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ANTIBACTERIAL ACTIVITY: A COMPARISM OF RIPE AND UNRIPE EXTRACTS OF TWO VARIETIES (RED AND YELLOW) OF CASHEW APPLE (*ANACARDIUM OCCIDENTALE)* ON SOME CLINICAL BACTERIAL ISOLATES

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ABSTRACT

The emergence of pathogenic bacteria that are resistant to several antibiotics has recently presented a significant challenge to the healthcare system. Therefore, an alternative source of antimicrobial agents is needed. The study aimed to compare the antibacterial activities of aqueous and ethanol extracts of ripe and unripe fruits of red and yellow varieties of *Anacardium occidentale* against *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Cold maceration method of extraction was used using water and ethanol. Antibacterial activities of the extracts against the isolates were evaluated by agar diffusion method while minimum inhibitory concentration (MIC) was determined using broth dilution method. In the determination of the antibacterial activities of all the extracts, the highest zones of inhibition were shown by ethanolic extracts of both ripe red cashew (25 mm) and yellow cashew (26 mm) against *Escherichia coli.* Both the aqueous and ethanolic extracts of unripe red and yellow cashew had no antibacterial effect against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* As well, both aqueous and ethanol extracts of ripe red and yellow cashew and ethanolic extract of ripe yellow cashew inhibited all the test organisms. Minimum inhibitory concentration ranged from 50 to 150 mg/ml. At 100 mg/ml, both ethanolic extracts of ripe red and yellow cashew were bactericidal to *Escherichia coli* while aqueous extract of ripe red cashew was bactericidal to *Escherichia coli* at 150 mg/ml. The antibacterial activities of *A. occidentale* apple (fruit) extracts underscore the credence to the efficacy of their use as traditional remedy against some human ailments.

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INTRODUCTION

Worldwide, microbial infections are a major factor leading to increased mortality and morbidity in human and animals [1]. Antibiotics have been used in the treatment of these infections for many years. However, the rise in antibiotic resistance by different bacterial strains in recent times has hindered the effectiveness of these medications [2]. As a result, treatment of microbial infections has posed an uphill task, and a lot of researches to find alternate treatments have been made [3].

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Despite advances in many biological domains and pharmaceutical microbiology, the dilemma of multidrug resistance and reemerging pathogens continue to persist [4]. Multidrug resistance in many microbial strains, on the other hand, is a primary reason why researchers are searching for new antimicrobial agents or modifying existing ones [5]. Researchers have shifted attention to phytochemicals to find viable alternatives to the existing synthetic drugs in the treatment of different diseases. Plants are a major source of innovative medicinal materials, and as a result, they have made significant contribution to human health and well-being. Phytomedicine, pharmacognosy, herbal science, and pharmaceutical chemistry are just a few of the fields where plants have proven their worth [6].

Medicinal plants are known to contain substance(s) with therapeutic properties including alkaloids, carotenoids, flavonoids, tannins, terpenoids, steroids, and glycosides [7]. These phytochemicals can be extracted from different plant parts including the bark, exudates, flowers, fruits, leaves, roots, twigs etc. Different plant species have been incorporated in the formulation of some proprietary drugs necessitating determination of their antimicrobial properties which will be useful in developing more effective formulations [4].

Anacardium occidentale Linn. (cashew) of the family Anacardiaceae is a tropical evergreen plat that is believed to originate from north-eastern Brazil. Different parts of the cashew plant have found useful in pharmaceutical and other industries [8]. Hence, the study aimed to compare the antibacterial activities of aqueous and ethanol extracts of ripe and unripe fruits of red and yellow varieties of *Anacardium occidentale* against *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Staphylococcus aureus*.

METHODS

Plant Collection and Preparation

Ripe and unripe red and yellow varieties of A*nacardium occidentale* apple were collected from cashew trees at Opi, Nsukka Local Government, Enugu State, Nigeria. The plant was identified and authenticated with the Voucher No UNH/04/0226E by a taxonomist in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Preparation of Extracts

The extracts were prepared from unpeeled, ripe and unripe red and yellow varieties of cashew apples that were soaked separately in sodium hypochlorite solution (0.5%) for 30 minutes and followed by washing in distilled water. Thereafter, they were blended in a homogenizer and stored in clean sterile capped bottles.

Aqueous Extraction

A portion (200 ml) of the crude ripe and unripe red and yellow varieties of the apple extracts were measured out separately into sterile glass beakers and labeled appropriately. A 100 ml of sterile distilled water was added to each and stirred at 30 minutes intervals for 3 h and thereafter, allowed to stand for 72 h following the method outlined by the Association of Analytical Chemists, AOAC (1990) [9]. The mixture was decanted and filtered through a very fine nylon sieve. This was preferred to filter paper to prevent the tannins being adsorbed to the filter paper. The clear crude filtrates (ripe and unripe, red and yellow varieties) were concentrated by evaporation to semi-solid state on a water bath at 100°C. The semi-solid extracts obtained were further concentrated using a rotary evaporator at 80^{o}C [10]. A stock solution of each extract (200 mg/ml) was obtained by reconstituting them in their respective extracting solvents, then filter-sterilized and thereafter stored at 4^{o}C in a refrigerator until needed. Varying concentrations of the extracts (25, 50, 100 and 150 mg/ml) were prepared from the stock solution.

Ethanolic Extraction

The same procedure as described earlier was carried out using 70% ethanol. The semi-solid extracts obtained were further concentrated using a rotary evaporator at 70^{o}C

Microbiological Studies:

Collection of the Test Organisms

The test organisms (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae and Staphylococcus aureus*) were collected from the Medical Diagnostic Laboratory, Department of Microbiology of University of Nigeria, Nsukka.

Antibacterial Activity of the Extracts:

Inoculum Standardization

Suspensions of the test organisms were prepared in a sterile nutrient broth (TM Media India). The nutrient broth was prepared following manufacturer's instructions. In test tubes, 2 ml of the broth were dispensed and sterilized in an autoclave at 121^{o}C for 15 minutes. After cooling, the sterile nutrient broth in the test tubes were inoculated with the organisms from the stock culture using a sterile wireloop (labelled appropriately) and incubated at 37^{o}C for 18 h. The broth turbidity was compared to 0.5 McFarland standard prepared by dispensing 0.1 ml H_2SO_4 and 50 µl into 9.9 ml of distilled water. This was mixed thoroughly to form a turbid mixture and stored in a dark place to avoid light. About 0.5 McFarland standard was used which is equivalent to 1.8× 10^8 bacterial suspension per ml.

Antibacterial Activity Assay

The antibacterial activity of ethanolic and aqueous extracts of ripe and unripe red and yellow A*nacardium occidentale* false fruits were evaluated against *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Staphylococcus aureus* by agar diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) [11]. The test organisms prepared and adjusted to McFarland standard (turbidity) were evenly streaked onto the surface of Mueller Hinton agar plate using sterile cotton swab stick. Wells (6 mm diameter) were made on the agar plates using a sterile cork borer. Thereafter, 0.1 ml plant extracts of different concentrations (25, 50, 100, 150 and 200 mg/ml) were introduced into the well and then incubated at 37°C for 18 h. Antibacterial activity was evaluated by measuring the zone of inhibition in millimeter (mm) using a transparent metre rule.

Determination of Minimal Inhibitory Concentration (MIC)

MIC was determined using the broth dilution method as described by CLSI [11]. A 0.5 ml of different concentrations (25, 50, 100, 150 and 200 mg/ml) of each of the extracts was dispensed into 9 ml of sterile broth in test tube (in duplicates) and properly labelled. Thereafter, 0.5 ml suspension of the test organism was inoculated into each concentration of the extracts. To serve as a control, a tube containing nutrient broth only was inoculated with the test organism as described above. All inoculated tubes were then incubated aerobically at 37^{o}C for 24 h. The least concentration of the extract that did not produce visible growth or turbidity was taken as the minimum inhibitory concentration.

Minimum Bactericidal Concentration (MBC)

The MBC of the extracts was determined by re-culturing all the broth culture in MIC above onto solid nutrient agar plates by streak method. The plates were thereafter incubated at 37^{o}C for 24 h and then examined for bacterial growth. The MBC was regarded as the lowest concentration that showed no growth [12].

RESULTS

Antibacterial Activity Tests

The results of the agar well diffusion tests done on all the eight extracts are shown in Figures 4-11.

At 150 mg/ml, ethanolic extract of ripe red cashew exhibited highest inhibition against *E. coli* with zone of inhibition of 25 mm, followed by *Pseudomonas aeruginosa* and *Staphylococcus aureus* (19 mm) and *Klebsiella pneumonia* (18 mm) (Figure 1). Similarly, inhibitory activity of aqueous ripe red cashew extracts against the isolates are in the order *Escherichia coli, Staphylococcus aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* with zones of inhibition of 22, 18, 17 and 16 mm respectively (Figure 2). Aqueous and ethanolic extract of unripe red cashew was inhibitory or showed activity against *Escherichia coli* and *Staphylococcus aureus* but did not show any activity against *Klebsiella pneumonia* and *Pseudomonas aeruginosa* Figures 3 and 4. *Escherichia coli, Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were found to be susceptible to ethanolic extract of ripe yellow cashew with inhibitory zone diameters of 26, 20, 18 and 17 mm respectively at 150 mg/ml (Figure 5). Aqueous extract of ripe yellow cashew exhibited similar inhibitory effect on all the test organisms except for *Klebsiella pneumonia* (Figure 6) *Escherichia coli,* and *Staphylococcus aureus* were susceptible to both ethanolic and aqueous extract of unripe yellow cashew while the extracts did not show any observable effect against *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Figures 7 and 8). In general, zone of inhibition decreases with decrease in extract concentration.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC results are shown in Table 1. MIC of all extracts against *Escherichia coli* ranged between 50 to 150 mg/ml. Similarly, MIC ranged between 100 to 150 mg/ml was observed in *Staphylococcus aureus.* MIC of both etanolic and aqueous extracts of the ripe apples ranged between 100 and 150 mg/ml against *Pseudomonas aeruginosa* and *Klebsiella pneumonia.* MBC was observed only in ripe red and yellow extracts against *Escherichia coli.* MBC was also observed in ethanolic extract of unripe red, ripe and yellow cashew against *Staphylococcus aureus.* MBC was not observed against *Pseudomonas aeruginosa* and *Klebsiella pneumonia* for all extracts (Table 2).

DISCUSSION

Medicinal plants have been utilized in recent years due to the emergence of antibiotics resistant microbes and the side effect of synthetic drugs. It can be inferred that the different extracting solvents used in this study did not impair the bioactive contents of the extracts. This observation is in agreement with the findings of Rajesh *et al.* [13] who carried out elementary phytochemical profiling of cashew using aqueous and ethanolic solvents. He found out that all the samples contained different phytochemicals at varying concentrations. Tannins detected in this study is similar to the findings reported by Bhairathi and Asna [14] and Okey-Ndeche *et al.* [15], who reported the presence of tannins in cashew plant. There are varying phytochemical contents among plants of the same species. This variance could be attributed to different species of the same plant, geographical location, soil moisture contents, nutrients, soil types and extraction medium.

In the determination of the antibacterial activities of all the extracts, ethanolic extract of both ripe red and yellow cashew apple exhibited higher activity against *Escherichia coli.* while ethanolic extract of ripe red cashew, aqueous extract of ripe red cashew and ethanolic extract of ripe yellow cashew inhibited all the test organisms. This finding is similar to the report of high antibacterial activity of aqueous and ethanolic extracts of *Anacardium occidentale* against *Escherichia coli* and *Staphylococcus aureus* by Okey-Ndeche *et al.* [15]. Also, our findings agreed with the report by Arekemase *et al*. [16] that ethanolic extract was more effective than aqueous extract of *A. occidentale*. Aderiye and David [17] reported high antibacterial activity of hot and cold aqueous fruit extract of *A. occidentale* against methicillin resistant *S. aureus* and *E. coli* O157:H7. In general, antibacterial activities of plant extracts are attributed to their content of bioactive substances.

At 100 mg/ml, ethanolic extracts of both ripe red and yellow cashew were bactericidal to *Escherichia coli* while aqueous extract of ripe red cashew was bactericidal to *Escherichia coli* at 150 mg/ml. In this present study, it was observed that there was little or no difference between the antibacterial activities of the red and yellow varieties of cashew. This could mean that the phytochemicals were present in roughly equal proportions

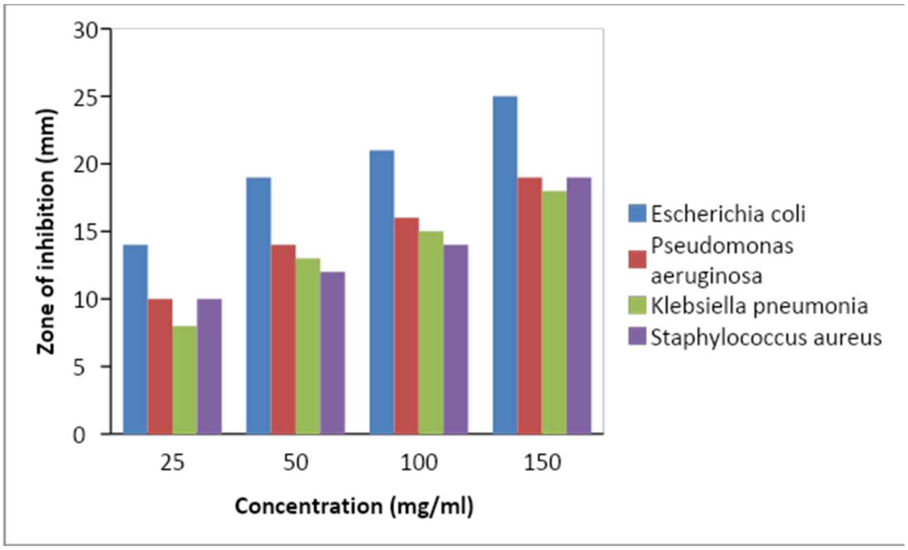

Figure 1: Antibacterial activity of ethanolic extract of ripe red cashew on clinical isolates

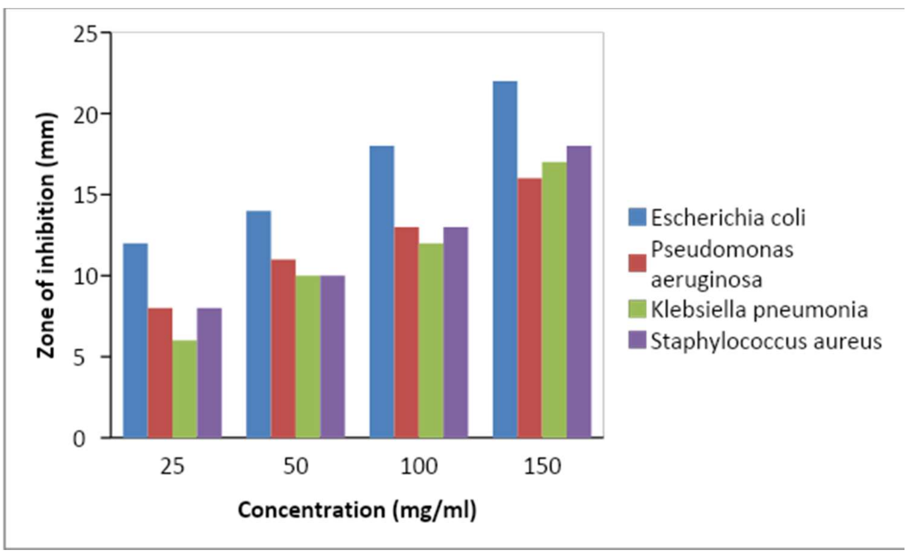

Figure 2: Antibacterial activity of aqueous extract of ripe red cashew on clinical isolates

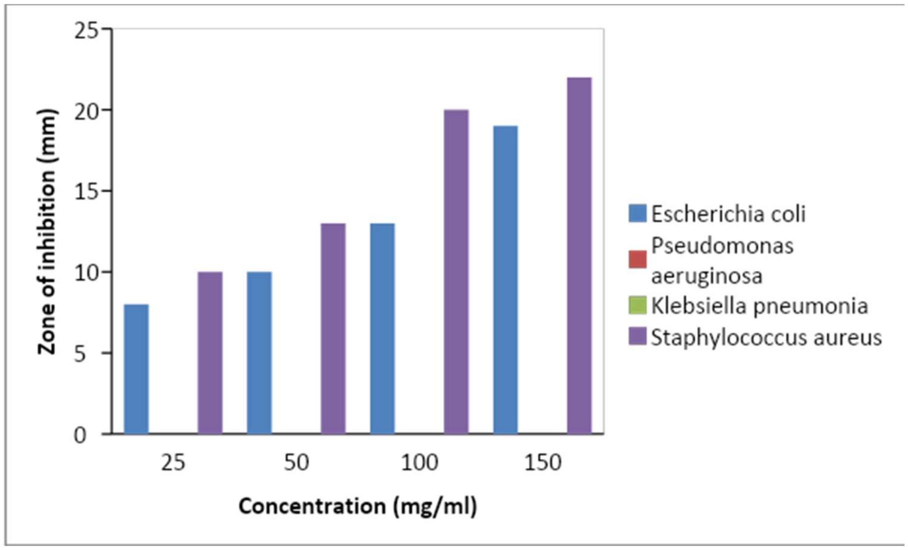

Figure 3: Antibacterial activity of ethanolic extract of unripe red cashew on clinical isolates

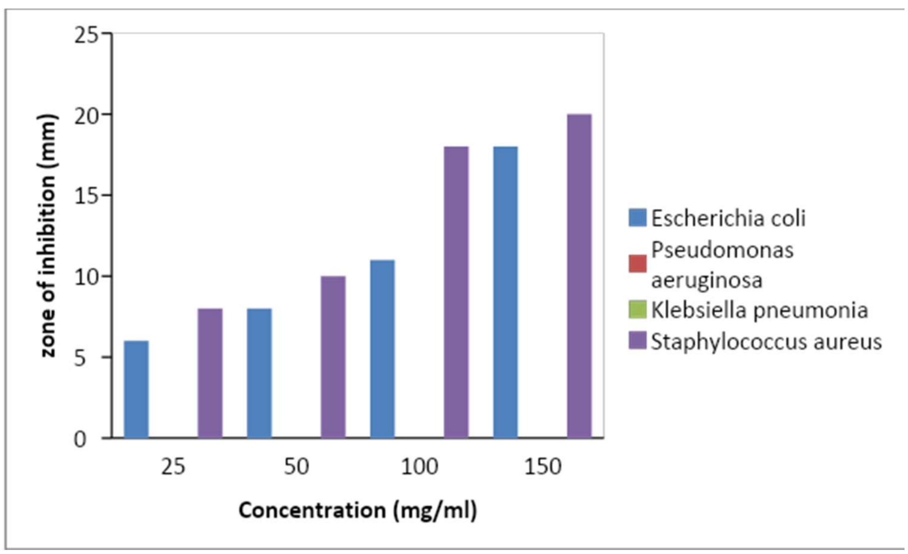

Figure 4: Antibacterial activity of aqueous extract of unripe red cashew on clinical isolates

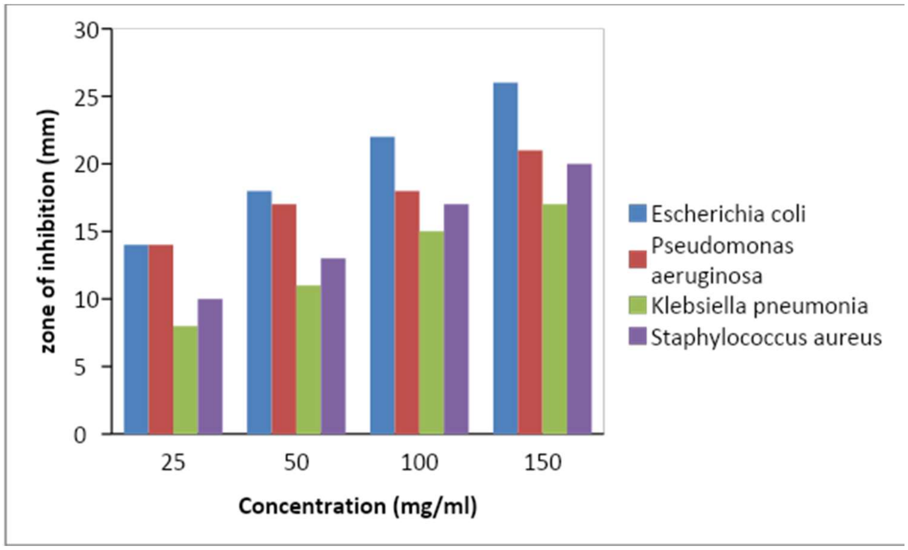

Figure 5: Antibacterial activity of ethanolic extract of ripe yellow cashew on clinical isolates

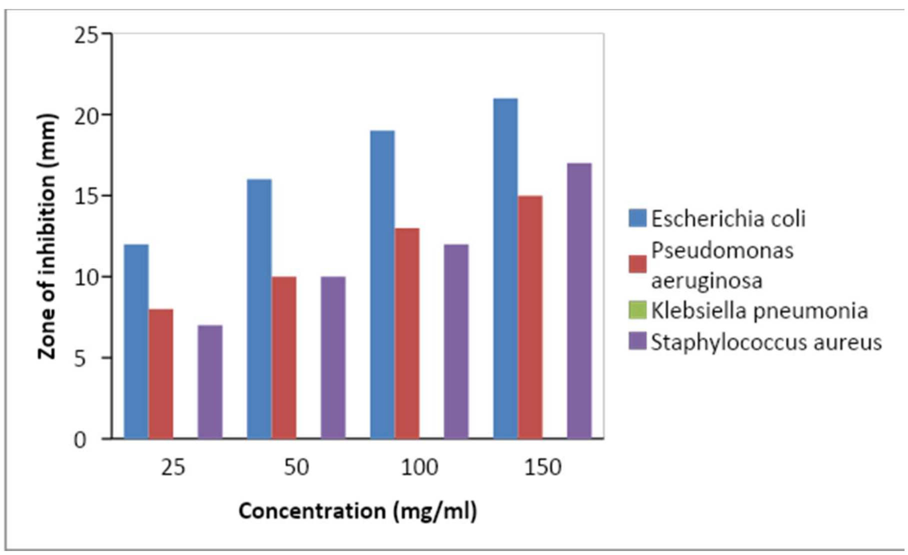

Figure 6: Antibacterial activity of aqueous extract of ripe yellow cashew on clinical isolates

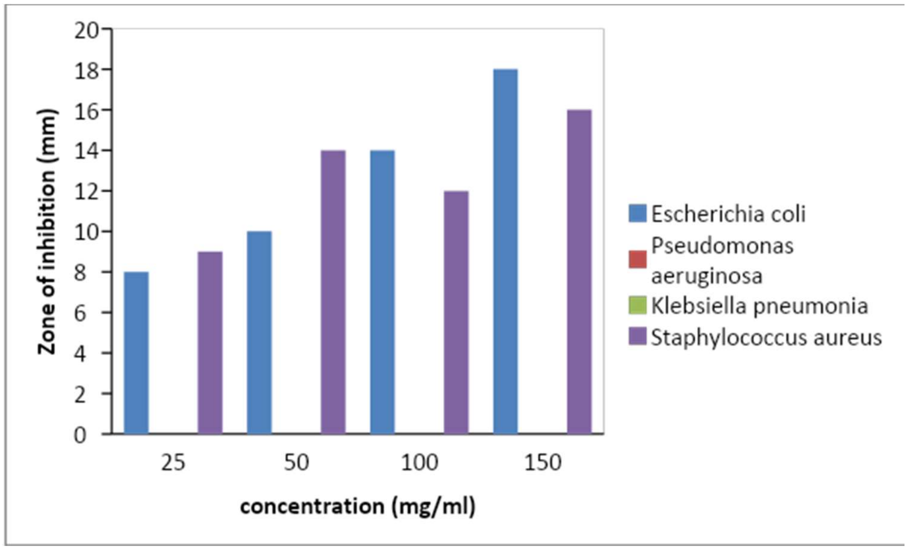

Figure 7: Antibacterial activity of ethanolic extract of unripe yellow cashew on isolates

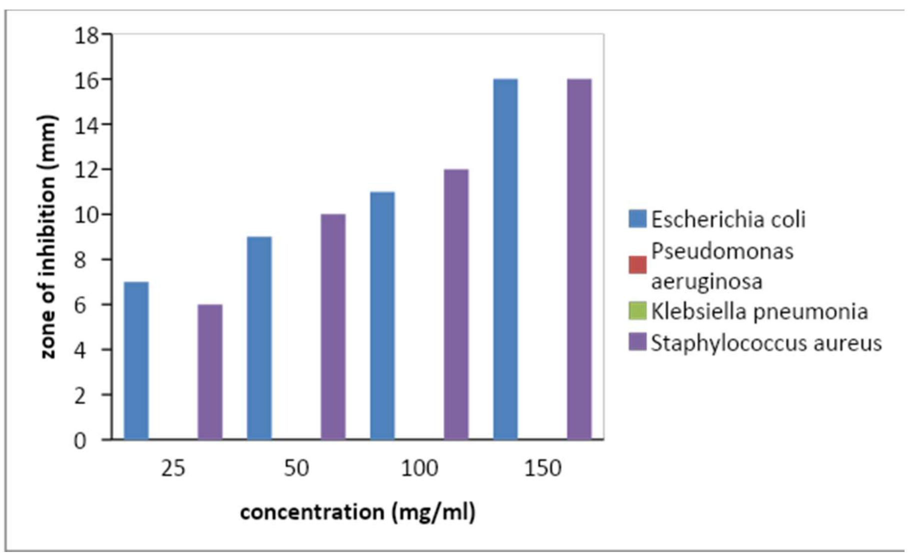

Figure 8: Antibacterial activity of aqueous extract of unripe yellow cashew on isolates

Table 1: MIC of all extracts on clinical isolates

Bacterial isolates	Minimum inhibitory concentration (mg/ml)							
	S1	S2	S3	S4	S5	S6	S7	S8
Escherichia coli	50	100	150	150	50	50	150	150
Pseudomonas aeruginosa	100	150	-	-	100	150	-	-
Klebsiellapneumoniae	100	150	-	-	100	-	-	-
Staphylococcus aureus	150	150	100	100	100	150	100	150

Legend:
S1: Ethanolic extract of ripe red cashew; **S2:** Aqueous extract of ripe red cashew
S3: Ethanolic extract of unripe red cashew; **S4:** Aqueous extract of unripe red cashew
S5: Ethanolic extract of ripe yellow cashew; **S6:** Aqueous extract of ripe yellow cashew
S7: Ethanolic extract of unripe yellow cashew; **S8:** Aqueous extract of unripe yellow cashew

Table 2: MBC of all extracts on clinical isolates

Bacterial isolates	Minimum bactericidal concentration(mg/ml)							
	S1	S2	S3	S4	S5	S6	S7	S8
Escherichia coli	100	150	-	-	100	150	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-
Klebsiellapneumoniae	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	150	-	150	-	150	-

Legend:
S1: Ethanolic extract of ripe red cashew; **S2:** Aqueous extract of ripe red cashew
S3: Ethanolic extract of unripe red cashew; **S4:** Aqueous extract of unripe red cashew
S5: Ethanolic extract of ripe yellow cashew; **S6:** Aqueous extract of ripe yellow cashew
S7: Ethanolic extract of unripe yellow cashew; **S8:** Aqueous extract of unripe yellow cashew

in both the red and yellow varieties. The ethanolic extracts in all ramifications had better antibacterial properties than the aqueous extracts. This suggests that ethanol could be a better solvent for the extraction of bioactive compounds in the cashew apple than distilled water as was also reported by Omojasola and Awe [18]. Again, it was observed that the ripe red and yellow varieties of cashew apples had better antibacterial properties than the unripe red and yellow varieties of cashew apples and this could be due to the presence of more phytochemicals in the ripe fruit than in the unripe fruit.

CONCLUSION

The traditional claims of the false fruit extracts of *Anacardium occidentale,* as an antimicrobial agent, have been confirmed, largely due to the fact that the false fruit extracts prepared from ripe and unripe, red and yellow varieties of cashews using ethanol and aqueous solvents, displayed antimicrobial activity against the test clinical isolates used in this study. If the active phytochemicals in the cashew apple capable of inhibiting and killing the growth of the test bacteria are analyzed and appropriately used in the medical formulations it would help in the in treatment of ailments of bacterial origin. Further studies need to be carried out to elucidate the potentials of proper application of the phytochemicals of this fruit extract in the treatment of gastrointestinal and upper respiratory tract infections.

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