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In Vitro Evaluation of the Antimicrobial Potentials of the Leaves Extracts of *Distemonanthus benthamianus* (baill) and Its Interaction with Amoxycillin

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Abstract **- This study was designed with the aim of evaluating the ethnomedicinal claims of the leaves of *Distemonanthus benthamianus* in the treatment of bacterial and fungal infection, hence, the methanol and aqueous extract of *Distemonanthus benthamianus* were evaluated for *in vitro* antimicrobial activities, using agar diffusion and tube dilution methods against standard strains of *Staphylococcus aureus* (NCTC 6571), *Echerichia coli* (NCTC 10418) for antibacterial activity and *Candida albicans* for antifungal activities. The results obtained confirmed that the extracts have potent antibacterial activity against the bacteria tested, with aqueous extract showing a greater activity. While *Candida albicans* was completely resistant to the extracts, confirming that the methanol and aqueous extracts of *Distemonanthus benthamianus* leaves have no anti candida effect. The minimum inhibitory concentration (MIC) of the methanol extract against *S.aureus* is 6.25mg/ml, against *E. Coli* is 12.50 mg/ml and aqueous extract against *S. aureus* is 3.125 and *E.Coli* 6.25. The minimum inhibitory concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of Amoxycillin against *S.aureus* is 0.0039mg/ml, against E.coli 0.00097mg/ml. The Minimum bactericidal concentration (MBC) for methanol extract against *S. aureous* is 12.50mg/ml, against *E. coli* 12.50mg/ml, MBC for aqueous extract against S. aureus is 3.125mg/ml, against E.coli 6.25mg/ml The extracts had no antifungal activity against *Candida albicans*.**

Key Words **- Bactricidal, Concentrations, Ethno medicinal, Infections, Inhibitory, Leaves, Minimum.**

INTRODUCTION

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being [1]. Their role is twofold in the development of new drugs: 1.They may become the base for the development of a medicine, a natural blue print for the development of new drugs or: 2.A phytomedicine to be used for the treatment of disease [1].

Traditional medicinal using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world (WHO, 2002) [2]. Medicinal plants have been used as folklore remedies over the years to treat, manage or control man's ailment. They contain large varieties of chemical substances that possess important therapeutic properties used in the treatment of these ailments. Also the problem of microbial resistance to commonly used antimicrobial agents has necessitated the search for newer and alternative compounds for the treatment of drug resistant infections and the high cost of conventional drugs, particularly in resource poor communities of the African continent has led to the increased use of plants as an alternative for the treatment of infectious diseases [3]. Several findings on the chemotherapeutic potentials of plants have shown that they can be sources of antimicrobial compounds of value [4].

Since the beginning of human civilization, medicinal plants have been used by mankind for its therapeutic value. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. The plant-based, traditional medicine systems continues to play an essential role in health care, with about 80% of the world's inhabitant relying mainly on traditional medicines for their primary health care [5].

India has several traditional medical systems such as ayurveda and Unani, which has survived through more than 3000 years, mainly using plant-based drugs. The materia medica of these systems contains a rich heritage of indigenous herbal practices that have helped to sustain the health of most rural people of India.

According to the World Health Organization (WHO, 1977) [6] "a medicinal plant" is any plant, which in one or more of its organ contains substances that can be sued for the therapeutic purposes or which, are precursors for the synthesis of useful drugs. This definition distinguishes those plants whose therapeutics properties and constituents have been established scientifically and plants that are regarded as medicinal but which have not yet been subjected to thorough investigation. The term

"herbal drug" determines the part/parts of a plant (leaves, flowers, seeds, roots, barks, stems etc) used for preparing medicine [7]. Furthermore, WHO (2001) [2] defines medicinal plants as herbal preparations produced by subjecting plants materials to extraction, fractionation, purification, concentration or other physical or biological processes which may be produced for immediate consumption or as a basis for herbal products.

The instant rising demanding of plant-based drugs is unfortunately creating heavy pressure on some selected high-value medicinal plant populations in the wild due to over-harvesting. Several of these medicinal plant species have slow growth rates, low population densities, and narrow geographic ranges [8], therefore they are more prone to extinction [9]. Conversely, because information on the use of plant species for therapeutic purpose has been possed from one generation to the next through oral tradition, this knowledge of therapeutic plants has economic changes [10]. Furthermore, the indigenous knowledge on the use of lesser-known medicinal plants is also rapidly declining. Continuous erosion in the traditional knowledge of many valuable plants for medicine in the past and the renewal interest currently, the need existed to review the valuable knowledge with the expectation of developing the medicinal plants sector [11].

Distemonanthus benthamianus : One of the big trees of the evergreen, semi-deciduous and secondary forest, to 40m high or more by 1.20m diameter of trunk, or slightly smaller; bole some what sinuous or straight, buttressing is variable from none to promine, sometimes with higher butt flares. Lower bole with yellowish bark with roundish scares of shedding scales; upper bark with a very characteristic red or reddish brown colour, especially on the upper part of the stem and on branches where exposed to the sun. Outer bark defoliating in very small, paper thin flakes overlaying a smooth green lenticellate inner layer. The fine flakes make the stem very smooth, hence the local name in Akwa Ibom "monkey can't climb". Slash thin and brittle, pale yellowish or pinkish, interspersed with while near the cambium, sticky. Fine ripple marks visible in bark and wood, fruits thin and papery, 7-13cm x 2-5-3.5cm, containing 2-3seeds.

MATERIALS AND METHODS

Test Organisms

The organisms used were standard strains of; *Staphylococcus aureus* (NCTC 6571), *Echerichia coli* (NCTC 10418) obtained from pharmaceutical microbiology laboratory, Faculty of pharmacy, university of Uyo. *Candida albican* (Clinical isolate from University of Uyo Health center.).

Reagents/Solutions/Drugs

N-hexane (Analar, BDH), Distilled water, dimethyl-sulphoxide (DMSO) BDH

Methanol (Analar, BDH), Ammonium hydroxide (J.T. Baker), Dragendorff reagen, Mayer's reagent, Hydrochloric acid, (Riedel-de-haen) 5% ferric chloride (Adrich), Formaldehyde, Potassium hydroxide Ammonia, Sulphuric acid (M&B), Benzene, Glacial acetic acid (M&B). All solvents and chemicals were of analytical grade.

Drug used

Amoxicillin capsule BP 500mg. Maxheal pharmaceuticals (India) Ltd

Culture Media

The culture media used include nutrient agar (Becton and Dickson

Co, USA), McConkey agar, nutrient broth No. 2, mannitol salt agar,

deoxycholate citrate agar, selenite F broth (Oxoid). All media were prepared according to manufacturer's instructions .

Sterilization of materials

Glasswares used were sterilized in hot air oven at about 160^0c for 1hour, media and bottles were sterilized in an autoclave of 120^0c for 15 minutes. Plastic containers and working bench were cleansed with concentrated disinfectant.

Maintenance and standardization of stock culture

The clinical isolate of *Candida albican* and *Staphylococcus aureus*-NCTC 6571 and *Echerichia coli*-NCTC 10418 were subcultured on sabouraud dextrose agar and nutrient broth respectively and incubated at 25^oC for 24 hours for Candida albican, bacteria was incubated at 37^0c for 24 hours

Collection and identification of plant

Leaves of *Distemonanthus benthamianus* was collected on July 7, 2012 at Itu Local Government Area of Akwa Ibom State, with voucher number UUH No. 43(a) and deposited at the Habarium of the Department of Pharmacognosy and natural medicine.

Extraction

The leaves of *Distemonanthus benthamianus* was air-dried and pulverised to obtain coarse powder.105g of the powder was macerated with methanol at room temperature for 72 hours using a macerating tank. It was filtered and the solvent removed by the use of a thermostat controlled water bath at a temperature of 40^0c to get the Methanol crude extract.

Preparation of various concentrations of extracts and drugs

The extracts were reconstituted in dimethyl-sulphoxide (DMSO) to obtain various concentrations thus: 1g of methanol extract was weighed and reconstituted with 10ml of solvent dimethyl-sulphoxide (DMSO) to give or obtain a concentration of 100mg/ml and 1g of aqueous extract was weighed and reconstituted with 10ml of DMSO to obtain a concentration of 100mg/ml. The solution was further diluted to yield varying concentrations of 50mg/ml, 25mg/ml and 12.5mg/ml solution. 10mg of amoxicillin was dissolved in 10ml of distilled water to give the stock solution with concentration of 1mg/ml.

Antimicrobial Sensitivity Test

The antimicrobial bioassay of the extracts was determined using the standard agar-well diffusion method [12]. The media were prepared according to manufacturer's instructions. 20ml each was aseptically poured into sterile petri dishes mixed with 0.0lml of the overnight broth culture of each of the test organism was introduced in each petri dish which has been appropriately labeled according to the type of extract and organism involved, a glass rod was used to spread the organism unto the plate. A 5mm sterile cork borer was used to remove cylindrical plugs from the agar plate to produce wells of same size as the cork borer; thereafter the reconstituted concentrations of the extract were introduced separately aseptically into the well using a sterile Pasteur pipette. In the remaining well of the petri dishes that had *staphylococcus aureus* and *Echerichia coli* earlier seeded in them amoxicillin was introduced, the plates were incubated at 37^0c for 24 hours for antibacterial activity and 48hours for antifungal activity. The plates were observed and the diameter of zone of inhibition was measured and recorded appropriately.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentration (MIC) was determined using the tube dilution method [13] [14]. A two-fold serial dilution of the methanol and aqueous extracts were aseptically carried out to give varying concentration ranging from 100 mg/ml-0.78125 mg/ml for both extracts and 1mg/ml stock concentration from 500mg amoxicillin capsule was used in preparing the various concentrations ranging from 0.5mg/ml - 0.000244mg/ml using two-fold dilution. All these preparation were done using nutrient broth as diluent. Each of the dilution was inoculated with 0.lml of the standardized inoculum separately. Thereafter, the tubes were incubated of 37^0c for 24 hours.

Determination of minimum bactericidal concentration

Non-turbid MIC tubes i.e. tubes without growth were selected. Aliquots (0.Iml) of inoculum from the non-turbid M.I.C. tubes were sub-cultured on solid nutrient agar plates by streaking. The plates were incubated at 37^0c for 24 hours. Also tubes without growth were re-incubated at 37^0c for 24 hours.

Evaluation of antibiotics synergy of extracts and amoxicillin

Using the agar- disk diffusion method or the Kirby- bauer disk diffusion method [15], the MIC and MBC of extracts and amoxicillin were prepared separately and a paper disc was saturated or impregnated with solution of the extracts and amoxicillin.

Nutrient agar, freshly prepared, was aseptically poured into sterile petri dishes and inoculated with 0.1ml of the test organism using a sterilized Pasteur pipette after which a sterilized glass rod was used to spread the inoculum evenly unto the solid nutrient agar absorbent. The filter paper which has been saturated with the M.I.C and MBC solution of extracts and amoxicillin were placed on the surface of the solid agar plate. The plates were incubated of 37^oc for 24 hours inhibition zone was observed and synergy is deduced from the size of the inhibition zone if it exceeds the summation of those of the single antibiotics.

RESULTS

Results Yield from Extraction

105g of the leaves of *Distemonanthus benthamianus* yield 28.79g of crude methanolic extract.

$$\%\text{yield} = \frac{\text{Dry weight of extract}}{\text{Weight of dried plant material}} \times \frac{100}{1}$$

$$= \frac{28.79\text{g}}{105} \times \frac{100}{1} = 27.419\%$$

ANTIMICROBIAL ACTIVITIES OF THE EXTRACTS AND AMOXICILLIN SENSITIVITY TESTS

Table (1) Zone of inhibition (mm) of extracts against *S. aureus*

Extracts (mg/ml)	100	50	25	12.5	6.25	3.125	1.562	0.781
Aqueous	32	30	29	26	20	5	NZI	NZI
Methanol	30	27	15	10	2	NZI	NZI	NZI

Table (2) Zone of inhibition (mm) of extracts against *E. Coli*

Extracts mg/ml)	100	50	25	12.5	6.25	3.125	1.562	0.781
Aqueous	30	27	25	10	3	NZI	NZI	NZI
Methanol	26	17	10	4	NZI	NZI	NZI	NZI

Table (3) Zone of inhibition (mm) of Amoxicillin against Isolates

Isolates	Amoxicillin mg/ml									
	0.5	0.25	0.125	0.625	0.0312	0.0156	0.0078	0.0039	0.00195	0.000975
S. aureus	47	44	40	37	34	30	25	10	3	NZI
E. coli	40	37	32	30	25	20	10	2	NZI	NZI

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Table (4) Zone of inhibition (mm) of extracts against *Candida albican*

Extract (mg/ml)	12.5	25	50	100
Aqueous	No zone of inhibition (NZI)			
Methanol	No zone of inhibition (NZI)			

Key:
NZI = NO ZONE OF INHIBITION

MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTRICIDAL CONCENTRATION (MBC) OF THE EXTRACTS AND AMOXICILLIN

Table (5) M.I.C and M.B.C (mglml) of Methanolic extract against the Isolates

			Concentration of Extract (mg/ml)				
Isolates	50	25	12.5	6.25	3.125	1.5625	0.7812
S. aureus	-	-	-*	-*	+	+	+
E. coli	-	-	-**	+	+	+	+

Table (6) M.I.C and M.B.C (mg/ml) of Aqueous Extract against the Isolates

	Concentration of Extract (mg/ml)						
Isolates	50	25	12.5	6.25	3.125	1.5625	0.7812
S. aureus	-	-	-	-*	-*	+	+
E. coli	-	-	-*	-*	+	+	+

Table (7) M.I.C and M.B.C (mg/ml) of amoxicillin against *S. aureus*

Amoxycillin (mg/ml)	S.aureus
0.5	-
0.25	-
0.125	-
0.0625	-
0.03125	-
0.015625	-
0.0078125	-
0.00390625	-
0.001953125	-
0.0009765625	-* *
0.00048828125	+

Table (8) M.I.C and M.B.C (mg/ml) of amoxicillin against *E. coli*

Amoxycillin (mg/ml)	E. Coli
0.5	-
0.25	-
0.125	-
0.0625	-
0.03125	-
0.015625	-
0.0078125	-
0.00390625	-*
0.001953125	-*
0.0009765625	+
0.00048828125	+

Keys:
+ = Growth
- = No Growth
* = Minimum Inhibitory concentration
* = Minimum Bactericidal Concentration

OMBINED EFFECT OF EXTRACTS AND AMOXYCILLIN AGAINST THE ISOLATES

Table (9) Amoxicillin in combination with the Extracts against *S. aureus*

Extract + Amoxicillin (mg/ml)	Zone of inhibition (mm)	Inference
	S.aureus	
Aqueous (MIC) + Amoxicillin (MIC) 3.125 0.00097	7	Additive
Methanol (MIC) + Amoxicillin (MIC) 6.25 0.00097	4	Indifference

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Table (10) Amoxicillin in combination with the Extracts against *S.aureus*

Extract + Amoxicillin (mg/ml)	Zone of inhibition	Inference
Aqueous (MBC) + Amoxicillin (MBC) 6.25 0.00097	8	Additive
Methanol (MBC) + Amoxicillin (MBC) 12.5 0.00097	7	Additive

Table (11) Amoxicillin in combination with the Extracts against *E. coli*

Extract + Amoxicillin (mg/ml)	Zone of inhibition	Inference
	E. coli	
Aqueous (MIC) + Amoxicillin (MIC) 6.25 0.00195	5	Indifference
Methanol (MIC) + Amoxicillin (MIC) 12.50 0.00195	4	Indifference

Table (12) Amoxicillin in combination with the Extracts against *E.coli*

Extract + Amoxicillin (mg/ml)	Zone of inhibition	Inference
Aqueous (MBC) + Amoxicillin (MBC) 12.50 0.00390	3	Indifference
Methanol (MBC) + Amoxicillin (MBC) 12.50 0.00390	3	Indifference

DISCUSSION

Extraction

The leaves were air-dried and pulverized before maceration to increase the surface area of contact of plant with extracting solvents, hence, increasing the rate and yield of extract [16].

ANTIMICROBIAL SENSITIVITY TEST

The extracts obtained were screened for antibacterial and antifungal activities using standard strains of *S.aureus, E. coli* and clinical isolates of *C. albican* to represent a desirable spectrum of microbes. Amoxycilin was also used in combination with the extracts for possible interactions.. The presence of zone of inhibition on the seeded agar plates showed that the plant extract posses broad spectrum antibacterial activity inhibiting the growth of both Gram positive (*S. aureus*) and Gram negative (*E. coli*) organisms. The extracts had no activity on the fungal organism (*C.albican*) at the concentration used.

The zone of inhibition for the S. aureus was greater than that of *E.coli*, while *C. albican* was found to be resistant to the extracts. From the sensitivity test result, it could be observed that the zone of inhibition is concentration dependent i.e. the higher the concentration, the greater the zone of inhibition and vice versa. Table 2-4 illustrates the concentrations which the extract and fractions were tested and their respective zone of inhibition and the zone of inhibition of the amoxicillin against the test organism.

Aqueous extract showed greater activity against both Gram positive and Gram negative organism but the difference is not significantly high. The extracts had no activity on *C. albican*. Amoxicillin showed a greater activity against the *S. aureus and E. coli* than the extracts at very low concentrations and the MIC and MBC lower than that of the extracts, showing that It is more potent than the extracts of the leaves of *Distemonanthus benthamianus* .

Antifungal activity

The antifungal screening test had no zone of inhibition against the extracts. This probably could be due to the fact that the cell wall of fungi resembles those of higher plants and hence limits of permeation of substances into them [17].

Synergy evaluation

This was carried out to find out if there will be an additive, potentiation or antagonist effect resulting from the combination. The result obtained showed little potentiation and some are additives, while some are indifferences Hence, the ultimate justification of the use of antibiotic combination will be based on a confirmation of the presence of synergism [18]. The extract if formulated into drug may be used in combination with the standard drug used (amoxicillin). The antibiotics combinations which are short of synergism are not accepted in antimicrobial chemotherapy [19] .

Statistical data analysis

SPSS software was used in statistical analysis of the various result obtained. The different concentration of extracts (12.5, 25, 50 and 100mg/ml) used against organisms had significant difference ($p < 0.05$) and also for amoxicillin in the activity significant difference is ($p<0.05$). From the analysis above, it could be deduced that the activity of the extract and fractions against *E. coli*, and *S. aureus* are not dose dependent

CONCLUSION

The leaves of *Distemonanthus benthamianus* exhibited significant antibacterial activity against some of the microorganism implicated in the pathogenesis of human

infections from Gram positive organisms and Gram negative but had no activity on fungal organism (*C. albican).* This activity was revealed during the antimicrobial sensitivity test. The zone of inhibition was found to be dose dependent and the extracts of the plant leaves are more effective in combination with Amoxicillin against S. aureus[20].

RECOMMENDATION

The leaves are therefore recommended for use as an antibacterial agent particularly against Gram positive organism, but more research should be carried out on the leaves to ascertain its safety and toxicity level and establish a safe dosage regiment.

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