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ASSESSMENT OF SECONDARY METABOLITES AND ANTIMICROBIAL ACTIVITIES OF FOUR SOLVENT EXTRACTS OF *Vernonia amygdalina* LEAVES ON SOME SELECTED PATHOGENIC MICROORGANISMS

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ABSTRACT:
Acetone, Hexane, Ethylacetate and Acetone / Hexane / Ethylacetate (50/30/20 v/v/v) extracts of *Vernonia amygdalina* leaves (Del, belonging to the family Asteraceae) were investigated for antimicrobial activities. Three different concentrations (100 mg/mL, 50 mg/mL and 25 mg/mL) of each extract were used against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25920 and *Candida albicans*. The disc diffusion method was used. Phytochemical screening and High Performance Liquid Chromatography (HPLC) analysis of constituents of the extracts were also carried out. Highest yield (14.25 %) after extraction was obtained for acetone extract followed by hexane extract (8.2 %), Acetone/Hexane/Ethylacetate extract (7.4 %) and ethylacetate (7.1 %) extract. Tannin, steroids, saponin, terpenoid, flavonoid, anthraquinones were present in each of the solvent extracts. Coumarin was present in both acetone and Acetone/Hexane/Ethylacetate extracts while glycosides were present only in the Acetone/Hexane/Ethylacetate extract. Protein was present in ethylacetate extract and absent in the other solvent extracts. The extracts revealed dose-dependent activities against all the test organisms. Highest range of inhibition zone (15.00 mm- 18.00 mm) was obtained for Acetone/Hexane/Ethylacetate extract at 25 mg/mL against the test organisms while lowest range of inhibition zone (7.00 mm- 10.00 mm) was obtained for acetone extract at 100 mg/mL. Various compounds (Oxalate, Phyrate, Epivernodalol, Vernodalol, Venonioside A, Vernonioside B, Vernodalin, Luteolin, Andrographolide, Andrographoside) were separated at different peak heights (mV) and at varying retention times from all the solvent extracts. The solvent extracts of *Vernonia amygdalina* can serve as good alternative to orthodox medicine in the treatment of infections associated with the three test organisms.

Keywords: *V. amygdalina*, Phyrate, Vernodalol, Pathogens, Antimicrobial activities, HPLC

INTRODUCTION:

The skin and mucous membrane are common routes of invasion of most pathogenic microorganisms, posing serious public health problems such as increased morbidity in patients that undergo surgical operations in developing countries [1,2], higher cost of health maintenance and even death. Some microorganisms associated with skin infections are Gram positive bacteria such as *Staphylococcus aureus* and Corynebacterium species, Gram negative bacteria such as *Escherichia coli* and *Enterobacter aerogenes* and yeast, *Candida albicans* [3].

Despite the vast production of a number of new antibiotics by pharmacological industries against pathogens, resistance among microorganisms has increased, probably due inappropriate use of the drugs resulting in the genetic ability of these microorganisms to transmit and adopt resistance to those drugs [4]. About 80 % of the populations in developing countries rely on medicinal plants [5,6] as alternative to orthodox medicine in their health care delivery system. In Nigeria, the majorities of citizens use medicinal plants and visit traditional medicine practitioners for their health care needs [7]. The greater interest in herbal medicine may be due to certain limitations of orthodox medicines in terms of high cost, limited availability, adulteration and associated toxicity [8].

Natural antimicrobials have been chosen from numerous medicinal plant parts (such as leaves, stems, barks, roots, bulks, corms, rhizomes, woods, flowers, fruits or the seeds). These parts are known to contain the highest concentration of active constituents which play important roles in bioactivity of medicinal plants and are used as new effective agents against drug resistance strains of microorganisms [9].

Medicinal herbs are commonly administered in many forms of preparations such as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such preparations have been popularly called galenicals, named after Galen, the second century Greek physician [10]. Some preparations are consumed in liquid form by the patient, as a tisane or a (possibly diluted) plant extract [11]. Determination of biologically active constituents from medicinal plants largely depend on plant part used as starting material, solvent used for extraction and extraction procedure [12]. Properties of a good solvent in plant extractions include, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate [13] . Organic solvents such as ethanol, acetone, and methanol are often used to extract bioactive compounds. A study reported that extraction of tannins and other phenolics was better in

aqueous-acetone than in aqueous-methanol [10,14]. Both acetone and methanol were found to extract saponins which have antimicrobial activity [12].

Vernonia amygdalina Del, commonly called bitter leaf, belongs to the family Asteraceae. It is a perennial plant predominantly found in Africa. It is known in different parts of Nigeria as '*Ewuro'* among the Yorubas, '*Onugbu'* among the Igbos and '*Chusar-doki*' or '*fatefate'* among the Hausas. Many Nigerian households attribute many folkloric uses to the leaves of *V. amygdalina*, including culinary herb in soup, as food vegetable, digestive tonic and as appetizer. Hausa females of Nigeria also consume *V. amygdalina* infusion to enhance sexual adequacies. A number of scientific findings have been reported on *V. amygdalina*. The plant had been reported to possess antimalarial, antimicrobial, antidiabetic, laxative, febrifuge, antihelminth properties [15]. The leaves have also been reported to have wound healing properties [16]. Oyugi *et al*. [17] showed that fractions of hexane, chloroform and n-butanol extracts of *V. amygdalina* inhibited the growth of human breast cancer cells even at concentrations of 25 mg/mL.

Despite the available information on *V. amygdalina*, the search for more information on medicinal plants has continued unabated. In addition, it is important to know that the type of solvent used in extraction of plants affect the type and amount of constituents detected in their extracts. Hence, the present study aimed at using different solvents with varying polarities to extract *V. amygdalina* leaves, determining the antimicrobial activities of the different solvent extracts of the plant, screening the different solvent extracts for secondary metabolites using preliminary phytochemical screening method and HPLC method to separate the active components in the solvent extracts.

METHODOLOGY:

Plant materials:

Fresh leaves of *Vernonia amygdalina* were harvested in Adewole area, Ilorin, Kwara State, Nigeria. They were authenticated at Herbarium Unit of Department of Plant Biology, University of Ilorin, where a voucher specimen was deposited (UILH/007/972).

Preparation of the crude extracts:

The leaves were carefully washed under running water and distilled water to get rid of dust particles and sand. They were completely dried in aerated place for 3-4 days and pulverized into fine powder using electric miller (Master Chef Blender, Mode MC-BL 1980, China). Crude extract of the leaves was obtained by soaking the powdered leaves in different solvents (1:10 w/v of plant material to solvent) of varying polarities; acetone (most polar), hexane (medium polar), ethyl acetate (least polar) and a combination of the three solvents (acetone/ hexane/ ethylacetate 5:3:2 v/v/v). The extraction was done for 48 hours at

room temperature with shaking at 150 rpm [18]. The homogenate obtained was subsequently filtered through Whatman No. 1 filter paper [19] and the filtrate was evaporated to dryness using rotary evaporator (Model RE Zhengzhou, Henan China). The concentrated extracts were then collected, weighed, packed in sterile air tight containers and labeled. Percentage yield of the extract was calculated and recorded. They were kept in the refrigerator at 4 ºC until needed for analysis.

Preparation of samples for HPLC:

Powdered dried leaves (1.0 g) of *V. amygdalina* were macerated with acetontrite/water (1:1; v/v, 10.0 mL), then centrifuged at 3000 rpm for 10 minutes. The material was filtered and the crude extract obtained was analyzed directly by HPLC-UV.

Sterility testing:

This was carried out using a modified method of Sule and Agbabiaka [20]. Serial dilution of 1.0 g of each extract was made to reduce the concentration. A quantity (2 ml) of each extract was inoculated into 10 mL of Muller Hinton broth, incubated at 37 ⁰ C for 24 hours. The absence of turbidity or clearness of the broth indicated sterility of the extract.

Preparation of stock solution of the extracts:

Diluent of 5.0 % Dimethylsulphoxide (DMSO) was prepared by adding 95 mL of water to 5 mL of DMSO. Stock solution of the extract was prepared by dissolving 2.0 g of extract into sterile test tube containing 20.0 mL of 5.0 % DMSO. The test tube was labeled as 100.0 mg/mL from which other working concentrations (50.0 mg/mL and 25.0 mg/mL) were prepared.

Preparation and Impregnation of discs:

Paper discs were prepared from good quality filter paper (What man No. 1). The discs were autoclaved for 15 minutes at 15 lbs pressure and allowed to cool. About 5 pieces of the sterilized paper discs were arranged using sterile forceps in sterile petri-dishes. Sterilized discs were aseptically impregnated with approximately 20 µl of 100 mg/mL concentrations of each solvent extract using mechanical pipettes. More sterilized discs were also aseptically impregnated with approximately 20 µl of other concentrations (50 mg/mL and 25 mg/mL) respectively. All impregnated discs were allowed to fully dry in incubator at 45 ⁰ C for 18-24 hours and were stored in labeled airtight containers at 20 ° C till they were ready for use [21]. The standard antibiotic discs used as positive control were Chloramphenicol and Gentamycin (Sigma, Saint Louis, MO, USA) for the bacterial strains and griseofulvin (Bristol, New York, USA) for yeast.

Test Organisms:

Three isolates used in this study were *Staphylococcus aureus* ATCC 25923, a Gram-

positive organism, *Escherichia coli* ATCC 25920, a Gram-negative Organism and a yeast, *Candida albicans*. They were obtained from the Microbiology Laboratory of University of Ilorin Teaching Hospital, maintained on Nutrient agar slant for the bacteria and Sabouraud Dextrose Agar slant for the yeast. They were cultured and sub-cultured to check for their purity, then their morphology and Gram staining was carried out to confirm their identity. The inocula were standardized using McFarland standard [22].

Preparation of McFarland turbidity standard:

This was prepared by mixing 99.5 ml of 1% sulphuric acid and 0.5 ml of 1.175% w/v barium chloride ($BaCl_2.H_2O$). The mixture was dispensed in 3-4 ml amount in tube.

Standardization of Inocula:

Colonies from microbial culture (3-5 colonies) was aseptically suspended into 5 mL of Mueller-Hinton Broth (MHB) using a sterile loop and incubated at 37 °C for 18 hours and yeast into Sabouraud Dextrose Broth (SDB) at 30 °C incubation period. The microbial growth were harvested using the same broth medium as used earlier (0.1 mL of the innoculum into 19.9 mL of the broth), the absorbance was adjusted at 580 µm and diluted to 0.5 McFarland turbidity equivalence (1.5×10^8 CFU/ mL) to obtain equivalence of 2×10^6 CFU/ mL for bacteria and 2×10^5 spore / mL for the yeast. This was used within 20 minutes of standardization [23].

Antimicrobial activities of the solvent extracts:

Antimicrobial activity of each solvent extract was tested using a modified disc diffusion method described by Ncube *et al*. [12]; a sterile cotton swab was dipped into the standardized *Escherichia coli* ATCC 25920 inoculums, pressed against the inside wall of the test tube to remove excess fluid and streaked uniformly on the surface of the solidified 20 mL Mueller-Hinton agar (MHA; Becton-Dickson, USA) for bacteria and Sabouraud Dextrose agar (SDA) for the yeast. The streaking was carefully done three times. The plates were rotated about 60 degrees to ensure even distribution of the inoculums on the agar surface, then allowed to dry for 5 min. Sterile filter paper discs loaded with the plant extract (100 mg/mL concentration) was placed on top of Mueller-Hilton agar plate (90 mm plate) using sterile forceps, the discs were pressed gently to ensure uniform contact with the agar surface. This was allowed to stand on the bench for 30 minutes for proper diffusion of extract from the disc into the inoculated medium and thereafter incubated at 37 °C for 24 hours for bacteria and at 30 ° C for 48 hours for *C. albicans*. This was repeated for *Staphylococcus aureus ATCC 25923* and for other concentrations (50 mg/mL and 25 mg/mL respectively). Control standard discs of chloramphenicol, gentamicine and griseofulvin for bacteria and yeast were set up. The diameters of zones of inhibition around the

discs were measured using a Vernier caliper (mm) and recorded. This study on the extracts and controls were carried out in triplicates to ensure reliability and the average of three replicates was calculated and recorded.

HPLC analysis:

A modular Shimadzu (Nexeramx) LC-10 system comprised of a LC-10AD pump, a CTO-10A column oven, a SPD-10A UV-DAD detector, a CBM-10A interface and a LC-10 Workstation was used. A LC-18 column (250 mm x 4.6 mm ID x 5 mm) from (Ubondapak , Bellefonte, USA) was employed at 30 ° C. Separations were done in the isocratic mode, using acetonitrile: water (40:60; v/v) at a flow rate of 1.0 mL per minute with an injection volume ("loop") of 10 µL, UV detection was at 254 nm.

RESULTS:

The percentage yield with the different solvents and the physical properties of the solvent extracts after evaporation are presented in Table 1. The *In vitro* antimicrobial assay result of different concentrations of the various solvent extracts against the test organisms is represented in Figure 1. A total of nine phytochemical constituents (Tannin, steroid, saponin, terpenoid, flavonoid, anthraquinones, protein, glycosides and coumarins) were screened for. Table 2 shows the constituents extracted by the four solvents used. The components separated by HPLC of the four solvent extracts of *V. amygdalina* leaves were visualized in the form of peaks in chromatograms. The results of the retention time and peak heights of the various components are presented in Table 3.

Table 1. Percentage yield of *V. amygdalina* Leaves after Evaporation

Solvents	Dry raw powder of plant (g)	Plant extract yield from dry raw powder (g)	Plant extract percentage yield (%)	Color
Acetone	200	28.5	14.25	Green
Hexane	200	16.4	8.2	Green
Ethylacetate	200	14.2	7.1	Green
Acetone / Hexane / Ethylacetate	200	14.8	7.4	Brown

Fig.1. In vitro Antimicrobial Effects of Different Concentrations of Various Solvent Extracts of *V. amygdalina* leaves against some Selected Pathogens

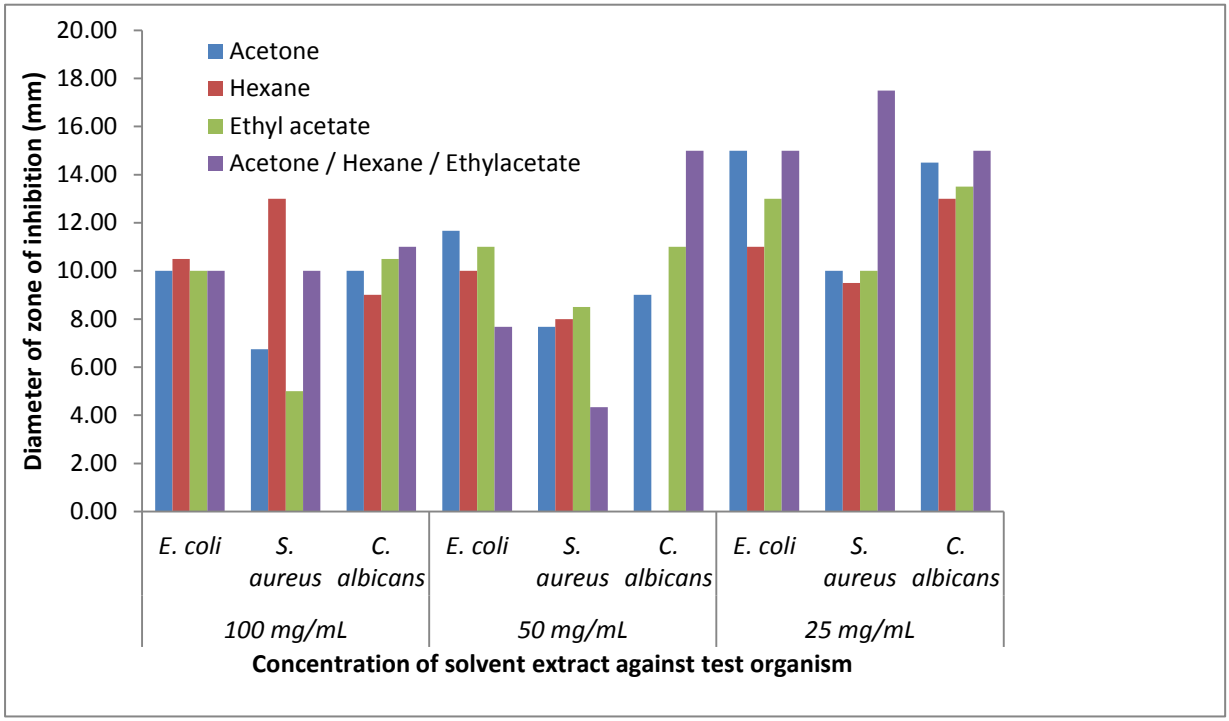

Table 2. Phytochemical constituents of four solvent extracts of *V. amygdalina* leaves

Composition	Acetone	Hexane	Ethylacetate	Acetone / Hexane / Ethylacetate
Tannin	+	+	+	+
Steroid	+	+	+	+
Saponin	+	+	+	+
Glycosides	–	–	–	+
Terpenoid	+	+	+	+
Protein	–	–	+	–
Flavonoid	+	+	+	+
Anthraquinones	+	+	+	+
Coumarins	+	–	–	+

Key: + = Present; – = Absent

Table 3. Retention time and peak heights in the different solvent extracts of *V. amygdalina* leaves separated at different intervals by HPLC method

COMPONENTS	Acetone		Ethylacetate		Hexane		Acetone / Hexane / Ethylacetate	
	Retention (Mins)	Peak height (mV)	Retention (Mins)	Peak height (mV)	Retention (Mins)	Peak height (mV)	Retention (Mins)	PEAK HEIGHT (mV)
OXALATE	1.266	34.054	1.266	33.477	1.267	33.348	1.266	32.751
PHYRATE	2.516	24.219	2.516	22.367	2.516	21.953	2.516	20.039
EPIVERNODALOL	2.750	24.994	2.750	22.904	2.750	22.437	2.750	20.278
VERNODALOL	4.450	11.959	4.450	8.136	4.450	7.281	4.450	3.333
VERNONIOSIDE A	4.700	11.786	4.700	7.707	4.700	6.796	*	*
VERNONIOSIDE B	5.466	6.921	5.466	2.215	*	*	*	*
VERNODALIN	11.050	158.213	11.050	153.998	12.166	47.515	11.050	149.459
LUTEOLIN	12.166	62.440	12.166	54.557	12.166	62.440	12.166	45.596
ANDROGRAPHOLIDE	13.700	38.239	13.700	26.675	13.700	16.345	13.700	9.278
ANDROGRAPHOSIDE	17.616	5.504	17.616	4.264	17.616	1.766	17.533	1.489

*= absent

DISCUSSION:

Findings in this study revealed that highest yield was obtained for acetone extract more than other solvent extracts. This is in accordance with the report of Sengel *et al*. [24] who reported that acetone was effective at extracting more phytoconstituents from plants than other solvents.

Tannin, steroid, saponin, terpenoid, protein, flavonoid, anthraquinones, glycosides and coumarins detected in the different solvent extracts in this work have been reported to play significant roles in bioactivity of medicinal plants since medicinal values of plant lie in these phytochemical compounds which produced a definite and specific action on the human body [25]. This result is also in line with the study conducted by Imaga *et al*. [26] who detected the presence of flavonoid, tannins, saponins, glycosides and terpenoids as the most predominant constituent in *Vernonia amygdalina* leaves extracts. Secondary metabolites (tannins, saponins, steroids, flavonoids, steroid, glycoside, anthraquinones, terpenoids) are suspected to be responsible for the bitter taste of this plant and they exert antimicrobial activity through different mechanism [27]. Flavonoids constituent exhibited a wide range of biological activities like antimicrobial, anti-inflammatory, antiangionic, analgesic, anti-allergic, cystostatic and antioxidant properties, anticancer activities [28, 29]. Hence, the compounds detected in the extracts may be responsible for the antimicrobial activities presented by zones of

inhibition observed against pathogenic microorganisms in this work.

Previous reports are on extraction of phenolic compounds typically from plant material using solvents as methanol, ethanol, acetone and ethyl acetate [30,31]. However, in the present study, the four solvents used for extraction of compounds in *V. amygdalina* leaves have been shown to affect the quantity and quality of the components separated by HPLC. This agrees with several studies that characterized bioactive compounds from *V. amygdalina* as flavonoids, saponins, alkaloids, tannins, phenolics, terpenes, steroidal glycosides, triterpenoids, and several types of sesquiterpene lactones [32]. In conformity with compounds separated in this work are the reports of Sobrinho *et al*. [33] and Erasto *et al*. [34] who variously revealed that the main bioactive constituents of the leaves of *V. amygdalina* are sesquiterpene lactones, vernonioside A1, vernonioside A2, vernonioside B1, vernonioside B2, vernodalin, vernolepin, vernomygdin, vernodalol, and vernodalinol. Oxalate, phytate, luteolin, andrographolide and andrographoside were detected in this study but were not listed in the reports given by those researchers.

Constituents were separated at sharper height peaks from acetone extracts more than from other solvents extracts. This is in concurrence with the study conducted by Michiels *et al*. [35] where acetone-based mixtures were also more effective solvents than the methanol-based mixtures for phenolic extraction yields from fruits and vegetables. However, in the report of Tomsone *et al*. [36], the best solvents for phenolic extraction from horseradish roots were ethanol and ethanol/water solutions [36].

Vernodalol, Vernonioside A, Vernonioside B and Vernodalin separated in the different solvent extracts of *V. amygdalina* leaves in this study justified their antimicrobial activities as presented by zones of inhibition obtained against the test organisms. Amodu *et al*. [37] and Luo *et al*. [38] variously reported that sesquiterpene lactones (vernodalinol, vernolepin, vernomygdin, hydroxyvernolide, vernolide and vernodalol) possessed antitumoral and antimicrobial properties, and exhibited significant bactericidal activity against bacteria. Isolated vernoniosides from *Vernonia amygdalina* leaves exhibited anti-inflammatory property and was used in the treatment of gastrointestinal disorder when tested with murine macrophage cell line and wild chimpanzees, respectively [39]. Presence of phenolic compounds in plants has been reported to play important role in shaping the biological properties of plants including antioxidant and antimicrobial properties [40].

All the solvent extracts of *Vernonia amygdalina* leaves showed varying concentration dependent activities against the test pathogens. Gill and Holley [41] reported that antimicrobial components (terpenoid, alkaloid and phenolic compounds) of plant extracts

interact with enzymes and proteins of cell membrane of microorganisms causing its disruption to disperse a flux of protons towards cell exterior which induces cell death or may inhibit enzymes necessary for amino acids biosynthesis. Findings in this study agrees with the findings of Owu *et al*. [42] who also reported dose-dependent contraction of ileums of guinea pigs induced with gastric secretion from aqueous extracts of *V. amygdalina*. However, lowest concentration (25 mg/mL) of all the solvent extracts showed higher activities with wider zones of inhibition against the tested pathogens than other concentrations (50 mg/mL and 100 mg/ mL), this may imply that the plant is highly potent even at reduced dosage.

Although, zones of inhibition obtained by the plant extracts was not as high as what was produced by the commercial drugs. The small molecular size possessed by antibiotics aids their solubility in diluents as this could enhance their penetration through the cell wall into the cytoplasm of the organism. Doughari *et al*. [43] reported that since antibiotics is in a refined state and plant extract in crude state, a higher antimicrobial activity may be recorded for commercial drugs.

CONCLUSION:

It could be deduced from this study that *V. amygdalina* leaves contain active constituents of pharmacological significance and extracts from the plant should be consumed at much reduced concentration so as to have a better treatment margin and reduced risk of dosage induced-toxicity. Results presented in this study also indicates that HPLC is a useful tool for assessment of components of different solvent extracts of plants, components which if properly screened could be harvested and used in the development of new drugs.

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