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Antibacterial Activity of *Verbena officinalis* (Linn) Crude Extract against Some Human Pathogens

Samuel Sahile[1], Metadel Habtamu[2], Subramanian Chandrodyam[1]*

[1]Post Box 196, Department of Biology, College of Natural and Computational Sciences, University of Gondar, Ethiopia

[2]Post Box 309, College of Teacher Education, Gondar, Ethiopia

*Corresponding Author E-mail: wlbsasu@gmail.com

ABSTRACT

The antibacterial effect of crude methanol extracts of V. officinalis was examined against six clinical isolates and standard bacterial strains using agar well diffusion and disc diffusion methods. The leaf extracts inhibited S. boydii, S. pneumoniae, and S. aureus and the root extract had moderate inhibition zones against S. boydii and MRSA. Strains like S. boydii, S. pneumoniae and S. aureus were more sensitive to methanol extracts than E. coli, P. aeruginosa and MRSA. The leaf extract was found to be more effective to inhibit the organism than the root extract. The MIC value of the leaf extracts equal to one dilution factor less than MIC value of root extract.

Key words: *Antibacterial, Extracts, Inhibition Zone, Human pathogen, V. officinalis*

INTRODUCTION

Nature has been a source of medicinal agents and a vast assay of illness and medicinal complaints has always been a real part of human condition[1]. The WHO[2] estimated that 80 % of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Globally there has-been an increased interest to identify compounds that are pharmacologically potent with low or no side effects for use in therapeutic purposes[3]. The remarkable contribution of plants to the drug industries was possible, because of large number of the phytochemical and biological studies all over the world[4].

Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism[5&6]. These compounds have significant therapeutic application against human pathogens including bacteria. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds[7&8]. Therefore, medicinal plants are finding their way into pharmaceuticals and food Supplements.

Recent progress to discover drugs from natural sources has resulted in compounds that are being developed to treat cancer, resistant bacteria and viruses and immunosuppressive disorders. Antimicrobial resistance is a major cause of significant morbidity and mortality globally. Ethno-medicine provides avenues for identification of compounds with antimicrobial properties and potential new antibiotics.

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Ethiopia has a significant portion of two of the world's biodiversity rich areas hot spot *i.e.* the eastern Afro-mountain Biodiversity Hotspot and the Horn of Africa-Biodiversity Hot Spot (Conservation International at www. biodiversityhotspots.org). these hotspots house a lot of the useful wild biodiversity, particularly which of medicinal plants[9]. More than one thousand identified medicinal plant species are reported in the Ethiopian Flora, however, many others are not yet identified. About 300 of these species are frequently mentioned in many sources. Ethiopia has rich medicinal plant lore and points out that almost all plants of the Ethiopian flora are used somewhere somehow medicinally[10].

Crude extracts of some Ethiopian medicinal plants show strong antimicrobial activities indicating that Ethiopian plants can serve as sources of effective drugs against diseases causing microorganisms. Therefore, the study of the antibacterial effects of *V. officinalis* may give a clue for the search of new antimicrobial agents for the treatment of infectious diseases in Ethiopia. Anti-inflammatory and anti-fungal properties of *V. officinalis* leaf extraction against plant pathogens were studied. Although *V. officinalis* is a valuable medicinal plant showing several biological activities, there hasn't been any significant study have been carried out to investigate the knowledge, on the crude extract of leaf and root of this species. The objective of this study was to assess the antimicrobial activities of methanol root and leaf extract of *V. officinalis in vitro* against some human pathogen.

MATERIAL AND METHODS

This cross sectional experiment was undertaken at University of Gondar, located in the North Western Ethiopia.

Plant material collection and extract preparation

V. officinalis is a perennial herb, usually of open habitats or bare ground on freely-draining, often calcareous soils. It is most frequent in rough grassland and scrub, on roadsides, and on sheltered coastal cliffs and rock outcrops; less often in quarries and gravel-pits, and on streamside, wood-borders and walls. The leaves and roots of *V. officinalis* were collected from the Genet Mountain situated at 13°09' North latitude and 37°28' East longitude with an altitude of 2160 masl. The voucher specimens were identified by using standard manuals[11]. The plant materials were washed using distilled water and hundred grams of root and leaf were cut into 1.5 cm pieces then combined with 500 ml of 1:10 aqueous methanol solvent. The mixture was pulverized with a stomacher for 2 min and mixed with solvent 500 ml methanol were added[12].

Test Organisms

Standard strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 6538), as well as Clinical isolates of *Steptococcus pneumoniae, MRSA and Shigella boydii* species were collected from Biomedical and Laboratory Science Research Center, University of Gondar and Ethiopian Health and Nutrition Research Institute (EHNRI). The microorganisms were kept at 4 ^{0}C for further use.

Standard Antibiotics

Gentamicin (10 µg/µl) in the form of liquid and Amoxicillin paper disc (10 µg/ml) was used as a positive control[13].

Inocula Preparation

The inocula preparation was carried out by direct colony suspension methods[13]. Three to five pure colonies were selected from an agar plate culture and blood agar for *S. pneumoniae* overnight culture. They were transferred in to a sterile capped glass tube containing 5 ml of sterile normal saline solutions and mixed by a vortex mixer. 0.5 McFarland turbidity standards were adjusted visually by comparing the test tubes. This was done by placing the inoculum tube and 0.5 McFarland standards against a paper with a white background and contrasting black lines in adequate light.

Agar Well Diffusion Method

Susceptibility tests were performed by agar-well diffusion method using the standard procedure[14]. For Disc diffusion the extracts were screened out for antimicrobial activity using the disk diffusion technique standard procedure[13].

Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentrations (MIC) of crude leaf and root extracts of the *V. officinalis* was performed using two fold broth dilution methods recommended by Committee for Clinical Laboratory Standards[13] and Determination of Minimum Bactericidal Concentration was recorded following standard procedures.

Data Analysis

ANOVA was used for determination of the antimicrobial susceptibility test of phytochemical extraction in the specific concentration. Values were expressed as mean plus or minus standard error of the mean (M ± SEM) by using SPSS version 20, 0 and presented in tables. P-values less than 0.05 were taken as statistically significant[15].

RESULTS

Agar well diffusion assay of the crude leaf and root extracts of *V. officenalis*

The antibacterial susceptibility of the crude leaf and root extracts of *V. officenalis* was observed using agar well and disc diffusion method by measuring the diameter of the growth inhibition zone (Table 1).

Table 1: Zone of inhibition at different concentrations of crud leaves and roots extract fractions of *V. officenalis* on *S .boydii*

Extract	Concentration(mg/ml)	Diameter of Zone of inhibition (mm)	
		IZE	Gentamicin
Root	100	2.39 ± 0.82	28.94±0.68
	200	3.74 ± 0.41	28.94±0.68
	300	3.97 ± 0.77	28.94±0.68
	400	4.86 ± 0.98	28.94±0.68
	500	13.73 ± 1.25	28.94±0.68
Leaf	100	1.85 ± 0.37	28.94±0.68
	200	3.26 ± 0.48	28.94±0.68
	300	6.08 ± 0.88	28.94±0.68
	400	10.21 ± 0.91	28.94±0.68
	500	14.58 ± 1.32	28.94±0.68

Mean ±SEM, P ≤ 0.05, IZE: Inhibition Zone of Extract

The mean zone of inhibition at different concentration levels of crude leaf and root extract fractions of *V. officenalis* increased proportionally as concentration increased which was statistically significant ($p< 0.05$) as shown in table 1. The range of inhibition zone observed from *V. officenalis* root extract ranged from (2.39 ± 0.82 mm) to (13.73 ± 1.25 mm) and leaf extract was ranged from (1.85 ± 0.37 mm) to (14.58 ± 1.32 mm) at 100 % (W/V) leaf extract indicates (2.39 ± 0.82 mm) followed by 200% (W/V) (3.74 ± 0.41 mm) which indicates as concentration increase IZD increases and its similar for root extract as directly proportional to the amount of concentrations.

Table 2: The antibacterial effect of leaf and root extract fractions of *V. officenalis* against some test organism using agar well diffusion method

Extract	Organisms	Diameter of Zone of inhibition (mm)			Mean $\bar{X}$	SEM	Gentamicin IZD(mm)
		A	B	C			
Leaf	*E. coli*	13.93	10.95	16.37	13.75	±1.56	37.58
	P. aeruginosa	15.63	16.4	13.89	15.33	±0.74	31.23
	S. boydii	22.73	20.98	19.84	21.18	±0.84	33.73
	S. aureus	19.73	20.59	19.84	20.08	±0.27	35.25
	S. pneumoniae	19.97	22.26	20.35	20.86	±0.70	27.27
	MRSA	13.82	14.84	12.61	13.75	±0.64	28.71
	$\ddot{X}$				15.52	±1.56	
	E. coli	8.97	9.88	10.68	9.84	±0.49	37.58
	P. aeruginosa	12.43	16.10	10.30	12.94	±1.69	31.23
	S. boydii	18.43	16.10	15.50	16.61	±0.93	33.73

Root	*S. aureus*	14.30	17.20	15.32	15.57	±0.81	35.25
	S. pneumoniae	19.88	13.43	15.33	16.21	±1.91	27.27
	MRSA	13.70	8.87	11.20	11.25	±1.39	28.71
	Ẍ				13.73	±1.25	

Mean ±SEM, P ≤0.05, IZD: Inhibition Zone Diameter, A: first test B: second test C: third test Ẍ: grand mean

As indicated in table 2, *V. officenalis* leaf extracts showed high degree of inhibition against the tested bacterial strains (21.18 ± 0.84 mm) and root extract had moderate inhibition zones (16.61 ± 0.93 mm), respectively. Which are statistically significant (p< 0.05) to both root and leaves extracts.

Among test strain most susceptible for extracts of leaf was *S .boydii* (21.18 ± 0.84mm**),** followed by *S. pneumoniae* (20.86 ± 0.70mm), *S. aureus* (ATCC 6538) (20.08 ± 0.27mm), followed by *P. aeruginosa* (ATCC 27853) (15.33 ± 0.74 mm), *MRSA* (13.75 ±0.64 mm), *and E. coli* (ATCC 25922) (9.84 ± 0.49) *respectively.* This was statistically significant (p < 0.05). Similarly, the most susceptible clinical strains for extracts of root was the same with leaf that is *S. boydii* (16.61 ± 0.93 mm), followed by, *S. pneumoniae (*16.21 ± 1.91mm), *S. aureus* (ATCC 6538) (15.57 ± 0.81mm) followed by *P. aeruginosa* (ATCC 27853) (12.94 ± 1.69 mm), *E. coli* (ATCC 25922) (13.75 ± 1.56mm) and *MRSA* (11.25 ± 1.39 mm) and this association was statistically significant (p < 0.05) from table 3.

Table 3: Zone of inhibition against test organisms of leaf and root extract fractions of *V. officenalis*

Extracts	Organisms	Diameter of Zone of inhibition (mm)	
		IZE	Gentamicin
Leaf	*E. coli***	13.75±1.56	37.58 ± 0.67
	*P. aeruginosa***	15.3±0.74	33.73 ± 0.71
	*S. boydii**	21.18±0.84	31.23 ± 0.50
	*S. aureus***	20.08±0.27	35.25 ± 0.4
	*S. pneumoniae**	20.86±0.70	27.27±0.68
	*MRSA**	13.75±0.64	28.71±0.32
Root	*E. coli***	9.84±0.49	37.58 ± 0.67
	*P. aeruginosa***	12.94±1.69	33.73 ± 0.71
	*S. boydii**	16.61±0.93	31.23 ± 0.50
	*S. aureus***	15.57±0.81	35.25 ± 0.4
	*S. pneumoniae**	16.21±1.91	27.27±0.68
	*MRSA**	11.25±1.39	28.71±0.32

**Clinical isolates, ***Standard strains, Mean ±SEM, P ≤0.05, IZD: Inhibition Zone Diameter*

Agar disc diffusion assay of the crude root and leaf extracts of *V. officenalis*

As indicated from table 4, *V. officenalis* leaf extracts showed high degree of inhibition against the tested bacterial strains (9.83 ± 0.36 mm) and root extract had moderate inhibition zones (8.75 ± 1.03 mm), respectively. Which are statistically significant (p<0.05) to both root and leaf extracts. The range of inhibition zone observed from *V. officenalis* leaf extract ranged from (1.20 ± 0.73mm) *MRSA* to (9.83 ± 0.36 mm) *S. aureus* and root extract was ranged from (3.18 ± 1.89 mm) *MRSA* to (8.75 ±1.03 mm) *S. boydii*.

Table 4: Antibacterial effect of leaf and root extract fractions of *V. officenalis* against test organism using agar disc diffusion assay

Extract	Organisms	Diameter of Zone of inhibition (mm)			Mean	SEM	Amoxicillin IZD (mm)
		A	B	C	*X*		
Leaf	*E.coli*	5.64	3.12	4.55	4.43	±0.72	16.32±1.21
	P.aeruginosa	0.0	7.27	6.32	4.53	±2.28	-
	S.boydii	8.05	11.10	10.00	9.71	±0.89	26.90±0.45
	S.aureus	10.24	9.10	10.17	9.83	±0.36	24.75±0.64
	S. pneumoniae	9.60	8.33	9.76	9.23	±0.45	26.26±0.70
	MRSA	0.0	2.54	1.08	1.20	±0.73	15.42±0.97
	Ẍ				6.49	±0.88	

	E. coli	5.42	2.10	4.3	3.91	±0.97	16.32±1.21
	P. aeruginosa	4.71	8.16	6.21	6.36	±0.99	-
	S. boydii	6.97	10.56	8.73	8.75	±1.03	26.90±0.45
	S. aureus	10.78	7.12	8.31	8.73	±1.07	24.75±0.64
	S. pneumoniae	7.64	7.71	7.53	7.62	±0.52	26.26±0.70
Root	*MRSA*	6.54	3	0	3.18	±1.89	15.42±0.97
	Ẍ				6.43	±0.66	

Mean ±SEM, P ≤ 0.05, IZE Inhibition Zone of Extract, A: first test B: second test C: third test Ẍ: grand mean

Among the test strains, for extracts of leaf *S. aureus* (ATCC 6538) was the most susceptible (9.83 ± 0.36 mm), followed by *S .boydii* (9.71 ± 0.89 mm**),** *S. pneumoniae* (9.23 ± 0.45 mm), *P. aeruginosa* (ATCC27853) (4.53 ± 2.28 mm), *E. coli* (ATCC 25922) (4.43 ± 0.72mm) and *MRSA* (1.20 ± 0.73 mm). This was statistically significant ($p<0.05$).

Similarly to the extracts of root among the test strains, S. *boydii* was the most susceptible (8.75 ± 1.03 mm), followed by, *S. aureus* (ATCC 6538) (8.73± 1.07mm), *S. pneumoniae* (7.62 ± 0.52 mm), *P. aeruginosa* (ATCC 27853) (6.36 ±0.99 mm), *E. coli* (ATCC 25922) (3.91 ± 0.97) and *MRSA* (3.18 ± 1.89 mm) respectively. This association was statistically significant ($p<0.05$) for *P. aeruginosa, E. coli* and *MRSA* respectively and no significance difference between strains of S. *boydii* ,*S. aureus* and *S. pneumoniae* from Table 4.disc diffusion assay reveled *MRSA* had greater IZD from root extract than leaf extract.

MIC values of *V.officenalis* leave and root crude extracts against test organisms.

The MICs were estimated by broth dilution technique. The test tubes were incubated at 37 ºC for each type of bacterial cultures. The MIC value of plant extracts of *V. officenalis* against the tested bacteria ranged from (3.91 mg/ml) leaf extract for *S. pneumoniae* and root extract for *S. pneumoniae* and *S. boydii* (7.81 mg/ml) respectively to (250 mg/ml) for standard strain of *E. coli*, and *MRSA*, respectively, (Table 5); Within the bacterial isolates *S. pneumoniae* were highly susceptible to the small MIC values of all extracts followed by *S. boydii* which was inhibited by the concentration of (7.81 mg/ml) on leaf and root extract. Isolates of *MRSA* and standard strain of *E. coli* were inhibited by a relatively high MIC values of (62.5 mg/ml) and (250 mg/ml) at leaf extract and (62.5 mg/ml) (125 mg/ml) root extract, respectively.

Table 5: The MIC values of *V.officenalis* root and *leaf* extract fractions against test organisms using two fold broth dilution methods

S#	Organisms	Plant extracts (mg/ml)	
		Leaf	**Root**
1	*E. coli* **	250	125
2	*P. aeruginosa* * *	125	-
3	*S. boydii**	7.81	7.81
4	*S. aureus** *	15.62	31.25
5	*S. pneumoniae**	3.91	7.81
6	*MRSA**	62.5	125

**Clinical isolates, **Standard strains*

The MBC values of *V. officenalis* leave and root crude extracts against test organisms.

The MBC values, which were determined by sub-culturing the samples on the appropriate medium having dilution values, are shown on table 6. Leaf extract of *V. officenalis* showed MBC values which ranged from (7.81 mg/ml) (against clinical isolate of *S. pneumoniae*) to (125 mg/ml) (against *MRSA*) respectively. The second important extract was root extract ranged from (15.62 mg/ml) against clinical isolate *S. pneumoniae* and *S. boydii* to (250 mg/ml) against *E.coli* and *MRSA* followed by standard *S. aureus* (62.5 mg/ml). The corresponding result of root and leaf extract is the same for clinical isolate strains of *S. boydii* at moderate concentration values.

Table 6: The MBC of *V. officenalis* leaf and root extract fractions against test organisms

S#	Organisms	Plant extracts (mg/ml)	
		Leaf	Root
1	*E. coli* **	-	250
2	*P. aeruginosa* * *	-	-
3	*S. boydii**	15.62	15.62
4	*S. aureus** *	31.25	62.5
5	*S. pneumoniae**	7.81	15.62
6	*MRSA**	125	250

**Clinically isolates and **Standard strains*

V. officenalis leaf and root extract fractions showed greater effects in suppressing bacterial growth in this study, with MBC values ranged from 7.81 mg/ml of leaf extract isolated strains of *S. pneumoniae* and strains of *MRSA* to 125 mg/ml of root extracts .Root and leaf extract showed similar value against standard strains of *P. aeruginosa*. An isolated strain of *S. boydii* (at 15.62 mg/ml from leaf and root extract) both extracts have the same value and standard strain of *S. aureus* (at 31.25 mg/ml from leaf extract). *S. aureus* is more sensitive to leaf extract than root extract but in case of *E. coli*, root extract is better to kill at lower concentration than leaf extract. *S.aureus* were the most sensitive strains next to S. *pneumonia,* strains (at 7.81 mg/ml from leaf extract) followed by *MRSA* from leaf extract at the value of 125 mg/ml.

DISCUSSION

Crude methanolic extract was used in the present investigation because most of the antimicrobial agents in plants are soluble in methanol[16]. A study by Alemayehu[12] show that methanol extract have more compounds than chloroform and petrol ether extracts up on TLC. In the present study, leaf extracts of *V. officenalis* showed the strongest antibacterial activity against 4 bacterial isolates and with mean zone of inhibition diameter of 21.18 ± 0.84 mm followed by root extract of plants with mean inhibition zone 16.61±0.93 mm. In this study, root extracts of *V. officenalis* showed maximum zone of inhibition 16.61 ± 0.93 mm against clinical isolate of *S. boydii* followed by 16.21 ± 1.91 mm and 15.57 ± 0.81 mm against clinical isolate of *S. boydii , S. pneumoniae* and standard strain of *S. aureus* (ATCC 2923), respectively. The least diameter of zone of inhibition recorded was 9.84 ± 0.49 mm against standard strain of *E. coli* next to clinical isolates of *MRSA* 11.25 ±1.39. The leaf extract showed stronger and broader spectrum of antimicrobial activity than root extract.

Other study conducted by Casanova *et al.*[17] indicated that ethanol, 50% methanol, ethyl acetate and aqueous leaf extracts of the *V. officenalis* from the standard bacterial pathogens of *S. aureus* and *S. typhi* showed maximum zone of inhibition (50 ±0.11 mm and 27 ± 0.06 mm), respectively and the least zone of inhibition was recorded by *P. aeruginosa* (12±0.2 mm). While a study conducted on sensitivity by Bayoub *et al.*[18] on ethanolic extract showed that aerial part extract is sensitive to *S. aureus, E. coliace* but insensitive to *E. coli*. There was a great variation in zone of diameters which could be the difference in the concentration of the active principles that might vary due to type of bacterial strains tested and solvents used for extraction process[19].

V. officenalis crude leaves extracts agar well diffusion demonstrated maximum zone of inhibition against standard strains of *S. aureus, P. aeruginosa* and *E. coli* (20.08 ± 0.27 mm), (15.30 ±0 .74 mm) and (13.75 ± 1.56 mm), respectively. The next bacterial strains inhibited were clinical isolates of *S. pneumonia, S. boydii,* and *MRSA* which had inhibition zones of (20.86 ± 0.70 mm), (21.18 ± 0.84 mm) and (13.75 ± 0.64 mm), respectively. The least inhibition zone was recorded from standard strain of *E. coli* and clinical isolates of *MRSA* (13.75 ± 1.56 mm) while S. *aureus* were the most inhibited strains among standard strains and among the clinical isolates most inhibited strain was *S. boydii* .Within the same leaf extract of *V. officenalis* disc diffusion assay had shown effective antibacterial activity of standard strains with range of zone of diameters *S. aureus* (9.83 mm) to *E. coli* (4.43 mm). The present study showed that the agar well diffusion had a high activity of extracts than the disc diffusion assay which is in agreement with the work of Olila *et al.*[20]. This is because; the free hydroxyl group present in the disc may prevent the diffusion of cationic polar compounds[21].

In the present study leave extract inhibit *P. aeruginosa, S. boydii* and *S. pneumoniae* effectively with different inhibition zone diameter whereas, a study conducted by Hernandez *et al.*[22], methanol leaves extracts of *V. officinalis* did not inhibit *P. aeruginosa, Shigella,* and *Streptococcus* but successfully inhibit *E. coli* and *S. aureus* with IZD (37.5mm). The difference of the above results encountered might be related to many factors such as age of the plant, plant part used, solvent concentration, tested strains used and extraction procedures followed[23].

The results of the present study showed, gram positive bacterial strains (*S. pneumoniae* and *S. aureus*) were more inhibited than gram negative ones in both plant extracts, while research carried out by Hernández *et al.*[22] showed lower activity against both types of bacteria. S. *pneumoniae* and *S. boydii* was the most susceptible bacterium to both leaf and root extracts, an observation that may be attributed to the absence of outer membrane of the organism which makes it more accessible to permeation by active principles of the extracts of *V. officenalis*[20]. In contrast the *P. aeruginosa* were the least susceptible to the extracts next to *E.coli*. This may be due to the fact that *P. aeruginosa* has intrinsic resistance from a restrictive outer membrane barrier and Trans-envelope multidrug resistance pumps (MDRs)[24&25].

Comparison of herbal preparations with standard antimicrobial drug was made by zone of inhibition obtained from each tested organisms. Zone of inhibition obtained by Gentamicine and Amoxicillin was greater than that of obtained by crude extracts of both plant preparations against the test organisms. Extract was effective but it had less potency than positive controls. However the negative controls has lower inhibition Zone than the extract.

In the present study, MBC equal to one dilution factor less than MIC value of the extracts and this statement is in agreement with Bayoub *et al.*[18]. This indicates that, leaf has a better antibacterial efficacy than root. In contrast leaf extracts and root extract had similar MBC values particularly in *S. boydii*.

CONCLUSION

V. officenalis crude leaves extracted by methanol possess a strong activity against the tested bacteria strains while crude root extracted by methanol has weak antibacterial effect. From the above results, it is concluded that *V. officenalis* used traditionally for metanolic extract as antibacterial. The standard drug Gentamicin was found to be two times effective on leaf extracts on agar well diffusion assay and three times effective on root extract agar well diffusion assay. The standard drug Amoxicillin were found to be 2-5 times effective on leaf and root extracts disc diffusion assay.

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