *Anacardium occidentale* fruits extracts (Cashew) on two Drug Resistant Bacteria

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ABSTRACT

This present study was aimed at investigating the phytochemical and antibacterial activities of both aqueous and ethanolic extracts of *Anacardium occidentale* (cashew) fruit on *Staphylococcus aureus* and *Escherichia coli*. The Phytochemical constituents of this medicinal plant were carried out using standard methods. Agar well diffusion method was used to determine the antibacterial activity of the plant extracts. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the plant extracts on the test isolates were determined by the Broth dilution method. Phytochemical analysis showed that Phenols, alkaloids, anthraquinolones, flavonoids, glycosides, tannins, glycoside, terpenoids and tannins were present in both ethanolic and aqueous extracts of *A. occidentale*. The antibacterial activities of aqueous and ethanolic fruit extract of *Anacardium occidentale* showed that the mean zone diameter of inhibition for *S. aureus* on the different extracts was between the range of 11mm to 28mm while that of *E. coli* was between 17mm to 29mm. The MIC of different extracts of *S. aureus* was between 6.25mg/ml to 25mg/ml while that of *E. coli* was also between 6.25mg/ml to 25mg/ml. The MBC of different extracts of *S. aureus* isolates were between the ranges of 100 to 150mg/ml while that of *E. coli* was also between the range of 100 to 150mg/ml. The ethanol and aqueous extracts of *Anacardium occidentale* (Cashew) fruits are rich in wide range of secondary metabolites. Both extracts exhibited bactericidal activities against *S. aureus* and *E. coli*. The antibacterial efficacy of the fruit extracts of *A. occidentale* lends credence to ethno-medicinal use of the plant to treat various ailments.

Key words: *Anacardium occidentale*, Ethanolic, Aqueous, *Staphylococcus aureus*, *Escherichia coli*

INTRODUCTION

Escherichia coli is a bacterium that is commonly found in the gut of humans and other warm-blooded animals. National Center for Emerging and Zoonotic Infectious Diseases [1] reported that most strains of *E. coli* are harmless. However, few are known to contaminate food. [2] Symptoms of disease include abdominal cramps, pains, bloody diarrhoea, and nausea. Fever and vomiting may also occur. Most individuals recover within 2 weeks, even though in a few cases the disease may become extremely dangerous. [2]

Staphylococcus aureus causes a variety of pyogenic (pus-forming) infections and toxinoses (microbial toxins) in humans. *Staphylococcus aureus* causes superficial skin lesions such as pimples or boils and more serious infections such as osteomyelitis and endocarditis. [3] It is an important community-acquired infections, nosocomial infections of surgical wounds and also, the most common cause of hospital acquired infection such as surgical wounds and *S. aureus* in hospitals are becoming increasingly resistant to

antibiotics. Mustapha [3] stated that lately, problems with microorganisms that are unaffected by drugs, side effects of orthodox drugs, and developing diseases where no medicines are obtainable, have inspired an awareness and curiosity in plants once again as a significant source of novel medicines.

Anacardium occidentale (Family Anacardiaceae), is a multipurpose tree of the tropics which attains a height of about 10-15m. [4] They grow on relatively dry soil in nature but in cultivation grow well in the tropical rain forest. The cashew tree produces many products and resources. The leaf, bark, and the apple are explored medicinally to treat variety of diseases in Nigeria. The tree is a native plant of Nigeria commonly called Kànjùù in Hausa. The leaves, stems and bark extracts are used extensively for the treatment of diarrhea, dysentery and colonic pain. [4] It has also been reported to possess anti-ulcerogenic, anti-diabetic and anti- inflammatory properties. [5] The ethanolic extracts of cashew nuts revealed the presence of various phytochemical compounds such as phenolic, triterpenoids, carbohydrate, xanthoprotein and flavonoids. [5] Phytochemicals are plant metabolites [6] which act as natural defence systems for host plants, and also provide characteristic colour, aroma and flavour in specific plant parts. They are a group of non-nutrient compounds that are biologically active when consumed by human. Many phytochemicals are health-promoting and can prevent many diseases. [7]

Cashew is majorly planted for its nut (about 10% of the cashew fruit) which is a highly valued commodity for its shell oil also known as cashew nutshell liquid (CNSL), while the apple is usually left on the farm to rot away. [8] Moreover, apart from direct consumption of the apple, there is no reported use of the apple in Nigeria despite various research efforts which has led to improved cashew production in the country with increase in the tonnage of cashew nuts being exported annually. [8] Therefore, this present study was designed to evaluate the presence of phytochemicals and antimicrobial activities of different solvent extracts of the cashew fruit tree.

MATERIAL AND METHODS

Collection, Identification and Preparation of Cashew apple

Ripe and fresh cashew (*A. occidentale*) fruits were plucked from the parent trees on Spiritan University farm land, Abia State Nigeria. The plant material was then authenticated at the Herbarium section of the Department of Botany, Nnamdi Azikiwe University Awka, Nigeria by a Botanist. The authenticated plant materials were rinsed with tap water and the nuts were dislodged manually. Afterwards, the apple was sliced with a laboratory knife and then pressed until drained. Thereafter, it was dried in an oven at 37 °C for two weeks. The dried samples were then ground into coarse powder with the aid of a mechanical grinder and were stored in clean air- tight containers, and kept in a cool, dry place until required for use.

Test organisms

Bacterial cultures of *Escherichia coli and Staphylococcus aureus* obtained from the laboratory section of the Department of Microbiology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria; were used as antimicrobial test organisms. Their identities were confirmed using cultural, morphological and biochemical tests as previously described by Oyeleke and Manga. [9] The bacterial isolates were maintained on nutrient agar slants at 4°C.

Biochemical Identification of the Test Organisms

Escherichia coli

The *E. coli* was placed on Eosin Methylene Blue agar for 18 hours. Colonies with green metallic sheen were observed which indicated a positive result for *E. coli.* [9]

Staphylococcus aureus

The *S. aureus* was placed on Mannitol Salt Agar (MSA) for 18 hours. Smooth circular colonies with yellow colour indicated a positive result for *S. aureus.* [9]

Standardization of the Tests Organism

The test organisms (*E coli and S aureus*) were standardized by the use of 24 hours old broth cultures prepared by inoculating the test organism into 5 ml of nutrient broth and the culture was adjusted to obtain 0.5 McFarland turbidity equivalent standards. [9]

Preparation of plant material and plant extracts

Two different fruit extracts namely aqueous and ethanolic were used for plant. They were prepared according to the methods of Oyeleke and Manga, [9]

Preparation of Aqueous fruit extract

Ten grams of dried ground fruit powder was dissolved in 100 ml of distilled water for 24 hours. The mixture was filtered using Whatman's filter paper No. 1 to obtain solution free of solids. The filtrate was concentrated by drying at 37°C and stored at 4°C.

Preparation of ethanolic fruit extract

Ten grams of dried ground fruit powder was dissolved in 100 ml of 95% ethanol for 24 hours. The mixture was filtered using Whatman's filter paper No. 1 to obtain solution free of solids. The filtrate was placed into evaporator to drive-off the solvent and stored at 4°C.

Extract Dilution

After preparation of the extract as described, aqueous and the ethanolic extract were reconstituted using sterile distilled H_2O to obtain concentrations of 200, 150, 100, 50, 12.5, 6.25 and 3.13 mg/ml.

Sterility test of the dried fruit extract

The dried fruit extracts (aqueous and ethanolic) were tested for growth of contaminants. One milliliter (1ml) of standard dried fruit extract was inoculated aseptically unto Nutrient Agar and incubated at 37^oC for 24hrs. The plates were observed for any sign of visible growth. No growth on the plates indicated/signified that the extracts were sterile. [9]

Qualitative phytochemical screening

The extracts of the plant material were subjected to qualitative phytochemical analysis for the presence of tannins, saponin, flavonoids, alkaloids and phenol which were carried out on the extracts using standard procedures as described by Harborne. [10]

Test for tannins

About 1 ml of extract was boiled in 20ml of water in a test and then filtered. A few drops of 0.1% ferric chloride was added and observed green or a blue – black coloration which confirmed the presence of tannin.

Test for saponin

About 5 ml of the extract was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously, then observed for the formation of emulsion which confirmed a positive presence of saponins.

Test for flavonoids

A 3 ml portion of 1% Aluminium chloride solution was added to 5ml of each extract. A yellow coloration was observed indicating the presence of flavonoids. 5 ml of dilute ammonia solution were added to the above mixture followed by addition of concentrated H_2SO4. A yellow coloration indicates a positive test for flavonoids.

Test for alkaloids

One milliliter of the extract was stirred with 5 ml of 1% aqueous HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1 ml of the filtrate was treated with a few drops of either Mayer's reagent (Potassium mercuric iodide-solution gave a positive test for alkaloids.

Test for steroids

A 2 ml portion of acetic anhydride was added to 2 ml extract of each sample followed by careful addition of 2 ml H_2SO_4. The color changed from violet to blue or green indicating the presence of steroids.

Test for terpenoids (Salkowski test)

About 5 ml of each extract was mixed with 2 ml of chloroform, and 3 ml concentrated

H_2SO_4 was carefully added to form a layer. A reddish-brown coloration of the interface was formed to show positive result for the presence of terpenoids.

Test for anthraquinone

About 5ml of extract was mixed with 10 ml benzene, filtered and 5 ml of 10% NH_3 solution was added to the filtrate. The mixture was shaken and the presence of violet colour in the ammoniac (lower) phase indicated the presence of anthraquinones.

Test for phenol

About 5ml of the extract was pipetted into a 30 ml test tube, and then 10 ml of distilled water was added to it. Two (2) ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol was also added and left to react for 30 min. The development of bluish-green colour was taken as a positive presence of phenol.

Test for glycosides (Keller-Kiliani test)

Five milliliter of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated deoxysugar characteristics of cardenolides which confirmed the presence of cardenolides. A violet-green ring appearing below the brown ring, in the acetic acid layer, indicated the presence of glycoside.

Antibacterial Assay

The antibacterial assay of the dried fruit extracts was carried out on the test isolates using Agar-well diffusion technique according to the methods of NCCLS. [11] The isolates were inoculated on the surface of freshly gelled sterile nutrient agar plates by streaking using sterilized swab stick. Wells were aseptically bored on each agar plate using a sterile cork borer (6mm) and were properly labelled. Fixed volumes (0.1 ml) of different concentrations of the extracts (aqueous and ethanolic) were then introduced into the wells in the plates, respectively. The last two wells were used as positive control well (filled with Ciprofloxacin, 5mg/ml) and a negative control well (filled with sterile water) respectively. The plates were allowed on the bench for 40 minutes for pre-diffusion of the extract to occur and then incubated at 37^{o}C for 24 hours. The resulting zone diameter of inhibition was measured using a transparent ruler calibrated in millimetres. The readings were taken to be the zone diameter of inhibition of the bacterial isolate in question at that concentration according to the methods of NCCLS. [11]

Minimum Inhibitory Concentration (MIC)

The MIC of the potent extracts was determined according to the macro broth dilution technique. Standardized suspensions of the test organism were inoculated into a series of sterile tubes of nutrient broth containing two-fold dilutions of leaf extracts and incubated at 37^{o}C for 24 hours. The MICs were read as the least concentration that inhibited the growth of the test organisms. [11] The lowest or least concentration of the extract that showed no growth in the test tubes was the MIC of the extract tested.

Minimum Bactericidal Concentration (MBC)

The MBCs were determined by first selecting tubes that showed no growth during MIC determination; a loopful from each tube was sub-cultured onto already gelled nutrient agar plates using spread plate technique and incubated for 24 hours at 37^{o}C. The least concentration, at which no growth was observed, was noted as the MBC. [11]

Mode of action of the extracts

All plates showing no visible growth on the nutrient agar (NA) indicated bactericidal effect of the concentration of the extract used. Plates showing light growth indicated the bacteriostatic effects of the extract concentration. Concentrations of the extracts showing moderate and heavy growth were considered to have no inhibitory effect on the organism. [12]

RESULTS

The phytochemical analysis is found on table 1. Phenols, alkaloids,

anthraquinolones, flavonoids, glycosides, tannins, glycoside, and terpenoids were present in both ethanolic and aqueous fruit extracts of *A. occidentale*.

Table 1: Phytochemical composition of *Anacardium occidentale* fruit extract

Phytochemical components	EE	AE
Steroids	-	-
Saponins	-	-
Phenols	+	+
Flavonoids	+	+
Glycosides	+	+
Tanins	+	+
Alkaloids	+	+
Anthroquinolones	+	+
Terpenoids	-	-

KEY: - = Absence EE = Ethanolic extract
+ = present AE = Aqueous extract

The antibacterial activities of aqueous and ethanolic fruit extract of *A. occidentale on S. aureus and E. coli* is found on table 2. The mean zone diameter of inhibition for *S. aureus* on the different extracts was between the ranges of 11mm to 28mm while that of *E. coli* was between 17mm to 29mm.

Table 2: Antibacterial activities of aqueous and ethanolic fruit extract of *A. occidentale* fruit extract on *S. aureus* and *E. coli*

Isolates	Mean zone diameter of inhibition (mm)						Extracts
S. aureus	28	23	17	12	31	0	AE
S. aureus	22	20	17	11	31	0	EE
E. coli	26	21	19	14	29	0	AE
E. coli	29	26	19	17	29	0	EE
	200	150	100	50	+C	-C	

Key: AE = Aqueous Extract
EE = Ethanolic Extract
+C = Positive control
-C = Negative control

The MIC of fruit extracts of *A. occidentale on S. aureus and E. coli* is found on table 3. The MIC of different extracts of *S. aureus* was between 6.25mg/ml to 25mg/ml while that of *E. coli* was also between 6.25mg/ml to 25mg/ml.

Table 3: Minimum Inhibitory Concentration (MIC) of *A. occidentale fruit extracts on S. aureus and E. coli*

	Concentration of Extracts(mg/ml)									
Isolates	200	150	100	50	25	12.5	6.25	3.13	Extracts	MIC
S. aureus	-	-	-	-	-	-	-	+	AE	6.25
S. aureus	-	-	-	-	-	-	+	+	EE	12.5
E. coli	-	-	-	-	-	-	-	+	AE	6.25
E. coli	-	-	+	-	-	-	+	+	EE	12.5

Key: AE = Aqueous Extract
EE = Ethanolic Extract

The MBC of fruit extracts of *A. occidentale* on *S. aureus* and *E. coli* is found on table 4. The MBC of different extracts of *S. aureus* isolates was between the ranges of 100 to 150mg/ml while that of *E. coli* was also between the ranges of 100 to 150mg/ml.

Table 4: Minimum Bactericidal Concentration (MBC) of *A. occidentale* fruit extracts *on S. aureus and E. coli*

	Concentration of Extracts(mg/ml)									
Isolates	200	150	100	50	25	12.5	6.25	3.13	Extracts	MBC
S. aureus	-	-	+	+	++	++	++	++	AE	150
S. aureus	-	-	-	+	+	++	++	++	EE	100
E. coli	-	-	+	+	+	++	++	++	AE	150
E. coli	-	-	-	+	+	++	++	++	EE	100

Key: AE = Aqueous Extract
EE = ethanolic Extract

DISCUSSION

Phytochemical analysis revealed the presence of alkaloids, tannins, anthraquinolones, glycosides, and phenols in both ethanol and aqueous extracts of *A. occidentale* dried fruit. Ayepola and Ishola [13] reported the presence of alkaloids; tannins and saponins in *A. occidentale* stem extract. Several studies have reported rich variety of secondary metabolites in *A. occidentale* extracts [14] (Rajesh *et al*., 2009). The pharmacological properties of medicinal plants have been attributed to their rich secondary metabolites. [14] Plants generally produce many secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs. [15,16]

The antibacterial analysis in this study showed that there was no significant

difference ($p \leq 0.05$) in the antibacterial effect of ethanol and aqueous extract against the test bacteria. This result is in disagreement with report of Arekemase *et al*., [17] who reported that ethanolic extract was more effective than aqueous extract. Aderiye and David [8] reported potent antibacterial effect of cold and hot water extract of *A. occidentale* against *E. coli* O167: H7 and methicillin resistant *S. aureus* (MRSA).

The antibacterial effect of the ethanol and aqueous extract against the test bacteria in this study could be attributed to the presence of the phytochemicals. Flavonoids have been reported to significantly affect the cell wall of the microorganisms which may invariably lead to the collapse of the cell wall and overall, affect the entire mechanism of the microbial cell. [18] Alkaloids have also been reported to be involved in antimicrobial activities. [19]

The minimum inhibitory concentration (MIC) of extracts against test *S. aureus and E. coli* in this study are higher than MIC reported by Arekemase *et al*. [17] The authors reported MIC of 0.313 and 0.625 mg/ml for reference strain of *S. aureus* and *E. coli* and 1.25 mg/ml against *S. aureus* isolated from food as against the 250 mg/ml recorded in this study. Onuh *et al*. [20] reported appreciable antimicrobial effect of the ethanol extract of *A. occidentale* against E. *coli*, *S. mutans*, *B. cereus*, *S. typhi*, and *C. albicans*. The authors also reported varying levels of phytochemicals in the leaves and stem bark of *A. occidentale*.

The result of the minimum bactericidal concentration (MBC) was similar to report of Arekemase *et al*., [17] who reported that the ethanolic extract was found to be bactericidal to all the test bacteria, while the aqueous extract was found to be bacteriostatic to the test bacteria.

CONCLUSION

The ethanol and aqueous extracts of *Anacardium occidentale* (Cashew) fruits are rich in wide range of secondary metabolites. Alkaloids, flavonoids, tannins, glycosides, anthraquinolones and phenols were found in both extracts. Both extracts exhibited bactericidal activities against *S. aureus* and *E. coli*. The antibacterial efficacy of the fruit extracts of *A. occidentale* were due to the presence of the secondary metabolites *and* lends credence to ethno-medicinal use of the plant to treat various ailments.

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