Nigerian Journal of Pharmaceutical and Applied Science Research, 5(2):11-17, August 2016

WEBSITE: www.nijophasr.com ***ISSN 1485-8059***

Phytochemical and *In Vitro* Antibacterial Evaluation Of The Aqueous, Methanol, Butanol and Petroleum Ether Crude Extracts of the Leaf of Boerhavia *Diffusa* (Nyctaginaceae)

Josephs Chimechefulam Godwin* and Dowe Ejiro

Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin City Edo state, Nigeria.

ABSTRACT

The Phytochemical screening and *in vitro* antimicrobial activity of the aqueous, methanol, butanol and petroleum ether crude extracts of *Boerhavia diffusa* against some clinical isolates were studied using standard phytochemical and microbiological methods. Organisms isolated from wounds, oral infections and urine samples were sourced from the Medical Microbiology laboratory of University of Benin Teaching Hospital (UBTH) and the standard strains of microorganisms from the Pharmaceutical Microbiology Department of University of Benin (UNIBEN). The organisms used for the study include, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Proteus vulgaris* and *Escherichia coli* as well as the standard strains; *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25923 and *Bacillus subtilis* NCTC 8236. The sensitivity of the clinical isolates and standard organisms was carried out using the ditch plate and agar well diffusion method. Results of preliminary sensitivity test using the ditch plate method showed lack of activity of the aqueous and petroleum ether extracts to the test organisms whereas the methanol and butanol extracts were active against all the test isolates except *P. vulgaris*. There was no significant difference in the zones of inhibition recorded for the methanol and butanol extracts. The standard strains of bacteria were more susceptible than their clinically isolated counterpart. However, the difference in susceptibility between the isolates and the standard strains was not statistically significant. The activity of the extracts was significant (**P<0.01) relative to the negative control (sterile distilled water). The difference in activity between the active extracts and the positive control (ciprofloxacin) was statistically significant (*P<0.05) at the concentrations used. Phytochemical screening results showed the presence of some secondary metabolites (anthraquinone, phenolics, tannins, steroids, carbohydrate, alkaloids and saponins) in the extract. The findings from this study showed the presence of pharmacologically important phytochemicals and the antimicrobial activity validates the therapeutic use of the plant.

Keywords: Phytochemicals, antibacterial, susceptibility, *Boerhavia diffusa*, resistance.

INTRODUCTION

The use of plants for ethnomedical treatment of various ailments has long been established. *B. diffusa* is the most popular among the family *Nyctaginaceae* with over 35 species (Apurba *et al*., 2012). The creeping herbaceous plant popularly called Pig weed or Hogweed. In Nigeria, it is called Akandom/Azeigwa, Etipoula and Babba-juji by the Igbo, Yoruba and Hausa ethnic groups respectively. It is a well known medicinal plant widely distributed in parts of Africa, Asia, America and Australia.

*Corresponding Author: **E-mail: godwin.josephs@uniben.edu; ejisdowey@yahoo.com**

Phone: +2348037199072 or +2348038093488

Parts of the plant including the leaves has been claimed to be effective in the treatment of different ailments like wound, inflammations, hypertension, diabetes, stress, dyspepsia, abdominal pain, inflammation, jaundice, enlargement of spleen, heart diseases, bacterial infections, infertility/impotence and epilepsy in traditional medicine (Orisakwe *et al*., 2003; Okoli *et al*., 2007). The therapeutic values of plants are due to substances produced by plants in the process of their adaptation to their environment. Some of these substances are secondary plant metabolites which serve chiefly as immunomodulators as well as having toxic effect on infection-causing microorganisms with little or no toxic effect on the host (Chaudhary and Dantu 2011). A combination of immune boosting properties as well as antimicrobial properties of medicinal herbaceous plant is a major advantage over conventional antibiotics and research to substantiate such claims becomes imperative.

The aim of this study is to evaluate the antimicrobial activity of *B. diffusa* against clinical bacterial isolates in comparison with some standard organisms, to compare the degree of activity due to the extraction solvents (methanol, distilled water, butanol and petroleum ether) and to screen for the presence of some secondary metabolites that contributed to the possible *invitro* activity of the various extracts.

MATERIALS AND METHODS

Collection and identification of plants

The leaves of the plant were collected from University of Benin environment between 10^{th}-20^{th} May, 2015 and authenticated by Dr. Uwumarongie Osamuyi Henry (A voucher specimen of the plant was deposited in the herbarium of Pharmacognosy Department University of Benin, Benin city, Nigeria with the herbarium number: UBPCGHB023-*Boerhavia diffusa*.

Preparation of plant extracts

The leaves were washed with distilled water and air dried for three weeks and subsequently pulverized into fine powders before maceration using methanol, distilled water, butanol and petroleum ether. After a period of 24 h of aqueous extraction and 72 h of methanol, butanol and petroleum ether extraction, the suspensions were filtered and the filtrate concentrated to dryness on a water-bath at controlled temperature (80^oC for the aqueous extract and 45^oC for the other solvents) to yield 35.5% aqueous, 6.4% methanol, 3.6% butanol and 2.2% petroleum ether extract. The extracts were stored in sterile sample bottles and kept in the refrigerator at 4°C prior to use.

Test organisms/Bacterial culture

The bacterial cultures used for this study were obtained from stock cultures of isolates characterized and authenticated from the treatment of oral swabs, wound swabs and urine samples collected from UBTH and Central hospital, Benin city while the standard bacterial cultures were from stock of American type culture collection (ATCC) United States of America. All the bacterial cultures used were obtained from authentic stock cultures of Department of Pharmaceutical Microbiology and Biotechnology laboratory, Faculty of Pharmacy, University of Benin, Nigeria. The clinical isolates used in this study were; *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Proteus vulgaris* and *C. albicans* while the standard strains used were; *Escherichia coli* ATCC 25922, *Staphylococus aureus* ATCC 25923 and *Bacillus subtilis* NCTC 8236.

Antimicrobial Assay

a.) Antibacterial activity of extracts

Overnight broth cultures of the organisms were adjusted to 0.5 McFarland standard to give an inoculums size of approximately 10^8 cfu/ml by one in hundred serial dilution (1:100). The ditch plate method described by Lalitha *et al*. (2004) was used for the preliminary sensitivity assay. A ditch was made in nutrient agar plates and molten nutrient agar was applied to seal the bottom of the ditch and each of the microbial dilutions were streaked across the ditch at right angles. The ditch was filled with 0.5 ml of 100 mg/ml concentration of the aqueous extract. The process was repeated for the methanol, butanol, petroleum ether extract and the positive control (1µg/ml Ciprofloxacin hydrochloride) as well as the negative control (Sterile distilled water). All experiments including controls were done in

duplicates and plates were incubated in an upright position for 24 h at 37^{o}C after which the diameter of clear zones were observed and measured.

b.) Antibacterial activity of extracts

Antibacterial sensitivity/potency of the active extracts following preliminary activity test was performed using the agar well diffusion method (Firas *et al*., 2008; Habamu *et al*., 2010). Six wells of 12mm in diameter were made into previously seeded Nutrient agar plates. Four of the wells were each filled with 0.1 ml of the extracts at varying concentration and the fifth well filled with 1µg/ml of Ciprofloxacin hydrochloride (positive control) while the sixth well was filled with distilled water (negative control). All plates were pre-incubated for 1 h to allow diffusion of test material before incubating overnight at 37^{o}C. The absence or presence of diameter of clear zone was observed, measured and recorded. The experiments were done in duplicates and the mean zones of inhibitions calculated.

Phytochemical Analysis

Qualitative screening of the phytochemical components of the plant extracts was carried out using the standard method described by Harbone (1998). Essentially, specific weight of the extracts was made up to 10 ml in a test tube and different reagents were added to specifications. Positive results were indicated by colour change and precipitate formation which were compared against standards. The extracts were tested for the presence of carbohydrates, alkaloids, tannins, saponins, anthraquinones, phenolics and steroids.

Statistical Analysis

Statistical analysis was performed using Paired t-Test and one way analysis of variance (ANOVA) to test the level of significance.

RESULTS

The qualitative phytochemical screening for bioactive secondary metabolites in various *B. diffusa* leave extracts revealed the presence of anthraquinones, carbohydrates, alkaloids, phenolic compounds, saponins, tannins and absence of steroids (Table 1).

Table 1: Phytochemical constituents in the various extracts of *B. diffusa* leave

Plant Constituents	Aqueous Extract	Methanol Fraction	Butanol Fraction	Petroleum ether extract
Carbohydrates	+	+	+	+
Anthraquinones	+	_	_	+
Alkaloids	+	+	+	+
Phenolic compounds	_	+	+	_
Steroids	_	_	_	_
Saponins	_	+	+	_
Tannins	_	+	+	_

Key: + = Present, - = absent

The preliminary antimicrobial sensitivity test revealed activity in the methanol and butanol leave extracts while the aqueous and petroleum ether leave extracts showed no inhibition (Table 2).

Results of the sensitivity of the active extracts against the test organisms at various concentrations are presented in Tables 3 and 4.

Table 2: Antibacterial activity of the extracts.

Organisms	Zones of Inhibition (mean ±S.E. mm)					
	Aqueous Extract	Methanol Extract	Butanol Extract	Pet. Ether Extract	Ciprofloxacin	Distilled
	(50 mg/ml)	(50 mg/ml)	(50 mg/ml)	(50 mg/ml)	1µg/ml	Water
S. aureus	–	23±0.6	25±1.3	–	30±1.2	–
P. aeruginosa	–	18±1.3	18±0.5	–	27±0.3	–
E. coli	–	20±1.6	21±1.3	–	29±0.6	–
K. Pneumoniae	–	21±0.3	23±0.0	–	28±1.0	
B. subtilis	–	22±0.5	24±1.5	–	28±0.3	–
P. vulgaris	–	–	–	–	24±1.5	–

Key: S.E. = Standard Error, – = No inhibition

Table 3: Antibacterial activities of methanol extract of *B. diffusa* at different concentrations

Organisms	Zones of Inhibition (mean ±S.E. mm)					
	Concentrations (mg/ml)				Ciprofloxacin	Distilled
	25	50	75	100	1µg/ml	Water
Staphylococcus aureus	19±1.0	23±0.0	27±0.0	32±0.0	30±1.0	–
Pseudomonas aeruginosa	18±0.3	24±1.0	26±0.3	30±0.0	28±1.0	–
Escherichia coli	21±1.6	25±1.0	29±1.0	32±0.5	26±0.3	–
Klebsiella pneumoniae	18±1.0	24±0.3	27±0.0	30±1.0	29±0.0	–
Proteus vulgaris	–	–	–	–	24±1.0	–
Bacillus subtilis	23±0.0	26±0.0	30±0.0	33±0.0	30±0.0	–
S. aureus ATCC 25923	21±0.6	25±1.0	28±0.0	33±1.0	32±0.0	–
E. coli ATCC 25922	20±1.0	23±1.0	30±1.0	32±0.0	27±1.0	–
B. subtillis NCTC 8236	21±0.9	24±0.0	31±0.0	33±0.0	33±0.2	–

Key: S.E. = Standard Error, – = No activity

DISCUSSION

The presence of Carbohydrate in all the plant extracts relates to its abundance in the leave of plants where photosynthetic activities majorly occur. Carbohydrates in plants may simply be a sugar or a more complex forms such as lipid or starch. They are food or energy source to both plants and animals. Some carbohydrates such as novel mono-substituted carbohydrate fatty acid (CFA) esters, lauric ether of methyl α-D-glucopyranoside and lauric ester of methyl α-d-mannopyranoside has inhibitory effect on some gram positive and gram negative bacteria (Nobmann *et al*., 2009). Alkaloids were also confirmed to be present in all the extracts. Alkaloids have been investigated for many pharmacological properties including antibacterial, antifungal, antiprotozoal, cytotoxic, antidiabetic and anti-inflammatory properties (Edeoga *et al*., 2005; Oduak and Lawrence, 2008). Anthraquinone was confirmed in the aqueous and petroleum ether extracts. The anthraquinones are a group of aromatic ring compounds with two or more ketone substitutions. Steroids was completely absent in the extracts. Saponins have foaming property and serve as mild detergent that

Table 4: Antibacterial activities of butanol extract of *B. diffusa* at different concentrations

Organisms	Zones of Inhibition (mean ±S.E. mm)					
	Concentrations (mg/ml)				Ciprofloxacin	Distilled
	25	50	75	100	1µg/ml	Water
Staphylococcus aureus	19±1.0	24±0.0	26±1.0	30±0.0	27±1.0	–
Pseudomonas aeruginosa	17±0.3	22±1.0	26±0.3	29±0.0	25±1.0	–
Escherichia coli	18±1.6	25±1.0	29±1.0	31±1.0	26±0.0	–
Klebsiella pneumoniae	18±1.0	25±0.0	28±0.0	33±1.0	27±0.0	–
Proteus vulgaris	–	–	–	–	25±0.0	–
Bacillus subtilis	22±1.0	26±1.0	30±0.0	34±0.0	29±0.0	–
S. aureus ATCC 25923	23±0.6	26±1.0	29±0.0	31±0.0	31±0.0	–
E. coli ATCC 25922	20±1.0	25±1.0	30±0.3	32±0.0	27±1.0	–
B. subtillis NCTC 8236	24±0.9	26±0.0	31±0.0	33±0.0	33±1.0	–

Key: S.E. = Standard Error, – = No activity

solubilize cell permeability barriers and consequent lysing of bacterial and fungal cells (Okwu, 2004). The presence of saponin in the methanol and butanol extracts may have contributed to their activity. Phenolic compounds was present in the methanol and butanol extracts. Phenolics are potent antimicrobial agents. The presence of phenolic compounds and tannins could possibly be the reason for the activity shown by the methanol and butanol extracts.

The petroleum ether and equeous extracts showed no activity against the test organisms at the concentration used whereas the result reported by akinibosun *et al*.(2009) showed that aqueous and ethanolic extracts of *B. diffusa* leaves had activity on *E. coli*, *S. aureus* and *P. aeruginosa* at varying concentration. The presence of the diluent (ethanol) and the extract mixture in their method may have contributed to the activity recorded for the aqueous extract. However, the methanol and butanol extracts showed a concentration dependent activity against the test organisms corroborates with the findings of Onwuliri and Dawand (2006), who found that increased concentrations of ethanol, methanol and butanol leave extract of *Moringa oleifera* resulted in increased sensitivity in the organisms. The degree of susceptibility varies among the bacterial as revealed by the Inhibition zone diameters (IZDs). The antimicrobial activity of extracts or antimicrobial agents is evident by the presence of growth inhibitory zones on seeded agar plate. The zone is measured as an index of the inhibitory action of the extract against the test microorganism (Ndukwe *et al*., 2005; Usman *et al*., 2005). Extracts were considered active at zone of inhibition diameter of >12mm. The study showed the highest IZDs of 33±0.0 mm at 100 mg/ml concentration on the average against *B. subtilis* especially among the standard strains (Table 3 and 4) and the least was 17±0.3 mm at 25 mg/ml concentration against *P. aeruginosa* while *P. vulgaris* was completely resistant to the extracts at the reference concentrations. This shows that microbial resistance is not restricted to synthetic/semisynthetic antibiotics alone but also to natural antimicrobial agents. This result is in agreement with the work of Oloyede *et al*.(2012) who reported *K. pneumoniae* and *P. vulgaris* resistant to Joolo herbal preparations to which five other genera of bacteria were susceptible. The phenomenon of resistance imposes serious constraints on the options available for the medical treatment of many bacterial infections. Bacterial resistance is a major clinical problem which has been observed with virtually every antimicrobial agent (Smith *et al*., 2000; Prescott *et al*., 2005 Ayepola, 2009). The same problem of resistance was slightly reflected even among the susceptible organisms when compared to the standard as it was observed that the IZDs of the isolates (*S. aureus*; 19±1.0, *E. coli*; 18±1.6 and *B. subtilis* 22±1.0) were slightly lower than those recorded for the

corresponding standard strains (*S. aureus* ATCC 25923; 23±0.6, *E. coli* ATCC 25922; 20±1.0 and *B. subtilis* NCTC 8236; 24±0.9) all at 25mg/ml concentration and almost similar pattern of susceptibility was observed for the various concentrations used (Table 4). This pattern of susceptibility/resistance may be due to previous exposure of bacterial organisms to different antibiotics/antibacterial agents prior to isolation. Apart from drug induced resistance, resistance may also be inherent which may be due to specific morphology of the bacteria which prevents the antibacterial agent from reaching the target site or production of degrading enzymes by the bacteria (Orisakwe *et al*., 2003; Chaudhary and Dantu, 2011). The gram-negative organisms were least susceptible while the gram-positive bacteria especially *B. subtilis* was the most susceptible to the butanol extract showing the highest IZD of 34±0.0 mm at 100mg/ml. Gram positive organisms are generally more susceptible to antimicrobial agents compared to gram negative organisms due to their cell morphology or additional permeability barrier especially by the possession of additional layer of lipopolysaccharides in addition to the meurin layer of gram negative bacteria (Prescott *et al*., 2005; Ramachandra *et al*., 2012). The extracts at 100mg/ml appear to be more active than the positive control, ciprofloxacin hydrochloride at the concentration used. However at lower concentration of the extracts especially at 25mg/ml which is about 24 times higher than the concentration of the standard antibiotics, it became very obvious that the positive control is far more active against the test organisms including *P. vulgaris* which resisted the inhibitory effect of the active extracts. The disparity between the extracts and the standard drug may be due to the pure nature of the standard antibacterial drug whereas the extracts is still in its crude state which requires a lot of purification in order to isolate the active compounds. In the study, distilled water was used to constitute various dilutions/concentrations of the extracts and it served as the negative control and clearly revealed that it wasn't a contributing factor to the activity of the active plant extracts.

CONCLUSION

The study has shown that *B. diffusa* leave extracts possesses a concentration-dependent activity against the test organisms as opposed to the negative control (Sterile distilled water). However, there was a significant difference (*P<0.05) in activity between the plant extract and positive control (ciprofloxacin hydrochloride). The study also showed lack of activity of the aqueous and petroleum ether extracts against the test organisms whereas the methanol and butanol extracts showed activity but the difference in activity between the two active extracts was not statistically significant. The standard microorganisms were more susceptible than the sourced clinical isolates but the difference in susceptibility was not statistically significant. *P. vulgaris* was completely resistant wheras *B. subtillis* was most susceptible. Phytochemical screening results revealed the presence of Carbohydrates, Phenolic compounds, anthraquinones, Tannins, alkaloids and saponins whereas steroids was completely absent. The antibacterial activity shown by this study, supports the use of *B. diffusa* leave extracts in treatment of various ailments in the society. It further shows the applicability of the plants as raw materials for medicament preparations. It is therefore recommended that more work on purification of active principles and the pharmacodynamics or pharmacokinetics be done.

AKNOWLEDGEMENTS

My profound gratitude goes to the Chief technologist of Pharmacognosy Department UNIBEN (Mrs F. Adesina) for her assistance especially in provision and constitution of phytochemical reagents and also to Mr. B. Osaze of Medical Microbiology Laboratory UBTH and to Dr. H.O. Uwumarongie for his role in authenticating the plant.

REFERENCES

Akinnibosun, F.I., Akinnibosun, H.A. and Ogedegbe, D. (2009). Investigation on the antibacterial activity of the aqueous and ethanolic extracts of the leaves of *Boerhavia diffusa* L. *Science World Journal* 4(2):1597-6343.

Apurba, S. A., Mahmuda, S. L., Jamaluddin, A.T., Amran, H. M, Repon, K. S., Farhana R. and Nishat, N. (2012). Phytochemical screening and *in vitro* bioactivities of the extracts of aerial part of *Boerhavia diffusa* Linn. *Asian Pacific Journal of Tropical Biomedicine*. 2(9): 673–678.

Ayepola, O.O. (2009). Evaluation of the Antimicrobial Activity of Root and Leaf Extracts of *Terminalia Glaucescens*. *Advances in Natural and Applied Sciences* 3(2): 188-191.

Chaudhary, G. and Dantu, P.K. (2011). Morphological, phytochemical and pharmacological, studies on *Boerhaavia diffusa* leave extracts. *Journal of Medicinal Plant Resources* 5(11): 2125–2130.

Edeoga, H.O., Okwu, D.E and Mbaebie, B.O. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology* 4(7): 685-688.

Firas, A., Bayati, L., Sulaiman, R. and Khudir, D.S. (2008). *In vitro* antimicrobial activity of *Salvadora persica* extracts against some isolated oral pathogens in Iraq. *Turkish Journal of Biol*ogy 32(409): 57-62.

Habamu, Y., Eguale, T., Wubete, A. and Sori, T. (2010). *In vitro* antimicrobial activity of some selected Ethiopian medicinal plants against some bacteria of veterinary importance. *African Journal of Microbiology Research* 4: 1230-1234.

Harbone, J.B. (1998). *Phytochemical methods: A guide to modern techniques of plant analysis*. 3rd Edition. Chapman and Hill, London. 279pp.

Nobmann, P., Smith, A., Dunne, J., Henehan, G. and Bourke, P. (2009). The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. *International Journal of Food Microbiology* 128(3): 440–445

Oduak, K. and Lawrence, C.E. (2008). Phytochemical screening of three selected chewing stick crude extract. *Journal of Asian Natural Products and Resources* 456 (76): 089-126

Okoli, R.I., Aigbe, O. Ohaju-Obodo, J.O. and Mensah, J.K. (2007). Medicinal herbs used for managing some common ailments among Esan people of Edo state, Nigeria. *Pakistan Journal of Nutrition* 6(5): 490–496.

Okwu, D.E. (2004). Phytochemicals and vitamin content of indigenous spices of South Eastern Nigeria. *Journal of Sustainable Agriculture and Environment* 6: 30-34.

Okwu, D.E. and Okwu, M.E. (2004). Chemical composition of *Spondias mombin* Linn.Plant parts. *Journal of Sustainable Agriculture and Environment* 6 (2): 140-147.

Oloyede, A.M., Aduramigba-Modupe, A.O., Efem, I.K. and Ejikeme, C. (2012). Evaluation of the antimicrobial and phytochemical properties of a herbal preparation. *Nature and Science* 10(7): 121-138.

Onwuliri, F.C. and Dawang, N.D. (2006). Antibacterial activity of aqueous and ethanolic leaf extract of drumstick plant (*Moringa oleifera* Lam.) on some bacterial species associated with gastrointestinal diseases. *Nigerian Journal of Botany* 19(2):272279.

Orisakwe, O.E., Afonne, O.J., Chude, M.A., Obi, E., Ilondu, N.A. and Dioka, C.E. (2003). Subchronic toxicity studies of the aqueous extract of *Boerhavia diffusa* leaves. *Journal of Health Science* 49(6): 444–447.

Prescott, L.M., Harley, J.P. and Klein, O.A. (2005). *Microbiology*. 6th edition. Mc-Graw Hills, New York.

Ramachandra, Y.L., Ashajyothi, C. and Padmalatha, R. (2012). *In vitro* antibacterial potential of *Boerhavia diffusa*. *International Journal of advances in Pharmacy, Biology and Chemistry* 1(3): 654-667.

Smith, M.A.L., Marley, K.A., Seigier, D., Sinletary, K.W. and Meline, B. (2000). "Bioactive properties of wild Blueberry fruits ". Journal of Food Science 65 (2): 543-449.

Tatsadjieu, L., Ngang, J., Ngassoum, M. and Etoa, F. (2003). Antibacterial and Antifungal activity of *Xylopia aethiopica*, *Monodora Myristica*, *Zanthoxylum Xanthoxyloides* and *Zanthoxylum Leprieurii* from Cameroon. *Fitoterapia* 74: 469-472.