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Original Research

Antibacterial activity of crude methanol extract of *Anacardium occidentale* leaf and its fractions on isolates of diarrhoea-causing pathogenic *Escherichia Coli* and *Salmonella enterica*

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ABSTRACT

This study investigated the antibacterial activity of *Anacardium occidentale* leaf crude extract and it's derived; petroleum, chloroform and methanol fractions on isolates of diarrhoea-causing pathogenic *Escherichia. coli*, and *Salmonella enterica*. The protocol described by Kupchan and Tsou as modified by Houghton and Raman was used to partition the crude extract. Field strains of *Salmonellae enterica* and *E. coli* enterobacteria, were obtained using standard procedures adopted by Mufandaedza.. Holes were bored in the agar, using a sterile borer which enabled the application of crude extract and the fractions, and thereafter, incubated for 24 hours. Results of the study showed that the crude extract elicited better ($p<0.05$) growth inhibitory (56.11 and 58.14%) effect on the *E. coli* and *S. enterica* isolates respectively, better than any of the derived fractions that recorded less than 30% activity, suggesting an additive, complementary or synergistic effect of individual composite fraction, rather than the role of a single biomolecular fraction in the leaf extract of *A. occidentale*, hence partitioning, adversely reduced or disintegrated the additive effect of the crude extract on enterogenic bacteria investigated. In conclusion, the crude extract of *Anacardium occidentale*, have shown good anti-bacterial activity, principally by inhibiting the growth of diarrhoea causing enterogenic *E. coli* and *Salmonella* organisms' growth *in vitro*, hence supporting its folkloric use in the management of enterogenic diarrhoea by traditional healers.

Keywords: Anacardium occidentale leaves; Antibacterial activity; synergism, Growth suppression

INTRODUCTION

The use of medicinal plants in the treatment of diseases in the developing Countries are rooted with traditional practices (Johansson *et al*., 2009), largely due to inability to afford the cost of conventional drugs as well as cultural prejudice to orthodox medications. Diarrhoea could occur in three (3) forms – secretory form (caused mostly by bacterial enterotoxins), exudative form (as a result of increased permeability of the intestinal mucosa either due to inflammation or infections) or osmotic form (commonly associated with mal-digestion or mal-absorption of food). Typically, most diarrhoea syndromes are combinations of these forms, and a good anti-diarrhoeal agent should be able to principally decrease intestinal secretions and motility or reverse the underlying problem such as pathogenic bacteria that produced the observed changes in secretions or motility (Tadesse *et al*., 2014). According to the United Nations International Children's Emergency Fund (UNICEF), over 5000 children die daily due to diseases with diarrhoeal complications (WHO and UNICEF, 2021). Resistance to antibacterial agents by most diarrhoea-causing pathogenic *Escherichia. coli* (*E. coli*), and *Salmonella enterica* has become a global concern in controlling diarrhoeas caused by bacterial enterotoxins (Farthing and Salam, 2012). *Anacardium occidentale* commonly known as cashew tree, is a multipurpose tree of the Amazon that grows up to 15 m high. The tree is small and evergreen, growing to 10-12 m (~32 ft) tall, with a short, often irregularly shaped trunk. The leaves are spirally arranged, leathery textured, elliptic to obovate, 4 to 22 cm long and 2 to 15 cm broad, with a smooth margin (Lim, 2012). The leaves of *A. occidentale* species possess a wide array of pharmacological properties, which reflect their health benefits, and confer their traditional uses as both food and medicine. Most of these

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health benefits includes; antibacterial, antiviral, antioxidant, cytotoxic, hypoglycaemic, hypolipidemic, anti-ulcerogenic, anti-hypertensive, analgesic and anti-inflammatory, and antifungal activities (Trevistan *et al.,* 2006; Abaas *et al.,* 2015). The leaf of the plant has been reported to possess antidiarrhoeal activity (Ezeigbo *et al.,* 2012) hence, it is imperative to investigate if the crude extract of *Anacardium occidentale* leaf and the partitions derived from the crude could be potent as an antibacterial agents on isolates of diarrhoea-causing pathogenic *E.coli*, and *Salmonella spp*.

The need for more natural alternative and better anti-bacterial agent has necessitated the continuous discovery and screening of newer compounds, particularly of plant origin. This study was designed to evaluate pharmacological evidence of the folkloric use of *Anacardium occidentale* plant as an antibacterial, and the efficacy of the derived partitions on two (2) diarrhogenic bacteria.

MATERIALS AND METHODS

STUDY LOCATION

This study was carried out in the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike (MOUAU). All procedures were carried out in strict compliance with the institutional ethical instructions for the work, as well as adequate consultations to the Ethical Committee and Experimental Ethic guidelines (Louhimies, 2002). Ethical approval number: MOUAU/CVM/REC/202224 was assigned to the study.

PLANT COLLECTION, EXTRACTION AND PARTITIONING

PLANT COLLECTION

Fresh leaves of *Anacardium occidentale* were collected within the premises of Veterinary College, MOUAU. They were authenticated by a taxonomist from the Department of Botany, MOUAU, with a voucher specimen number MOUAU/CVM/VPP2017/017, assigned to the leaf sample.

EXTRACT PREPARATION

The leaves were separated from debris, washed in running tap water, exposed to early morning mild sunlight for 3 consecutive days) and subsequently dried in a Gallenkamp hot air oven (Weiss Technik UK) set at 40°C to obtain crisp leaf samples, before being pulverized into coarse powder using a stainless steel laboratory blender (Ansah *et al.,* 2011). The pulverized leaves were weighed and macerated in MeOH (1:5 w/v) for 72 hours and with intermittent shaking every 2 hours to ensure thorough mixing. The mixture was filtered through Whatman filter paper (No. 1), and concentrated under reduced pressure using a rotary evaporator (Cole-Parmer type N-1110, China).

PARTITIONING OF THE EXTRACT

The protocol used by Kupchan and Tsou (1973) as modified by Houghton and Raman (1998) was employed. About 40 grams of the crude extract was dissolved in 360 ml of analytical grade of methanol (1:9 ratio). Thereafter, the resultant solution was then partitioned successively in equal volume of immiscible organic solvents of increasing polarity, first petroleum ether (Pet Ether) which was well shaken, then chloroform ($CHCl_3$). Before adding the next solvent, the partition formed by the former was decanted. Thereafter the next solvent was added, well shaken and allowed to settle. At the end, all the three (3) fractions obtained (methanol, chloroform and pet ether fractions) were evaporated to dryness using a rotary evaporator. The percentage yield (w/w) of the crude extract and the derived partitions were calculated using the formula:

$$\textbf{\% Yield} = \frac{\text{Weight of material extracted}}{\text{Weight of plant material}} \times \frac{100}{1}$$

PHYTOCHEMICAL SCREENING

The weighed crude extract and the partitions obtained were screened for its phytochemicals (qualitatively)according to previously described assays by Trease and Evans (1989); Sofowora (1996); Harborne (1998); Brusotti *et al* (2014).

ANTI-BACTERIAL ACTIVITY

For the antibacterial activity, Field strains of diarrhoea-causing *Salmonella enterica* and *E. coli* used in this study were cultured and isolated according to the standard procedures (Mufandaedza *et al.,* 2006) in the Microbiology Laboratory, Michael Okpara University of Agriculture Umudike. The two isolates were sub cultured frequently every 15 days and maintained on nutrient agar slants before use. Antibacterial activity of all extract (the crude and the three partitions) samples were tested by modified Agar well method, as described by Zabin *et al*. (2012). Inoculum suspension of each of the isolate was spread on two different petri dishes (*E. coli* and *Salmonella enterica*), using sterile L-shaped glass rod, and four Wells of 0.5 cm in diameter were made on each media plate labeled A-D representing the four graded (0.1, 0.2, 0.4 and 0.8 mg/ml) concentrations of the crude extract of *Anacardium occidentale* leaf and 100 µl of each concentration, were used to fill the bored wells aseptically. The plates were placed at room temperature for an hour to allow diffusion of extract into the agar. Then the plates were incubated for 24 hours at 37˚c. Above procedures were repeated for the three derived partitions (methanol, chloroform and petroleum ether), and the experiment was performed in triplicates and the results were tabulated by measuring the diameter of inhibitory zone using a transparent meter rule at the end of 24 hours.

$$\text{The percentage suppression of growth zone} = \frac{\text{Zone of inhibition (mm)}}{\text{Total length of growth zone (mm)}} \times \frac{100}{1}$$

STATISTICAL ANALYSIS

Data obtained from this study were subjected to One-way analysis of variance (ANOVA), and were presented as means ± standard errors of mean. The concentration- dependent anti-bacterial activity (percentage inhibition zones (IZ), and percentage growth suppression) were measured, analysed and result presented in Tables. Differences in the means were obtained using Duncan post-hoc statistics. Statistical confidence was set at 95 % ($P < 0.05$). Graph pad (prism 5) statistical package was used.

RESULTS

YIELD OF THE PARTITIONING

The plant derived partitions yielded 8 grams (40 %), 7 grams (35 %) and 4 grams (20 %) of methanol, chloroform and petroleum ether fractions, respectively.

PHYTOCHEMICAL

The phytochemical screening of *A. occidentale* leaf crude extract revealed the presence of alkaloids, glycosides, flavonoids, tannins, saponins, anthraquinone, steroids, phenolics, resins, terpenoids, and cardiac glycosides (Table I).

Table I. Phyto-chemical screening of crude extract and derived partitions of *A. occidentale* leaf

Metabolites	Crude extract	Methanol fraction	Petroleum ether Fraction	Chloroform Fraction
Alkaloids	+	+	+	+
Glycosides	+	+	+	-
Flavonoids	+	+	+	+
Tannins	+	+	-	+
Saponins	+	+	+	+
Anthraquinone	+	-	-	+
Steroids	+	+	+	+
Phenolic acid	+	-	-	+
Resins	+	-	+	-
Terpenoids	+	+	-	+
Cardiac Glycosides	+	+	-	-

Key: + means present, - means absent

ANTIBACTERIAL ACTIVITY

The antibacterial activity of the crude extract on the growth of the isolates of *Salmonella spp*. and *E. coli* are presented in Table I|. The result showed that the crude extract of *A. occidentale at* the graded concentrations tested evoked a concentration dependent ($p<0.05$) anti-bacterial activity against isolates of *E. coli* and *salmonella* organisms. At the highest concentration (0.8 mg/ml) of the crude extract, a 54.44% and 57.22% inhibition of growth of *E. coli* and *Salmonella* (Table 1) organisms, respectively were achieved, and were comparable ($p>0.05$) with the activity of the reference drug, (Ampicillin, 250 mg/ml) which inhibited the bacteria growth by about 56.11% on *E. coli* media plate and 59.44% on *Salmonella spp.* media plate. The result comparing the crude extract with the derived fractions showed that the crude extract gave better ($p<0.05$) growth inhibition, followed by chloroform fraction (27.55; 28.11%) and methanol fraction (17.38; 20.16%), on *E. coli* and *Salmonella spp.* plated media respectively (Table II). The petroleum ether fraction recorded the least ($p<0.05$) growth inhibition by 15.33%. However, the chloroform fraction suppressed the growth zone of *E. coli* and *Salmonella* organisms better than other fractions.

Table II: Showing the anti-bacterial activity of the crude extract of *Anacardium occidentale* at different concentrations on the growth of *E.coli* and *Salmonella spp.*

Treatment	Length of growth zone (mm)	Zone of Inhibition (mm)	% Suppression of Growth zone
E. coli			
0.1 mg/ml	18.00	3.33 ± 0.33^c	18.50
0.2 mg/ml	18.00	4.00 ± 0.57^c	22.22
0.4 mg/ml	18.00	6.60 ± 0.30^b	36.66
0.8 mg/ml	18.00	9.80 ± 0.57^{ab}	54.44
Ampicillin (250 mg/ml)	18.00	10.10 ± 0.55^a	56.11
$R^2 = 0.922$			
Salmonella spp			
0.1 mg/ml	18.00	5.33 ± 0.33^c	26.61
0.2 mg/ml	18.00	5.66 ± 0.66^c	31.44
0.4 mg/ml	18.00	7.66 ± 0.30^b	42.55
0.8 mg/ml	18.00	10.30 ± 0.33^a	57.22
Ampicillin (250 mg/ml)	18.00	10.70 ± 0.55^a	59.44
$R^2 = 0.871$			

Note: Values are presented as Mean ± S.E (Standard Error). Different superscript letters indicate significant differences ($p<0.05$) between groups.

DISCUSSION

The phytochemical analysis of the *A. occidentale* leaf crude extract revealed the presence of alkaloids, glycosides, flavonoids, tannins, saponins, anthraquinone, steroids, phenolics, resins, terpenoids, and cardiac glycosides, with anthraquinone and phenolic acids phytochemicals absent in methanol and petroleum partitions, while glycosides and resins were not detected in the chloroform partition (Table 1). Konan *et al*. (2007) and Razali *et al*. (2008), have reported that *A. occidentale* leaf and the bark are rich in polyphenols, alkaloids, anthraquinone, and terpenoids, and are used in the ethno-medical management of gastrointestinal tract disorder and hypertension in West Africa and South America. The antimicrobial activity recorded in some plant extracts have been attributed to some of the secondary metabolites (Palombo, 2006), and most of these phytochemicals viz: anthraquinone, phenolics, resins, terpenoids and glycosides have been found to possess strong antimicrobial activities (Fridous *et al*., 1990; Khanahmadi *et al*., 2010; Kaul & Pattan, 2011; Holler *et al*., 2012; Neilsen *et al*., 2012), to both Gram –ve and +ve organisms because of their ability to be toxic to microorganisms by inhibiting the enzymes which are essential for the growth of microorganism (Syed *et al*., 2011), degrade the cell wall (Karsha & Lakshni, 2010; Riss *et al*., 2015), interact with the composition and disrupt cytoplasmic membrane (Russel, 2002; Seasotiya & Dala, 2014), damage membrane protein and interfere with membrane integrated enzymes (Alamsher, 2009; Nalubega *et al*., 2011), cause leakage of cellular components (Subhas *et al*., 2010), coagulate cytoplasm, deplete the proton motive force (Narayan, 2012; Djeussi *et al*., 2013), change fatty acid and phospholipids constituents (Lopez *et al*., 2005), impair enzymatic mechanism for energy production and metabolism (EL-Mahmood *et al*., 2010), alter nutrient uptake and electron transport (Sasidharan *et al*., 2011). The findings from this study showed that the crude extract of *A. occidentale* leaf had mild (with 56.11 and 59.14%) antibacterial inhibitory actions on the growth of *E. coli* and *Salmonella enterica* respectively, at 0.8 mg/ml concentration (Plates; 1- 4), compared wiith 56.11 and 59.14% antibacterial activities of the standard drug (Ampicillin, 250mg/ml) against *E. coli* and *Salmonella enterica* respectively, suggesting that the phytoconstituents present in this plant extract may have similar antibacterial effect as the Ampicillin drug used. The presence of anthraquinone, resins, terpenoids, glycosides and phenolic compounds in the crude extract *Anacardium occidentale* leaf is suspected to have contributed to its antimicrobial activity against these two Gram -ve strains (*Salmonella enterica* and

Table III: Comparing the anti-bacterial activity of the crude extract and the different fractions of *Anacardium occidentale* on the growth of *E.coli* and *Salmonella spp.*

Treatment (0.8 mg/ml conc.)	Length of growth zone (mm)	Zone of Inhibition (mm)	% Suppression of Growth zone
E. coli			
Methanol fraction	18.00	3.13 ± 0.06^{c}	17.38
Pet ether fraction	18.00	2.76 ± 0.14^{c}	15.33
Chloroform fraction	18.00	4.96 ± 0.08^{b}	27.55
Crude extract	18.00	9.80 ± 0.57^{a}	54.44
Salmonella spp			
Methanol fraction	18.00	3.63 ± 0.08^{c}	20.16
Pet ether fraction	18.00	3.13 ± 0.08^{c}	17.38
Chloroform fraction	18.00	5.06 ± 0.12^{b}	28.11
Crude extract	18.00	10.30 ± 0.33^{a}	57.22

Note: Values are presented as Mean ± S.E (Standard Error). Different superscript letters indicate significant differences ($p<0.05$) between groups.

Plate 1. Antibacterial activity of the crude extract of *A. occidentale* (A – 0.8 mg/ml; B – 0.4 mg/ml; C – 0.2 mg/ml; D – 0.1 mg/ml) on plated media of *E. coli.* (Mg×40)

Plate III. Antibacterial activity of the crude extract of *A. occidentale* (A – 0.8 mg/ml; B – 0.4 mg/ml; C – 0.2 mg/ml; D – 0.1 mg/ml) on plated media of *Salmonella spp* (Mg×40)

Plate V. Comparing antibacterial activity of *A. occidentale* crude extract and derived fractions (A – crude extract, 0.8 mg/ml; B – chloroform fraction, 0.8 mg/ml; C – methanol fraction, 0.8 mg/ml; D – petroleum ether fraction, 0.8 mg/ml) on plated media of *E. coli.*

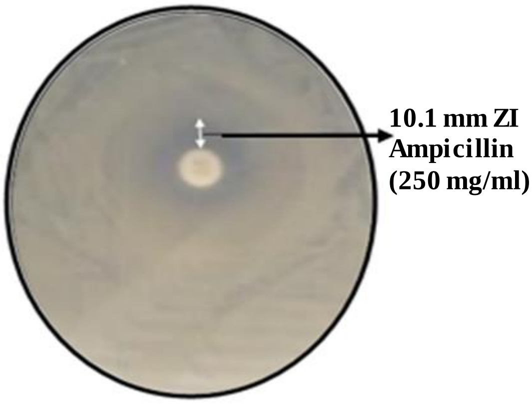

Plate II. Ampicillin standard drug (250 mg/ml) on plated media of *E. coli.* (Mg×10)

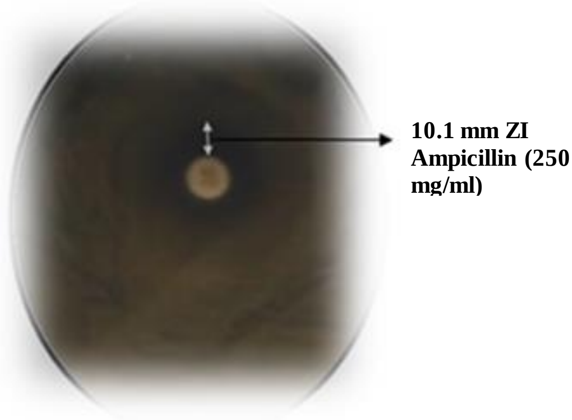

Plate IV. Ampicillin standard drug (250 mg/ml) on plated media of *E. coli.* (Mg×10)

Plate VI. Comparing antibacterial activity of *A. occidentale* crude extract and derived fractions (A – petroleum ether fraction, 0.8 mg/ml; B – crude extract, 0.8 mg/ml; C – methanol fraction, 0.8 mg/ml; D – chloroform fraction, 0.8 mg/ml) on plated media of *Salmonella spp*.

E coli). These findings supported previously reported studies done by different researchers such as Rodriguez *et al*., (2001); Lopez *et al*., (2005); Akindele and Adeyemi, (2006); Gurinder and Daljit, (2008); Saralaya *et al.,* (2010); Hossain *et al.,* (2012) and Santos *et al.,* 2013), using other plant extracts that contains high amount of these compounds.

The different fractions did not elicit an appreciable (< 50 %) anti-bacterial activity in both colonies at the same 0.8 mg/ml concentration, compared with the crude form of the extract, however, the chloroform partition gave 27.55, and 28.11% inhibitory activity on the growth of *E. coli* and *Salmonella enterica* at 0.8 mg/ml concentration respectively, better than other partitions (Table II; Plates; 5 and 6), thus, suggesting that the crude extract may be more effective in managing *Salmonella enterica* induced diarrhoea than those of *E. coli*. The dissociation of these important bio-molecules present in this plant leaf, noted for their anti-microbial activities (Konan *et al*. (2007) and Razali *et al*. (2008), via partitioning could partly, be one of the reasons for the ineffectiveness of the partitioned extracts on the two isolates, compared with crude extract which had better antibacterial effect on the two Gram –ve organisms tested (Table II & III).

CONCLUSION

The crude extract of the leaf of *A. occidentale* have shown to possess significant antibacterial activity against the two Gram -ve strains of diarrhoea-causing *Salmonella enterica* and *E coli*, organisms better than its derived fractions, indicating that the action of the plant extract appears to be reduced after fractionation via solvent-solvent partitioning.

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