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Full length research paper

Comparative Study of Antibacterial Activity of Juice, Acetone, Methanol and Ethanol Leaf extract of *Andrographis Paniculata* (King of Bitters)

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The rate of antimicrobial resistance of bacteria has justified the need for continuous search for other sources of drug especially from natural plant. Claims of medicinal properties including antibacterial activities of *Andrographis paniculata* extract sprang from traditional use and also of its ethanol leaf extract, we therefore compare several methods of extraction to see the one with most anti-bacterial activities among the various preparations.The plant extract was prepared both by crushing (raw juice/liquid extract) and use of solvents (ethanol, acetone and methanol). Bacteria isolates from human specimens were collected and identified. The agar diffusion method was used to test the antibacterial properties of the extract with standard antibiotic discs and with *Bridelia* extract for comparison. Forty-nine bacteria isolates (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Klebsiellaoxytoca*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Yersinia intermidis*) were tested. Both the raw juice extract and extraction with various solvents failed to show any appreciable inhibition of bacteria growth. The standard antibiotic discs and *Bridelia* extract produced zone of inhibition. Various extracts of *Andrographis paniculata* did not exhibit appreciable antibacterial activity.

Keywords:*Androgaphis paniculata* extract; antibacterial activity; Zone of inhibition; Agar diffusion; Gram positive; Gram negative.

INTRODUCTION

Increasing rate of antimicrobial resistance of bacterial has justified the need for continuous search for other sources of drug especially from natural plant sources. There are several claims of various medicinal properties including antibacterial activities of *Andrographis paniculata* extracts, which actually sprang from traditional use. In Africa, a large proportion of the population still believes and relies on the use of herbal preparations for treatment of various diseases and ailments with claims of beneficial responses. *A.paniculata* is one of the plants used for treating febrile illnesses possibly due to malarial and bacterial infections in local communities in Nigeria. It is locally called 'Jogbo' because of its bitterness but popularly called 'Mejemeje' (seven-seven) among 'Yoruba' speaking natives in Nigeria because an average dosage comprises of seven leaves eaten raw once or twice daily for about five days in the treatment of febrile illness or chronic debility and some herbalist also recommend it for treatment of hypertension.

Antibacterial activities of both aqueous and ethanolic leaf extract have been reported (Abubacker and Vasantha, 2010; Vinothkumar *et al*., 2010; Singha *et al*., 2003). It is a common belief that any bitter leaves are medicinal and must be useful in treating fevers. Other bitter plants so administered in 'Yoruba' land include 'Oruwo' (*Morinda lucida*), 'Dongo-yaro' (Neem tree – *Azadirachta indica*), 'Ewuro' -Bitter leaf (*Vernonia auriculifera*), Quinine tree (*Cinchona officinalis*) to mention a few. *A. paniculata* is an erect annual herb extremely bitter in taste in all parts of the plant body. The plant is known in north-eastern India as "king of bitters", and belongs to the family *Acanthaceae*. It is widely cultivated in Southern Asia; it grows erect in moist shady places with glabrous leaves and white flowers with rose-

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Figure 1: One of the *Andrographis paniculata* plant in the source garden

purple spots on the petals. The stem is dark green, 0.3-1.0m in height, 2-6mm in diameter; the leaves are glabrous, up to 8.0cm long and 2.5cm broad (Zhang, 2004; Niranjan *et al*., 2010) (Figure 1).

The phyto-constituents of *Andrographis paniculata* plant which has been well elucidated include; diterpenes, flavonoids, iterpenoid, lactones, alkanes, ketones, aldehydes, andrographolides (which is the major constituent), paniculides, farnesols, polyphenols, arabinogalactan, and several other sub-units of andrographolides (Niranjan *et al.,* 2010; Akbar S., 2011).Mostly the leaves and roots have been traditionally used over the centuries for different medicinal purposes in Asia and Europe as a folklore remedy for a wide spectrum of ailments or as an herbal supplement for health promotion. The Indian pharmacopoeia narrates that it is a predominant constituent of at least 26 Ayurvedic formulations (Mishra *et al.,* 2007; Akbar S, 2011). It is also used for a wide assortment of other conditions such as digestive complaints including diarrhoea, constipation, intestinal gas, colic, and stomach pain (Gupta *et al*., 1990) and liver conditions (Chturvedi *et al.,* 1983; Trivedi and Rawal, 2001). Other applications include infections like leprosy, *pneumonia*, *tuberculosis*, and HIV/AIDS (Calabrese *et al*., 2000) and for skin conditions (Hancke *et al*., 1995; Melchior *et al*., 2004) while some people use it for sinusitis, sore throat, and allergies (Thamilikitkui *et al*., 1991; Zhanel *et al*., 1991) and also as astringent, bacteria killing agent, painkiller, fever reducer, anti-malarial and treatment for worms (Kumar *et al*., 2004; Rahman *et al*., 1999, Wiart *et al*., 2005; Wongsawad *et al.,* 2011).Other uses include treatment of snake and insect bites, loss of appetite, kidney problems and prevention of heart disease and diabetes and an inherited condition called familial Mediterranean fever and cancers (Zhao *et al*., 2008; Kumar *et al*., 2004).Laboratory evidences have been provided for its anti-diarrhoeal, anti-malarial, antiviral and anti-cancer properties (Calabrese *et al.,* 2000; Zhao *et al*., 2008; Rahman *et al.,* 1999; Wiart *et al*., 2005; Prajjai *et al.,* 2003). However, there are very few studies on its often-claimed antibacterial properties and most of the studies gave conflicting report on the antibacterial activity of different extracts of the leaf. While Vinothkumar and colleagues 2010 claimed that the aqueous leaf extracts showed good activity against both Gram positive and Gram negative bacteria, Leelarasamee *et al*.,1990 and Voravuthikunchai and Limsuwan 2006 reported no antibacterial activity. In our environment, the traditional application has been raw consumption of the leaves for general maintenance of health -"blood purifier", or for the treatment of hypertension, heart disease and febrile illnesses and the plant was used as an infusion, decoction, or powder, either alone or in combination with other medicinal plants. In this modern time, there is a need to standardize the dosage of herbal remedies, this could not be done unless we are able to determine the extent of the activity (ies) of the extracts and afterwards determine the appropriate dosage even before drug formulation that may take several years to come.

This study therefore aims at evaluating the antibacterial activity of the various extract of the leaves of *Andrographis paniculata* against human bacterial isolates in order to verify its widespread ethno medicinal application as antibacterial and also compare several methods of extraction to see the one with most anti-bacterial activities among the various preparations. This was to be followed by the determination of the minimum

TABLE 1: The diameter zone of inhibition (mm) of different extracts o f *A. paniculata, Bridelia ferruginea* and commercial antibiotic discs.

Name of Organism (N)	Average Zone of inhibition with extracts and commercial antibiotic discs in Milimeters.										
	CN (30µg)	CIP (5µg)	AMC (30 µg)	CRO (30 µg)	AK (30 µg)	CFM (5µg)	AcetExtr.	Met. Extr.	Ethan. extr	Juice	*Bridelia* extr
S. aureus (22)	21	22	18	16	24	19	0	0	0	0	20
K. pneumoniae (6)	0	26	10	22	21	16	0	0	0	0	17
K. oxytoca(2)	8	24	10	18	21	16	0	0	0	0	17
E. coli (8)	18	24	15	17	19	18	0	0	0	0	11
P. mirabilis (2)	18	22	10	16	24	10	0	0	0	0	12
P. vulgaris (2)	21	18	12	16	23	14	0	0	0	0	12
S. typhi (2)	12	22	0	35	16	16	0	0	0	0	16
P. aeruginosa. (3)	0	18	8	19	22	12	0	0	0	0	12
Yersinisintermidis	14	18	10	16	12	18	0	0	0	0	06
Acinetobacterbaumani	12	12	10	8	18	16	0	0	0	0	06

CN=Gentamicin; CIP=Ciprofloxacin; AMC= Amoxycillin-Clavulanic acid; CRO=Ceftriaxone; AK=Amikacin; CFM= Cefipime; Acetextr. = Acetone extract; Meth. Extract= Methanolic extract; Ethan. Extract= Ethanolic extract;*Brideli = Bridellia ferruginea* Benth extract.

inhibitory concentration (MIC), but this was not done because the extracts did not exhibit any appreciable antimicrobial activity.

MATERIALS AND METHODS

Collection and Extraction of the leaves

The leaves were collected fresh from the chief researcher's garden early in the morning from a small portion of land where it was planted in Moniya-Ibadan, Oyo State, Nigeria, (Figure1) and divided into portions, for crushing (raw liquid/juice extract), ethanol, acetone and methanol extraction following standard procedure. (Romanik *et al*., 2007, Ajaiyeoba *et al*., 2006).Briefly described, the fresh leaves for crushing was washed in ordinary tap water and crushed with an enamel mortal and pestle and the juice without adding any water, was collected by squeezing and then filtered with a fine cloth sieve and used same day. The portion for alcohol (ethanol, methanol and acetone) extraction was air-dried in a shady environment and latter blended to powder with electric blender. The powdered leaves of *Andrographis paniculata* were extracted separately by soaking about 20g in200mls of the different solvents (absolute ethanol, methanol and acetone) for maximum of 72 hours, this was to allow full extraction of the leaf constituents. The extracts were collected, and carefully filtered using Whatmanfilter No.1 and concentrated using a rotatory evaporator and poured into petri dish and dried on water bath at low temperature. The extract were weighed and then stored in a screw cap universal bottle and kept in the refrigerator until used. Isolation and Purification of bacteria colonies (Barrow & Feltham, 1995, Cheesbrough 2006). A total of Forty-nine bacteria isolates (22 *Staphylococcus aureus*, 8 *Escherichia coli*, 6 *Klebsiella pneumonia* and 2 *Klebsiellaoxytoca*, 2 *Proteus vulgaris*, 2 *Proteus mirabilis*, 2 *Salmonella typhi*, 3 *Pseudomonas aeruginosa*, 1 each of *Acinetobacter baumannii* and *Yersinia intermidis*) were tested in the study. They were isolated from different human specimens sent to the laboratory of Department of Medical Microbiology, University College Hospital, Ibadan, for microscopy, culture and sensitivity. Samples of each bacterium were collected and stored on nutrient slopes and sub-cultured on Chocolate and MacConkey agar and subsequently on Nutrient agar (for biochemical tests) all the plates were incubated overnight and for a maximum of 24hrs at 37C. The characteristics morphology, colour and odour of the colony growths were verified. Gram staining was done in order to characterize each isolate into either Gram-negative or Gram-positive. Catalase and coagulase tests were conducted to identify *S. aureus*, oxidase test was conducted for *Pseudomonas sp*. and further identification was done using Microbact 24E (Oxoid), for Gram negative bacteria.

Evaluation of the Antimicrobial activity of extract of *Andrographis paniculata* leaves (Bauer and Kirby 1966, CLSI 2010)

The agar diffusion method was used to test the antibacterial properties of the extracts with standard antibiotic discs also included for comparison. The pure isolates of these bacteria were inoculated into sterile peptone water and incubated for 4-6hrs at 37C and diluted with normal saline to produce 0.5 McFarland' s standard.

Mueller-Hinton agar was prepared in a sterile condition: The agar was punctured with an agar borer in 4 different sites for the different extracts. The plate was then inoculated with 0.5 McFarland's standard broth culture using sterile swab sticks dipped in the bacterial broth and after removing excess fluid was uniformly swabbed on the punctured agar plate. Application of extracts and commercial antibiotic discs on bacterial culture: The extracts were used as test while three to four commercial antibiotics discs were applied as control and another plant extract (*Bridelia ferruginea Benth*) was also included as control. About 0.05mL of each of the extract was carefully dispensed into the agar wells and the commercial antibiotic discs were placed firmly at regular interval. All the plates were then incubated for 24hrs at 35-37C and the diameter of zone of inhibition (ZD) was measured with a mathematical set divider and placed on a ruler and recorded in millimetre. The different preparations were tested separately against the different organisms and the results were recorded. The average ZD for the different species of isolates was calculated (Table1).The MIC, which was proposed for any of the extracts that produces appreciable inhibition, was not carried out since none of the extract of *Andrographis paniculata* produces noticeable zone of inhibition.

RESULTS

Antibacterial susceptibility studies of acetone, methanol, ethanol and juice extracts of *A. paniculata* against *S. aureus*, *Klebsiella species*, *E. coli*, *Proteus spp*., *S. typhi*, *P.aeruginosa*, *Acinetobacter baumannii* and *Yersinia intermidis* are summarized in Table 1.

The effect of the extracts and antibiotic discs on *E. coli*: The juice extract and other forms of extract of *A. paniculata* did not inhibit the growth of any of the isolates of *E. coli*, as there was no zone of inhibition, signifying that strains of *E. coli* isolates tested are all resistant to it despite good agar diffusion (Fig. 2). *E. coli* isolates were susceptible to gentamycin, ciprofloxacin, and amikacin commercial antibiotics and *Bridellia ferruginea* extract with appreciable zone of inhibition (Table 1).

The effect of the extracts on *Pseudomonas aeruginosa*: All the tested *Pseudomonas aeruginosa* were resistant to the various extract of *Andrographis paniculata* but showed appreciable susceptibility to amikacin 22mm (18-25mm), ciprofloxacin 18mm (14-26mm); Ceftriaxone 19mm (12-26mm) but resistant to augmentin- 8mm (0-14mm) and gentamicin 0mm in diameter, and also susceptible to extract of *Bridelia Klebsiella sp*.: *Klebsiella* species *(K. pneumoniae* and *K. oxytoca*) were resistant to various extract of *Andrographis paniculata*, but showed appreciable susceptibility to ciprofloxacin, and amikacin, and ceftriaxone commercial antibiotic discs ranging between 16-26mm and also to *Bridelia* extract.

The effect of the extracts on *Staphylococcus aureus*: All the 22 *Staphylococcus aureus* strains tested were resistant to the various extract of *Andrographis paniculata*. The bacteria were susceptible to *Gentamicin*, and Amikacin and appreciable susceptibility to ciprofloxacin and other discs with zone of inhibition ranging from 16- 19mm and good response to *Bridelia* extract.

The effect of the extracts on *Proteus species*: The four isolates of *Proteus* did not respond to the extracts of *Andrographis paniculata*. But all of them showed some response to Amikacin and ciprofloxacin commercial antibiotic discs and even to *Bridelia* extract. (Table 1)

The effect of extract on *Salmonella typhi*: The extracts did not inhibit the growth of *Salmonella typhi* but the isolates were inhibited by ciprofloxacin and ceftriaxone commercial discs and it also showed good response to *Bridelia ferruginea*.

The effect of the extract on *Yersinia* and *Acinetobacter*: The extracts could not inhibit the isolates, which also showed reduced susceptibility to commercial antibiotic discs including Amikacin while *Bridelia ferruginea* extract produced only 6mm zone of inhibition.

DISCUSSION

Antimicrobial agents have been used in clinical practice for over 40 years (Zhanel *et al*., 1991). However, there has been a considerable level of resistance to these agents and it is pertinent to continue to search for newer antibacterial agents especially from natural sources such as herbs. In the present study, in vitro antibacterial activity of *A. paniculata* was tested against 22 Gram positive and 27 Gram negative bacterial isolates by agar diffusion method. The juice which represents the commonest form usually consumed for treatment of febrile illnesses failed to inhibit the in-vitro growth of both Gram positive and Gram negative bacteria lisolates tested despite a good diffusion into the agar (Figure 2). Other forms of extraction using acetone, methanol and ethanol also failed to exhibit antibacterial effect on all the 49 different isolates from various human specimens. Our findings are discordant with Vinothkumar and colleagues [2] who reported that the aqueous extract of *Andrographis paniculata* exhibit good antibacterial activity

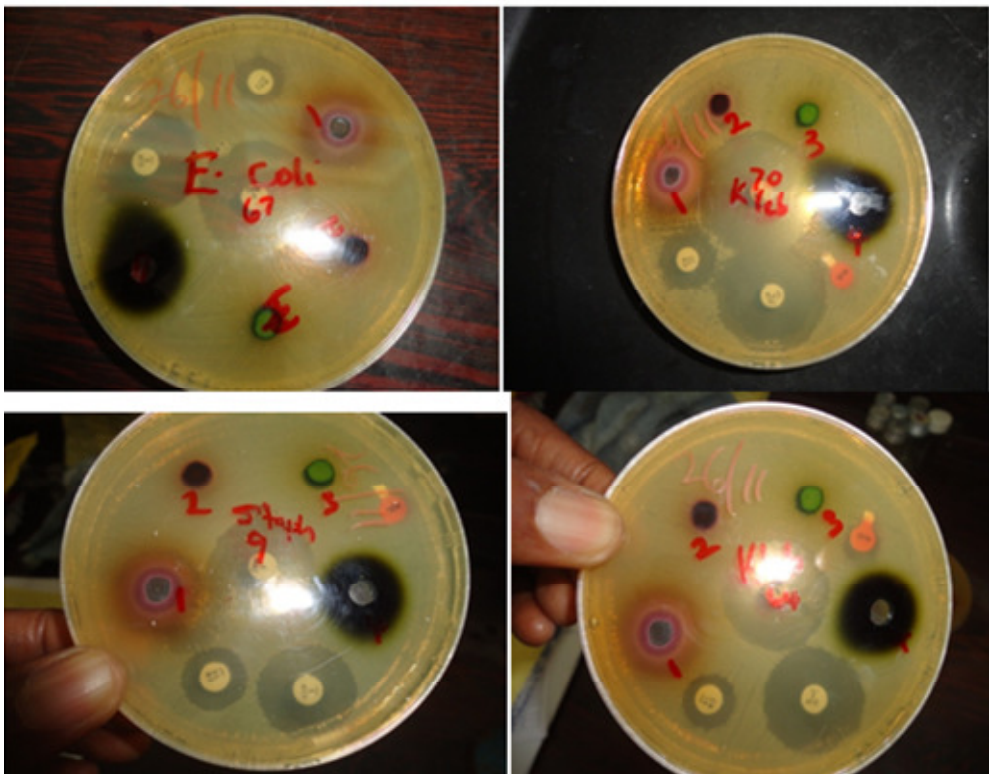

Figure 2a: Agar plates of Organisms and susceptibility to *Andrographis paniculata* extracts 2, 3, and 4 (in red marker), 1 is *Bridelia* extract.

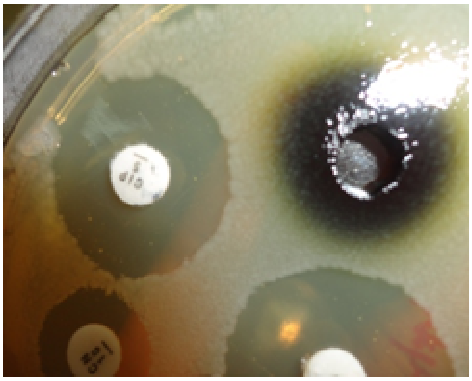

Figure 2b: The extract diffused well in the agar but does not inhibit growth

against different isolates including *E. coli*, and *Pseudomonas aeruginosa*. The study by Vinothkumar was conducted on standard organism, which might explain why they recorded intermediate activity (0-13mm) against the tested organisms.

However, our study corroborates the findings of Leelarasamee and colleagues who also reported undetectable anti-bacterial activity of *A. paniculata* against Gram negative and Gram positive organisms both in-vitro and ex-vivo even at a “high concentration that may not be clinically feasible”. Sankar and colleagues, 2010 who also worked on clinical isolates reported that the aqueous extract of *A. paniculata* was not found to be active against all organisms tested, while methanol extract exhibited low antimicrobial activity against *E. coli*, *Klebsiella pneumoniae*, *Bacillus cereus* and *Vibrio cholerae* and is inactive against rest of the test organisms. Voravuthikunchai and Limsuwan in 2006 also

reported that ethanol extract was devoid of significant activity against enterohemorrhagic strains of *E. coli*, while Sule *et al* in 2011 in Malaysia reported varying responses by strains of human skin disorders. Meanwhile, with these conflicting findings from different geographical locations, it is possible to think that the source (s) of the plant may contribute to its biological activities; however, a quality control test on *Andrographis paniculata* Nees which compared the plant grown in Nigeria with that grown in India confirmed key similarities between the Nigerian and Asian plant (Ameh *et al*., 2010).The biological activities of *A. paniculata* as reviewed by Niranjan *et al*., showed that the active component such as dehydroandrographolide, neoandrographolide has very good anti-inflammatory properties which might be due to effect of andrographolide on the expression of inducible nitric oxide synthase (iNOS) and other inflammatory cytokines. The antibacterial activity reported might not be as a result of actual killing/inhibition of the organisms but from the anti-inflammatory property. This deduction is supported by the findings of Liu et al. (Liu *et al.,* 2007a and b) who found that oral administration of neoandrographolide (100-150mk/kg) reduces the increased vascular permeability (induced by acetic acid) in mice. Neoandrographolide at concentrations (30-90µM) significantly inhibited the productions of nitric oxide (NO) and prostaglandin E2 in bacterial lipopolysaccharide (LPS) stimulated-murine macrophages without inducing cytotoxicity. It is therefore possible to conclude that anti-inflammation may be an alternative explanation for antibacterial activity attributed to *Andrographis paniculata*.

In conclusion, the various extracts of *Andrographis paniculata* failed to exhibit inhibitory effect on various clinical isolates tested, further study on the whole plant, its chemical constituents and their subunits is strongly indicated.

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Authors contributions

HOD developed the concept and designed the experiment and wrote the manuscript. OO and BA participated in the laboratory activities, OO participated in writing the initial draft of the manuscript. All authors read the final draft and none have declared any conflict of interest.

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