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NATURAL DRUGS

PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITIES OF *VERNONIA AMBIGUA, VERNONIA BLUMEOIDES* AND *VERNONIA OOCEPHALA* (ASTERACEAE)

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Abstract: Some *Vernonia* species (*Vernonia ambigua, Vernonia blumeoides* and *Vernonia oocephala*) used in Northern Nigerian traditional medicine, were subjected to phytochemical screening using standard procedures. The antibacterial activity using the disc diffusion method as outlined by the NCCLS was carried out on *Klebsiella pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Corynbacterium ulcerans,* methicillin resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi, Pseudomonas aeruginosa, Shigella dysentriae, Proteus mirabilis* and *Pseudomonas fluorescence*. The results of the antibacterial activity as indicated by zone of growth inhibition ranged from 14 to 27 mm for the crude ethanol extracts and chloroform fractions of the *Vernonia* species being studied. The activity of chloroform fraction of *V. blumeoides* was higher on *C. ulcerans* and *K. pneumoniae* (27 mm), while the chloroform fractions of *V. oocephala* and *V. ambigua* were more active on *P. mirabilis* (27 mm) and *S. typhi* (22 mm), respectively. It is worth of mention that the chloroform fractions of the three *Vernonia* species demonstrated activity (20 mm) against MRSA. The minimum inhibitory concentration (MIC) values ranged from 1.25–2.5 mg/mL for all the organisms tested. The MIC of 1.25 mg/mL exhibited by the chloroform fractions on both Gram positive and negative bacteria indicates broad spectrum activity of the *Vernonia* species being studied. Phytochemical screening of the extracts/fractions revealed the presence of steroids/terpenes, saponins, flavonoids, alkaloids, tannins and glycosides. The antibacterial activity exhibited in this study may be attributed to flavonoids, saponinss or sesquiterpene lactones. The overall results indicate that the extracts/fractions are potent antibacterial preparations at least *in vitro*. This lends credence to the use of these plants for the treatment of various infectious diseases.

Keywords: phytochemical, antibacterial activities, *Vernonia ambigua, Vernonia blumeoides, Vernonia oocephala*, Asteraceae

The use of plant continues to play essential roles in traditional medicine for the treatment or management of various human diseases, especially in rural Africa where infectious diseases are endemic due to poverty and poor sanitations. In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of the children with high fevers, is the use of herbal medicines at home (1). The importance of plants in medicine remains even of greater relevance with the current global shift to obtain drugs from plant sources, as a result of which attention has been given to the medicinal value of herbal remedies for safety, efficacy and economy (2, 3). Plants constitute an important source of active natural products which differ widely in terms of structure and therapeutic properties. The continued investigation into the secondary plant metabolites for anti-infective agents has gained importance in recent years because of the alarming increase in resistance of pathogenic microorganisms to existing antibiotics. For instance, the incidence of methicillin resistant *Staphylococcus aureus* (MRSA) continue to increase tremendously across the globe and poses enormous therapeutic problems. In Nigeria, Kenya and Cameroon, the rate of MRSA spread was about 21–30% in 2003 (4). However, prevalence rate of

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about 69% of MRSA isolates was obtained in a study among healthy women in Zaria, Nigeria in 2005 (5). Multidrug resistant *Salmonella typhi* emerged in 1987 and has spread throughout the Indian subcontinent, South East Asia and sub-Saharan Africa (6). The worldwide escalation in both community and hospital-acquired antimicrobial-resistant bacteria is threatening the ability to effectively treat patients, emphasizing the need for continued surveillance, prudent infection control, and new treatment alternatives (7–9). Therefore, the need to develop efficient and safe drugs from plant sources is of great importance, because continued investigation of plants secondary metabolites has led to important breakthrough in pharmacology and has helped tremendously in the development of modern pharmacotherapeutics in Africa and other parts of the world (10, 11).

Vernonia species are important herbal recipes in African ethnomedicine, for instance in Madagascar *V. polytricholepis* has been widely used for fever and respiratory problems and *V. nudicaulis*, for venereal diseases (tea of the whole plant). The root of *V. nigritiana* is used in Senegal as an emetic, diuretic, used against fever, dysentery and intestinal worms. The Mongo tribes in Congo-DR use a bark decoction of *V. conferta* and *V. jugalis* as a bathe for bloody diarrhea. In Nigeria, *V. amygdalina* is used for gastrointestinal disorders, as a general tonic and appetite stimulant, for skin diseases and as a medication for fever, dysentery, malaria, diabetics and constipation (12–15). It is of particular interest to note that *Vernonia* species are widely used to treat malaria (16). Plants of the *Vernonia* are characterized by two or three whorls of pappus bristles on the achene, eligulate florets in generally oblong heads and many series of involucral bracts on the receptacle (17). These members of the Asteraceae family are important as weeds, ornaments and as green vegetables. They are referred to as the bitter genus. *Vernonia ambigua, Vernonia blumeoides* and *Vernonia oocephala* are distributed across Northern Nigeria and are widely used in traditional medicine for the treatment of various human ailments including parasitic (malaria) and infectious diseases (personal communication). Plants of the *Vernonia* genus produce characteristic compounds such as sesquiterpene lactones, with several reported biological activities, such as fungistatic (18), and cytotoxic activities (19, 20). Some other compounds have been isolated from *Vernonia*, including flavonoids (21), steroids (22) and polysaccharides (23). Because no pharmacological or phytochemical study has been reported on these *Vernonia* species, this work was designed to evaluate the phytochemical and antibacterial properties of the plants in order to establish the scientific basis for some of their therapeutic properties in folkloric use.

EXPERIMENTAL

Plant material

Fresh plants of *Vernonia ambigua, Vernonia blumeoides,* and *Vernonia oocephala* were purchased in medicinal plants market at Zaria city, Kaduna State, Nigeria in the month of September, 2008. The plants were taxonomically authenticated by U. S. Gallah at the herbarium of Department of Biological Sciences, Ahmadu Bello University, Zaria. Voucher specimen numbers 1137, 1784 and 1334, respectively, were deposited there for future reference. The aerial parts of plant samples were air-dried for two weeks and grounded to powder using mortar and pestle. The samples were kept in plastic container until required for analysis.

Extraction

A 250 g of the powdered sample (aerial part) of each plant was extracted exhaustively with 50% ethanol (cold extraction) for two weeks. The extract was filtered using Whatman filter paper no. 2, and concentrated at reduced pressure using the rotary evaporator at 45^{o}C, which afforded 12.5 g, 23.4 g and 25.5 g of aqueous ethanol extracts of *Vernonia ambigua* (VA), *Vernonia blumeoides* (VB) and *Vernonia oocephala* (VO), respectively. Ten grams of each crude extract was suspended in distilled water and partitioned with chloroform (200 mL × 3) which afforded 6.2, 5.5 and 6.8 g of the chloroform portions of VA, VB and VO, respectively.

Phytochemical screening

Phytochemical screening to detect the presence of secondary metabolites such as alkaloids, flavonoids, saponins, tannins, glycosides, anthraquinones, cardiac glycosides, steroids and triterpenes was carried out according to standard procedures as reported by Sofowora (24) and Evans (25).

Test for glycoside ($FeCl_3$ test)

To about 0.5 g of the extract/fraction, 5 mL of conc. H_2SO_4 was added and boiled for 15 min. This was then cooled and neutralized with 20% KOH. The solution was divided into two portions. Three drops of ferric chloride solution was added to one of the portions, and a green to black precipitate indicated phenolic aglycone as a result of hydrolysis of glycoside.

Test for free anthraquinones (Bontrager's test)

Small portion of the extract was shaken with 10 mL of benzene and filtered. Five milliliters of 10% NH_3 solution was added to the filtrate and stirred. The production of a pink-red or violet color indicates the presence of free anthraquinones.

Test for saponins (Frothing test)

Small quantity of the extract was dissolved in 10 mL of distilled water. This was then shaken vigorously for 30 s and was allowed to stand for 30 min. A honey comb formed for more than 30 min indicates saponins.

Test for steroids and triterpenes (Lieberman-Burchard's test)

Equal volume of acetic anhydride was added to the extract. One milliliter of conc. H_2SO_4 was added downside the tube and the color change was observed immediately and later. Red, pink or purple color indicates the presence of triterpenes while blue or blue-green indicates steroids.

Test for flavonoids (Shinoda test)

About 0.5 g of the extract was dissolved in 1.5 mL of 50% methanol and warmed on steam bath. Metallic magnesium and 5 drops of concentrated hydrochloric acid were added. A red or orange color indicates the presence of flavonoids aglycone.

Test for tannins (Ferric chloride test)

About 0.5 mL of extract was dissolved in 10 mL of distilled water and then filtered. Few drops of ferric chloride solution were added to the filtrate. Formation of a blue-black precipitate indicates hydrolyzable tannins and green precipitate indicates the presence of condensed tannins.

Test for alkaloids (Dragendoff's test)

To about 0.5 g of each extract, 1% diluted HCl (20 mL) was added in a conical flask, heated on a steam bath and then filtered. The filtrate was made alkaline with 28% NH_3 solution and then extracted with chloroform (3×5 cm^3). The combined $CHCl_3$ extracts were concentrated and treated with equal volume of 1% HCl. Dragendorff's reagents (2 mL) were added and occurence of orange-red precipitate indicated the presence of alkaloids.

Test organisms

Ten clinical bacterial strains: *Klebsiella pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Corynbacterium ulcerans,* methicillin resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi, Pseudomonas aeruginosa, Shigella dysentriae, Proteus mirabilis* and *Pseudomonas fluorescence* were obtained from the Department of Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH) Shika. The isolates were purified on nutrient agar (OXOID) plates and characterized using standard microbiological and biochemical procedures (26, 27). The MRSA strains used in this study were clinical isolates from urethral swab, seminal fluid, urine, high virginal swab, blood, skin and sputum of patients with symptoms of *S. aureus*-associated diseases. The isolates were identified by standard method (26). The disc diffusion method outlined by the NCCLS (28) was used with 1 µg oxacillin disk (Oxoid). The zones of inhibition were measured after incubation at 35°C for 24 h. Isolates with zones diameter = 10 mm were considered methicillin resistant. The organisms were maintained on agar slope at 4°C and subcultured for 24 h before use.

Determination of antibacterial activity

The disc diffusion method was used (29). Stock solution (100 mg/mL) of each extract and fractions were prepared using the extractants. Discs (6 mm diameter) were prepared using Whatman filter paper and sterilized by autoclaving. The blank sterile discs were placed on the inoculated Mueller Hinton Agar (MHA) surface and impregnated with 15 µL of stock solutions (1500 µg/dics). Antibiotic discs of ampiclox (75 µg/dics) and streptomycin (30 µg/dics) were used as positive control, whereas discs of the extracting solvents were used as negative control. The plates were incubated at 37°C for 24 h. All tests were performed in triplicate and the antibacterial activities were expressed as the mean diameter of inhibition zones (mm) produced by the plant extracts.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using micro-broth dilution methods as outlined by NCCLS (30). Dilutions (1–9 mg/mL) of concentrations of extract and fractions that exhibited sensitivity against the test organisms were prepared using test tubes containing 9 mL of double strength broth. The test tubes were inoculated with the suspension of the standardized inocula. These were incubated at 37°C for 24 h and observed for growth. The minimum inhibitory concentrations (MICs) of the extracts/fractions for each test organism were regarded as the lowest concentration that inhibited visible growth of the test organisms.

Table 1. Phytochemical screening of extracts.

Plant constituent	*Vernonia ambigua*		*Vernonia blumeoides*		*Vernonia oocephala*	
	EE	CF	EE	CF	EE	CF
Alkaloids	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Saponins	+	–	+	–	+	–
Tannins	+	-	+	–	+	–
Steroids/terpenes	+	+	+	+	+	+
Anthraquinones	–	–	–	–	–	–
Glycosides	+	–	+	–	+	–

EE = ethanol extract, CF = chloroform fraction, + = positive, and – = negative

Table 2. Antibacterial susceptibility test.

Test organisms	Zone of inhibition of growth (mm)							
	V. ambigua		*V. blumeoides*		*V. oocephala*		Ampiclox (75µg/dics)	Streptomycin (30 µg/disc)
	EE	CF	EE	CF	EE	CF		
S. aureus	18	20	22	24	18	20	20	27
MRSA	14	20	17	20	20	20	NT	NT
S. pyogenes	16	18	16	18	16	24	0	22
C. ulcerans	16	19	24	27	20	22	NT	NT
S. typhi	20	22	18	20	0	0	0	0
S. dysenteriae	0	0	14	18	0	0	NT	NT
P. mirabilis	0	0	0	0	22	27	NT	NT
P. aeruginosa	18	20	0	0	0	0	26	0
P. fluorescence	0	0	0	0	0	0	NT	NT
K. pneumoniae	16	19	22	27	20	25	0	20

EE = ethanol extract, CF = chloroform fraction, MRSA = methicillin resistant *Staphylococcus aureus*, NT = not tested

RESULTS

Phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids/terpenes and glycosides in the crude extracts/fractions of the *Vernonia* samples. However, saponins, tannins and glycosides were found absent in the chloroform fractions of all the samples. Anthraquinones were absent in the extracts and fractions of all the three *Vernonia* plants being investigated (Table 1). The results of the antibacterial activities showed that the plant extracts/fractions exhibit remarkable activity against the test organisms (*C. ulcerans, K. pneumonia, Staphylococcus aureus, P. mirabilis, S. typhi*, *S. dysentriae*, MRSA and *P. aeruginosa*) with zone of growth inhibition ranging from 14 to 27 mm. The susceptibilities of *P. aeruginosa, S. dysentriae* and *P. mirabilis* were only found in each case to the extract/fraction of *V. ambigua, V. blumeoides* and *V. oocephala*, respectively. The MIC values ranged from 1.25–2.5 mg/mL for all the organisms tested (Table 3). *P. fluorescence* was, however, resistant to the extracts/fractions of all the *Vernonia* samples (Table 2).

DISCUSSION AND CONCLUSION

Phytochemical screening of plant products or extracts aimed to evaluate the antibacterial properties may be of immense importance in the discovery of new therapeutic agents, especially at this time when the scientific community is preoccupied with searching for alternative treatment to combat the

Table 3. Determination of minimum inhibitory concentration (MIC).

Test organisms	Minimum inhibitory concentration (mg/mL)					
	Vernonia ambigua		*Vernonia bluemoides*		*Vernonia oocephala*	
	EE	CF	EE	CF	EE	CF
S. aureus	2.5	1.25	1.25	1.25	2.5	1.25
MRSA	2.5	1.25	2.5	1.25	1.25	1.25
S. pyogenes	2.5	2.5	2.5	2.5	2.5	1.25
C. ulcerans	2.5	2.5	1.25	1.25	1.25	1.25
S. typhi	1.25	1.25	2.5	1.25	NT	NT
S. dysenteriae	NT	NT	2.5	2.5	NT	NT
P. mirabilis	NT	NT	NT	NT	1.25	1.25
P. aeruginosa	1.25	1.25	NT	NT	NT	NT
K. pneumoniae	2.5	2.5	1.25	1.25	1.25	1.25

EE = ethanol extract, CF = chloroform fraction, MRSA = methicillin resistant *Staphylococcus aureus*

increasing threat of drug resistant microorganisms. The result of antibacterial evaluation of the aerial part of *Vernonia ambigua, Vernonia blumeoides* and *Vernonia oocephala* showed that the chloroform fractions were more active than the crude ethanol extracts against the test organisms (Table 2). Difference in polarity of solvents is perhaps responsible for the difference in solubility of plant active principles. It means that the antibacterial agent(s) have concentrated in the chloroform fractions hence variation in degree of activity. The susceptibility of *C. ulcerans* and *K. pneumonia* to chloroform fraction of *V. blumeoides* demonstrate higher (27 mm) antibacterial activity in terms of growth inhibition of the test organisms. It is interesting to note that the ethanol extract and chloroform fraction of *V. oocephala*, as well as the chloroform fractions of *V. ambigua* and *V. blumeoides*, demonstrated good antibacterial activity against the MRSA. The significance of this outcome is the efficacy at which the fractions demonstrate the observed activity, which may be a milestone in the continued search and development of newer drugs or phytotherapeutic agents against the MRSA.

The ethanol extracts and chloroform fractions of the *Vernonia* species demonstrated activity against both Gram negative (*P. aeruginosa*, *S. typhi*, *S. dysentriae* and *P. mirabilis*) and Gram positive bacteria (*S. aureus*, *C. ulcerans* and *S. pyogenes*) (Table 2). The susceptibility of *P. aeruginosa* to only the extract and fraction of *Vernonia ambigua* may be a pointer to its potential as a drug against this organism. Infections caused by *Pseudomonas* species such as mastitis are often difficult to combat (31). The fact that *P. mirabilis* was susceptible to only the extract/fraction of *V. oocephala* indicates the potency of the plant against diseases caused by the organism (Table 2). *Proteus mirabilis* is a pathogenic Gram-negative bacterium that frequently causes kidney infections, typically established by ascending colonization of the urinary tract (32, 33). The organism produces a variety of unique virulence factors that contribute to its pathogenicity and persistence in the human host (34).

Low MIC indicates the minimum inhibitory concentration required to inhibits the growth of the test organism, which translate to high potency of the extract or fraction. The MIC of 1.25 mg/mL exhibited by the chloroform fractions on both Gram positive and negative bacteria indicates broad spectrum activity of the *Vernonia* species being studied. The order of potency was: *V. oocephala* > *V. blumeoides* > *V. ambigua* (Table 3). The results of our findings compared or even surpassed the report of antibacterial activity of bark extracts of *V. tenoreana* which exhibits MICs of 10, 15 and 20 mg/mL for various test organisms (35). Similar report revealed the antibacterial potency of active leaf extract of *V. amygdalina* with MICs of 22.5–26.0 mg/mL and 19.8–26.4 mg/mL for the Gram-negative and Gram-positive isolates tested (36). Owing to their popular use as remedies for many infectious diseases, plants with secondary metabolites such as alkaloids, saponins, terpenoids, flavonoids, sesquiterpene lactones and steroids have been found to have antimicrobial properties *in vitro* (37–39). Thus higher antibacterial activity exhibited by the chloroform fractions in this study may be attributed to flavonoids or steroids/terpenes. These major chemical constituents have been identified and characterized

from most *Vernonia* species (40–44). Although the mechanisms of action of bioactive constituents of the *Vernonia* species being studied may be difficult to speculate; however, many antibacterial agents may exhibit their action through inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (45). It is probable that the antibacterial agent(s) in the fractions of the *Vernonia* might act *via* some of the above mechanisms.

The overall results indicate that the extracts and fractions are potent antibacterial preparations at least *in vitro*. However, *in vivo* evaluation may be required to ascertain that active concentrations of the extract when absorbed may remain bioactive for the time to completely kill the pathogens. Further phytochemical and pharmacological studies are challenging task in order to better understand the effects of these important pharmaceutical resources. Plants of the *Vernonia* genus (Asteraceae) may prove to be a rich source of compounds with potential antimicrobial activities. Bioactivity guided isolation, purification, characterization and structural elucidation of the active constituents from the *Vernonia* species is on-going in our laboratory.

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