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Full Length Research Paper

In-vitro screening of antibacterial potentials of *Aspilia africana* leaves.

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Abstract
In-vitro screening of antibacterial potentials of aqueous, ethanolic and methanolic extracts of *Aspilia africana* leaves was carried out using agar well diffusion method. Data obtained from this study indicated that the leaf extracts of *A. africana* possessed antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella pneumonia*e. The methanolic and ethanolic extract of *A. africana* showed antibacterial activity with the diameter of zone of inhibition of 9.0 - 16.0 mm and 7.17 - 14.17 mm against *S. aureus*, 8.0 – 14.67 mm and 8.83 - 13.17 mm against *K. pneumonia* and 7.0 - 14.0 mm and 7.50 - 12.33 mm against *P. aeruginosa* respectively. Methanolic extract of *A. africana* was observed to be more potent, inhibiting all isolates thus showing higher antibacterial activity than the ethanolic and aqueous extracts. The efficacy of the extracts was further exhibited as the *S. aureus* used for the study, was resistant to the positive control (gentamicin) while been sensitive to the extracts. The test organisms were not sensitive to the extractants alone. The efficacy of the extracts towards inhibition of the micro organisms increased with increased concentration. The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the extracts on the test organisms also increased in the following order; aqueous < ethanolic < methanolic. Findings from this study further showed that plants are potential sources of new drugs for treating infections caused by these antibiotic resistant clinical pathogens.

Keywords: Antibacterial activity, *A. africana*, agar well diffusion assay, methanolic extract.

INTRODUCTION

Aspilia africana (Pers.) C. D. Adams var *africana* C. D. Adams [family compositae] is a common weed of field crops in West Africa found in fallow land especially in the forest zone it is a scrambling perennial herb varying in height from 60cm to about 1.5m depending on rainfall. The flowers are showy yellow florets and the fruits are bristly and minutely hairy with four angled schemas about 5mm long (Akonbundu and Agyakwa, 1998). Plant materials continue to play an important role in the maintenance of human health as over 50% of all modern chemical drugs originates from natural plant sources. Several plants are now being used in part or as a whole to treat many diseases. Active components of these plants are now being investigated, extracted and developed into drugs with little or no negative effects or contra-indication (Oluyemi *et al.*, 2007). Rural dwellers in most parts of the world do not depend on the orthodox medicine for the cure of diseases and ailments. This is because most of the modern equipment's are expensive and service delivery too expensive to afford. As a result of this, a

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larger section has resorted to the use of traditional medicines.

Aspilia africana is one of the many indigenous plant used by tradomedical practitioner in Nigeria to cure certain illness. It is known as 'organgila' in Ibo, 'tazalian' in Hausa, 'yungung' in Yoruba, 'Edemedong' in Efik (Single, 1965).

It has several indigenous uses for its leaves and flower but most notable is its use to stop bleeding and fast healing of wound. Thus the reason why it is referred to as "haemorrhage plant" due to its ability to stop bleeding from fresh wounds (Esimone *et al*., 2005).

Phytochemical analysis of the plant leaf extract revealed the presence of a number of beneficial phytochemicals such as terpenoids, saponins, flavonoids, phenols and tannin. *A. africana* leaf is also rich in crude protein and minerals (Abii and Onuoha, 2011). It also contains biologically active substances that are antiviral, fungicidal and antibacterial. These biological activity is due to the presence of thiarubrines, a derivative of 1, 2 – dithiocyclohexa – 3, 5 – diene (Masato and Wu, 1994). These strong antibiotic properties thus validate its applications as an antibacterial agent, for disease prevention and treatment in herbal and ethno-pharmaceutical practices.

Pseudomonas aeruginosa is a gram-negative, aerobic, rod-shaped bacterium with unipolar motility (Ryan and Ray, 2004). It is an invasive and toxigenic bacterium that commonly causes disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. It is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics due to its ability to thrive on most surfaces thus making it a major nosocomial pathogen (Brooks *et al.,* 2004). The symptoms of its infections are generalized inflammation and sepsis. If its colonization's occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal (Brooks *et al.,* 2004).

Staphylococcus aureus is a gram positive cocci frequently found as part of the normal skin flora on the skin and nasal passages (Brooks *et al.,* 2004). It is estimated that 20% of the human population are long-term carriers of *S. aureus*. *S. aureus* is the most common species of Staphylococci that causes infections. *S. aureus* is a successful pathogen due to a combination of bacterial immune – evasive strategies. One of these strategies is the production of carotenoid pigment staphyloxanthin, which is responsible for the characteristic golden colour of *S. aureus* colonies. This pigment acts as a virulence factor, primarily by being a bacterial antioxidant which helps the microbe evade the reactive oxygen species which the host immune system uses to kill pathogens (Clauditz *et al*., 2006) (Liu *et al*., 2005).

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan and Ray, 2004) It is clinically the most important member of the Klebsiella genus of *Enterobacteriacea*. Member of the Klebsiella genus typically express 2 types of antigen on their cell surface. The first O antigen is a component of the lipopolysaccharide of which 9 varieties exist. The second is K antigen a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for serogrouping (Podschun and Ullman, 1998). *K. pneumoniae* is also known to be an agent of nosocomial infections (Brooks *et al.,* 2004).

The above discussed microorganisms are both indigenous flora as well as pathogens of human thus making them suitable candidate for *in vitro* study on the antibacterial efficacy of *A. africana.*

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In this study, we investigated the antimicrobial efficacy of *A. africana* extracts on *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* in an attempt to further give further scientific backing to various tradomedical claims and uses of the leaves of *A. africana*.

MATERIALS AND METHOD

Collection and identification of plant sample: Fresh leaf samples of *Aspilia africana* (Pers.) C. D. Adams var *africana* C. D. Adams [family compositae] were obtained from Ayanji in Ovia North-East Local Government Area of Edo State. It was identified properly and authenticated with reference to the Herbarium sheets (Voucher number 234) available at the Herbarium of the Department of Plant Biology and Biotechnology, University of Benin, Benin, Nigeria.

Source and maintenance of test organisms: Pure culture of test organisms used in the project; *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were obtained from the Medical Microbiology Unit of the University of Benin Teaching Hospital.

Preparation of Extract: The fresh leaf samples were washed with distilled water and then surface sterilized with 0.1 % mercuric chloride for few seconds. Afterwards the plant materials were washed thoroughly with distilled water (3 times) (Merinal *et al*., 2012). Sterilized leaf samples were properly sundried, ground and extraction was done at the Department of Pharmacognosy, University of Benin. Aqueous, methanol and ethanolic extraction of the plant material was prepared as described by Oyagade *et al*., (1999). These were carried out by suspending 25grams of the finely ground leaves in 125 millilitre of distilled water or 95% ethanol or methanol. The hot water extraction was done at 80°c in a water bath for 1/2hours. The ethanolic and methanolic extraction was done at 28±1°c for 120hours. The extracts were then decanted and filtered through a Whatman filter paper. The filtered extract was then sterilized using a membrane filter and evaporated to dryness at 45°c. the residues obtained were reconstituted in 95% ethanol at stock concentration of 0.2g/ml. the extract solution were then stored in the refrigerator at 4±2°c until used (Omojosola and Awe, 2004).

Standardization of microbial inoculums: Five inoculums of the organism growing as pure culture in a nutrient agar plates were inoculated into 10 ml of nutrient broth in a test tube aseptically. The mouth of the tube was covered with cotton wool and wrapped with aluminium foil followed by incubation at 37 ^{o}C for 24 hours.

Antibacterial Assay: Fifteen ml of molten sterile nutrient agar was poured into Petri dishes. After solidification, an overnight broth culture of *S. aureus*, *P. aeruginosa* and *K. pneumoniae* was introduced unto the surface of a sterile plate each and sterile glass spreaders were used for even distribution. Wells were made aseptically with a 7 mm sterile cork borer and 0.1 ml of the test solutions of different concentration were introduced into well. The extract was allowed to diffuse into the medium for about 1 hour and then incubated aerobically for 24 hours at 37^{0}C. One well contained extractant (methanol, ethanol or distilled water); the antibiotic Gentamicin served as negative and while water served as positive controls in each plate. The plates were examined for zones of inhibition, which indicate the degree of susceptibility of the test organism. The antimicrobial activity of water, methanol and ethanolic extract were measured and compared with the activity of the extractants in the control well.

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Determination of Minimum Inhibitory Concentration (MIC): MIC of the aqueous and ethanolic extracts of this plant was determined by solution of the extract to various concentrations of 25, 50, 100 and 200 mg/ml. Nine ml of sterile peptone water was dispensed into each test tube, then 1ml of each of the extract at different concentrations were introduced and mixed in a test tube, 0.1ml of inoculums was added to each tube. The tubes were incubated aerobically at 37 ^{0} C for 24 hours. Two control tubes were maintained for each test batch. These included antibiotic control (tube containing extract and the growth medium without the inoculums) and organism control (the tube containing the growth medium and the inoculums). The lowest concentration (higher dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as MIC.

Determination of Minimum Bactericidal Concentration (MBC): MBC was determined by sub – culturing test solution which showed no detectable growth (no turbidity) after 24 hours incubation onto fresh drug free nutrient agar and incubated further for 24 hours to determine the MBC of the extract required to kill the organism. These concentrations were indicated by the failure of the test organism to grow on the recovery media after incubation (that is not show growth after incubation) indicating a bactericidal effect.

RESULTS

The antibacterial effectiveness of the leaf extracts at concentrations of 200mg/ml, 100mg/ml, 50mg/ml, and 25mg/ml as compared with the activity of Gentamicin was shown in Table 1. The antibacterial activity of the conventional antibiotic was higher than most of that of the plant extracts except for those of the methanolic extract which exhibited higher zones of inhibition. The methanolic extract of *A. africana* showed the highest antibacterial activity with the diameter of zone of inhibition of 9.0 - 16.0 mm against *S. aureus*, 8.0 - 15.0 mm against *K. pneumonia*, and 7.0 - 14.0 mm against *P. aeruginosa.* The ethanolic extract of *A. africana* showed the antibacterial activity with the diameter of zone of inhibition of 7.0 - 14.0 mm against *S. aureus*, 9.0 - 13.0 mm against *K. pneumonia*, and 8.0 - 12.0 mm against *P. aeruginosa.*

Table 2 shows the MIC and MBC of the methanolic, ethanolic and aqueous extract of the plant. Generally the methanolic extract showed greater antibacterial activity compared to its corresponding extract in the ethanolic and aqueous extract. The methanolic extract showed the highest activity against *S. aureus,* then *K. pneumoniae,* followed by *P. aeruginosa*.

Table 1: **Antibacterial activity of *A. africana* plant extracts on the test organisms**

Concentration (mg/ml)	*S. aureus*			*K. pneumoniae*			*P. aeruginosa*		
	Aqueous (mm)	Ethanol (mm)	Methanol (mm)	Aqueous (mm)	Ethanol (mm)	Methanol (mm)	Aqueous (mm)	Ethanol (mm)	Methan (mm)
200	7.00 ± 0.00	14.17 ± 0.73	16.00 ± 0.58	8.10 ± 0.44	13.17 ± 0.44	14.67 ± 0.33	6.83 ± 0.60	12.33 ± 0.33	14.00 ± 0
100	0	12.33 ± 0.33	14.00 ± 0.00	0	12.33 ± 0.33	13.17 ± 0.17	0	11.50 ± 0.29	11.67 ± 0
50	0	11.00 ± 0.33	12.00 ± 0.00	0	8.83 ± 0.44	10.50 ± 0.32	0	7.50 ± 0.29	11.13 ± 0
25	0	7.17 ± 0.17	9.00 ± 0.29	0	0	8.00 ± 0.29	0	0	7.00 ± 0.
Positive control (Gentamicin)		0			17.00 ± 0.29			13.50 ± 0.29	
Negative control (Extractant)		0			0			0	

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Table 2: **MIC and MBC of the extracts against test organisms.**

Isolates	Aqueous		Ethanol		Methanol	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
S. aureus	200	0	100	200	25	100
K. pneumonia	200	0	25	0	25	0
P. aeruginosa	200	0	50	0	25	0

DISCUSSION

The data obtained from this study indicates that the leaf extracts of *Aspilia africana* possesses antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella pneumonia*e. It was shown in this study that the methanolic extract of *A. africana* was superior to that of ethanolic and aqueous extract**.** The antibacterial activity of the crude plant extracts on the test organisms justified the active principle or ingredient observed in herbal physician in their preference for the local gin as extractant in the preparation of crude drugs from medicinal plant materials. When these solvents are used as herbal extractants, it may be possible that bioactive substances that are less soluble in water would then be dissolved by the solvent (Oyagade *et al*., 1999). In the antibacterial sensitivity test, methanolic extract of *A. africana* exhibited the most outstanding antibacterial activity against *S. aureus with* an inhibiting effect of 16.00 ± 0.58 mm at 200mg/ml concentration is higher than others at the same concentration. The susceptibility of the *S. aureus* strain to ethanolic and methanolic extracts in this study is noteworthy as the said *S. aureus* was an antibiotic resistant strain as it was resistant to the conventional antibiotic, Gentamicin used. This further demonstrates the antibacterial potency of plant extracts against conventional antibiotic resistant pathogens. The methanolic extract of *A. africana* showed growth inhibitory effect for all the concentrations (i.e. 25, 50, 100 and 200 mg/ml) against *K. pneumoniae* in which it has its diameter of zone of inhibitory effect of 14.67 ± 0.33mm at 200 mg/ml concentration, followed by *P. aeruginosa.* The sensitivity of the test organisms to the extracts of *A. africana* in this study corresponds with the work of Adeniyi and Odufowora (2000) that showed that extracts of *A. africana* possessed a broad spectrum antibacterial activity against both gram positive and gram negative bacteria. The aqueous extract of all these plant did not exert much antibacterial effect on the organisms; it shows its highest zone of inhibition against *Klebsiella pneumoniae* with a zone of inhibition of 8.10 ± 0.44 mm at 200 mg/ml. This is possibly due to the failure of the active ingredient to dissolve in it and all the sensitive extracts were more at higher concentrations than lower concentration. It was also observed that the sensitivity of organisms to the extracts increased with increase in concentration of the extracts.

Failure of some of the extract to exert antibacterial effect or the test organism is not enough to conclude that the leaf does not contain substances that can exert antibacterial activity against the test organism because the potency of extract depends on the method used to obtain the

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extract and some other factors. Research has shown that the age of plant when harvested and the season of plant determine the amount of the amount of the active constituents and thus the active ingredients of plants can vary in quality and quantity from season to season (Sofowora, 1996). The inability of the negative control (the extractants) to inhibit the growth of the test organisms in this study goes to attest that the antimicrobial activity of the extracts can only be attributed to the plants only.

The aqueous extract did not exert any bacteriocidal effects on the organisms as it was only able to inhibit their growth. This is in contrast to the methanolic and ethanolic extracts that exerted both inhibitory and bacteriocidal effects on the test organisms. The methanolic and ethanolic extract was both bacteriostatic and bactericidal at a concentration of 25 mg/ml, 100 mg/ml for methanol and 100 mg/ml and 200 mg/ml for ethanol on *S. aureus*. The comparison of the activity of the plant extract with conventional antibiotics, such as gentamicin confirmed reports by other workers (Esimone *et al.*, 2005) that constitutional antibiotics are more active than plant extracts.

CONCLUSION

This study demonstrated methanol to be a stronger extractant than ethanol and water in the extraction of active substances from *A. africana* leaves for antibacterial purposes. The antibacterial activity of the extract could be enhanced if the active substances in them are purified. Research laboratories are therefore enjoined to work hand in hand with traditional herbal practitioners so that while the traditional healers from their historic knowledge provide preliminary information on the uses of medicinal plant, the scientific basis for the efficacy of the extracts is also studied by the laboratory scientist, so that proper advice can be given on how the drugs should be prepared and administered. This plant therefore holds a promise as a potential source of new drug for treating infections caused by these clinical pathogens.

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