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EVALUATION OF ANTIBACTERIAL ACTIVITIES OF GRAVIOLA (*ANNONA MURICATA)* LEAVE AND STEM BARK EXTRACTS AGAINST CLINICAL ISOLATES OF *SALMONELLA SPP AND ESCHERICHIA COLI*

Makeri, H.K.[1]*, Maikai, V.A.[2] and Muhammad, A.[2]
[1]Department of Animal Health and Husbandry, College of Agriculture and Animal Science, Ahmadu Bello University, P.M.B. 2134, Mando Road, Kaduna. Nigeria.
[2]Department of Basic Sciences, College of Agriculture and Animal Science, Ahmadu Bello, University, P.M.B. 2134, Mando Road, Kaduna. Nigeria.

ABSTRACT
Graviola (Annona muricata) Nigeria it is commonly called "Mama" in Hausa language is a tropical plant found in Nigeria in the Sudan- Guinea Savannah vegetation zone has been reported to be used in the treatment of various types of ailments. The present study investigated the antibacterial properties of the stem- bark and leave extracts of the plant against clinical isolates of Escherichia coli and Salmonella spp using agar cup method. Results showed that petroleum ether extract of stem bark and leave had (> 12mm) growth inhibition against E.coli and Salmonella spp. at 50-100 mg/ml concentrations compared to the methanol and aqueous extracts which did not inhibit growth. The minimum inhibitory concentration (MIC) of petroleum ether extract of stem bark and leave was 12.5 mg/ml. It therefore showed that petroleum ether of Annona muricata had antibacterial activities against E.coli and Salmonella spp. thereby justifying its usage ethnopharmacologically in the treatment of infections.
Key words: Annona muricata, E.coli, Salmonella spp, Growth inhibition

INTRODUCTION

Infections by organisms such as *Salmonella* spp and *E.coli.*have various clinical manifestations in man and animals (Delong and Pace, 2001). In man, infections are associated with the consumption of animal or poultry products such as, eggs and poultry meat. Other serotypes e.g. *Salmonella pullorum* and *Salmonella gallinarum* cause severe clinical diseases in poultry but not in humans (Rybicki, 1990). Infections in poultry are through eggs (transovarial), or feeds, water contamination, incubators or from bird to bird. Colibacillosis on the other hand, is a disease caused by *E.coli* which is considered an opportunistic pathogen and a normal inhabitant of the intestinal tract (Barnes *et al.,* 2003). *E.coli* is a common pathogen of poultry, with faecal contamination of eggs, feed and water commonly occurring (Delong and Pace, 2001). The control and treatment of diseases caused by these organisms in livestock and poultry relies almost entirely on the use of conventional drugs, such as furazolidone,sulphonamides, gentamicins, streptomycins *e.t.c.* However, escalating cost, circulation of fake and adulterated drugs, non availability of conventional drugs and development of resistance had led to the increasing use of herbal remedies by African small holder livestock producers and pastoralists (Abdu *et al.,* 2000). History also provides evidence of the relationship of plants and medicine (Raghevendra *et al.,* 2006). Traditional veterinary practices continue to play important roles in livestock health and management in Nigeria and Africa (Kudi and Myint, 1999). An urgent need arises to discover new antibacterial compounds with diverse chemical structures and novel mechanisms of action against infectious diseases. Plant based antibacterials represent a vast untapped source of medicines offering enormous therapeutic potential (Tagboto and Townson, 2001).

Graviola is also known with other common names as, Sour sop, in Nigeria it is commonly called "Mama" in Hausa language. It is a small upright green tree, 5 – 6 meters high, with large glossy and green leaves. It produces a large heart shaped edible fruit that is 15- 20 cm in diameter. It is yellow to green in colour and has white flesh inside. *Annona muricata* (Graviola) has been reported to be traditionally useful in the control of tumors (Wu *et al.,* 1995; Nicolas *et al.,* 1997; Chang *et al.,* 2001; Yuan *et al.,* 2003), antimicrobial activity (Sundarrao *et al.,* 1993; Betancur-Galvis *et al.,* 1999; Takashi *et al.,* 2006), anti-parasitic, anti-malarial activities (Alali *et al*., 1998; Jaramillo e*t al.,* 2000; Luna *et al.,* 2005). This study evaluates the antibacterial activity of extract of stem –bark and leaf extracts of *Annona muricata*.

MATERIALS AND METHODS

Plant collection and Identification

Plant parts were collected from Mando in Igabi Local Government Area of Kaduna state, Nigeria. The plant parts were taken to the Department of Biological Sciences, Ahmadu Bello University, Zaria, where it was identified and a voucher number (1726) specimen deposited.

Plant Processing

The leaves and stem bark were harvested and washed with tap water and air dried at room temperature for 19 days. They were then pulverized using a Kenwood blender and sieved. The powdered leaf and stem bark were stored in labelled polythene bags and kept in a cupboard prior to usage.

Extract Preparation

One hundred (100 gm) gram of the leaf and stem bark powders were separately weighed into a thimble and there after transferred into a Soxhlet extractor and extracted sequentially with petroleum ether (40-60^{0}C), methanol and water. The extracts were individually collected after each extraction and concentrated using a crucible over a water bath set at 40^{0}C. The solvent free extracts were then weighed and stored in brown bottles at 4^{0}C.

Preparation and Standardization of Inoculum.

The bacterial species are *E.coli* and *Salmonella* spp.They were clinical isolates obtained from the Veterinary Teaching Hospital (VTH), Ahmadu Bello University, Samaru - Zaria. From the slant cultures of the identified organisms (*E. coli* and *Salmonella sp*), a colony was suspended with a sterile wire loop into a sterile bijou bottle containing sterile distilled water and the opacity was then matched with that of McFarland turbidity standard which corresponded to 10^6 CFU/ml (Colony Forming Unit per Millilitre).

Preparation of various concentrations of extracts.

The method of NCCLS, (1997) was used. One gram (1 gm) each of extract was dissolved in 10 ml of sterile distilled water for aqueous and methanolic extracts while petroleum ether extract was dissolved in N-N-Dimethyl formamide to give the stock solution . The dissolved extracts (stock solution) were serially diluted to give 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml concentrations.

Sensitivity testing of the extracts.

The method of Nostro *et al*., (2000) was used. A quantity (20 ml) of sterile Mueller- Hilton agar was poured into sterile Petri dishes to solidify. Using a sterile syringe, 0.1 ml of the standardized inocula (test organisms) was inoculated onto four petri dishes in duplicate, representing the various concentrations and for each of the extracts. The bacterial suspension was spread with the aid of a sterile swab stick. Using a cork borer (8mm in diameter), four wells were made in each agar plate. All plates were appropriately labeled according to the corresponding concentrations and extract used. Using a sterile syringe, 0.1ml of the diluted extracts was placed into the wells and the plates left on the work bench for 2 hrs for the extracts to diffuse into the agar before incubation at 37^{0}C for 24 hrs. Ciprofloxacin antibiotic sensitivity disc (10 µg) from (Oxoid, Hampshire, England) was used as a control against test organisms. After incubation, the plates were observed for evidence of inhibition (appearance of clear zones that are completely devoid of growth around wells). The diameters of the zones were measured using a calibrated rule in millimeters.

Determination of MIC

The method of National Committee for Clinical Laboratory Standards (1997) was used. The inocula were prepared from overnight broth cultures and adjusted to turbidity equivalent to 0.5 McFarland standard. A stock solution of 0.2 g/ml was made and the serial dilutions of 100, 50, 25 and 12.5 mg/ml were prepared from it. Four (4 ml) of each dilution was incorporated in 16 ml of the appropriate melted agar medium and poured into each of the petri dishes. Each dish was sectioned into four. A loopful of the diluted culture of each test organism was inoculated by streaking on the surface of each section of the petri dish. The dishes were incubated at 37^{0}C for 24 h. A control was also set which contained only nutrient agar and the test organism. The MIC was defined s the lowest concentration of the extract inhibiting the visible growth of each organism.

Phytochemical screening of the leaves and stem-bark extract of *Annona muricata*

This was out carried out standard procedures of Harborne (1973), Trease and Evans (1989) and Sofowora (1993).

RESULTS

The plant *Annona muricata* is shown (Plate 1). The petroleum ether extract of stem bark and leave showed (> 12 mm) growth inhibition zones against *E.coli* and *Salmonella spp* at concentrations of 25-100 mg/ml (Table 1), (his appears to be dose dependent compared to the aqueous extracts of stem bark and leave with (< 8 mm) growth inhibition to the test organisms at the highest concentration of 100 mg/ml. There was no growth inhibition with methanol extracts of the leaves. The reference drug ciprofloxacin showed (> 30 mm) growth inhibition at 10 µg concentration. The minimum inhibitory concentration (MIC) again showed that the petroleum ether extracts at a concentration of 12.5 mg/ml (Table 2), were able to inhibit growth, while the methanolic and aqueous extracts failed to show any activity. The phytochemical screening showed that the aqueous and methanol leave and stem bark extract had absence of alkaloids, anthraquinones, flavonoids and phenols. While alkaloids and anthraquinones were present in the petroleum ether extract of leaf and stem bark.

Plate 1: *Annona muricata* plant

Table 1. Antibacterial activity of extracts of *Annona muricata*

Extract/Drug	Concentration mg/ml	Mean Zone of Inhibition (mm)	
		E.coli	***Salmonella***
Petroleum ether stem bark	12.5	0.0	13.0
Extract	25.0	12.0	15.0
	50.0	13.0	17.0
	100.0	17.0	23.0
Petroleum ether leave extract	12.5	9.0	12.0
Extract	25.0	11.0	15.0
	50.0	13.0	17.0
	100.0	15.0	20.0
Methanolic stem bark	12.5	0.0	0.0
Extract	25.0	0.0	0.0
	50.0	9.0	9.0
	100.0	13.0	11.0
Methanolic leave extract	12.5	0.0	0.0
	25.0	0.0	0.0
	50.0	0.0	0.0
	100.0	0.0	0.0
Aqueous stem bark extract	12.5	0.0	0.0
	25.0	0.0	0.0
	50.0	0.0	0.0
	100.0	0.0	0.0
Aqueous leave extract	12.5	0.0	0.0
	25.0	0.0	0.0
	50.0	0.0	0.0
	100.0	0.0	0.0
Ciprofloxacin	10 µg	33.0	27.0

Values greater than 9 mm indicates activities.

Table 2. Minimum Inhibitory Concentration (MIC) of leaf and stem bark extracts of *Annona muricata*

Extract/Drug	Concentration mg/ml	Mean Zone of Inhibition (mm)	
		E.coli	*Salmonella*
Petroleum ether stem bark extract	12.5	-	-
	25.0	-	-
	50.0	-	**++**
Petroleum ether leave extract	12.5	+++	++
Extract	25.0	+	-
	50.0	-	-
	100.0	-	-
Methanolic stem bark	12.5	+++	+++
Extract	25.0	+	+
	50.0	-	-
	100.0	-	-
Methanolic leave extract	12.5	+++	+++
	25.0	+++	+++
	50.0	+++	+++
	100.0	+++	+++
Aqueous stem bark extract	12.5	+++	+++
	25.0	+++	+++
	50.0	+++	+++
	100.0	+++	+++
Aqueous leave extract	12.5	+++	+++
	25.0	+++	+++
	50.0	+++	+++
	100.0	+++	+++

Key: - No Growth, + Very fair Growth, ++ Moderate Growth, +++ Abundant Growth

Table 3: Phytochemical Screening of Leaves and Stem Bark Extract of *A. muricata*

Pytochemical	Leaf			Stem Bark		
	Aqueous	Methanol	Petroleum ether	Aqueous	Methanol	Petroleum ether
Alkaloids	-	-	+	-	-	+
Anthraquinone	-	-	+	-	-	+
Carbohydrates	+	+	+	+	+	+
Cardiac Glycosides	+	+	+	+	+	+
Flavonoids	-	-	-	-	-	-
Phenols	-	-	-	-	-	-
Saponins	+	+	-	+	+	-
Steroids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+

Key: + Present: - absent

DISCUSSION

The *in-vitro* study to determine the antibacterial activities of *Annona muricata* shows the therapeutic potential of petroleum ether extracts against *E.coli* and *Salmonella* species. Theresults highlight the fact that the activity was resided in the petrol ether extract and not the water extract. Water is the activity, the work showed that methanol extract did not show any activity against the bacteria. This work tends to agree with (Hassan *et al.*, 2006) who reported activity of chloroform extract of *B. augustifolia* onbacteria. Tepe *et al.,* (2004) similarly reported that *S. cryptantha* and *S. multicaulis* oils showed antimicrobial activity. The petroleum ether extract of *A. muricata* was active against gram negative bacteria which is similar to Rabe and Van Staden, (1997) who reported that plant extracts were active against gram negative bacteria. The solvent used by traditional herbalist to extract active substances from plants due to its availability (Shale *et al.,* 1999). The findings contradict this, as the active ingredient is resided in the petroleum ether extract of the plant.The results also contradict Pathak *et al*., (2003) who reported that methanol extract of leaf of *Annona muricata* had antibacterial phytochemical screening revealed presence of alkaloids and anthraquinones in the petroleum ether extracts of the leave and stem bark. *A.muricata* has been reported to be rich in miscellaneous lactones and isoquinoline alkaloids (Alali *et al.,* 1992; Wu *et al.,* 1995). It has also been reported that *A.muricata* is known to contain a large number of biologically active compounds and chemicals generally referred to as annonaceous acetogenins which include quinolines and isoquinolines, annopentocins, annomuricins, coreximine and reticuline isolated from the leaf, barks

and seeds of *Annona muricata* (Alali *et al.,* 1992; Wu *et al.,* 1995; Nicolas *et al.,* 1997; Chang *et al.,* 2003). The activity noticed could be due to either of these chemicals. The demonstration of antibacterial activity of *A. muricata* reveals a good potential lead. The study supports the traditional usage of the plant parts which contains compounds with antibacterial properties that can be used as antibacterial agents in the development of new drugs for the treatment of infectious diseases.

CONCLUSION

Though the results are preliminary, the work has shown that the petroleum ether extract of stem bark and leaf of *Annona muricata* had activity against *E.coli* and *Salmonella* species.

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