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CYTOTOXICITY AND ANTIBACTERIAL ACTIVITY OF FRUITS EXTRACTS OF *Xylopia aethiopica* AGAINST SOME SELECTED BETA-LACTAMASE PRODUCING BACTERIA

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ABSTRACT
Xylopia aethiopica commonly known as the West African pepper tree is consumed as food condiment and also used traditionally in the treatment of cough, bronchitis, dysentery and female sterility. Air-dried and ground fruits of *Xylopia aethiopica* were extracted using ethanol and the recovered extract was partitioned to give chloroform, water, methanol and n-hexane fractions. The five extracts were screened for antibacterial activity against strains of ß-lactamase producing bacteria: *Escherichia coli, Proteus sp., and Klebsiella pneumonia* as well as cytotoxicity against brine shrimp larvae. The result of the antibacterial screening showed that all the extracts exhibited selective activity against *Proteus sp.*, the interface-2 demonstrated the highest activity with diameter of zone of inhibition of 14mm at 1000µg/disc. On the other hand, methanolic extract showed high cytotoxic activity in the brine shrimp lethality bioassay with LC_{50} value of 3.4µg/mland moderate antibacterial activity with diameter of zone of inhibition of 12mm at 1000µg/ml.
Keywords: *Xylopiaaethiopica*; Extracts; antibacterial activity; ß-lactamase producing bacteria; cytotoxicity; brine shrimp lethality bioassay.

INTRODUCTION

Xylopia aethiopica is an evergreen aromatic tree of the family, Annonaceae. Plants of the family, annonaceae are well known as good source of biologically active natural products example, annonaceous acetogenins which have been developed as anticancer drugs, commercial insecticides and cosmetic additives (Guoen *et al.*, 1997). Over the decades, beta-lactam antibiotics such as penicillins and cephalosporins have been widely used in the treatment of infections caused by Gram-negative bacteria but resistance against this group of antibiotics among pathogenic bacteria has become a global problem. The most important mechanism for this resistance is the production of an enzyme called the beta-lactamase (Saeide *et al.*, 2015). The beta-lactamases are widely distributed in both Gram-positive and Gram-negative bacteria but is produced at highest concentrations by certain Gram-positive bacteria and in particular by *Bacillus cereus*. The enzymes protect these organisms against the action of beta-lactam antibiotics making them inactive by opening the beta-lactam ring (Ambler, 1980). There is therefore the need to discover new antimicrobial compounds from plants to effectively inhibit beta-lactamases. It is expected that plants are safe, have low cost and are effective against antibiotic resistant bacteria. Consequently, one of the objectives of this researchis to conduct antibacterial activity assay against resistant bacteria (beta-lactamase producing bacteria)

However, some natural products are known to carry toxicological properties. In view of this, numerous research studies have been developed in order to determine the toxicity of medicinal plants. The most convenient method for the preliminary assessment of toxicity of natural products is the brine shrimp lethality assay (Mentor *et al.*, 2014). Brine Shrimp Lethality Assay (BSLA) is used to detect the presence of compounds that have cytotoxic effects on cell line.Cell cytotoxicity refers to the ability of certain chemicals or mediator cells to destroy living cells.Through the evaluation of cytotoxic activity against brine shrimps, novel cytotoxic, antitumor and pesticidal compounds can be isolated from potential plant sources (Lilybeth and Olga, 2013). In this bioassay, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivity. The method utilizes a simple zoological organism, Brine shrimp nauplii (*Artemia salina*) as the effective and most extensively studied in the *Artemia* species. Brine toxicity is closely correlated with the toxicity for higher animal models which makes the assay an excellent predictive tool in the determination of toxic potentials of plant extracts in humans (Mentor *et al.*, 2014).

These assays were intended to establish the presence of cytotoxic natural products and compounds capable of inhibiting the growth of ß-lactam antibiotic resistant bacteria.

MATERIALS AND METHODS

General

The reagents used were purchased from Sigma-Aldrich. The glasswares used were washed with detergents, rinsed with distilled water and oven dried before use. Sterile distilled water and normal saline were used for the antibacterial assay. Clinical isolates of the three bacterial strains (*Escherichia coli*, *Klebsiella pneumoniae* and *Proteus sp.*) used for the antibacterial assay were obtained from the Department of Microbiology, Bayero University Kano. Brine shrimp eggs were purchased from Sigma-Aldrich and stored in a refrigerator until needed

Collection of Plant Material

The plant material was collected from Niger State in December, 2011 by Malam Ali Garko. It was further identified in the Department of Biological Sciences, Bayero University, Kano

Extraction of Plant Material

The extraction and fractionation of air-dried and ground plant materials were carried out according to the method described by Fatope *et al.,* (1993). 1000g of the plant material was percolated with 3L of 96% (v/v) ethanol for a period of two weeks at room temperature with frequent agitation. The percolate was decanted and filtered using a Whatman filter paper. The filtrate was concentrated at reduced pressure using a Rotary evaporator (Buchi R200) at 40^{o}C and then allowed to further air-dry. The dried crude extract was weighed and labeled as F_1.

Fractionation of Crude Extract

The ethanol (crude) extract was partitioned with chloroform-water in 1:1, 200ml volume ratio. This leads to the formation of three different extracts; Water extract, F_2, Interface extract, F_3 and Chloroform extract, F4. The water extract was discarded. The chloroform extract (F_4) was washed with saturated solution of sodium chloride and then with distilled water. It was then air-dried and weighed.

The dried chloroform extract obtained above was further partitioned between 10% aqueous methanol and n- hexane in equal ratio of 200ml volume. The resulting of aqueous methanol, an interface and n-hexane fractions were concentrated at reduced pressure and weighed. They were labeled as F_5, F_6 and F_7 respectively.

Antibacterial Screening of the Partitioned Fractions

The antibacterial screening was carried out using disc diffusion method according to standard procedure of Kirby-Bauer as described by Cheesebrough, 2000.

Preparation of the Media

Nutrient agar was prepared by dissolving 28g of the solid nutrient agar in 1000ml distil water and sterilized in an autoclave at 110^{o}C for 45 minutes. It was then poured aseptically into petri dishes and allowed to solidify until needed.

Preparation of Extracts Impregnated Paper Discs

One gram of each extract was dissolved in 1ml of dimethylSulphoxide (DMSO) to yield 1.0g/ml (10^6µg/ml) solution. This was labeled as stock solution. From the stock solution, 0.1ml was transferred into a sterile bijou bottle containing 0.9ml DMSO which gave a concentration of 10^5µg/ml solution. 0.1ml was transferred into another bijou bottle containing 0.9ml DMSO which gave a concentration of 10,000µg/ml and the resulting solution was further diluted to give a concentration of 1000µg/ml. A hundred sterile discs each of 6mm diameter punched from a Whatman No.1 filter paper using a paper punch were dispensed into each bijou bottle containing extracts of different concentration and shaken. Each disc takes up 0.01ml to make the required disc potency. This resulted into impregnated discs of 1000, 100 and 10µg/disc for each of the fractions. The discs were stored in a refrigerator until needed (Hussein and Deeni 1991).

Preparation of the Inoculum

Active cultures of the test organisms were prepared in nutrient agar plates from stock cultures that were maintained on nutrient agar slant at 4^{o}C and incubated at 37^{o}C. Microbial cultures, freshly grown at 37°C were appropriately diluted in test tubes containing sterile normal saline solution to match 0.5 Mcfarland standard described by Cheesebrough, (2000). The Mcfarland standard was prepared by mixing 0.6ml of 1% (w/v) dehydrated barium chloride solution with 99.4ml of 1% (v/v) sulphuric acid solution. This was labeled as the standard inoculum. Two loopfuls of the standard inoculum were evenly streaked onto the prepared nutrient agar plates. Discs of different concentrations as well as Gentamycin (10µg/disc) which was used as positive control were aseptically placed and pressed firmly onto each plate using a sterile forceps at about 40mm apart. After 15 minutes free diffusion time, the plates were of incubated at 37^{o}C for 24 hours. Diameters of zones of inhibition were measured using a millimeter rule and recorded to the nearest whole number.

Brine Shrimp Lethality Test (BSLT)

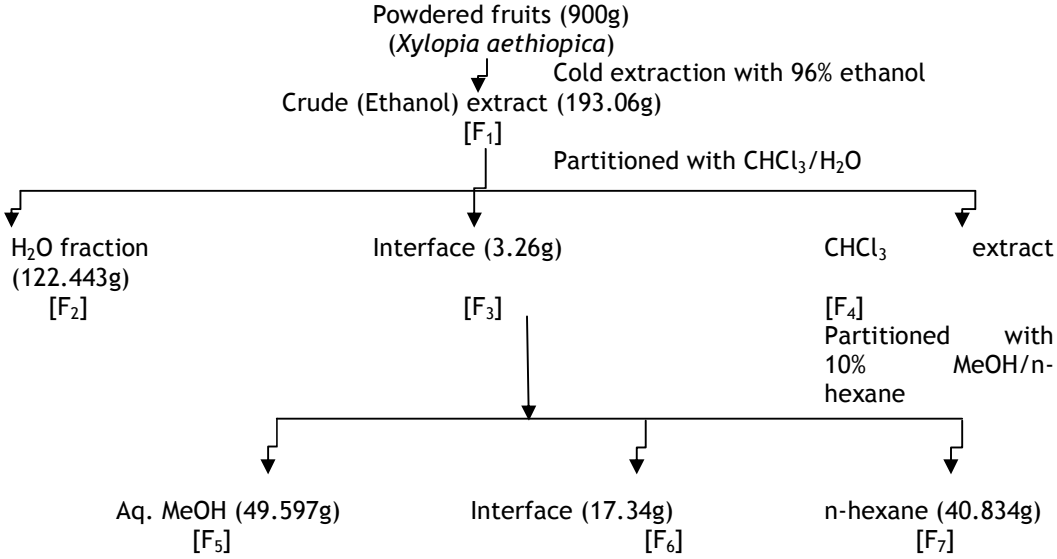
Screening of extracts and column fractions against brine shrimp larvae was carried out according to the method described by Meyer *et al.,* (1982) and Fatope *et al.*, (1993).

Brine shrimp eggs were added to 250ml instant sea water in a hatching chamber. The eggs were allowed to hatch for 48hours. Samples were prepared by dissolving 20mg of each of the extract in 2ml of methanol. This was labeled as the stock solution. From the stock solution, aliquots of 500µl, 50µl and 5µl were pipetted and poured into separate vials which are equivalent to concentrations of 1000µg, 100µg and 10µg/ml of the samples respectively. Usually three vials for each concentration were prepared making a total of 9 vials per extract plus a control vial containing sea water and shrimps without the extract. The solvent in each vial was evaporated at room temperature overnight. Two drops of DMSO (dimethyl sulphoxide) were added to each vial plus 3ml of sea water. 10 shrimps were transferred to each vial using a Pasteur pipetteand the volume of the liquid in each vial was adjusted to 5ml with instant sea water. After 24 hours, the numbers of surviving shrimps were counted and the LC_{50} was determined at 95% confidence interval using regression analysis (Arias and Mulla, 1975).

RESULTS AND DISCUSSION

Extraction and Fractionation of Plant Material

The extraction of the powdered fruits of *Xylopia aethiopica* was carried out using 96%v/v ethanol and the resulting extract was partitioned to give chloroform, interface, 10% aqueous methanol and n-hexane fractions (fig. 5). About 21.5% of the plant material went into the ethanol which on partitioning between chloroform/water, about 64% of the ethanol fraction went into chloroform. Further partitioning of the chloroform extract resulted in about 41% and 33% of the extract going into methanol and n-hexane respectively. This showed that the fruits extracts of the plant contains reasonable amount of medium polar and non-polar compounds. The formation of an interface was also observed (Table 1).



Scheme 1: Extraction and Fractionation Procedure of the Powdered Fruits of the Plant

Table 1: Weights of Extracts Recovered and their Physical Properties

Extracts	Colour	Texture	Weight (g)
F_1 [crude extract]	Dark greenish-brown	Solid	193.06
F_3[interface-1]	Dark brown	Solid	3.26
F_4 [$CHCl_3$ extract]	Dark green	Semi-solid	122.443
F_5 [methanol extract]	Dark green	Solid	49.957
F_6 [interface-2]	Dark brown	Semi-solid	17.34
F_7 [n-hexane extract]	Pale green (bright and transparent)	Oily-like	40.834

Screening of Extracts for Brine Shrimp Lethality Test (BSLT)

The brine shrimp test is used for the evaluation of cytotoxic effects of plant extracts. The activity is expressed in LC_{50} values in µg/ml at 95% confidence interval. Plants having LC_{50}values >1000µg/ml are considered inactive whereas plants having LC_{50} values <1000µg/ml are considered active.

The cytotoxicity of the extracts against brine shrimp larvae was evaluated and the result expressed in LC_{50}values of µg/ml (Fatope *et al.*, 1993). The methanol extract demonstrated the highest activity with an LC_{50} value of 3.4µg/ml. This showed a polarity independent activity which is inconsistent with the polarity of the tested extracts in the order: hexane<chloroform<ethanol<methanol<interface (Table 2).

Table 2: Results of Brine Shrimp Lethality Test of Extracts

Extracts	Replicates	Initial number of larvae	Total number of survivors			Total number of deaths			LC50
			1000	100	10	1000	100	10	
F_1(crude extract)	3	10	0,0,0	5,4,3	7,6,3	10,10,10	5,6,7	3,4,7	19.7(43.0-5.3)
F_4(chloroform extract)	3	10	4,3,3	6,6,5	7,8,8	6,7,7	4,4,5	3,2,2	185.5(986.1-59.8)
F_5(methanolic extract)	3	10	0,0,0	2,2,1	4,3,3	10,10,10	8,8,9	6,7,7	3.4(11.7-0.1)
F_6(Interface-2)	3	10	1,1,0	4,4,3	7,6,5	9,9,10	6,6,7	3,4,5	25.0(55.9-6.8)
F_7(n-hexane extract)	3	10	0,0,0	2,2,1	6,5,5	10,10,10	8,8,9	4,5,5	12.4(24.8-3.6)
Control	1	10	10	10	10	0	0	0	-

Key: 1000, 100 and 10 are the concentrations of the extracts in µg/ml

Screening of Extracts for Antibacterial Assay

The antibacterial test is used to determine the efficacy of plant extracts against bacteria. It is expressed in terms of zone of inhibition (Figure 6) which showed a significant reduction in the bacterial growth. The plant extract with wider zone of inhibition is considered to be highly active against the tested bacteria and vice-versa.

The antibacterial activities of Extracts were determined and the diameter of zones of inhibition produced by the extracts against the tested organisms was recorded. All the extracts showed selective activity against *Proteus sp*. Interface-2 exhibited the highest activity with diameter of zones of inhibition of 14mm, 7mm and 7mm at 1000, 100 and 10µg/disc respectively. Methanol and ethanol fractions showed similar activities with diameter of zones of inhibition of 12mm each at 1000µg/disc. Chloroform fraction on the other hand showed moderate activity with diameter of zones of inhibition of 10mm, 7mm and 7mm at 1000, 100 and 10µg/disc respectively. N-hexane fraction was found to be inactive on all tested bacteria(Table 3). The zone of inhibition increased with increase in concentration of the extracts, this showed a concentration dependent activity which is consistent with the polarity of the tested fractions in the order: hexane<chloroform<ethanol<methanol<interface.

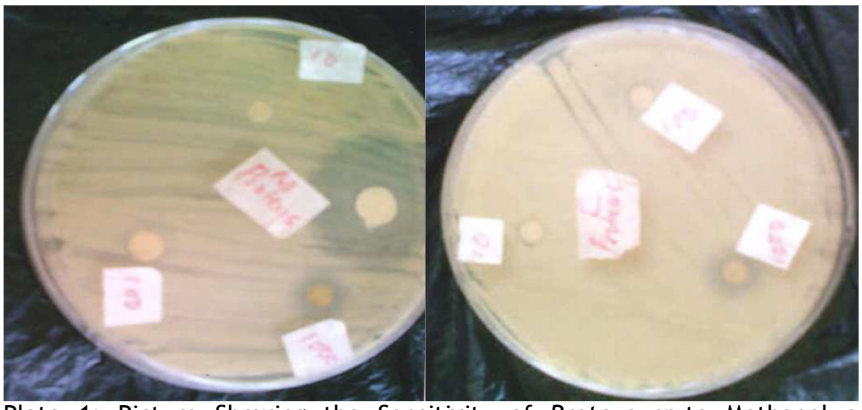


Plate 1: Picture Showing the Sensitivity of *Proteus sp.*to Methanol and Chloroform Extracts Respectively.

Table 3: Result of Antibacterial Screening of Extracts

Extracts	Conc. (µg/disc)	Test organisms		
		Proteus sp.	*Escherichia coli*	*Klebsiella pneumonia*
n-hexane [F_7]	1000	-	-	-
	100	-	-	-
	10	-	-	-
Methanol [F_5]	1000	12mm	-	-
	100	-	-	-
	10	-	-	-
Interface-2 [F_6]	1000	14mm	-	-
	100	7mm	-	-
	10	7mm	-	-
Ethanol [F_1]	1000	12mm	-	-
	100	-	-	-
	10	-	-	-
Chloroform [F_4]	1000	10mm	-	-
	100	7mm	-	-
	10	7mm	-	-

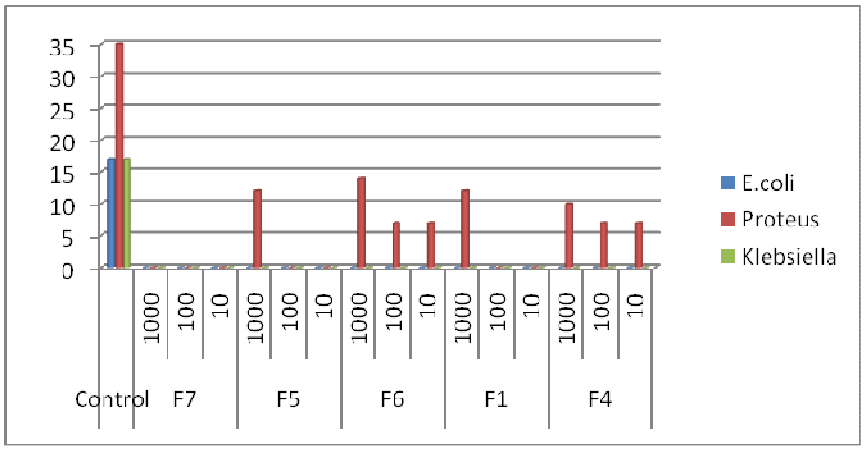


Figure 7: Chart Showing the Result of Antibacterial Activity of Extracts

CONCLUSION

Fruits of *Xylopia aethiopica* plant has been established to be cytotoxic in the brine shrimp test. Since this assay correlates well with anticancer and insecticidal activity, the fruits of this plant should be investigated for possible development as anticancer or insecticidal agent. The moderate selective activity against *Proteus sp* of the extracts provides basis for further development of more potent antibiotics.

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