



COMBINED ANTIBACTERIAL ACTIVITY OF HONEY BEES (*APIS FLORAE*) AND *EMBELIA SCHIMPERI* EXTRACTS AGAINST STANDARD AND CLINICAL ISOLATED BACTERIA

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ABSTRACT

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Despite tremendous progress in drug development and treatment, infectious diseases caused by bacteria are still a major threat to human and animal health mainly due to drug resistances. These the ever-increasing threats demand a global effort to search for novel solutions. Different parts of plants and bee's honey have been used, separately or in combination, as a source of therapeutic agents in traditional medicine since ancient time due to bioactive compounds they contain. Therefore the objective of this study is to determine the antibacterial activity of crude extract of *Embelia schimper* leaves and combined with bee's honey (*Apis florae*) against some standard and clinical pathogenic bacteria. The *In-vitro* antibacterial activity of the extracts was tested at different concentrations by using agar well diffusion method and measuring zone of inhibition. The data was analyzed by ANOVA. Both the clinical and standard bacteria showed sensitivity to *E. schimper* with inhibition zones ranging from 7.17 ± 0.29 to 28.96 ± 0.25 . The ethanol extract of *E. schimper* leaves and ethanol extract blended with honey have showed significant inhibition against the tested organisms than the chloroform extract and chloroform extract blended with honey respectively. The inhibition zone was lower against *S. aureus* during both the Chloroform leaf extract and the chloroform blended with honey. The highest inhibition zone was observed against *E. coli* with the ethanol leaf extract and against *K. pneumonia* with the ethanol mixed with honey. Therefore, the *E. schimper* leaves extract and synergy with honey has witnessed broad spectrum antibacterial activity.

Contribution/Originality: This study contributes to the existing literature of the antibacterial effects of leaf extracts of *E. schimper*. It is the only study that investigated the synergetic effects of leaves of *E. schimper* with honey. Therefore, the synergy of the leaves with honey has shown promising antimicrobial effect.

1. INTRODUCTION

Medicinal plant extracts have been playing a vital role in the treatment of many diseases including microorganism infections since ancient time (Sukanya *et al.*, 2009). It is well-known that native healers not solely use remedies made from one single plant species however conjointly prepare specific mixtures of various healthful plants to treat, oral diseases, wounds, and skin disorders. This traditional medical knowledge is useful not only for community healthcare but also for future search and development of effective drugs (Sukanya *et al.*, 2009; Desalegn, 2014).

Microbial diseases continued to be the major threats to the world in spite of the efforts and progress in developing new medications (Cos *et al.*, 2006). The impact of microbial diseases has very substantial problem in developing countries like Ethiopia where there's restricted access to standard medications. The main problem encountered with antibiotics in clinical use is resistances, high toxicity, unaffordable costs and low efficacy. Currently, the ever-increasing threat, urges a world effort to look for novel solutions (Theuretzbacher, 2012; Lee, 2015). Many clinical drugs are derived from natural remedies. Most traditional healers use medicinal plant parts blended with honey or butter. This indicates that blended remedies have synergetic effect in treating microbial infections (Rios and Recio, 2005).

Embelia is a Scandent or climbing shrub or tree, 2-13 m tall; as indicated in Figure 1 appendix part, branches with prominent lenticels, young parts with rust-colored hairs, becoming glabrous leaves alternate or in clusters at end of branches belongs to the family Myrsinaceae (Sebsebe and Hedberg, 2003). In Ethiopia, the bark or fruits from *E. schimperi* are combined with other species, such as *Albizia anthelmintica*, *Guizotia abyssinica*, *Glinus lotoides* and *Hagenia abyssinica*, mixed with water and taken as a Taenicide or used as a disinfectant which suggests that the pharmacological effect of this plant can be increased by interaction with other plant ingredients (Doughari *et al.*, 2008; Zafar *et al.*, 2010). The use of honey as a traditional remedy for microbial infections dates back to ancient times. Research has been conducted on honey, which has demonstrated to be effective against several human pathogens, including *Escherichia coli* (*E. coli*), *Enterobacter aerogenes*, *Salmonella typhimurium*, *Staphylococcus aureus* (Lusby *et al.*, 2005). In some parts of Ethiopia traditional healers use combinations of honey and parts of *E. schimperi* to treat microbial infections but it is not investigated scientifically. The present study was, therefore, aimed to see synergic antibacterial effect of leaf extract of *E. schimperi* and honey against standard and clinical isolated bacteria.

2. MATERIALS AND METHODS

2.1. Study Area

The study was conducted at Microbiology Laboratory, Department of Biology, University of Gondar; Gondar, Ethiopia. Gondar lies between 9° to 13° 45'N latitude and 36° to 40° 30'E longitude. It has variable altitudes that range from 550 m (in western lowland) to 4,620 m (Semen Mountain in North) above sea level and it is the homeland of Walia ibex and Ethiopian wolf (Central Statistical Authority, 2008).

2.2. Plant Material Collection and Identification

The leaves of *E. schimperi* were collected from University of Gondar garden. The plant species were identified by Taxonomist. Then, the collected plant material was washed with tap water and rinsed with distilled water. The leaves were placed in dark place protected from sunlight and open air at room temperature in Microbiology laboratory until it dries out.

2.3. Preparation of Plant Extracts

The dried leaves were powdered with a mechanical grinder. The powder was stored in a sterile brown bottle at room temperature under dark place (Shahidi, 2004). The 20g dried leaves powder of *E. schimperi* was macerated with 100mL chloroform and ethanol, and shaken on shaker (Edmund Buhler GMBH) shown in Figure 2 for 72hr to extract the active compound. Then, the solution was filtered using Whatman no.1 filter paper into previously washed, dried, labeled and weighed beakers (Chulet *et al.*, 2010). The filtrate was then evaporated on oven at 35°C. The extract was dissolved in 3% tween eighty to achieve 50% concentration (500mg/mL) prior to the test. The antibacterial activities of the crud extract of the plants were tested against selected organisms (Aladesanmi *et al.*, 2007).

2.4. Inoculums Preparation for Antimicrobial Test

The tested strains, *E. coli* ATCC 2592, *K. pneumonia* ATCC 700603, were standard strains and *S. typhi* and *S. aureus* were clinical isolate. The standard strains as well as the clinical isolates were obtained from stocks of culture collections maintained in microbiology laboratory of department of Medical microbiology, University of Gondar. The bacteria were maintained in triptone Soya broth with 20% glycerol and kept at a temperature below 4°C. Sub-culturing was done on nutrient agar during the experiment. Two to three isolated overnight cultured colonies of each organism were transferred aseptically by sterilized wire loop into 5 ml sterile normal saline in a test tube and mixed thoroughly, using vortex mixer (GEMMY Model: VM-300p), for uniform distribution and compared with 0.5 McFarland standard (Shahidi, 2004).

2.5. Honey Samples

The natural honey used in this experimental study was purchased from local market. The honey was diluted in distilled water to achieve 50% concentration (500mg/mL) prior to the test.

2.6. Preparation of 0.5 Mcfarland Turbidity Standards

The McFarland 0.5 turbidity standard was prepared by adding 50 µL of a 1.175% (wt/v) barium chloride dihydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution to 9.95 mL of 1% (v/v) sulfuric acid. The 0.5 McFarland standards were vigorously agitated to turbidity on a vortex mixer before use. The 0.5 McFarland standards are comparable to a bacterial suspension of 1.5×10^8 colony-forming units (CFU)/mL (NCCLS, 1993).

2.7. Antibacterial Activity Determination

2.7.1. Agar Well Diffusion

Muller Hinton agar was prepared by dissolving the required amounts of the powder in distilled water and boiled to mix thoroughly and autoclaved. The sterilized media was aseptically poured in to sterile Petri-plates in a laminar air flow and allowed to dry at room temperature for 30 minutes (López *et al.*, 2011). Bacterial suspension was prepared to a density of 1.5×10^8 colony-forming units (CFU)/mL by comparing with 0.5 McFarland standards in sterile normal saline. Aliquots of the organism (100µL) were aseptically transferred to Muller Hinton agar using a micropipette and seeded evenly by using sterilized cotton swab. On each plate, equidistant wells were prepared with a 6 mm diameter sterilized cork borer, 2 mm from the edge of the plate (Shahidi, 2004). Hundred micro liter of plant extract (500 mg/ml) was aseptically introduced into a respective agar wells. Gentamycin were used as positive controls and distilled water was included as negative control. This was followed by allowing the agar plate on the bench for 40 minutes for pre-diffusion followed by incubation at 37°C for 24 hrs. The experiment was performed in triplicate. Those extracts showing any inhibition at all were noted for further tests for the quantitative assessment of their activity.

2.7.2. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the extracts was determined for extracts that showed ≥ 10 mm diameter inhibition zone according to methods described by Shahidi (2004) and Akinyemi *et al.* (2005). The test was performed using macro-tube dilution method; the extract solution (500 mg/ml) was serially diluted to 1:2 then 1:4 mg/ mL concentrations with sterilized distilled water. The extract was then aseptically introduced to a tube containing sterilized and specified volumes of Nutrient broth. The inhibition of growth was determined by comparing a tube which contains pure and inoculated nutrient broth after 24 hr incubation at 37°C and the minimum concentration that inhibited growth was considered as MIC value of the extract (Parekh *et al.*, 2006).

Experimental data was collected in biology laboratory and the collected data was analyzed using SPSS version 20 software. Mean and standard deviations of the triplicates analysis were calculated using analysis of variance

(ANOVA) to determine the significance difference between the mean followed by Duncan's multiple range test ($p < 0.05$). The statistically significant difference was defined as ($p < 0.05$).

3. RESULT

Ethanol and chloroform extracts of Leaves of *E. schimperi* were evaluated *in vitro* for antibacterial activity by agar well diffusion method as it has been shown in appendix part of Figure 3 and its result has been described in Tables below. Variable degree of inhibition zones against *E. coli*, *K. pneumonia*, *S. thyphi* and *S. aureus* has been shown. As it was presented on Table 3.1 below the antibacterial activities of crud extracts of *E. schimperi* prepared by ethanol extraction has showed statistically significant difference ($p < 0.05$) in growth inhibition activity than the chloroform extract against *E. coli* standard strain but no statistically significant difference was observed against *S. thyphi* with both extracts. Ethanol extracts of *E. schimperi* has shown comparable activity against *E. coli* standard strain when compared with positive control drug gentamycin. *E. schimperi* ethanol extract showed better activity than chloroform extract against *S. aureus* standard isolate.

Table-3.1. Antibacterial activities of organic extracts of *Embelia schimperi* using agar well diffusion method.

Organism	Zone of inhibition (mm) \pm standard deviation			
	E. schimperi extract		Positive control	Negative control
	Ethanol	Chloroform	Gentamycin	3% tween80
<i>E. coli</i>	15.33 \pm 2.08 ^{2a}	9.33 \pm 1.53 ^b	16.56 \pm 0.51 ^a	00.00
<i>K. pneumonia</i>	14.5 \pm 0.5 ^a	11.70 \pm 0.61 ^b	28.96 \pm 0.25 ^c	00.00
<i>S. thyphi</i>	10.83 \pm 0.76 ^a	10.67 \pm 0.58 ^a	15.03 \pm 0.40 ^b	00.00
<i>S. aureus</i>	12.67 \pm 2.31 ^a	8.83 \pm 0.29 ^b	17.33 \pm 0.35 ^c	00.00

Values are means of triplicate determinations values within the same row followed by different supper scripts (letters a, b, c) are significantly different at ($p < 0.05$).

Table 3.2 below shows ethanol and chloroform extracts of *E. schimperi* blended with honey. *E. schimperi* extract mixed with honey showed statistically significant difference against the tested organisms when compared with chloroform extract mixed with honey. Statistically significant ($p < 0.05$) inhibition zones difference was observed on the antimicrobial sensitivity activities by the mixture of chloroform extract with honey compared to positive control drug Gentamycin against the tested organisms but no significant difference was observed with mixture of ethanol extract with honey and Gentamycin ($p \geq 0.05$) against *E. coli* and *S. thyphi*. When compared to control drug gentamycin, against *K. pneumonia* greater inhibition zone was seen than ethanol and chloroform extracts mixed with honey.

Table-3.2. Antibacterial activities of organic extracts of *Embelia schimperi* ethanol and chloroform extracts mixed honey using agar well diffusion method.

Organism	Zone of inhibition (mm) \pm standard deviation			
	E. schimperi extract		Positive control	Negative control
	Ethanol with honey	Chloroform with honey	Gentamycin	3% tween80
<i>E. coli</i>	15.77 \pm 0.25 ^a	7.83 \pm 0.29 ^b	16.65 \pm 0.61 ^a	00.00
<i>K. pneumonia</i>	21.00 \pm 0.00 ^a	10.83 \pm 1.04 ^b	28.96 \pm 0.25 ^c	00.00
<i>S. thyphi</i>	14.60 \pm 0.53 ^a	9.50 \pm 0.50 ^b	15.03 \pm 0.20 ^a	00.00
<i>S. aureus</i>	11.50 \pm 1.32 ^a	7.17 \pm 0.76 ^b	17.33 \pm 0.35 ^c	00.00

Values are means of triplicate determinations values within the same row followed by different supper scripts (letters a, b, c) are significantly different at ($p < 0.05$).

4. DISCUSSION

Embelia schimperi is one of the common medicinal plants as reported by local healers (Rondevaldova *et al.*, 2015). *In vitro* antibacterial activity of *E. schimperi* had previously been studied in different areas which show antimicrobial effect in comparison with other plants used for the treatment of different diseases (Lulekal *et al.*, 2014). Although the plant parts were used traditionally as an anti-infective preparation in combination with other plant parts (d'Avigdor *et al.*, 2014) to the best of our knowledge there is no report on its *in vitro* antimicrobial activity in

combination with bee's honey. In this study, the efficacy of the extract and its mixture with honey against different bacterial species was evaluated *in vitro*. Different zones of inhibition were recorded from each crude extract and synergistic effect of the extract blended with honey at different concentrations for each species of bacterium. Regarding the efficacy of the crude extract, the highest zones of inhibition was observed against *E. coli* with Ethanol extract and the lowest was against *S. aureus* with chloroform extract. Concerning the effectiveness of the blend, the highest inhibition zone was witnessed against *K. pneumonia* with the ethanol extract mixed with honey and the lowest was witnessed against *S. aureus* with chloroform extract mixed with honey. Thus, the antibacterial activities of the ethanol crude extract and that blended with honey has shown promising potential against the tested bacterium and was found to be almost comparable to the standard control drug gentamycin.

According to Okwori *et al.* (2007) the formation of clear inhibition zone of ≥ 10 mm diameters around the wells was regarded as significant susceptibility of the organisms to the extract. The current study has shown a result that can witness the antibiotic potential of the tested plant crude extract and that blended with honey. The highest mean zone of inhibition against all the bacterial strains tested ranged between 7.17mm and 21.00mm and the positive control produced mean zone of inhibition between 15.03mm and 28.96mm, but the negative control has not produced any zone of inhibition.

The level of antimicrobial activity exhibited by plant material depends on many factors, including the plant part, moisture content, harvesting process, preparation process, strains of the tested bacterium and type of the solvent used to extract the active compound (Ramesh *et al.*, 2001; Wendakoon *et al.*, 2012). For instance, relatively high temperatures that can be generated during tissue grinding can denature chemical constituents. In addition, the extraction solvent, the time of maceration, may affect the level and composition of secondary metabolites extracted from plant tissues (Wendakoon *et al.*, 2012). In our study, there was higher and statistically significant antimicrobial inhibition zone difference. The susceptibility of the tested bacterium was higher to the ethanol crude extract and its blend with honey than that of the chloroform. The extraction solvents used in this study could have caused variation in the antimicrobial activity results. According to Motamedi *et al.* (2010) report, the solubility of active principles in plant materials varies according to extraction solvent used, which might be related to differences in antimicrobial effect of plant extracts.

Moreover, the sensitivity differences between Gram-positive and Gram negative bacteria to the extract of different medicinal plants might be due to the structural and compositional differences in membranes between the two groups (Hodges, 2002). In contrary of other considerable studies, the current study has indicated that the tested plant extracts has exhibited better activities on Gram-negative bacteria than on Gram-positive bacteria. However, as several studies confirmed, Gram-negative bacteria having an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to antimicrobial chemical substances and act as a diffusion barrier and making them less susceptible to the antimicrobial agents than are Gram-positive bacteria (Nostro *et al.*, 2000; Hodges, 2002). In spite of this permeability differences, however, some of the extracts have still exerted some degree of inhibition against Gram-negative organisms as well. Indeed, Gram negative bacteria are more resistant to antibiotics because they possess impermeable outer membrane; consequently, the levels of antibiotics in the cell are reduced (Bockstael and Aerschot, 2009).

On the other hand, in this study half of the tested bacterium was clinical isolates and the lower inhibition growth was observed during the test. This revealed that, since they have exposure to standard drugs, they might have relatively higher resistance that the standard tested bacterium. As Togan *et al.* (2014) described possible differences in susceptibility patterns between standard and clinical strains, in which clinical strains may represent current isolates responsible for clinical disease and spread of resistance.

The antimicrobial effects of different Ethiopian honeys were already evaluated and witnessed as it has greater mean inhibition zone against bacteria (Ewnetu *et al.*, 2013). Similarly this study has evaluated the synergistic effect of the plant crude extract blended with honey. As aforementioned, *E. schimperi* is well known its usage as herbal

medicine and most traditional healers prefer to use medicinal plant combined with honey to act synergistically for the treatment of many infections (Nwankwo *et al.*, 2014). The finding of the current research has indicated that ethanol extract of leaves of *E. schimperi* blended with honey has shown the higher antibacterial activity far better than ethanol crude extract and chloroform extract blended with honey. The higher inhibition zone of ethanol crude extract blended with honey was against *K. pneumonia* which was standard bacteria.

The inhibitions of ethanol extract of plant leaves were enhanced by mixing with honeys due to their synergistic antibacterial property as already reported (Omoya and Akharaiyi, 2011). The antibacterial action of honey is believed to be due to acidity, osmolarity, hydrogen peroxide generation and phytochemical components (Molan, 1992). Furthermore, the antimicrobial effects of different honeys might be related to Phytochemicals such as Phenolic acids (benzoic and cinnamic acids) and flavonoids (flavanones, flavanols) which were reported for significant contribution of the antibacterial capacity of honey that varies greatly depending on the floral sources (Gheldof *et al.*, 2002). Many, but not all, of the bacterial strains commonly encountered by humans are killed by flavonoids, even though the mechanism is not yet known (Farnesi *et al.*, 2009). These synergistic antimicrobial effects of honey with Ethanol plant extract mixtures might be related chiefly to the increase in volume of these flavonoids in the mixture (Molan, 1992).

5. CONCLUSION

The tested plant has showed broad spectrum antibacterial activity as confirmed by high zone of inhibition. Both the *E. schimperi* leaves crude extract with ethanol and chloroform, and blended with honey has witnessed antibacterial potential against the tested pathogens. This finding suggests that there is possibility to isolate potential antibacterial drugs from the medicinal plant and the honey. The antibacterial activity of *E. schimperi* leaves extract is more effective when it is blended with honey than using the crude extract alone. Therefore, the use of mixture of honeys with *E. schimperi* extracts for treatment of bacterial infection is recommended. Phyto-chemical screening of the tested plant have to be done to detect secondary metabolites that are responsible for the antibacterial activity and its toxicity and their synergy with honey needs to be tested.

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APPENDIX



Figure-1. *Embelia schimperi* locally called Enkoko in Amharic
Source: picture taken from the garden during its collection.



Figure-2. Plant extraction procedure.

Source: picture taken at the period of plant extraction by the author.

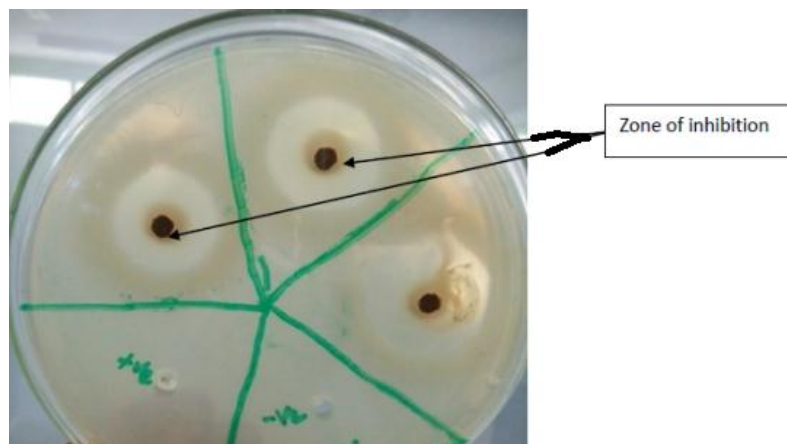


Figure-3. Variable degree of inhibition zones.

Source: picture by author during sensitivity test.

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