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## IN-VITRO ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACT OF *VERBENA OFFICINALIS*

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**ABSTRACT:** The leaf of *Verbena officinalis* was collected, air dried under the shed, powdered, soaked in and extracted with 80% methanol. The crude extract was tested for its *in-vitro* antibacterial activity against *S. aureus* and *E. coli* using agar disc diffusion method. Antibacterial assay was made using extract concentrations of 250 mg/ml, 500 mg/ml, and 1000 mg/ml. The extract showed a concentration-dependent zone of inhibition against the test bacteria. The strongest activity (largest zone of inhibition, 20.38 mm) was obtained at the concentration of 1000 mg/ml against *S. aureus*. The minimum inhibitory concentrations (MICs) of *V. officinalis* against *S. aureus* and *E. coli* were determined to be 0.019 mg/ml and 0.313 mg/ml respectively. Phytochemical analysis was also conducted on the powdered leaf of the plant. Seven secondary metabolites including chromophores, polyphenols, saponin, phytosterols, flavonoids, cardiac glycosides and tannins were detected from this medicinal plant. The observed antibacterial activity could be attributed to the presence of these secondary metabolites which have been associated with varying degrees of antibacterial activities. In conclusion, the plant was found to exhibit an encouraging level of antibacterial activity. Further, studies are thus warranted to determine the antibacterial activities of the different chemical isolates of the plant. Also, *in-vivo* studies should be carried out to support these findings and to determine the safety and other pharmacokinetic profiles.

**INTRODUCTION:** Despite tremendous progress made in medicine, infectious diseases caused by bacteria, fungi, viruses, and parasites remain major health threats globally. Their impacts are particularly massive in developing countries where there is widespread unavailability of modern medicines and a high rate of emergence of drug resistance<sup>1</sup>.

The development of drug resistance, as well as lack of availability of certain antibiotics, has to lead to the search of new antimicrobial agents mainly from plant extracts with the goal to discover new chemical entities, which overcome these problems<sup>2</sup>. Current research on naturally occurring molecules and products primarily focus on plants since they are locally available, less costly, and can be selected based on existing ethnomedicinal claims for their uses<sup>3</sup>. *Verbena officinalis* (family Verbenaceae) is a weed that is found in semiarid environments<sup>4</sup>. It is a perennial plant which has been used for centuries as herbal medicine and health supplement in both Western and Eastern countries<sup>5</sup>.

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The plant is claimed to have an antidepressant and anticonvulsant effect. It is also suggested for its use for the treatment of jaundice, cough, cold and digestive problems <sup>6</sup>. This plant has traditionally been used in Ethiopia for treatment of tonsillitis and pneumonia <sup>7</sup>. Hence, it is imperative to evaluate the claimed efficacies of parts of this plant scientifically. Phytochemical analysis has also not been done on samples collected from the study area. Therefore, the objective of this study was to determine the antibacterial activity of leaf extract of *Verbena officinalis* and to identify its secondary metabolites.

## MATERIALS AND METHODS:

**Plant Material Collection and Preparation of Extract:** The whole part of the plant, *Verbena officinalis*, was collected from around Mekelle City from June to September 2013. The test plant was taxonomically identified at the herbarium in the Department of Biology, Mekelle University **Fig. 1**. The leaf of the plant was air-dried at room temperature under the shed, and crushed by grinder machine. The crude extract was prepared according to the procedure described by Shewit *et al.* <sup>8</sup> The dried leaves were powdered, measured and macerated three times in 80 % methanol for about 72 h. After 72 h, the filtrate was concentrated by rotary evaporator under reduced pressure. The crude extract was kept in a closed container in a refrigerator at 4°C until used for the test.



**FIG. 1: PICTURE OF VERBENA OFFICINALIS (PHOTO TAKEN IN THE MICROBIOLOGY LABORATORY)**

**Bacterial Inoculums Preparation:** Two species of bacterial isolates, *Staphylococcus aureus* and *Escherichia coli*, were used for this test. The test bacteria were obtained in their lyophilized form from the National Veterinary Institute of Ethiopia.

The bacteria were then revived on nutrient broth. Finally, the revived bacteria were grown on selective media, *i.e.* *Staphylococcus aureus* on manitol salt agar and *Escherichia coli* on MaConkey for confirmation. Then, the confirmed bacteria were sub-cultured on nutrient agar to get separated colonies. Finally, the separated colonies were suspended in sterile 0.85% saline solution.

## Antibacterial Sensitivity Test:

**Disc Diffusion:** The standard disc diffusion assay method adapted from Taylor *et al.*, <sup>9</sup> was used for this study. The turbidity of actively growing bacterial suspension was adjusted to 0.5 McFarland standards. Then, the bacteria adjusted to 0.5 McFarland standards were inoculated on Muller Hinton Agar using sterile swabs. The plant extract was dissolved in dimethylsulfoxide (DMSO). Then, discs which were prepared from sterilized filter paper were impregnated with the dissolved extracts.

The extracts were prepared at three different concentrations (250 mg/ml, 5000 mg/ml, and 1000 mg/ml). For negative control, discs impregnated with DMSO were used, and a standard antibiotic (cefoxitin 30 µg/disc) was used as positive control. Each extract concentration was tested eight times. The discs were applied on the cultured dish by sterile forceps approximately equidistance to each other. Then Petri dishes were incubated for 20 hours at 35 °C. Finally, diameters of the zones of inhibition as indicated by clear area were measured by using the digital caliper.

## Minimum Inhibitory Concentration (MIC)

**Determination:** The minimum inhibitory concentration was determined by microdilution method according to Andrews <sup>10</sup>, with slight modifications. Briefly, 25 µl of the plant extract (20 mg/ml of DMSO) and 20 µl of bacterial suspension (adjusted to 0.5 McFarland standards) were added to 200 µl of broth in the first two wells of 96-well plates. Then, this procedure was repeated by two-fold serial dilutions of the plant extract across the rows up to the 10<sup>th</sup> column.

Cefoxitin was used as a positive control, inoculated wells of antibiotic-free broth were used as negative control, and un-inoculated wells of antibiotic-free broth were used to check sterility. Then the plates

were covered with plate sealing tape and incubated at 37 °C for 20 h. Finally, the lowest concentration of the plant extract at which there was no visible bacterial growth was taken as minimum inhibitory concentration.

**Phytochemical Analysis:** Phytochemical analysis of the plant was conducted to determine the identity of secondary metabolites. The analysis was done according to the standard procedures described by Sofowora<sup>11</sup>.

**Data Processing and Analysis:** Data on the diameter of zones of inhibition was analyzed by SPSS Version 17 software. Zones of inhibition were expressed as mean  $\pm$  SD. Statistical analysis was also undertaken by one-way analysis of variance (one way ANOVA) test coupled to Least Significant Difference (LSD) to compare results from different extract concentrations, and also the effectiveness of the test plant against the different bacteria. The result was considered statistically significant at 95% confidence level and P-value < 0.05.

## RESULTS AND DISCUSSION:

**The Yield of Crude Extract:** Initial screening of plants for possible antimicrobial activity was carried out by first preparing crude extracts obtained using 80% (v/v) methanol. The total yield of the crude extract obtained was 8.92%.

**Phytochemical Screening:** In the phytochemical investigation of *Verbena officinalis* leaves, seven secondary metabolites including chromophores, polyphenols, saponin, phytosterols, flavonoids, cardiac glycosides, and tannins were detected **Table 1**. These metabolites are widely distributed

in the plant kingdom, with several biological activities. Similarly, Bilia *et al.*, also reported that the main components of *Verbena officinalis* are iridoids, phenylpropanoids, flavonoids, and terpenoids<sup>12</sup>. Another phytochemical study conducted by Verma and Siddique also indicated that the plant *Verbena officinalis* contains flavonoids and terpenoids<sup>6</sup>.

Phenolic compounds isolated from medicinal plants strongly inhibited the growth of *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia coli*<sup>13</sup>. Sellami *et al.*, also confirmed the antibacterial activity of flavonoid from extracts of different spices of plants by using the agar well diffusion method<sup>14</sup>. The antimicrobial activity of *Verbena officinalis* was also confirmed by Casanova *et al.*<sup>15</sup> who reported that flavonoids isolated from *Verbena officinalis* showed antifungal inhibition of up to 87%.

Secondary metabolites detected in this medicinal plant are known to have an antimicrobial activity which hints that the antibacterial activity of *Verbena officinalis* is due to the presence of these compounds. For example, Gnananickam and Mansfield<sup>16</sup> reported the antimicrobial activity of flavones and isoflavones against *Staphylococcus epidermidis*. Similarly, Nancy *et al.*, confirmed that flavonoids isolated from *V. officinalis* showed antimicrobial activity against both gram negative and gram-positive bacteria<sup>17</sup>. The antimicrobial action of *V. officinalis* is due to more than one component. In such cases, the major component is not only responsible for the antimicrobial activity, but it can be the result of the synergistic effect of different components<sup>4</sup>.

**TABLE 1: PHYTOCHEMICAL SCREENING OF LEAF OF VERBENA OFFICINALIS**

No.	Type of secondary metabolite detected	Color obtained during the test	Test result
1	Chromophores	Yellow to red precipitate	(+) positive
2	Polyphenols	Green blue	(+) positive
3	Saponins	Honeycomb froth	(+) positive
4	Phytosterols	Reddish brown	(+) positive
5	Carotenoids	Colorless	(-) negative
6	Phenolic glycosides	Brown	(-) negative
7	Free anthraquinones	Colorless	(-) negative
8	Flavonoids	Orange precipitate	(+) positive
9	Cardiac glycosides (by Salkowski)	Rose color and reddish blue brown at the interface	(+) positive
10	Tannins	Brown	(+) positive
11	Alkaloids: By Dragendorff's solution	Orange but no precipitate	(-) negative
	By Meyer's solution	Colorless and no precipitate	(-) negative

**Antibacterial Activity of *Verbena officinalis* Crude Extract:**

The results of the antibacterial activity of the methanol extract obtained from *Verbena officinalis* are presented in **Table 2**. The extract showed a concentration-dependent zone of

inhibition which is statistically significant ( $p < 0.05$ ). The tested plant showed a higher zone of inhibition ( $p < 0.05$ ) against *S. aureus* than *E. coli* at all concentrations.

**TABLE 2: MEAN ZONE OF INHIBITION OF VERBENA OFFICINALIS CRUDE EXTRACT AGAINST *S. AUREUS* AND *E. COLI* AT DIFFERENT CONCENTRATIONS**

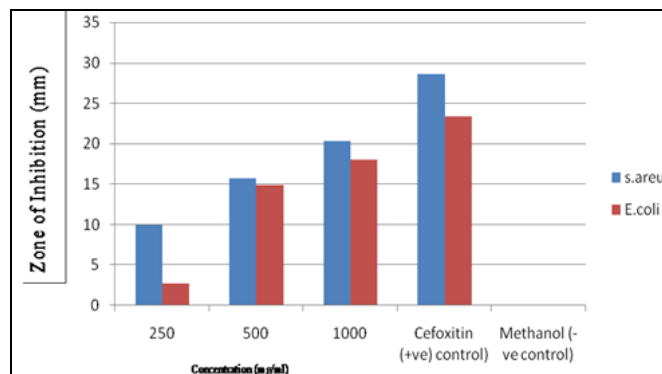
Test Chemical	Conc. (mg/ml)	Zone of inhibition (mm) (Mean ± SD)			
		<i>S. aureus</i>	p-value	<i>E. coli</i>	p-value
<i>V. officinalis</i> extract	250 (0.25 µg/disc)	10.00 ± 1.85		2.75 ± 1.83	<0.05
	500 (0.5 µg/disc)	15.71 ± 4.5	P<0.05	14.875 ± 2.748	>0.05
	1000 (1 µg/disc)	20.38 ± 5.39676		18.000 ± 5.237	>0.05
Cefoxitin (+ve) control)	30 µg/disc	28.62 ± 1.18		23.375 ± 1.4078	<0.05
DMSO (-ve control)	0	0		0	
		P<0.05		P<0.05	

Results of this study showed that crude *Verbena officinalis* leaf extract has varied antibacterial activities against both of the tested organisms. This suggests that the extract of this plant has a broad spectrum of antibacterial activities. This correlated with the finding of previous studies that reported the presence of antimicrobial substances within the leaves of the plant as described by Kubo *et al.*<sup>18</sup> However, these antibacterial activities were observed to be significantly lower than that of cefoxitin, the standard antibacterial drug used as positive control in this study. This could probably be because the sample extracts used in the test were crude preparation which may not necessarily contain enough of the active chemical. Further purification and fractionation are thus needed to yield more isolated active compounds<sup>19</sup>.

The methanol extract of *V. officinalis* showed better antibacterial activity (zone of inhibition 20.38 mm) against *S. aureus* and *E. coli* at the highest concentration used (1000 mg/ml), and the largest zone of inhibition was observed against *S. aureus*. This result strongly agrees with the findings of Hammer *et al.*<sup>20</sup> who previously reported that *V. officinalis* has good antimicrobial activity against gram-positive bacteria only, and possible discrepancies could be due to changes in phytochemical contents of the plant growing in geographically diverse locations.

Results of this study are also supported by the findings of Maune *et al.*, who reported bactericidal effects of a herbal product containing *V. officinalis* against gram-positive as well as gram-negative bacteria (*Staphylococcus aureus* and *Escherichia*

*coli*)<sup>21</sup>. The maximum zone of inhibition recorded for crude extract of *Verbena officinalis* 20.38 mm and 18 mm against *S. aureus*, and *E. coli* respectively is also in accordance with that of Mustafa *et al.*, where they reported the zone of inhibition of the crude extracts of the tested plants was in the range of 14-26 mm<sup>22</sup>.



**FIG. 2: ZONE OF INHIBITION OF CRUDE EXTRACT OF *V. OFFICINALIS* AT DIFFERENT CONCENTRATIONS AGAINST *E. COLI* AND *S. AUREUS***

As shown in **Fig. 2**, a wide magnitude of variation in antibacterial activity was detected among the tested concentrations as well as variation in sensitivity among the tested bacteria. *Verbena officinalis* crude extract showed significant activities against both species of test bacteria at all concentrations used in this study.

The minimum inhibitory concentration of crude *Verbena officinalis* leaf extract (shown in **Table 3**) was found to be 0.019 mg/ml and 0.313 mg/ml against *S. aureus* and *E. coli* respectively. According to CLSI<sup>23</sup>, *S. aureus* ATCC® 25923 and *E. coli* ATCC® 25922 were found to be susceptible to ceftriaxone and cefoxitin at MIC of 0.008 mg/ml



respectively. This shows that the MIC of this plant extract is higher as compared to standard antibiotics which might be because the plant extract is crude containing only traces of the potentially active ingredients. The finding of our study is in line with that of Balungile *et al.*, where an

alcoholic extract of some plants showed MIC values that range from 0.39 to 0.78 mg/ml against different bacterial species<sup>24</sup>. Mustafa *et al.*, has also reported that MICs of some plant extracts ranged from 8 to 14.2 mg/ml against drug-resistant bacteria<sup>22</sup>.

**TABLE 3: MINIMUM INHIBITORY CONCENTRATION OF *VERBENA OFFICINALIS* CRUDE EXTRACT AGAINST *S. AUREUS* AND *E. COLI***

Test bacteria	Extract Concentration (mg/ml)											MIC (mg/ml)	
	10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.019	0.009		
<i>S. aureus</i>	-	-	-	-	-	-	-	-	-	-	-	+	0.019
<i>E. coli</i>	-	-	-	-	-	-	+	+	+	+	+	+	0.313

**CONCLUSION:** This study confirmed the antibacterial activity of leaves of *Verbena officinalis*, a plant which is also claimed in folk medicine as being useful in the treatment of diseases caused by bacteria.

According to the results of this study, it can be concluded that *Verbena officinalis* leaf extract showed a broad spectrum of antibacterial activity as evidenced by the high zone of inhibition and good MIC results against both *S. aureus* and *E. coli*. These results provide evidence that some of the secondary metabolites present in the leaves *V. officinalis* might indeed be potential sources of new antibacterial agents.

The finding of this study also hints that potential lead molecule can be isolated from the tested plant that can be used as a base for the synthesis of other effective antibacterial drugs. Therefore, the authors recommend further studies to be done on isolated compounds and fractions of the plant for antimicrobial activity and toxicological screening. Moreover, *in-vivo* studies should also be conducted to confirm the safety and efficacy of this plant's secondary metabolites.

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**CONFLICT OF INTEREST:** Nil

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