

Full Length Research Paper

Antimicrobial activity of *Thymus schimperi* Ronninger (Lamiaceae) against standard and clinical isolates of human pathogenic bacteria

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Thymus schimperi Ronninger (Lamiaceae) locally known as Tosign, is a multipurpose endemic plant that has been used for various remedies as constituents of traditional medicine in Ethiopia. The objective of this study is to evaluate antibacterial activity of water, ethanol, methanol and chloroform extracts of *T. schimperi* using agar well diffusion and broth dilution methods against human pathogenic bacterial strains. Amongst the solvents used for this study, chloroform extract possess the highest potential of inhibiting the growth of all bacteria under study at concentrations of 50 mg/ml while ethanol and methanol extract fail to inhibit three gram negative bacteria, namely: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (clinical isolate) and *Shigella flexneri* (ATCC 12022) at the same concentration. Water extract did not show any zone of inhibition on all test bacteria as compared to other solvents. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed only for chloroform extracts that showed inhibition against all test organisms. This study revealed that, the highest inhibition with chloroform extract was exhibited against Methicillin-Resistant *S. aureus* (MRSA) with mean zones of inhibition of 22.6±2.5 mm whilst the minimum inhibition zone was observed for *E. coli* with mean zone of inhibition of 14.6±2.3 mm. The MIC value ranged from 6.25 to 12.5 mg/ml while the MBC value ranged from 6.25 mm to 25 mg/ml. This study clearly indicates that the crude chloroform extract of *T. schimperi* showed highest antibacterial activity against all studied bacterial strains as compared to the three solvents used in this study. Thus, further study and characterization of active compounds of chloroform extract of this plant is required.

Key words: Antibacterial activity, MBC, MIC, *Thymus schimperi*, Tosign, zone of inhibition.

INTRODUCTION

Herbal drugs have got official recognition and gained a lot of acceptance worldwide due to their high therapeutic

worth, fewer side effects, and economic value (Gupta et al., 2010; Kumar et al., 2010). Ethiopia has between 650

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and 1,000 medicinal plants, comprising about 10% of the entire flora of the country (Fulas, 2007). The current literature survey revealed that a large number of these plants have long been in use by local people; however, many of them are lacking modern scientific investigations. It has been widely claimed that about 80% of Ethiopian population rely on traditional medicine (Birhanu et al., 2015; Geleta et al., 2015). According to World Health Organization, majority of the population in developing countries like: Ethiopia (90%), Benin (80%), India (70%), Rwanda (70%), Uganda (60%), and Tanzania (60%) extensively use traditional medicines in health care (WHO, 2003).

The life-threatening infection of pathogenic microorganisms has increased worldwide and has become the cause of mortality in many of developing countries (Al-bari et al., 2006; WHO, 2015). The increasing prevalence of multi-drug resistant strains of bacteria and the appearance of strains with reduced susceptibility to antibiotics resulted in the formation of untreatable bacterial infections that open the door to search for new source of medicine (Rojas et al., 2006; Sosa et al., 2010; Biadlegne et al., 2014). Based on pre-existing indigenous knowledge, the numbers of modern drugs have been prepared from existing natural sources and many more had showed promising results.

Thymus species are well known for their medicinal importance because of their biological and pharmacological properties. The substances extracted from thyme especially the phenolic components *thymol* and *carvacrol* showed antibacterial activity against gram-positive and gram-negative bacteria due to their effects on the bacterial membrane (Asfaw et al., 2000). Because of its antibacterial activity, thyme is also useful as an antiseptic for the urinary tract, mouth and skin wounds. Tea and decoction prepared from thyme have successfully been used against gastro-intestinal complaints. Thyme oils are remedies to expel intestinal parasites, particularly hookworm (Mufti, 2011). *Thymus schimperi* (locally called Tosign) was found to have significant antioxidant activity and food preservative effect (Hailemariam and Emire, 2013). However, it is not well investigated on the modern scientific grounds. Keeping in view the common use of *T. schimperi* in traditional medicine, the present study was designed to evaluate its antibacterial activity against some human pathogenic bacteria to bridge information gap pertaining in the community.

MATERIALS AND METHODS

Plant material

Partially dried 2 kg leaves of *T. schimperi* R. (Lamiaceae) were purchased from Addis Ababa, Capital city of Ethiopia, and brought to Biotechnology laboratory of Gondar University. The plant material was properly screened from unwanted woody parts, giving 1.5 kg final weight. The scientifically authenticated leaves were then fully dried for 10 days at room temperature.

Preparation of plant extracts

The cleaned and dried leaves of Tosign were grinded using a grinder. The obtained powder was passed through a sieve (pore size: 30 μ m diameter) and made ready for extraction. Four different solvents were used for extraction namely; chloroform, ethanol, methanol, and distilled autoclaved water. About 100 g of powder was taken and mixed with 300 ml of each solvent sequentially. The extraction was done using orbital shaker with continuous shaking for 8 h per day for 3 consecutive days. The extract was filtered using Whatman #1 filter paper. The debris was discarded and filtrate was evaporated under reduced pressure at 40°C. Finally the extract was dried; stock solution of 100 mg/ml was prepared in 50% Dimethyl sulfoxide (DMSO) (Anas et al., 2008), vortexed well, labeled and stored at 4°C in refrigerator until used. Chloroform crude extract was dissolved by the help of microwave for about one minute.

Preparation of test organisms

Both gram positive and gram negative bacterial strains: namely; *Escherichia coli* (ATCC 25922), methicillin resistant *Staphylococcus aureus* (MRSA), *Shigella flexneri* (ATCC 12022), *S. aureus* (ATCC 25923), *Klebsiella pneumoniae* (clinical isolate), *S. pneumoniae* (ATCC 63), and *S. pneumoniae* (clinical isolate) were used. The test microorganisms were grown on nutrient agar at 37°C for 24 h. The standard 0.5 McFarland known to form 1.5×10^8 CFU/ml was prepared by taking two to four colonies in normal saline solution following standard procedure (Andrews, 2006).

Antibacterial activity assay

The antibacterial activity of water, ethanol, methanol, and chloroform extracts of *T. schimperi* were determined using agar well diffusion method (Taye et al., 2011). The inoculums were prepared by taking overnight bacterial culture and adjusting to 0.5 McFarland standard in 0.9 % autoclaved NaCl (Normal saline). For sensitivity test, 38 g of Muller Hinton Agar was dissolved in 1000 ml distilled water and autoclaved at 121°C for 15 min. The media was then poured into sterilized petri-dishes with uniform thickness and the agar was allowed to set at ambient temperature under laminar hood until solidification. The inoculums were spread evenly on the surface of solidified Muller Hinton agar with the help of sterilized cotton swab. On each plate, six equidistant wells were made with a 6 mm diameter sterilized cork borer. Then 100 μ L of each plant extract adjusted to the same concentration (50 mg/ml) was aseptically added into a respective well. Chloramphenicol (30 μ g/disc) and Vancomycin (30 μ g/disc) were used as a positive control whilst 50% DMSO was used as a negative control. This was followed by allowing the agar plate to stay for 30 min under laminar hood and then incubated at 37°C for 24 h. The formation of clear inhibition zone of ≥ 7 mm diameters around the wells were taken as significant susceptibility measurement. The experiment was prepared in triplicate and mean value was used for further analysis.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined for extracts that showed inhibition zone of ≥ 7 mm diameter and for extract that inhibited the growth of all tested bacteria at concentration of 50 mg/ml. Among the extracts, only chloroform extract inhibited the growth of all tested microorganisms. The test was performed by using standard methods: agar well diffusion and microtiter plate (micro-tube dilution method). In former method, double serial dilution was employed from 50 mg/ml to obtain 1:2,

1:4, 1:8, 1:16, 1:32, and 1:64 in order to get 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mg/ml concentration of extract respectively using 50% DMSO. Then 100 μ L of diluted extract was added to prepared wells on Mueller Hinton agar and followed by identifying MIC concentration. In later method, the same principle was followed as above serial dilution except dilution was made in 1ml of nutrient broth. A 30 μ L of standard suspension of test organisms were then added to each labeled concentration. Control was prepared with no inoculation of test organisms. The micro-tube was incubated at 37°C for 24 h and the presence of growth was evaluated by observing the bacterial turbidity of each tube before and after incubation and comparing the tube to the control.

Minimum bactericidal concentration (MBC)

For MBC, dilutions with no visually visible growth were taken and sub cultured on Mueller Hinton agar, and incubated for 24 h at 37°C. The concentration that resulted in no visible growth was then taken as MBC value.

Data analysis

Data was analyzed using statistical software package SPSS version 16.0 and presented as means \pm standard deviation (SD). The one-way ANOVA was performed to examine the differences among test organisms and P value <0.05 was considered to be statistically significant.

RESULTS

The results of this study are presented in Table 1, and Figure 1. A 50 mg/ml concentration of all the four extracts prepared was tested for antimicrobial activity. Water extract did not show any antibacterial activity against all tested bacteria. Methanol and ethanol extract failed to inhibit the growth of: *E. coli*, *S. flexneri*, and *K. pneumonia* (clinical isolate). However, ethanol and methanol extract inhibited all studied gram positive bacteria; *MRSA*, *S. aureus*, *S. pneumoniae* (standard) and *S. pneumoniae* (clinical isolate). Methanol extract showed higher ($P < 0.05$) inhibition zone against the tested bacteria as compared to ethanol extract (Table 1). It is worth mentioning that chloroform extract showed 100% inhibition against all test bacteria with higher inhibition zone for most of test bacteria except for standard *S. aureus* (17 mm).

The maximum inhibition zone of ethanol extract (19 mm) was recorded for *MRSA* and standard *S. pneumoniae* while the minimum was obtained for standard *S. aureus* (17 mm). Likewise, the maximum inhibition zone for methanol extract was obtained for *MRSA* (19.3 mm) whilst the minimum was obtained for standard *S. aureus* (18.3 mm). For chloroform extract, the maximum inhibition zone was recorded for *MRSA* (22.7mm) while the minimum value was for standard *E. coli*. Hence, the currently evolving *MRSA* showed highest inhibition zone with chloroform extract as compared to other test bacteria (Table 1).

Standard antibiotic chloramphenicol (30 μ g/disc) and

vancomycin (30 μ g/disc) were used as positive control while 50% DMSO was used as negative control. As compared to standard antibiotics, chloroform extract showed high inhibition value than vancomycin (19 mm) for *MRSA* (22.7 mm) and standard *S. flexneri* (19.7 mm). Likewise, the chloroform extract resulted in higher zone of inhibition than chloramphenicol for Clinical isolate *K. pneumoniae*. Similar to water extract there was no inhibition with negative control and the data was not included in Table-1.

The MIC and MBC test was only conducted for chloroform extract because of the higher inhibition value recorded and its strong potential against all tested bacterial strains. The MIC value ranged from 6.25 mg/ml to 12.5 mg/ml. Five of test bacteria (*MRSA*, *S. flexneri*, *K. pneumoniae*, *S. pneumoniae* standard and clinical isolate) got high MIC value (12.5) with both micro dilution and agar well diffusion method, whilst only two of test organisms (*E. coli* and *S. aureus*) showed lowest MIC value (6.25 mg/ml). However, the maximum MBC value was 25 mg/ml, while the minimum value was 6.25mg/ml. The highest MBC value (25 mg/ml) was obtained for three test bacteria (*K. pneumoniae*, clinical *S. pneumoniae* and standard *S. pneumoniae*). The lowest MBC value (6.25 mg/ml) was recorded only for *E. coli*. The MBC and MIC value of *E. coli*, *MRSA*, and *S. flexneri* were found to be similar, whilst the remaining has different values for both tests (Figure 1).

DISCUSSION

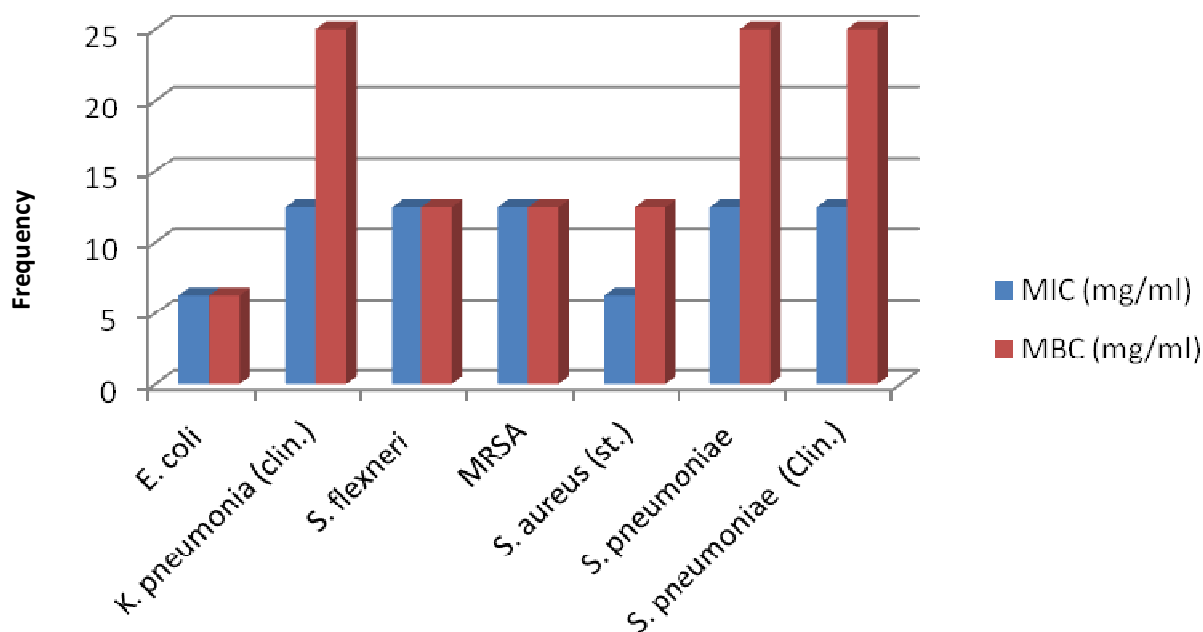
Ethno-botanical screenings have been found to offer information on the importance of traditional medicines especially for those that do not have enough scientific evidence to prove their traditional use. This study is a continuation of earlier work justifying the utilization of Ethiopian folk medicine (Taye et al., 2011; Asressu, 2013). In the present study, attempts were made to validate the use of *T. schimperi* as antimicrobial agent and to substantiate the earlier findings (Hailemariam and Emire, 2013). To the best of our knowledge, there was no earlier work conducted on these bacterial strains. Though, local people have been using Tosign as cultural remedies, however, the information available is very minimal on this indigenous herb since its availability is restricted to Ethiopia (Asressu, 2013).

The results of this study, clearly indicate that ethanol and methanol extracts could not inhibit the growth of all gram-negative bacteria (*E. coli*, *S. flexneri* *K. pneumoniae*). However, both extracts were effective against the remaining gram-positive bacteria. The observed difference in antibacterial activities between gram-negative and gram-positive were attributed due to the differences in composition and structure of bacterial outer membrane and cell wall which are among primary site of drug action in these organisms (Kenneth and George, 2004). Outer membrane of gram-negative

Table 1. Mean inhibition zone of four solvent extracts from *T. schimperi* at concentration of 50 mg/ml on different test bacteria.

Test organism	Inhibition zone (mm) Mean±S.D				Control	
	Water extract	Ethanol extract	Methanol extract	Chloroform extract	C30	V30
<i>E. coli</i> (ATCC 25922)	0.0	0.0	0.0	14.6±2.3	25	28
MRSA	0.0	19.0±3	19.3±1.5	22.7±2.5	26	20
<i>S. flexneri</i> (ATCC 2022)	0.0	0.0	0.0	19.7±0.5	27	19
<i>S. aureus</i> (ATCC 25923)	0.0	17.0±3.4	18.3±2.8	17.0±2.6	20	19
<i>K. pneumonia</i> (clinical isolate)	0.0	0.0	0.0	18.3±2.0	15	20
<i>S. pneumoniae</i> (ATCC 63)	0.0	19.0±1	19.7±1.1	20.7±3.0	24	21
<i>S. pneumoniae</i> (clinical isolate)	0.0	18.7±.05	19.0±1.0	19.3±1.1	21	20

Statistically significant: *P<0.05; **P<0.01; ***P<0.001 (One way ANOVA). C30 = Chloramphenicol, V30 = Vancomycin.

**Figure 1.** Mic and BMC values of chloroform extract against all tested bacteria.

bacteria is rich in lipopolysaccharide which can hinder penetration of different antibiotic molecules (Kenneth and George, 2004; Abdollah et al., 2010). However, in this study the chloroform extract of *T. schimperi* demonstrated total inhibition of all gram-negative as well as gram-positive tested bacteria. These results are in agreement with an earlier study where twenty different solvents were evaluated and chloroform was found to be the best solvent for the extraction of non-polar biological active compounds that were lethal to many bacteria (Harmala et al., 1992).

The aqueous extract of *T. schimperi* showed no antibacterial activities against all tested bacteria. This is not surprising since plant extracts from organic solvents have been found to give more consistent antimicrobial activity as compared to water extract (Parekh et al.,

2005). The results gotten in this study are supported by previous studies where water was not found to be a suitable solvent for the extraction of antibacterial compounds from medicinal plants. Nonetheless, organic solvent, such as methanol and ethanol were suggested to be better than water as a solvent for antimicrobial agent extraction (Majhenic et al., 2007; El-Safey et al., 2011). Various reports suggested that water soluble flavonoids are not important as antimicrobial activity, though, water soluble phenolics were found to exhibit antioxidant potential (Nang et al., 2007). The aqueous extract of *Leonotis ocymifolia* was also found not to inhibit the growth of any bacterial species (Habtamu et al., 2010). The antimicrobial activity study on another species of genus thyme, *T. serpyllum* indicated that the aqueous extracts did not show any significant activity (Mufti, 2011).

The antimicrobial activities of many plants can be attributed due to the presence of high concentrations of *carvacrol*, which is known to occur at very high concentrations in many plant oils, including the members of the *Labiatae* family, such as *T. serpyllum* (Bounatirou et al., 2007). *T. schimperi* contained important antifungal substances such as *thymol*, *linalool*, and *carvacrol* (Lakew, 2011). The pharmacological actions of the plant extracts were suggested to be parallel to their *carvacrol* contents (Aydin et al., 2007). *Carvacrol* is considered to be biocidal, resulting in bacterial membrane perturbations. Furthermore, *carvacrol* might cross the cell membranes, penetrate the interior of the cell and interact with intracellular sites critical for antibacterial activities (Cristani et al., 2007).

The activity of the plant extract against both gram-positive and gram-negative bacteria might indicate the presence of broad-spectrum antibiotic compounds in that plant (Vaghasiya and Chanda, 2007). Chloroform extracts which resulted in higher inhibition zone were compared to vancomycin (22.7 and 19 mm on MRSA, 19.7 and 19 mm on *S. flexneri*). At the same time, it showed high inhibition zone (18.3 mm) than chloramphenicol (15 mm) on *K. pneumoniae*. Similar studies, conducted on other medicinal plants were shown that their antibacterial activity was comparable to positive controls and even some times higher than that (Ahmet et al., 2004). The fact that this plant extract was being active against both standard and clinical isolates; it is an indication that it can be a source of very potent antibiotic substances that can be used against multidrug resistant microorganisms.

Methicillin-Resistant *S. aureus* (MRSA) continued to be a major pathogen causing infections in hospitals and in the community, and are increasingly isolate in hospitals worldwide starting from its initial isolation in the UK in 1961 (Udo et al., 2006). Interestingly, this study indicated the highest inhibition of MRSA with chloroform extract when compared to other bacterial strains studied. Hence, there was no doubt that highly powerful anti MRSA substances would be isolated in future from medicinal plants like *T. schimperi*.

Conclusion

Traditionally, *T. schimperi* (Tosign) has been used in various liquid and solid foods as flavor and medicinal uses. Our result indicated that it is a promising source of antimicrobial agents specially when extracted using chloroform. The broad antimicrobial activity of chloroform extract indicates the presence of highly active antimicrobial agents that can treat wide spectrum of human pathogens including the resistant ones. Thus, Tosign might represent an inexpensive source of natural antibacterial substances for use in treating various diseases, drug design as well as to prevent the growth of bacteria and extend the shelf life of the processed food.

In nutshell, from this present study, further information could be generated from several angles to validate the utility of this plant for medical application. The need to characterize and describe the antimicrobial activities, and investigate the suitability of these antimicrobial properties in practical applications is also important.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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