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Evaluation of Malawian Vernonia glabra (Steetz)Vatke leaf and *Securidaca longepedunculata* (Fresen) root extracts for antimicrobial activities

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ABSTRACT

The aqueous, ethanol and acetone crude extracts and dichloromethane, hexane, ethyl acetate and n-butanol fractions of the *Securidaca longepedunculata* roots and *Vernonia glabra* leaves were studied for their antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans* using agar well diffusion method. The phytochemical presents, as well as the minimum inhibitory concentration (MIC) and minimum bacteriacidal/fungicidal concentration (MBC/MFC) of the extracts were also determined using standard methods. Results obtained indicated that *Vernonia glabra* leaves acetone extracts had excellent antimicrobial activity against *E. coli*, *P. aeruginosa* and ethanol extracts against *S. aureus*. The n-butanol fractions had the best activity against *E. coli* and *S. aureus*, dichloromethane fraction against *P. aeruginosa* and ethyl acetate fraction against *C. albicans*. For *S. longepedunculate* root ethanol extracts showed best activity against *E. coli*, acetone extract against *P. aeruginosa* and aqueous extract against *C. albicans*. The n-butanol fractions had best activity against *E. coli*, *P. aeruginosa* and *C. albicans*. These results verified the claims by traditional healers in Malawi that the plants extracts treats bacteria related ailments such as diarrhoea and could be a potential source for development of phytomedicine.

INTRODUCTION

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 (Gurib-Fakim, 2006). Multiple drug resistance has become a real problem in pharmacotherapeutics due to an increasing number of diseases exhibiting various levels of drug resistance (Henry, 2000). Furthermore, the development of synthetic drugs has slowed down as a result of drug resistance necessitating investigation of new antimicrobial drug (Shah, 2005). Consequently, this has created a new renewed interest in the search for new drugs in order to combat resistance.

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Herbal medications and phytochemical screening of various plant species for medicinal leads are now receiving much attention. Some of the herbal medicines that are being considered as a source of new antibacterial drugs have been time-tested for thousands of years and are comparatively safe for both human use and the environment (Fazly-Bazzaz et al., 2005). The plant genetic resource base in Africa has an enormous potential to provide diverse chemical, enzymes and genes that has remained unexploited at industrial scale for production and design of new pharmaceutical products (Cragg and Newman, 2001).

Securidaca longepedunculata belongs to the family of Polygalaceae widely distributed in Western and Southern Africa and almost all the parts of the plant are reported to be used in disease management (Ojewole, 2008). The plant is a savanna shrub with twisted bole or slender erect branches and grows up to 30ft high and in Malawi, the leaves and roots are used to treat wounds, coughs, venereal diseases, diarrhoea, snake bites, bilharzias and

other ailments (Morris, 1991; Msonthi and Magombo, 1983). *Vernonia glabra* belongs to the family of Asteraceae and is herbaceous perennial plant with flowers grouped in dense clusters at the tip of the stem and grows up to 4-5ft high. It is widely used in Malawi for treatment of pneumonia and stomach ailments (Morris, 1991). The aim of the present investigation was to evaluate the antibacterial and antifungal effects of *S. longepedunculata* roots and *Vernonia glabra* leaves extracts on four pathogens *(Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans) that* have been. proven to be major causal organisms of various human infections. And the study was carried out along with the standard drugs (Chloromphenical for bacteria and Fluconazole for fungus).

MATERIAL AND METHODS

Plant material

The medicinal plants *Vernonia glabra* and *S. longepedunculata* were collected from Zomba and Machinga districts and identified by Mr. I.H. Patel at Malawi Herbarium and Botanical Gardens with voucher specimen numbers 34810(Masiye, Zomba 15˚19□S 35˚18□E) and 887(EJ Tawakali and I.H. Patel, Machinga 15˚07□S 35˚27□E) respectively. The roots and leaves were separately shade dried, finely powdered using a blender and kept in airtight polyethylene bags at room temperature in the dark until used.

Microorganisms

Escherichia coli, Pseudomonas aeruginosa, *Staphylococcus aureus* and *Candida albicans* were obtained from the Department of Microbiology, University of Malawi. The test strains were maintained on nutrient agar slant at 4ºC and sub-cultured on nutrient broth for 24hrs prior to testing.

Direct extraction and solvent-solvent extraction of plant material

One gram of finely powdered sample was extracted three times with 10ml of solvents (acetone, ethanol and distilled water) with vigorous shaking. The extracts were decanted after centrifuging at 5300 x g for 10 minutes and solvents removed at room temperature (Eloff, 1998).

For solvent-solvent extraction, 100grams of finely powdered plant material was extracted with 1 litre of acetone (technical grade-Merck) in macerating bottle. The bottle was shaken for 1hr, 6hr and 24hr on shaking machine and extracts decanted. Six grams of the extract collected was fractionated using solvents of varying polarities (dichloromethane, hexane, butanol, ethyl acetate, aqueous methanol and water). All the solvents were removed under reduced pressure using rotary evaporator at 45ºC and dried under room temperature.

Determination of antimicrobial activity

The agar well diffusion method (Trease and Evans, 1983) was used to assay the extracts for antimicrobial activity. 0.2ml of 1 in 100 dilutions of bacterial and fungal cultures (2.5×10^5 cfu ml^{-1}) was added to 20ml of the melted and cooled Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) respectively. The contents were mixed by gentle swirling movements before being poured into sterile petri dishes. After solidification of agar, wells (6mm) was bored in each plate. 100µl of each extract dissolved in acetone was poured into appropriately labelled well.

Diameter of zones of inhibitions were determined as an indication of activity after incubating the plate at 37ºC for 24hrs for bacteria and at 25ºC for 72hrs for fungi. Acetone was included in each plate as negative control while chloromphenical and fluconazole were used as positive control for bacteria and fungi respectively. Activity index for each extract was calculated.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal (MBC/MFC)

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity. Modified micro broth dilution method (Basri and Fan, 2005) was followed for determination of MIC values. A loopful of the bacterial and fungal cultures from the slant were inoculated in the nutrient broth and incubated at 37 ± 1^0C for 24 hours for bacteria and at 25ºC for 72hrs for fungi. The fresh broth (20ml) was seeded with 0.25ml of the 24 hour broth cultures and a two-fold serial dilution method followed. The extracts were dissolved in acetone to obtain a 10mg/ml solution. A 0.2ml solution of the plant extract was added to 1.8ml of the seeded broth to forms the first dilution. 1ml of first dilution was diluted further with 1ml of the seeded broth to produce the second dilution, and the process repeated until six such dilutions were obtained. A set of tubes containing only seeded broth were kept as control and suitable solvent controls also maintained. After incubation for 24 hours at 37 ± 1^0C, the tube with no visible growth of the microorganism was taken to represent the Minimum Inhibitory Concentration (MIC) of the test sample which was expressed in mg/ml.

Determination of Total activity (TA)

Total Activity is the volume at which test extract can be diluted with the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1g plant material by the MIC of the same extract or compound isolated and expressed in ml/g (Eloff, 2004).

Phytochemical screening

The plant extracts were screened for the presence of flavonoids, Alkaloids, Saponins Terpenoids, Steroids and sterols, Tannins and Phenols using Sofowora and Harborne methods. Flavonoids were identified by adding 5ml of dilute ammonia solution to an aqueous filtrate of the extract, followed by the addition of concentrated sulphuric acid. A yellow coloration observed in the extract indicated the presence of flavonoids. Alkaloids were tested by adding a few drops of acetic acid to 5ml of an extract followed by modified Dragendroff's reagent

(potassium iodide mixed with bismuth oxynitrate) and mixed well. An orange red precipitate formed indicated the presence of alkaloids. Saponins were tested by boiling 2 grams of the powdered sample in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate mixed with 5ml of distilled water and was shaken vigorously for a suitable persistent froth. The frothing mixed with 3 drops of olive oil and shaken vigorously, and formation of emulsion observed indicated the presence of saponins. Terpenoids detection was carried out by mixing 5ml of plant extract with 2ml of chloroform and concentrated sulphuric acid added to form a layer. A reddish brown coloration of the interface formed showed the presence of terpenoids. Steroids and sterols were identified by adding 2ml of acetic anhydride to 0.5g of plant extract with 2ml of sulphuric acid. Colour change from violet to blue green in the sample indicated the presence of steroids and sterols. Tannins were identified by boiling 0.5gram of the powdered sample in 20ml of distilled water in a test tube and then filtered. A few drops of 0.1% ferric chloride added and observed for brownish green or a blue-black coloration indicated the presence of tannins. And phenols were identified by adding few drops of drops of methanol and ferric chloride solution to the plant extract mixed. A blue green or red colour indicated the presence of phenol.

Determination of phytoconstituents by High Performance Liquid Chromatography (HPLC)

Further determination of phytoconstituents were performed using a Shimadzo LC-10AD HPLC system(Japan), equipped with a Shimadzo SPD-10AV UV-VIS Spectrophotometer detector with a thermostted flow cell and a selectable two wavelengths of 190-370nm or 371-600nm with SCL -10A system control. The detector signal was operated at ultraviolet wavelength detection at 254nm. An Agilent ZORBEX SB-C18 (Agilent Technologies, USA) column (3.5µm, 4.6mm × 150mm, i.d.) was used for chromatographic separation. The injection volume of 20µL was used. The isocratic mobile phase comprised methanol: acetonitrile (60:40). Analysis was performed at a flow rate of 0.6mL/min. 10mg of the plant extracts were dissolved in appropriate solvents filtered through Whatmann paper No. 1 into volumetric flask and made up to 25ml.

RESULTS

Antimicrobial activity (assessed in terms of inhibition zone and activity index) of the plant extracts tested against different microorganisms were recorded in Table 1. Excellent activity for *V. glabra* were observed in crude extracts of acetone IZ: 13mm, AI: 0.5±0.07, ethanol IZ: 15mm, AI: 0.68±0.06 and acetone IZ: 14mm, AI: 0.54±0.04 for *E. coli, S. aureus, P. aeruginosa* and *C. albicans* respectively.and fractions of n-butanol IZ: 17mm, AI: 0.63±0.17 and IZ: 12mm, AI: 0.46±0.07, dichloromethane and ethyl acetate IZ: 12mm, AI: 0.46±0.07 and ethyl acetate IZ: 15mm, AI: 0.58±0.27 for *E. coli, S. aureus, P. aeruginosa* and *C. albicans* respectively. For *S. longepedunculate*, the best activity were observed in crude extracts of ethanol IZ: 12mm, AI: 0.40±0.07, acetone IZ: 14mm, AI: 0.47±0.02 and aqueous IZ: 10mm, AI: 0.33±0.22 for *E. coli, P. aeruginosa* and *C. albicans* respectively and for fractions of n-butanol IZ: 11mm, AI: 0.42±0.23, IZ: 13mm, AI: 0.50 and IZ: 12mm, AI: 0.42±0.22 for *E. coli, P. aeruginosa* and *C. albicans* respectively. There was no significant activity reported against *S. aureus* for *S. longepedunculate* root extracts.

The range of MIC and MBC/MFC of extract evaluated for bioactivity in diffusion assay recorded were in Table 2. For *V. glabra* crude extracts, the lowest MIC values were recorded in acetone for S. *aureus* MIC 0.3125mg/ml while in fractions of n-butanol for *P. aeruginosa* MIC 0.156mg/ml. For *S. longepedunculate*, the lowest MIC values were recorded in acetone for S. *aureus* MIC 0.3125mg/ml while in fractions of n-butanol for *E. coli,* MIC 0.3125mg/ml.

Amount of extracts isolated from plant parts and total activity (TA) were calculated and recorded in Table 3a and 3b. Total activity is the volume at which test extract can be diluted with the ability to kill microorganisms. Ethanol was best solvent for extracting compounds from both *V. glabra* (105.2mg/g) and *S. longepedunculate* (185.8mg/g). Total activity (TA) for *V. glabra* obtained for *P. aeruginosa* were 168ml/g while for *S. longepedunculate*, aqueous extracts were 136ml/g, 272ml/g and 136ml/g for *E. coli, S. aureus* and *P. aeruginosa* respectively.

The acetone extract was resolved into 6 different fractions as shown in a schematic representation of the solvent-solvent resolution in Figure 1. Hexane was best solvent for solvent-solvent resolution from both *V. glabra* (403.2mg/g) and *S. longepedunculate* (571mg/g). n-butanol fractions total activity (TA) for *V. glabra* obtained were 154.56ml/g, 1.61% loss for *E. coli,* 645.12ml/g, 3.36%loss for *S. aureus* and 1238ml/g, 25.8%loss for *P. aeruginosa* while for *S. longepedunculate*, ethyl acetate fractions were 636.8ml/g, 3.3% loss for, *S. aureus and* 1731.2ml/g, 72.13%loss, 865.6ml/g, 4.5%loss and 432.8ml/g, 9.01%loss for *E. coli, P. aeruginosa* and *C. albicans* respectively.
The qualitative phytochemical screening of the *V. glabra* leaves and *S. longepedunculate* root extracts showed the presence of alkaloids, flavonoids, saponins, steroids and phytosterols, and phenolic compounds as in Table 4. Flavonoids and saponins were more abundant in *S. longepedunculate* root extracts compared to *V. glabra* leaves extract; this confirms the presence of the phytoconstituents responsible for antimicrobial activity in the leaf and root bark extracts.

The best way for chemical characterization of herbal compounds is HPLC fingerprint analysis as it provides qualitative and quantitative information on the herbal plant extracts (Bauer, A.W., et al. 1960). In the present investigation, analysis of ethanol extracts of *V. glabra* leaves and *S. longepedunculate* roots showed 9 and 11 fingerprint profiles peaks respectively as indicated in Figure 2 and 3. *V. glabra* leaves fingerprint profile showed 1 major peak at 1.89min(92.057%) while *S. longepedunculate* roots showed 2 major peaks at 1.77min(85.819% and 2.45min(12.056%).

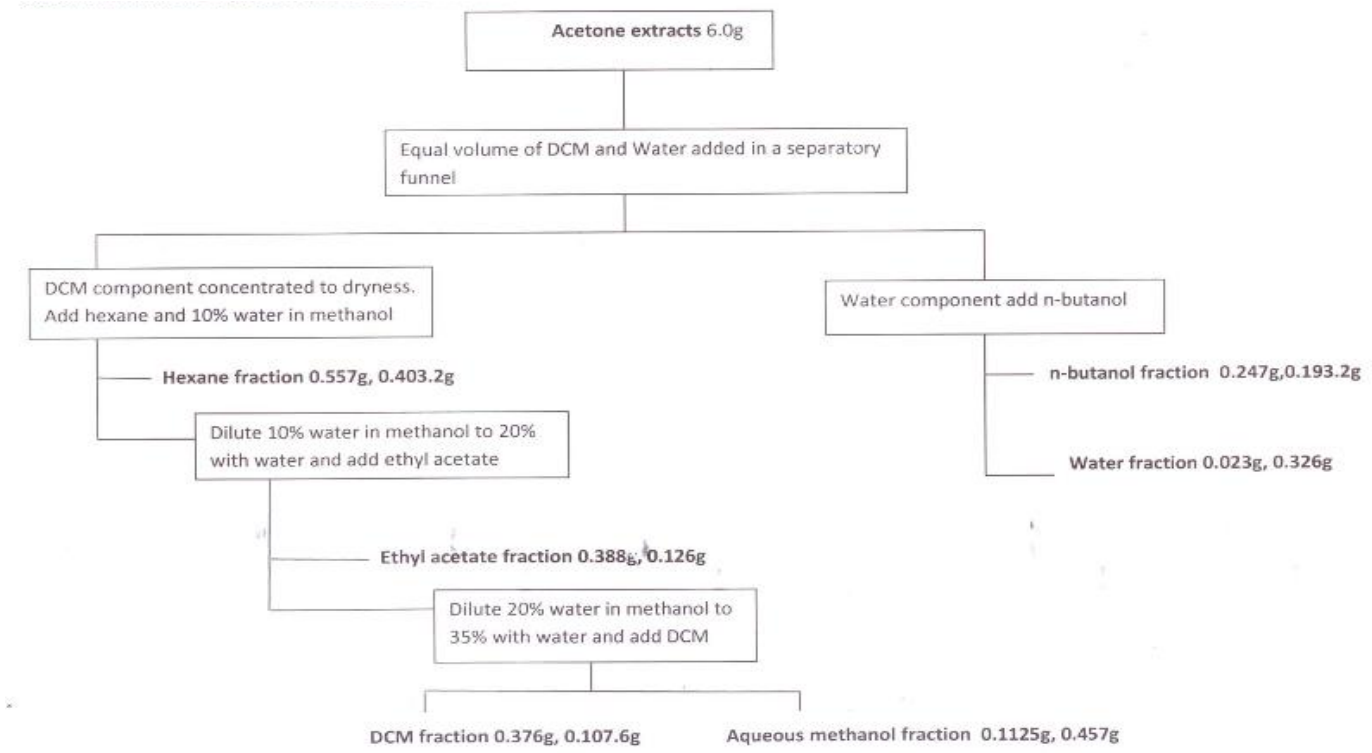


Fig. 1: stepwise procedure for fractionation of *V.glabra* (steetz) vatke leaf and *Securdaca longepedunculata* (fresen) foot extracts. Text in bold represent final collected fractions.

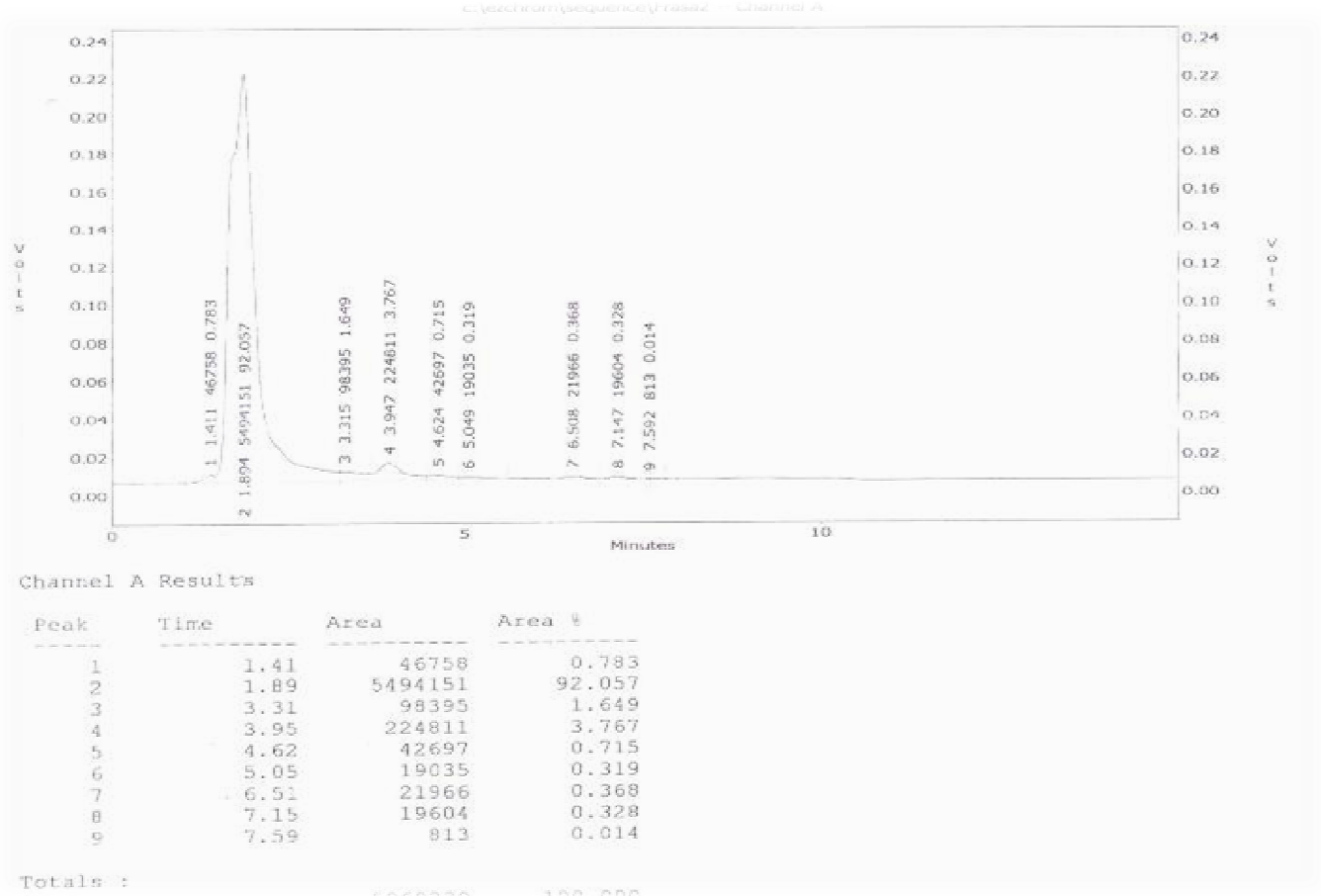
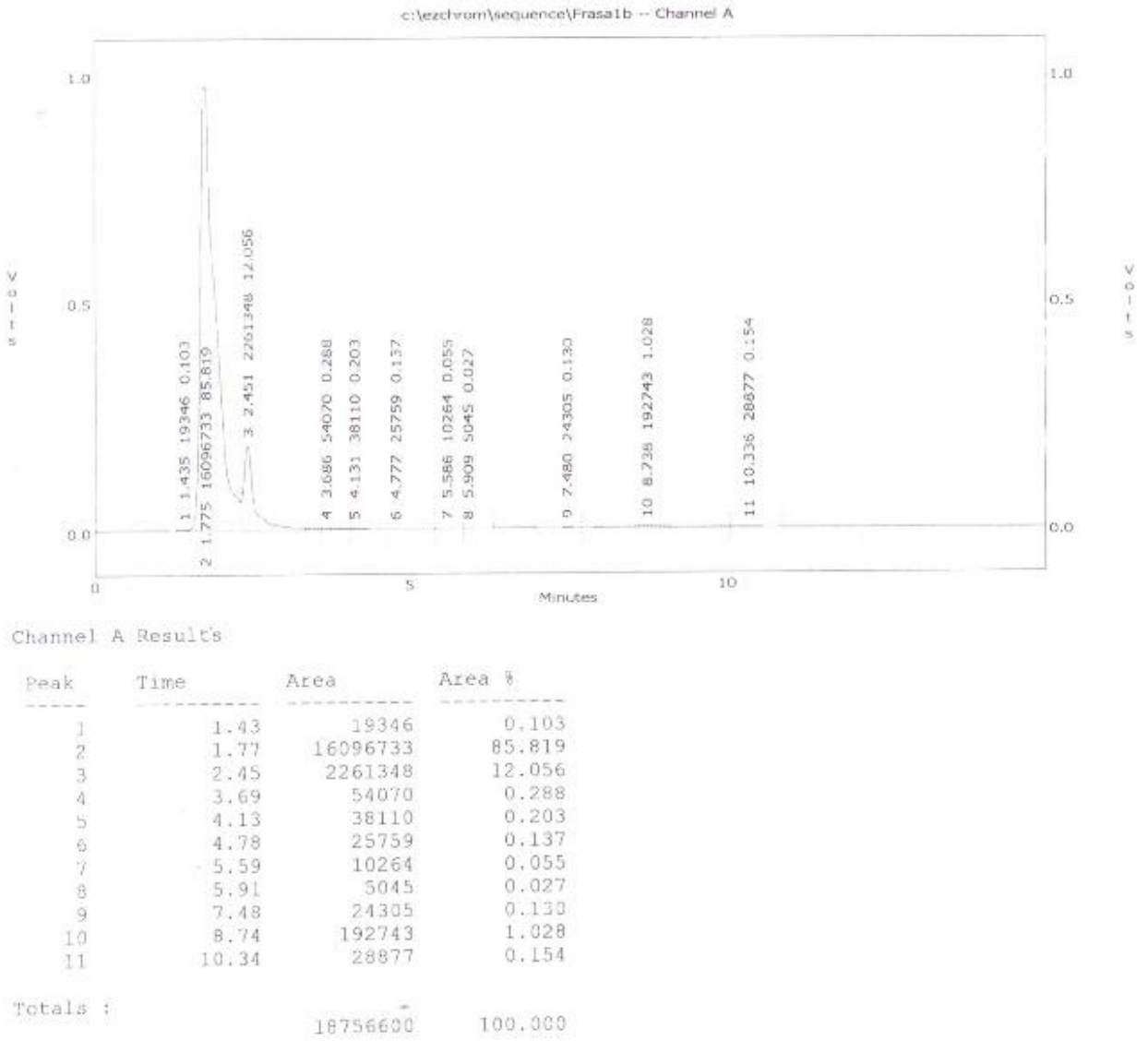


Fig. 2: HPLC fingerprint of *Vernonia glabra (*Steetz)Vatke leaf extracts.



Channel A Results

Peak	Time	Area	Area %
1	1.43	19346	0.103
2	1.77	16096733	85.819
3	2.45	2261348	12.056
4	3.69	54070	0.288
5	4.13	38110	0.203
6	4.78	25759	0.137
7	5.59	10264	0.055
8	5.91	5045	0.027
9	7.48	24305	0.130
10	8.74	192743	1.028
11	10.34	28877	0.154
Totals :		18756600	100.000

Fig. 3: HPLC fingerprint of *Securidaca longepedunculata (Fresen) root* extracts.

Table. 1: Antimicrobial activity of crude extracts and fractions of *Vernonia glabra (*Steetz)Vatke leaf and *Securidaca longepedunculata* (Fresen) root extracts.

Plant	Plant part	Extract	Test microorganism							
			E. coli		*S. aureus*		*P. aeruginosa*		*C. albicans*	
			IZ(mm)	AI	IZ(mm)	AI	IZ(mm)	AI	IZ(mm)	AI
V. glabra	Leaves	Acetone	13	0.5±0.07	12	0.46±0.08	14	0.54±0.04	-	-
		Ethanol	9	0.3±0.05	15	0.68±0.09	10	0.33±0.09	-	-
		Aqueous	11	0.52±0.12	9	0.50±0.03	12	0.43±0.12	9	0.3±0.18
		DCM fraction	15	0.58	10	0.38	12	0.46±0.12	13	-
		hexane fraction	8	0.31	-	-	-	-	7	0.27
		n-butanol fraction	17	0.65	12	0.46	9	0.35	13	0.5
		Ethyl acetate fraction	13	0.5	9	0.35	12	0.46	15	0.58
S. longepedunculata	Root	Acetone	10	0.33±0.08	8	0.4±0.03	14	0.47±0.02	-	-
		Ethanol	12	0.4±0.07	9	0.45±0.07	8	0.27±0.06	-	-
		Aqueous	7	0.33±0.25	7	0.33±0.21	7	0.39±0.15	10	0.33±0.22
		DCM fraction	8	0.31	9	0.35	11	0.42	9	0.35
		hexane fraction	-	-	9	0.35	-	-	-	-
		n-butanol fraction	11	0.42±0.23	-	-	13	0.5	12	0.42±0.22
		Ethyl acetate fraction	10	0.38	12	0.46±0.01	11	0.42	9	0.35

IZ = Inhibition zone in mm (mean value; including 6mm diameter of hole/disc), AI = Activity Index (IZ developed by extract/IZ developed by standard), ± = SEM, (-) = No activity, Extracts assayed in triplicate,

Table. 2: MIC and MBC/MFC values of crude extracts and fractions of *Vernonia glabra (*Steetz)Vatke leaf and *Securidaca longepedunculata* (Fresen) root extracts

Plant	Plant part	Extract	Test microorganism							
			E. coli		*S. aureus*		*P. aeruginosa*		*C. albicans*	
			*MIC	*MBC	*MIC	*MBC	*MIC	*MBC	*MIC	*MFC
V. glabra	Leaves	Acetone	0.625	1.25	0.313	0.625	1.25	2.50	-	-
		Ethanol	1.25	2.5	1.25	2.50	0.625	1.25	-	-
		Aqueous	2.5	5	0.625	1.25	0.625	1.25	-	-
		DCM fraction	-	-	0.625	1.25	0.3125	0.625	1.25	
		n-hexane fraction	-	-	-	-	-	-	-	-
		n-butanol fraction	1.25	2.5	0.625	1.25	0.156	0.3125	-	-
		Ethyl acetate fraction	2.5	5	0.625	1.25	0.625	1.25	2.5	-
S. longeped unculata	Root	Acetone	2.5	5	0.313	0.625	0.3125	0.625	1.25	2.5
		Ethanol	5.00	10.00	2.5	5	2.5	2.5	-	-
		Aqueous	1.25	2.5	0.625	1.25	1.25	2.5	-	-
		DCM fraction	0.625	1.25	1.25	2.5	0.625	1.25	2.5	5
		hexane fraction	2.5	5	-	-	-	-	-	-
		n-butanol fraction	0.3125	0.625	1.25	2.5	0.625	1.25	1.25	2.5
		Ethyl acetate fraction	0.625	1.25	0.625	1.25	1.25	2.5	-	-

**in mg/ml*

MIC= Minimum Inhibitory Concentration, MBC/MFC= Minimum Bactericidal/Fungicidal Concentration

Table 3a: Quantity and Total Activity of *Vernonia glabra (*Steetz)Vatke leaf and *Securidaca longepedunculata* (Fresen) root extracts

Plant	Plant part	Extract	Amount of extract mg/g dried plant part	Total Activity(ml/g)			
				E. coli	*S. aureus*	*P. aeruginosa*	*C. albicans*
V. glabra	Leaves	Acetone	39.2	62.7	125.4	31.4	-
		Ethanol	105.2	84.2	84.2	168.3	-
		Aqueous	102	-	-	-	-
S. longepedun culata	Root	Acetone	34.8	-	111.4	111.4	-
		Ethanol	185.8	37.2	74.3	74.3	-
		Aqueous	170	136	272	136	-

Total Activity = $\frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$

Table 3b: Quantity and Total Activity of fractions of *Vernonia glabra (*Steetz)Vatke leaf and *Securidaca longepedunculata* (Fresen) root extracts

Plant	Plant part	Extract	Amount of extract (mg)	Total Activity(ml/g) and % loss							
				E. coli		*S. aureus*		*P. aeruginosa*		*C. albicans*	
				TA	% Loss	TA	%Loss	TA	%Loss	TA	%Loss
V. glabra	Leaves	Acetone extract	6000	9600		19200		4800		-	
		DCM fraction	107.6	-	-	172.16	0.9	344.32	7.17	-	-
		hexane fraction	403.2	-	-	-	-	-	-	-	-
		n-butanol fraction	193.2	154.56	1.61	645.12	3.36	1238.46	25.8	-	-
		Ethyl acetate fraction	126	50.4	0.5	201.6	1.05	201.6	4.2	-	-
S. longepedun culata	Root	Acetone extract	6000	2400		19200		19200		4800	
		DCM fraction	464.2	742.72	30.95	371.36	1.9	742.72	3.87	185.68	3.87
		hexane fraction	571	228.4	9.52	-	-	-	-	-	-
		n-butanol fraction	541	1731.2	72.13	432.8	2.25	865.6	4.5	432.8	9.01
		Ethyl acetate fraction	398	636.8	26.5	636.8	3.3	318.4	1.66	-	-

Total Activity = $\frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$

Table. 4: Data showing the preliminary phytochemical screening of *Vernonia glabra (*Steetz)Vatke leaf and *Securidaca longepedunculata (Fresen) root* extracts

Phytochemical	*Vernonia glabra*	*Securidaca longepedunculata*
Flavonoids	+++	+++
Alkaloids	+	++
Saponin	++	+++
Steroids & Phytosterols	+	+
Tannin	-	+
Phenolic compounds	+	++
Terpenoids	-	+

DISCUSSIONS

Results of the present study showed that ethanol and acetone crude extracts and dichloromethane, ethyl acetate and n-butanol fractions of *V. glabra* leaves and *S. longepedunculate* roots extracts tested inhibited the growth of selected bacteria and fungi, indicating the broad spectrum bioactive nature against *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*. The high antimicrobial activity of *S. longepedunculate* roots extracts were also reported by Ajali and Chukwu(2004), Arnold and Gulumian(1984) which attributed these activities to the presence of flavonoids and saponins while antimicrobial activity of *V. glabra* were reported by Achola and Munenge(1996) and antitumour activity by Jakupovic, (1985). Flavonoids are known to be synthesized by the plants in response to microbial infection in nature so it is not surprising in this research that they are very effective against a wide array of microorganisms (Bennett, R.N., Wallsgrove, R.M., 1994). Their activity is probably due to their ability to complex with extracellular and soluble proteins and other components of cell walls. Similarly, the lipophilic nature of flavonoids may also disrupt microbial membrane (Tsuchiya, et al., 1996).

It was also observed that most of the extracts were active against Gram positive bacteria due to the fact that Gram negative bacteria have a cell wall outer membrane which appears to act as a barrier to many substances including synthetic and natural antibiotics (Tortora et al., 2001).

In the present investigation, IZ, AI, MIC, MBC/MFC and TA have been evaluated for *V. glabra* leaves and *S. longepedunculate* roots extracts. For most of the extracts MIC values recorded were very low indicating strong bioefficacy. It can be observed from the results that the plant-based traditional medicine system continues to play an essential role in health care with about 80% of the world inhabitants relying mainly on traditional medicine for their primary health care (Farnsworth et al., 1985). Consequently, there is need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plant resources.

CONCLUSION

The *V. glabra* leaves and *S. longepedunculate* roots extracts and fractions displayed potent and relevant pharmacological activities with considerable antibacterial and antifungal activity against selected Gram positive and Gram negative bacteria and fungi. The results obtained show that the compound could be of pharmaceutical interest for therapeutical application as complementary antibacterial and antifungal agents in infectious disease. The fingerprint profile obtained by HPLC can further be used for identification of phytoconstituents understudy. Therefore, further work is recommended on crude extracts and fractionation of bioactive compounds to unravel its structures and indicate their exact potential to inhibit several pathogenic microbes and development of a novel broad spectrum antimicrobial herbal formulation. Also, further evaluation of the cytotoxicity effect of the compounds and extracts needs to be undertaken to justify claimed use by traditional healers to treat various diseases.

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