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Preliminary study of phytochemical constituents and antimicrobial activity of two varieties of cashew apple grown in Nigeria

Faith Iguodala Akinnibosun[1] and Adedayo Michael Oyetayo[2*]

[1]Department of Microbiology, University of Benin, Benin City, Nigeria.
[2]Department of Science Laboratory Technology, Rufus Giwa Polytechnic, Owo, Nigeria.
Email: michealococcus@gmail.com

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ABSTRACT

Aims: The specific aim of this study was to investigate the phytochemical constituents and antimicrobial activities of cashew apple (*Anacardium occidentale*) grown in Nigeria.
Methodology and results: The cashew apples (red and yellow) were plucked directly from parent tree, sliced and drained in a press. Thereafter, it was dried, grounded and extracted using solvent percolation. The extracts were screened for the presence of phytochemicals and antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aspergillus fumigatus* and *Candida albicans* using agar well diffusion method while the minimum inhibitory concentration was done using tube dilution technique. Alkaloids, tannins, flavonoids, steroids, glycosides, terpenoids, phenols and anthraquinones were found in the two varieties at varying degree. However, saponin was not detected in either of the variety. The antimicrobial activities of the red and yellow cashew apples were comparable at all the concentrations used. Also, these activities were concentration dependent in all the samples as increased zones of inhibition were observed as the concentrations increased. There was a significant ($p \leq 0.05$) difference between the antimicrobial activities of the different extracts. The largest inhibition zones were recorded against *E. coli* (22.33±0.15 mm) and *K. pneumoniae* (24.33±0.01 mm) for red cashew apple ethanol extracts at 30mg/mL while *A. fumigatus* (5.33±0.00 mm) showed the least zone of inhibition against the same extract. Ethanol extracts recorded the highest inhibitory activities against all the test organisms, followed by aqueous extract while n-hexane extract had the least inhibitory activity on the organisms. The least MIC recorded was 2.5 mg/mL and it was obtained against *S. aureus* (Red aqueous extract, red and yellow ethanol extracts), *E. coli* and *K. pneumoniae* (red and yellow ethanol extracts) while the highest MIC was 30 mg/mL recorded against *B. subtilis* (yellow n-hexane extract). In all, the ethanol extracts of the cashew apples showed comparable antimicrobial activities with the controls at 30 mg/mL.
Conclusion, significance and impact of study: The result of this investigation confirms the presence of bioactive substances in cashew apple which may be responsible for its antimicrobial activities against selected microorganisms, consequently, supporting the folkloric use of the apple in the treatment of various diseases.

Keywords: Phytochemical, antimicrobial, cashew apple, inhibition

INTRODUCTION

The plant kingdom has been the best source of remedies for curing many diseases; this is why medicinal plants have played a key role in the worldwide maintenance of health. There therapeutic effects are attributed to the presence of phytochemicals which are the natural bioactive compounds found in plants. These phytoconstituents work with nutrients and fibers to form an integral part of human defense system against various diseases and stress conditions (Nair, 2010). Phytochemical are basically divided into two (primary and secondary) constituents according to their function in the plant metabolism. Primary constituents comprise common sugars, amino acids, proteins and chlorophyll, while secondary constituents consist of alkaloid, flavonoids, saponin, phenolics etc (Opawale *et al*., 2015).

Natural products of higher plants are an important source of therapeutic agents; therefore many research groups are currently screening the different activities of plants (Mothana *et al*., 2010). Throughout Africa, poor rural communities use their knowledge about plants to protect field crops, stored grain and livestock from damage caused by pests. Similarly, many plants have known efficacy for control of diseases and disease vectors. This information has been passed down from generation to generation and offers an effective, low cost, sustainable and environmentally benign pest

*Corresponding author

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management strategy (Nair, 2010). Africa is endowed with rich biodiversity and indigenous knowledge system about bioactive plants whose beneficial values have not been fully exploited in tropical medicine and agriculture.

Cashew (*Anacardium occidentale* Linn.) (Bailey, 1961) a tropical evergreen tree plant is one of these highly underutilized/underexploited plants (FAO, 2013). 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. 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.

All parts of the plant like leaves, nuts, fruit, root and bark have been used in traditional medicine to manage variety of ailments. The leaf is reported to be used as aphrodisiac, anthelminthic, antitumor, ascites, cures fever, ulcer, leucoderma, skin diseases, dysentery, piles, loss of appetite as mentioned in Ayurveda (Dhavan, 2002). The root is considered purgative and the fruit is mainly used as antidiarrheal. The tar from the bark is used as a counter irritant. As an external application, it was recommended in leprosy, ring worm, and ostinate ulcer. It is powerfully rubefacient and vesicant and requires to be used with caution (Kokate, 1994). Therefore, this present study was designed to assess the presence of phytochemicals and antimicrobial potential of different solvent extracts of the cashew apple.

MATERIALS AND METHODS

Collection, identification and preparation of cashew apple

Ripe and fresh cashew (*A. occidentale*) fruits were plucked from the parent trees on Agbi farm land, Owo, Ondo state Nigeria in March, 2017. The plant material was then authenticated at the Herbarium section of the Department of Forest Resources Technology and a voucher specimen (AOF1702) was deposited (in the same Department) Rufus Giwa polytechnic, Owo. The authenticated plant materials were rinsed with tap water and the nuts were dislodged manually. Afterwards, the apple were 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 microorganisms

The test microorganisms used for this analysis include (Bacteria) *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and (Fungi) *Candida albicans* and *Aspergillus fumigatus*. They were all collected from the Medical Microbiology Laboratory and Parasitology Department, Federal Medical Center, Owo, Ondo State, Nigeria in February, 2017. Organisms were sub-cultured and maintained on agar slants.

Reagents and chemicals

All reagents and chemicals were obtained from the Department of Microbiology, Federal University of Technology, Akure, Ondo State Nigeria. They were Nutrient agar (Lab M, Lankershire, U.K.) for culturing bacteria and Potato Dextrose agar (Oxoid, U.K.) for fungal culture.

Extraction of the cashew apple

One hundred gram of the powdered sample was soaked in 200 mL of different solvents (water, ethanol and n-hexane) for 72 h with intermittent stirring using sterile spatula. The plant extracts were then filtered through Whatman No.1 filter paper into bijou bottles and then dried using rotary evaporator at a temperature of 50 °C to yield crude extracts (Opawale *et al*., 2015). Different concentrations of the extracts were prepared by diluting 0.1 g, 0.2 g and 0.3 g of the extracts in 100 mL of 0.01% Tween-20 to obtain concentrations of 10 mg/mL, 20 mg/mL and 30 mg/mL respectively (Hag *et al*., 2012).

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 were carried out on the extracts using standard procedures as described by Harborne (1984).

Test for tannins

One milliliter of extract was boiled in 20 mL 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. Ten milliliter of the filtrate was mixed with 5 mL 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% aluminum chloride solution was added to 5 mL of each extract. A yellow coloration was observed indicating the presence of flavonoids. A 5 mL of dilute ammonia solution were added to the above mixture followed by addition of concentrated H_2SO_4. A yellow coloration indicates a positive test for flavonoids.

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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)

Five 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

Five milliliter 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

Five milliliter of the extract was pipetted into a 30 mL test tube, and then 10 mL of distilled water was added to it. 2 mL of ammonium hydroxide solution and 5 mL of concentrated amyl alcohol were 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-Killani 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 a positive presence of cardenolides. A violet green ring appearing below the brown ring, in the acetic acid layer, indicated the positive presence of glycoside.

***In vitro* antimicrobial susceptibility test**

The extracts obtained from the test plants were screened against the test organisms by agar well diffusion method (Perez *et al*., 1990). A 25 mL aliquot of nutrient and Potato Dextrose agar was poured into different Petri plates. When the agar solidified, each test organism was inoculated on the surface the appropriate plates (1×106 CFU/mL) using a sterile glass spreader and allowed to sink properly. Subsequently, the surface of the agar was punched with 6 mm diameter cork borer into wells and a portion of 50 µL of each of the extract concentrations was filled into the wells. Control wells containing the same volume of 30% Dimethyl sulphoxide (DMSO) served as negative control, while Chloramphenicol (50 µg) and Myconazole (100 µg) was used as positive control for bacterial and fungal the plates respectively and the plates were incubated at 37 °C for 24 h (bacteria) and 27 °C for 72 h (fungi). Each experiment was carried out in triplicates and the diameter of the zones of inhibition was then measured in millimeters.

Minimum Inhibitory Concentration (MIC)

The MIC of the plants extracts were determined by double dilution broth methods of (Idu and Igeleke, 2012). Two-fold serial dilutions of the extracts were prepared in nutrient broth (bacteria) and Potato Dextrose broth (fungi) to achieve a decreasing concentration ranging from the least concentration that produced clear zone of inhibition (100 mg/mL to 1.56 mg/mL). All tubes with the controls were labeled accordingly. Each dilution was seeded with 1 mL of standardized inoculums (1.0 × 106 CFU/mL) and incubated at 37 °C for 24 h. A tube containing only seeded broth (i.e. without plant extract) was used as the positive control while the un-inoculated tube was used as negative control. The lowest concentration of each extract that showed a clear of inhibition when compared with the controls was considered as the MIC.

Data analysis

Data were presented as mean±standard error (SE). Significance difference between different groups was tested using two-way analysis of variance (ANOVA) and treatment means were compared with Duncan's New Multiple Range Test (DNMRT) using SSPS window 7 version17.0 software. The significance was determined at the level of $p \leq 0.05$.

RESULTS

The phytochemical constituents of the two varieties of cashew grown in Nigeria is presented in Table 1 where alkaloids, tannins, flavonoids, steroids, glycosides, terpenoids, phenols and anthraquinones were found in the two varieties at varying degree. However, saponin was not detected in the solvent extracts used for either of the variety. Moreover, ethanol extracts contain more of these compounds in abundance followed by the aqueous extracts while most of the phytocompounds detected in n-hexane extracts were in trace amounts. Furthermore, the phytochemical's presence were comparable in both varieties except for anthraquinones which appeared to be present more in the red variety than in the yellow.

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Table 1: Phytochemical constituents of red and yellow cashew apple extracts.

Phytochemical	Aqueous		Ethanol		n-hexane	
	Red	Yellow	Red	Yellow	Red	Yellow
Alkaloids	++	++	++	++	+	+
Tannins	++	++	+++	+++	+	+
Saponins	-	-	-	-	-	-
Flavonoids	++	+	+++	++	+	-
Steroids	+	+	+	+	+	+
Glycosides	+	+	++	++	+	+
Terpenoids	-	-	+	+	-	-
Phenols	++	++	+++	+++	++	++
Anthraquinones	++	+	++	+	-	-

-, not detected; +, present in trace amount; ++, present in moderate amount; +++, present in abundance.

The results of the antimicrobial activities of the various extracts of the red and yellow cashew are presented in Figures 1 to 6. The antimicrobial activities were concentration dependent in all the samples as increased zones of inhibition were observed as the concentrations increased. Here, all the test bacteria were susceptible to the aqueous extract at all the concentration used while only the yeast (*C. albicans*) showed susceptibility to the extracts whereas, the mould *A. fumigatus* did not show susceptibility at any of the concentration of the aqueous extracts. *E. coli* and *K. pneumoniae* showed the highest susceptibility to the aqueous extracts at the highest concentration used (30 mg/mL). The antimicrobial activities of the aqueous extract of the two varieties of cashew were comparable at all the concentrations used with the red cashew apple aqueous extract showing 12.00±0.10, 10.33±0.01, 18.67±0.00, 20.00±0.02 and 15.33±0.01 mm zones of inhibitions against *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae* and *C. albicans* respectively while 12.67±0.01, 11.00±0.05, 18.33±0.00, 19.00±0.02 and 16.00±0.00 mm were recorded against the organisms respectively for yellow cashew apple aqueous extract (Figures 1 and 2).

Moreover, Figure 3 and 4 shows the antimicrobial activities of ethanol extracts of the two varieties of cashew apple where it was observed that *E. coli* and *K. pneumoniae* showed the highest susceptibility to the extracts at all the concentrations used. Also, all the organisms showed susceptibility to the ethanol extracts of both varieties at the highest concentration used (30 mg/mL) with red cashew apple ethanol extract showing 17.33±0.00, 15.00±0.02, 22.33±0.15, 24.33±0.01, 5.33±0.00 and 15.00±0.12 mm zones of inhibitions against *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *A. fumigatus* and *C. albicans* respectively while yellow cashew apple extract had 17.33±0.00, 15.00±0.01, 21.00±1.00, 23.00±0.01, 4.33±0.02 and 15.67±0.10 mm zones of inhibition respectively against the test organisms.

In addition, the antimicrobial activities of n-hexane extracts of the two varieties of cashew apple is presented in Figures 5 and 6 which reveal a rather low activity against the test organisms at all he concentrations used.

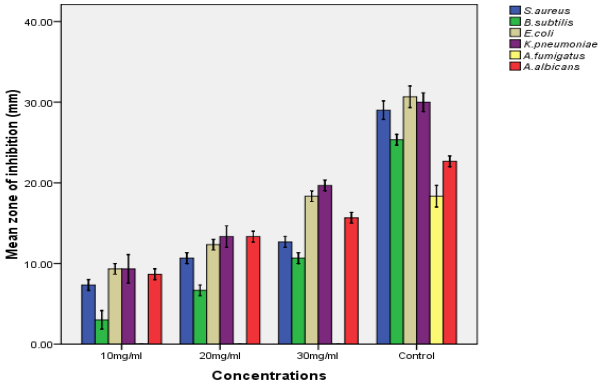

Figure 1: Antimicrobial activity of aqueous extract of red cashew apple.

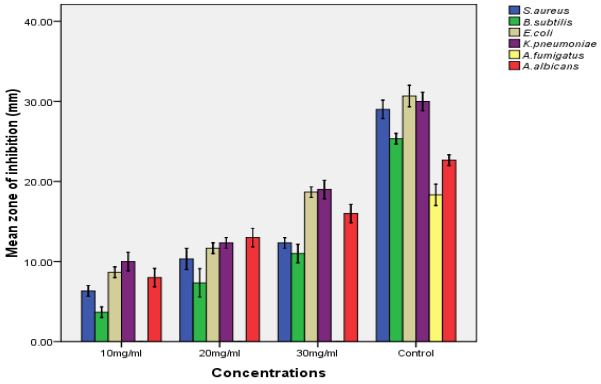

Figure 2: Antimicrobial activity of aqueous extract of yellow cashew apple.

The two varieties were not active against *A. fumigatus* at any of the tested concentrations while *B. subtilis* was only susceptible at 30 mg/mL. However, red cashew apple n-hexane extract showed 9.00±0.00, 6.67±0.10, 10.33±0.00, 11.00±0.00 and 8.67±0.10 mm zones of inhibitions against *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae* and *C. albicans* respectively whereas yellow cashew apple extract recorded 8.67±0.00, 5.00±0.00, 9.67±0.01, 10.33±0.00 and 15.67±0.10 mm zones of inhibition respectively against the organisms.

The minimum inhibition concentration (MIC) of the various extracts of the two varieties of cashew apple is presented in Table 2. The least MIC recorded was 2.5 mg/mL and it was obtained against *S. aureus* (red aqueous extract, red and yellow ethanol extracts), *E. coli* and *K. pneumoniae* (red and yellow ethanol extracts) while the highest MIC was 30 mg/mL recorded against *B. subtilis* (yellow n-hexane extract) and *A. fumigatus* (yellow ethanol extract). Albeit the MIC of the aqueous and n-hexane extracts of the two varieties of cashew apple were not detected against *A.fumigatus*.

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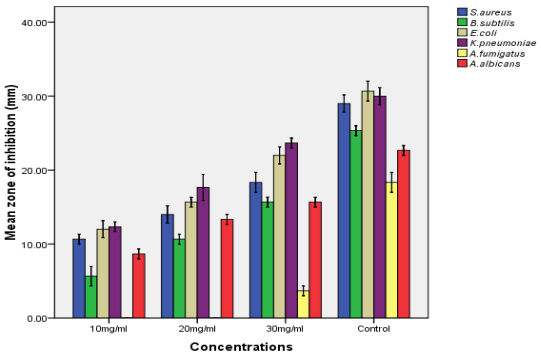

Figure 3: Antimicrobial activity of ethanol extract of red cashew apple.

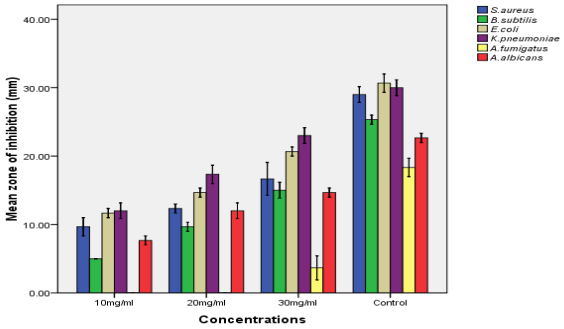

Figure 4: Antimicrobial activity of ethanol extract of yellow cashew apple.

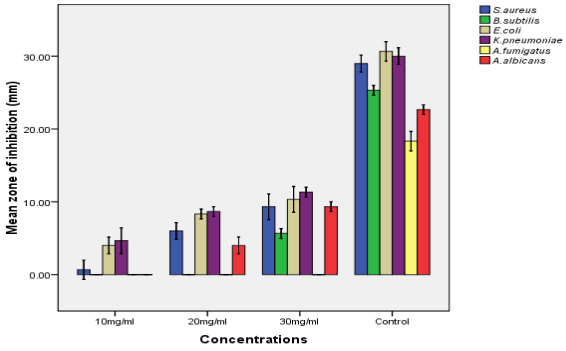

Figure 5: Antimicrobial activity of n-hexane extract of red cashew apple.

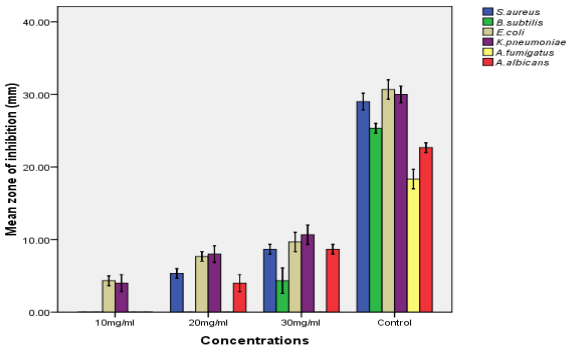

Figure 6: Antimicrobial activity of n-hexane extract of yellow cashew apple.

DISCUSSION

Medicinal plants are rich in secondary metabolites which are potential sources of drugs and of therapeutic importance. The evaluation of various plants according to their traditional uses and medical values based on their therapeutic efficacy lead to the discovery of newer and recent drugs for treating various ailments. This fact forms the basis for the development of new drugs from various plant sources (Abulude *et al*., 2010).

Phytochemicals are natural bioactive compounds produced by plants as secondary metabolites that work with nutrients to protect against pathogenic attack (Pallav *et al*., 2014). Although, pharmaceutical industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased and has become a global concern (Silva *et al*., 2007). The increasing failures of chemotherapeutics and antibiotics resistance exhibited by pathogenic microbial infectious agents have led to the screening of several medicinal plants for their potential antimicrobial activity (Parekh and Chanda, 2006). Duke (2001) had emphasized the advantages of medicinal extracts as being low cost, fewer side effects and easily accessible to rural people.

The preliminary qualitative phytochemical investigations of two varieties of cashew apple using different types of extraction solvents (water, ethanol, n-hexane) revealed the presence of alkaloids, flavonoids, steroids, phenols, tannins, terpenoids, glycosides and anthraquinones in while saponins was absent.

Table 2: MIC of different extracts of red and yellow cashew apple (mg/mL).

Organism	Aqueous		Ethanol		N-hexane	
	Red	Yellow	Red	Yellow	Red	Yellow
S. aureus	2.5	5.0	2.5	2.5	10.0	15.0
B. subtilis	10.0	10.0	5.0	10.0	25.0	30.0
E. coli	5.0	5.0	2.5	2.5	7.5	10.0
K. pneumoniae	5.0	5.0	2.5	2.5	7.5	10.0
A. fumigatus	ND	ND	10.0	30.0	ND	ND
A. albicans	5.0	5.0	5.0	5.0	15.0	20.0

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ND, not detected.

The presence of these phytochemicals in the cashew apple extracts is an indication of their potential antimicrobial activities as they play important roles in their bioactivity (Trease and Evans, 2002). Aiswarya *et al*. (2011) has previously reported the presence of some of these phytochemicals in cashew apple while Braga *et al*. (2007) asserted that alkaloids, tannins, flavonoids and phenolic compounds are the most important bioactive constituents of plants. Cowan (1999) also reported in his work that plants rich in phytoconstituents like alkaloid, flavonoids, tannins, terpenoids and steroids have antibacterial properties. Plants rich in flavonoids and tannins are reported for their antibacterial activities which is accomplished by inactivating enzymes while tannins and other compounds of phenolic nature are also classified as active antimicrobial compounds (Akinjogunla *et al*., 2012).

All the extracts exhibited different degrees of antimicrobial activity against tested strains. The plant materials showed significant ($p \leq 0.05$) differences in their antimicrobial activities. The ethanol and aqueous extracts of the red and yellow cashew apple were found to be more active against the test organisms compared with the hexane extracts. This might be due to the differences in the level of the various secondary metabolites present in the extracts as well as the extracting ability of the solvents. This corroborates the observations of Gonçalves and Gobbo (2012). The zones of inhibition obtained from the ethanol extracts of both varieties of cashew apple at 30 mg/mL were comparable to those of the antibiotics (Chloramphenicol and Myconazole) used as positive controls. Naczk and Shahidi (2004) stated that the observed differences between activities of plant extracts may be due to insolubility of active compounds in some solvents or the presence of inhibitors to the antimicrobial components which could have accounted for the poor antimicrobial activity of n-hexane extracts of cashew apple in this study.

The demonstration of antimicrobial activity against both Gram-positive and Gram-negative bacteria may be indicative of the broad-spectrum antibiotic compounds in the extracts of plant material. All the extracts recorded the highest inhibition zone on *E. coli* and *K. pneumoniae* suggesting that cashew apple may be a source of remedy for gastrointestinal infections caused by these organisms and related microorganisms. Polar metabolites like flavonoid, coumarins, anthraquinones, phenols and glycosides have been found to possess important antimicrobial properties (Smania *et al*., 1999).

In fungi assay, all the extracts of the two varieties of cashew recorded appreciable zones of inhibitions on *C. albicans* however, only ethanol extract had little inhibitory effect on *A. fumigatus*. This may be due to the difference in the physiology of the two fungi, since *A. albicans* is a yeast while *A. fumigatus* is a mould capable of producing resistant spores. The sensitivity of *C. albicans* to the ethanol extracts of the cashew apple has lend credence to the chemotherapeutic potentials of this plant part in the treatment of diseases such as oral and vaginal candidiasis (Agedah *et al*., 2010).

The extracts of the cashew apple inhibited both bacteria and fungi growth, but their effectiveness varied with concentration. Both Gram-positive and Gram-negative bacteria were more sensitive to the active extracts than the fungi. The antibacterial activity trend may be attributed to the presence of secondary metabolites of various chemical types present in the plant material either individually or in combination (Ranathunga *et al*., 2010). The significant ($p \leq 0.05$) activity of the cashew apple extract *C. albicans* correlate with the work of Liu *et al*. (2011) who confirmed similar activity of the cashew apple. Kubo *et al*. (1999) also confirmed the activity of the cashew apple extracts on *H. pylori*.

One of the important observations from this study is the susceptibility of the versatile bacterium *Staphylococcus aureus* which is major nosocomial pathogen with low intrinsic susceptibility to antimicrobial agents and very high ability to acquire resistance respectively was significantly inhibited by the aqueous and ethanol extracts of cashew apple that are comparable to the positive controls and could be further exploited for chemotherapeutic agents that could be used against infections caused by multiple antibiotic resistant strains that are very common in Nigeria.

The minimum inhibitory concentration (MIC) is the least concentration of the extracts that visibly inhibit growth of organisms. It is an important diagnostic tool because it helps in confirming resistance of microorganisms to antimicrobial agents.

It was observed that the antimicrobial activity of the cashew apple extracts depends on the concentrations and the tested microbial strains. Interestingly, it was found that *S. aureus*, *E. coli* and *K. pneumoniae* were the most sensitive test microorganisms with the lowest MIC values against the cashew apple extracts. In terms of antifungal activity, the lowest MIC value of 5 mg/mL was recorded by the ethanol and aqueous extracts of the two varieties of the cashew apple against *A. albicans* while a moderate value of 10 mg/mL was obtained against *A. fumigatus* by red ethanol extract. The values of MIC obtained for this plant materials are lower to those reported by lower than those reported by da Silva *et al*. (2016) for the leaf, flower and stem bark extracts of cashew plant.

CONCLUSION

From the results of this study, the two varieties of cashew apple contain bioactive substances like alkaloids, tannins, flavonoids, steroids, glycosides, terpenoids, phenols and anthraquinones at varying degree while saponins was absent. Moreover, the cashew apple extracts were active against some pathogenic microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Candida albicans* and *Aspergillus fumigatus*. Furthermore, ethanol extracts were found to be more effective with broad spectrum antibacterial and antifungal activities compared to the

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aqueous and hexane extracts. The inhibitory effect of the potent plant extracts against pathogenic bacterial and fungal strains suggests the plant material could be a potential candidate for bioprospecting for antimicrobial development for the treatment of ailment caused by these pathogens.

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