World Applied Sciences Journal 32 (8): 1600-1610, 2014
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2014.32.08.146

Synergistic Antibacterial Effect of *Sida rhombifolia* Leaf Extracts and *Apis mellifera* Honey Against Standard and Drug Resistant Clinical Isolated Pathogenic Bacteria

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Abstract: Combination of *Sida rhombifolia* and honey is traditionally useful antimicrobial substances for treatment of urinary tract infections. The objective of this study was to investigate synergistic antibacterial activity of *S. rhombifolia* and honey against multidrug resistant pathogenic bacteria. Solvent crude antimicrobial extracts of *S. rhombifolia* were tested against pathogenic bacteria using agar well diffusion method. Amoxicillin, tetracycline and vancomycin were used as positive controls. Minimum inhibitory and bactericidal concentrations of crude extracts were determined. The inhibition zone of ethanol, methanol and chloroform extracts of *S. rhombifolia* ranged from 21.17 to 24.17 mm against *S. Pneumoniae* and *E. coli* (clinical isolates) were significantly ($P \leq 0.05$) greater than the inhibition zone range from 16.50 to 18.83 mm against MRSA and *K. pneumoniae* (clinical isolates). Inhibition zone of combination of *S. rhombifolia* leaf crud extracts and honey (25.00 - 25.67 mm) against *K. pneumoniae* (clinical isolate) was significantly ($P \leq 0.05$) greater than the rest drug resistant pathogenic bacteria. Inhibition zone of the synergistic antibacterial effect of mixture of *S. rhombifolia* extracts and honey against pathogenic bacteria was significantly ($P \leq 0.05$) greater than antibacterial agents of *S. rhombifolia* and honey used separately. Thus, this might be the basic reason why local community widely used combination of *S. rhombifolia* and honey for treatment of various pathogenic bacteria. Further *in-vivo* study of synergic antimicrobial activity of *S. rhombifolia* extract and honey is recommended to come up with comprehensive knowledge for treatment of multidrug resistant pathogenic bacteria.

Key words: Honey · Multidrug resistant · Pathogenic bacteria · *Sida rhombifolia* · Synergistic effect

INTRODUCTION

Over the last few decades, a great interest has developed in searching for antimicrobial drugs from natural plant products. This interest primarily arises from the belief that drugs derived from plants are safe and dependable compared with synthetic drugs that may have adverse effects on host besides their high cost. Natural antimicrobials came from a wide array of sources including plants, animals and microorganisms [1]. Researchers have so far discovered over 10,000 biologically active compounds of microbial origin [2].

Recently, much attention has been directed toward extracts and biologically active compounds isolated from popular plant species and also scientists advance in their search for new bacterial targets to attack bacteria evolve. Because currently, many bacterial pathogens are becoming resistant to the existing antibiotics due to their misuse or repeated use of antibiotics in the treatment of infectious diseases [3-5].

Ethiopian climatic condition is rich in growing various plants having medicinal property. So, Ethiopian biodiversity has excellent research and development opportunity. The wider acceptability of herbal therapeutics by the society in relation to modern drugs is another driving force for rapidly ongoing research activities in the field of alternate medical systems. Literature survey revealed that the plant *S. rhombifolia* belonging to family malvaceae possess insecticidal and antimicrobial properties [6].

Sida rhombifolia has considerable reputation for its medicinal value in traditional medicine. The plant is much used for poulticing ulcers, boils, swellings, broken bones, cuts, herpes and styles and for a skin application in

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chicken pox [7]. The roots and stems are useful in fever, heart disease, piles and all kinds of inflammation [8]. An infusion of the root is given in dysentery [9]. It is applied to the abdomen for abdominal complaints [10]. The plant is also useful for treatment of tuberculosis [11-12]. Leaves are used as a diuretic [12] and also in treatment of skin rashes [13]. Stem is also employed as edemulescent and emollient [14]. Traditionally the plant is used for urinary tract infections and infected wounds [15].

Sida rhombifolia is a perennial or sometimes annual plant in the family malvaceae. Malvaceae is a cosmopolitan family of herbs, shrubs and trees. Modern research carried out on the malvaceae plants revealed that most of the plants belonging to this family are medicinally important as they contain biologically active compounds. The vernacular name of *S. rhombifolia* is ''Gorjejit'' in Amharic and different names are given in different localities of English speakers, such as Queensland hemp, sida hemp, Cuba jute, arrow leaf sida, broom jute sida and French speakers Chanvre du Queensland, herbe dure [16]. It is widely distributed in the tropics and occurs in almost all countries of tropical Africa. It is cultivated as a fiber crop [17] in Niger, India, Australia, America, Democratic Republic Congo and the Central African Republic. In Cameroon a watery maceration of the leaves is drunk as an antihypertensive agent, as a sedative, against sexually transmitted diseases and to cure diarrhea [18].

Apis mellifera honey is gaining acceptance by the medical profession for use as an antibacterial agent for the treatment of ulcers, bed sores, bacterial gastro-enteritis, eye infection and other surface infections resulting from burns and wounds [19-20]. Honey increases the sensitivity of microorganisms to antibiotics and decreases the microbial resistance to antibiotics [21]. In many cases it is being used with success on infections not responding to standard antibiotic and antiseptic therapy. Its effectiveness in rapidly clearing up infection and promoting healing is not surprising in light of the large number of research findings on its antibacterial activity. None of the reports in the medical literature; however, mention any selection of the honey used for the treatment of infections. Although it is recognized that honey has antibacterial activity, it is not generally realized that there is a very large variation in the antibacterial potency of different honeys and that the antibacterial properties can be easily lost by inappropriate handling and storage of honey [22]. In Ethiopia, although the local community uses *S. rhombifolia* leaf and honey mixture in various combinations to treat different types of diseases, still there is no any scientific report about the synergistic effect of *S. rhombifolia* and honey for the treatment of many pathogenic bacterial infections. Therefore, there is a need to investigate the synergistic antibacterial effect of *S. rhombifolia* and honey mixture for the treatment of many different standard and drug resistant clinical isolates of pathogenic bacterial strains. As a step in this direction, this study was focused on one of the selected medicinal plant i.e. *Sida rhombifolia* in combination with honey. The objective of the study was to investigate the synergistic antibacterial activity of *S. rhombifolia* leaf crude extracts and honey against standard and drug resistant clinical isolates of pathogenic bacteria.

MATERIALS AND METHODS

Description of the Study Area and Duration of the Study: The study was conducted in Amhara National Regional State, North Gondar Zone, at University of Gondar, Department of Biotechnology (Microbiology Laboratory) from December 1, 2012 to April 30, 2013. Gondar town is located at 748 Km from Addis Ababa to North West part of Ethiopia. It is located at latitude and longitude of 12°36'N and 37°28'E with an elevation of 2133 meters above sea level. The study design was experimental using appropriate methods such as determination of antibacterial activities, MIC and MBC.

Collection and Identification of Plant Material and Source of Honey: A fresh and healthy leaf sample of *S. rhombifolia* was collected from "Shinta River" in the month of December. The plant was selected based on the indigenous knowledge of the local traditional healers. A voucher specimen of this plant was identified at the national botanical plant herbarium of Addis Ababa University, Department of Biology. And also pure, unprocessed and un-boiled commercial honey that obtained from East Gojjam, Debre Markos local area in Ethiopia was used in this study. The honey sample produced by *Apis mellifera* was first filtered with sterile gauze to remove debris and highly pure (100.0%) honey was referred to as "neat".

Preparation of Plant Extracts: The leaf of *S. rhombifolia* was thoroughly washed in running tap water to remove debris and dust particles and then rinsed in distilled water. The leaf was dried in the laboratory in an open air at room temperature for about seven days and protected from sun light. Once completely dry, the leaves were grounded to a fine powder using an electronic blender and the powder was stored in a clean bottle at room temperature in dark place. The dried and powdered plant leaf (50 g) was extracted with each 300 ml of ethanol, chloroform and

methanol in a separate flask and then agitated for about 36 h on the orbital shaker. The extracts were filtered through Whatman No. 1 filter paper and centrifuged at 1000 rpm for about 15 minutes and then concentrated in a vacuum at 40°C using a rotary evaporator. Each extract was transferred to glass vials and kept in refrigerator at 4°C until use [2]. Solutions of honeys were prepared by 50% (v/v). Filtered and purified honey was considered as 100%. Each sample of honey was diluted sterilized water to give final concentrations of 50, 25, 12.5 and 6.25 % and then kept at 0°C for further investigation.

Sources of the Tested Organisms: Microorganisms used in this study included eight different bacterial strains, of these; four strains were Gram-positive bacteria such as *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (ATCC 63), Methicillin Resistant *Staphylococcus aureus* and *Streptococcus pneumoniae* (clinical isolates). The rest four strains were Gram-negative bacteria namely *Klebsiella pneumoniae* (clinical isolate), *Escherichia coli* (ATCC2592), *Escherichia coli* (clinical isolate) and *Shigella flexneri* (ATCC 12022). The standard test pathogenic bacteria were obtained from Department of Biotechnology, University of Gondar and those drug resistant clinical isolated pathogenic bacteria were collected from Gondar College of Medicine and Health Sciences Hospital. The bacterial cultures were maintained in their appropriate nutrient broth at 4°C until use.

Preparation of Inoculum: The tested microorganisms were separately cultured on nutrient agar at 37°C for 24 h by using streak plate method. Then, three to five well-isolated overnight cultured colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a flamed wire-loop and the growth was transferred into a screw-capped tube containing 5 ml of nutrient broth. The broth cultures (test tubes) were incubated without agitation for 24 h at 37°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard. The turbidity of the actively growing broth culture was adjusted with sterile saline to obtain turbidity optically comparable to that of the 0.5 McFarland turbidity standards 1.5×10^8 CFU/mL) [15].

Antibacterial Activity Testing of S. Rhombifolia Leaf Extracts and A. Mellifera Honey Using Agar Well Diffusion Assay: According to Andrews [23] 0.5 McFarland standards was prepared. Pathogenic test bacteria were inoculated into 5 ml of nutrient broth (Himedia) and incubated at 37°C for 24 h. The cultures were aseptically swabbed on the surface of sterile nutrient agar plates using a sterile cotton swab. Suspensions of the bacterial isolates were made in sterile normal saline and adjusted to the 0.5 McFarland's standard. Small volume (100 Microliter) of bacterial suspensions were added to each Mueller Hinton (MH; Oxoid, England) agar plate and then evenly seeded and streaked by means of sterile swab on the agar plate surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum and finally, the rim of the agar was swabbed. Agar wells were prepared by using a sterilized cork borer with 6 mm diameter, 4 mm deep and about 2.5 cm apart to minimize overlapping of zones [24]. By using a micropipette, 50% (v/v) of 100 Microliter of *S. rhombifolia* leaf extracts, honey and mixture of them were carefully added to the respective wells in the plate in triplicate and the antibiotic discs were dispensed with a dispensing apparatus (sterile pair of forceps) onto the surface of the inoculated agar plate and pressed down to ensure complete contact with the agar surface. Crude extracts and antibiotic discs were allowed to diffuse for about 40 minutes before incubation and then the plates were incubated in an upright position at 37°C for 24 h. After overnight incubation, the diameters of inhibition zones were measured in mm using a plastic ruler, which was held on the back of the inverted plate and the results were recorded. Antibiotic discs (Amoxicillin 25 Mcg, Tetracycline 30 Mcg and Vancomycin 30 Mcg) were served as positive controls while sterile distilled water was used as negative control.

Determination of the Minimum Inhibitory Concentration (MIC): The minimal inhibitory concentration (MIC) values of leaf extracted from *S. rhombifolia,* honey and mixture of them were determined based on broth macro- test tube dilution method. In the determination of MIC, 30 sterile screw-capped test tubes were placed on a suitable rack in three rows i.e., 10 tubes in each row and labeled each of them including the negative and positive control test tubes. Extracts and honey were diluted to concentrations (50%, 25%, 12.5% and 6.25%). The first test tube was contained 50 % extract (4 ml of crude extract and 4 ml of nutrient broth), the second one was contained 25 % extract (2 ml of extract and 6 ml of nutrient broth), the third one was contained 12.5 % extract (1 ml of extract and 7 ml of nutrient broth) and the last one was also contained 6.25 % extract (0.5 ml of extract and 7.5 ml of nutrient broth) to bring 8 ml. To each dilution of honey, *S. rhombifolia* and a mixture of both, 100 Microliter of the bacterial inoculum

was carefully dispensed in sterile screw-capped tubes which consist of crude extracts and nutrient broth. Nutrient broth with bacterial inoculation but no any extract (positive control tubes) and nutrient broth only with no bacterial inoculation (negative control tubes) were included for every test microorganism to demonstrate an adequate microbial growth over the course of the incubation period and media sterility, respectively. Then tubes were incubated aerobically at 37 °C for 24 h and examined for bacterial growth. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (turbidity) after overnight incubation was recorded as the MIC.

Determination of the Minimum Bactericidal Concentration (MBC): To determine the MBC, macro-test tubes used in the MIC, which did not show any visible growth of bacteria after the incubation period were sub-cultured on to the surface of the freshly prepared Mueller Hinton Agar (MHA) plates and incubated at 37°C for 24 h. The MBC was recorded as the lowest concentration (highest dilution) of the extract that did not permit any visible bacterial colony growth on the agar plate after the period of incubation [25].

Data Analysis: All data were analyzed using the program SPSS software package version 16.0 for windows. Means and standard deviations of the triplicates analysis were analyzed by one - way analysis of variance (ANOVA) to determine the significance differences between the means followed by Duncan's multiple range test. The statistically significant difference was defined as $p \leq 0.05$.

RESULTS

Analysis of Antibacterial Sensitivity Testing

Evaluation of S. Rhombifolia Leaf Crude Extracts and its Synergetic Effect with Honey Against Test Pathogenic Bacteria: The diameter of inhibition zone of *S. rhombifolia* leaf ethanol, methanol and chloroform crude extract was evaluated against standard and clinical isolated pathogenic bacteria and had shown in Table 1. The mean inhibition zone of ethanol crude leaf extract of *S. rhombifolia* (17.67 mm) against *K. pneumoniae* (clinical isolate) was significantly ($P \leq 0.05$) less than the rest extractants. Whereas the inhibition zone (19.50 mm) of methanol crude leaf extract of *S. rhombifolia* against *S. aureus* (ATCC 25923) was significantly ($P \leq 0.05$) greater than the inhibition zones (18.00-18.50) of chloroform and

Table 1: Mean inhibition zones of *S. rhombifolia* leaf crude extracts (by different solvents) and synergetic effect of honey against standard and clinical isolated pathogenic bacteria

Test organisms	Solvents used for extraction	Inhibition zone of crude leaf extracts (mm)	Inhibition zone of mixture (v/v) of crude leaf extracts and honey (mm)
K. pneumoniae (clinical isolate)	Et	(17.67±0.58)^{a}	(25.83±1.04)^{b}
	Met	(18.50±1.50)^{ab}	(25.67±0.58)^{b}
	Ch	(18.83±1.04)^{ab}	(25.00±1.00)^{a}
S. aureus (ATCC 25923)	Et	(18.50±0.50)^{ab}	(27.83±2.02)^{a}
	Met	(19.50±0.87)^{b}	(28.50±0.50)^{b}
	Ch	(18.00±0.87)^{a}	(27.83±2.02)^{a}
S. pneumoniae (ATCC 63)	Et	(19.33±1.15)^{a}	(28.67±1.15)^{bc}
	Met	(22.33±0.76)^{b}	(29.00±1.73)^{c}
	Ch	(19.33±1.15)^{a}	(28.00±1.50)^{a}
E. coli (ATCC2592)	Et	(22.50±0.50)^{a}	(26.50±3.97)^{ab}
	Met	(25.50±0.87)^{c}	(25.50±2.78)^{a}
	Ch	(24.17±4.65)^{bc}	(25.83±1.44)^{a}
S. flexneri (ATCC 12022)	Et	(25.83±1.61)^{c}	(27.83±1.44)^{bc}
	Met	(24.83±1.76)^{bc}	(25.83±2.36)^{a}
	Ch	(23.67±1.53)^{a}	(27.50±1.32)^{b}
MRSA (clinical isolate)	Et	(18.00±1.32)^{b}	(25.00±0.00)^{ab}
	Met	(17.50±0.87)^{b}	(24.67±0.58)^{a}
	Ch	(16.50±0.50)^{a}	(25.33±1.15)^{ab}
S. pneumoniae (clinical isolate)	Et	(21.17±1.26)^{a}	(24.67±0.58)^{a}
	Met	(23.00±0.00)^{b}	(25.50±0.87)^{b}
	Ch	(21.17±1.26)^{a}	(24.33±0.58)^{a}
E. coli (clinical isolate)	Et	(22.33±0.58)^{a}	(24.33±0.58)^{a}
	Met	(24.17±1.89)^{b}	(25.67±1.15)^{b}
	Ch	(24.00±1.73)^{b}	(24.67±0.58)^{a}

Values were means of triplicate determinations. Values of the same column followed by different letters are significantly different at ($p \leq 0.05$).

Table 2: The mean inhibition zone of honey against standard and drug resistant clinical isolated pathogenic bacteria

Test organisms	Inhibition zone of honey (in mm)
*K. pneumoniae (*clinical isolate)	(17.00±1.00)^{ab}
S. aureus (ATCC 25923)	(20.00±1.00)^{c}
S. pneumoniae (ATCC 63)	(21.00±1.00)^{c}
E. coli (ATCC2592)	(19.17±1.04)^{c}
S. flexneri (ATCC 12022)	(19.00±2.00)^{bc}
MRSA (clinical isolate)	(15.67±0.58)^{a}
S. pneumoniae (clinical isolate)	(17.00±1.00)^{ab}
E. coli (clinical isolate)	(15.33±0.58)^{a}

Values were means of triplicate determinations. Values of the same column followed by different letters are significantly different at ($P \leq 0.05$).

ethanol crude leaf extracts of *S. rhombifolia.* There was no statistically significant ($P \geq 0.05$) difference between the zone of ethanol and chloroform crude leaf extracts of *S. rhombifolia* against *S. pneumoniae* (ATCC 63) but the inhibition zone of methanol crude leaf extract of *S. rhombifolia* (22.33 mm) against this test organism was significantly ($P \leq 0.05$) greater than ethanol and methanol crude leaf extracts. The inhibition zone (22.50 mm) of *S. rhombifolia* crude leaf extract with ethanol solvent against *E. coli* (ATCC2592) was significantly ($P \leq 0.05$) less than the inhibition zones of methanol and chloroform crude leaf extracts of *S. rhombifolia.* Whereas the inhibition zone (23.67 mm) of chloroform crude leaf extract of *S. rhombifolia* against *S. flexneri* (ATCC 12022) was significantly ($P \leq 0.05$) less than the rest extractants. The inhibition zone (17.50-18.00 mm) of methanol and ethanol crude leaf extracts of *S. rhombifolia* against MRSA (clinical isolate) was significantly ($P \leq 0.05$) greater than the inhibition zone (16.50 mm) of chloroform crude leaf extract. But there was no statistically significant difference between ethanol and chloroform crude leaf extracts of *S. rhombifolia* against *S. pneumoniae* (clinical isolate) as well as methanol and chloroform crude leaf extracts against *E. coli* (clinical isolate) (Table 1).

The mean inhibition zones of mixture (v/v) of each crude leaf extracts of *S. rhombifolia* and honey were presented on Table 1**.** The inhibition zone of chloroform crude leaf extract of *S. rhombifolia* and honey mixture (25.00 mm) against *K. pneumoniae* (clinical isolate) was statistically ($P \leq 0.05$) less than the inhibition zone (25.67-25.83 mm) of methanol and ethanol crude leaf extracts of *S. rhombifolia* and honey mixture (Table 1).

But there was no statistically significant difference between ethanol and chloroform crude leaf extracts of *S. rhombifolia* and honey mixture against *S. aureus* (ATCC 25923). Whereas the inhibition zone (28.00 mm) of chloroform crude leaf extract and honey in mixture against *S. pneumoniae* (ATCC 63) was significantly ($p \leq 0.05$) less than the rest solvents used in combination with honey. The inhibition zone (25.50-25.67 mm) of methanol crude leaf extract of *S. rhombifolia* and honey mixture against *S. pneumoniae* (clinical isolate) and *E. coli* (clinical isolate) was significantly ($p \leq 0.05$) greater than the inhibition zones of ethanol and chloroform crude leaf extracts of *S. rhombifolia* and honey mixture.

Inhibition Zone of Honey Against Test Pathogenic Bacteria: The diameter of zone of inhibition of honey against tested pathogenic bacteria was presented on Table 2. The mean inhibition zones of *A. mellifera* honey ranged from (19.00 - 21.00 mm) against test pathogenic bacteria. Inhibition zone of *S. flexneri* (ATCC 12022), *E. coli* (ATCC2592), *S. aureus* (ATCC 25923) and *S. pneumoniae* (ATCC 63) were significantly ($P \leq 0.05$) greater than the mean inhibition zone of the rest pathogenic bacteria, but within these organisms the inhibition zone of honey against *E. coli* (ATCC2592), *S. aureus* (ATCC 25923) and *S. pneumoniae* (ATCC 63) were significantly ($P \leq 0.05$) far greater than the rest test organisms.

Comparison of Ethanol, Methanol and Chloroform Crude Leaf Extracts of S. rhombifolia and Commercial Antibiotic Discs Against Gram-negative and Gram-positive Pathogenic Bacteria: The inhibition zone of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* and commercial antibiotic discs against Gram-negative and Gram-positive pathogenic bacteria had shown on Table 3a and Table 3b, respectively. The inhibition zone of antibiotic discs such as Amoxicillin 25 Mcg, Tetracycline 30 Mcg and Vancomycin 30 Mcg (0.00 mm) against *K. pneumoniae* (clinical isolate) and *E. coli* (clinical isolate) were statistically ($p \leq 0.05$) less than the mean inhibition zones of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* (17.67 - 24.17 mm) (Table 3a). And also the inhibition zone of Amoxicillin 25 Mcg, Tetracycline 30 Mcg and vancomycin 30 Mcg (0.00 mm) against *S. pneumoniae* (clinical isolate) as well as the zone (13.50 mm) of vancomycin against MRSA (clinical isolate) were

Table 3a: Comparison of inhibition zone of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* and commercial antibiotic discs against Gram-negative test organisms

		Diameter of mean inhibition zones (mm)				
			Positive controls		-ve control	
Test organisms	Solvents used for extraction	plant leaf crude extracts	AMX	TE	VA	SDW
*K. pneumonia (*clinical isol)	Et	17.67±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0
	Met	18.50±1.50^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0
	Ch	18.83±1.04^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0
E. coli (ATCC2592)	Et	22.50±0.50^{bc}	19.50±0.50^{b}	15.83±0.76^{a}	29.67±0.29^{d}	0
	Met	25.50±0.87^{c}	19.50±0.50^{b}	15.83±0.76^{a}	29.67±0.29^{d}	0
	Ch	24.17±4.65^{c}	19.50±0.50^{b}	15.83±0.76^{a}	29.67±0.29^{d}	0
S. flexneri (ATCC12022)	Et	25.83±1.61^{c}	20.17±0.29^{b}	14.67±0.58^{a}	25.33±0.58^{c}	0
	Met	24.83±1.76^{c}	20.17±0.29^{b}	14.67±0.58^{a}	25.33±0.58^{c}	0
	Ch	23.67±1.53^{c}	20.17±0.29^{b}	14.67±0.58^{a}	25.33±0.58^{c}	0
E. coli (clinical isol).	Et	22.33±0.58^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0
	Met	24.17±1.89^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0
	Ch	24.00±1.73^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0

Values were means of triplicate determinations. Values of the same column followed by different letters are significantly different at ($P \leq 0.05$).

Table 3b: Comparison of inhibition zone of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* and commercial antibiotic discs against Gram-positive test organisms.

		Diameter of mean inhibition zones (mm)				
			Positive controls		-ve control	
Test organisms	Solvents used for extraction	Plant leaf crude extracts	AMX	TE	VA	SDW.
S. aureus (ATCC 25923)	Et	18.50±0.50^{b}	14.00±1.00^{a}	19.00±1.00^{b}	21±1.00^{c}	0
	Met	19.50±0.87^{bc}	14.00±1.00^{a}	19.00±1.00^{b}	21±1.00^{c}	0
	Ch	18.00±0.87^{b}	14.00±1.00^{a}	19.00±1.00^{b}	21±1.00^{c}	0
S. pneumoniae (ATCC63)	Et	19.33±0.58^{b}	14.83±1.26^{a}	20.67±1.61^{bc}	25.50±1.50^{d}	0
	Met	22.33±0.76^{c}	14.83±1.26^{a}	20.67±1.61^{bc}	25.50±1.50^{d}	0
	Ch	19.33±1.15^{b}	14.83±1.26^{a}	20.67±1.61^{bc}	25.50±1.50^{d}	0
MRSA (clinical isolation)	Et	18.00±1.32^{c}	0.00±0.00^{a}	0.00±0.00^{a}	13.50±0.32^{b}	0
	Met	17.50±0.87^{c}	0.00±0.00^{a}	0.00±0.00^{a}	13.50±0.32^{b}	0
	Ch	16.50±0.50^{c}	0.00±0.00^{a}	0.00±0.00^{a}	13.50±0.32^{b}	0
S. pneumoniae (clinical isolation)	Et	21.17±1.26^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0
	Met	23.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0
	Ch	21.17±1.26^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0

Values were means of triplicate determinations. Values of the same column followed by different letters are significantly different at ($P \leq 0.05$).
Key: Et = Ethanol, Met = Methanol, Ch = Chloroform, AMX = Amoxicillin, TE = Tetracycline, VA = Vancomycin, -ev = Negative and SDW = Sterile distilled water.

statistically ($P \leq 0.05$) less than the inhibition zone of *S. rhombifolia* leaf crude extracts (Table 3b). At the same time, mean inhibition zone of the antibiotic disc amoxicillin 25 Mcg (14.00 mm) against *S. aureus* (ATCC 25923) was statistically ($P \leq 0.05$) less than the mean inhibition zone of the crude leaf extracts of *S. rhombifolia* (18.00 - 19.50 mm). Whereas the mean inhibition zone of the antibiotic disc of vancomycin 30 Mcg (25.50 mm) to *S. pneumoniae* (ATCC63) was statistically ($P \leq 0.05$) greater than the mean inhibition zones of chloroform, ethanol and methanol crude leaf extracts of *S. rhombifolia* (19.33-22.33 mm). The inhibition zone of chloroform, methanol and ethanol crude leaf extracts (23.67 - 25.83 mm) against *S. flexneri* (ATCC12022) were statistically ($p \leq 0.05$) greater than the inhibition zones of the antibiotic discs Tetracycline 30µg and Amoxicillin 25µg (14.67 - 20.17 mm).

Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): Minimum inhibitory concentration and minimum bactericidal concentration of different concentrations of crude leaf extracts of *S. rhombifolia*, honey and mixture of each crude leaf extracts and honey (v/v) was investigated. As shown in Fig. 1a below, MIC of ethanol

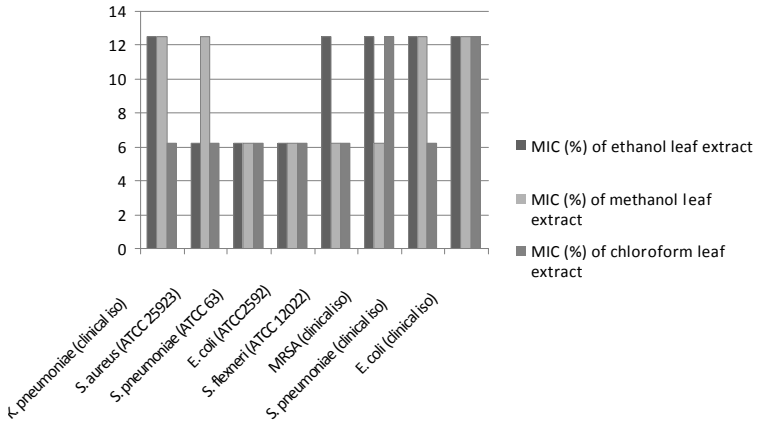

Fig. 1a: MIC determination of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* against standard and drug resistant clinical isolated pathogenic bacteria.
Key: iso = isolate), *MRSA* = Methicillin resistant *Staphylococcus aureus*

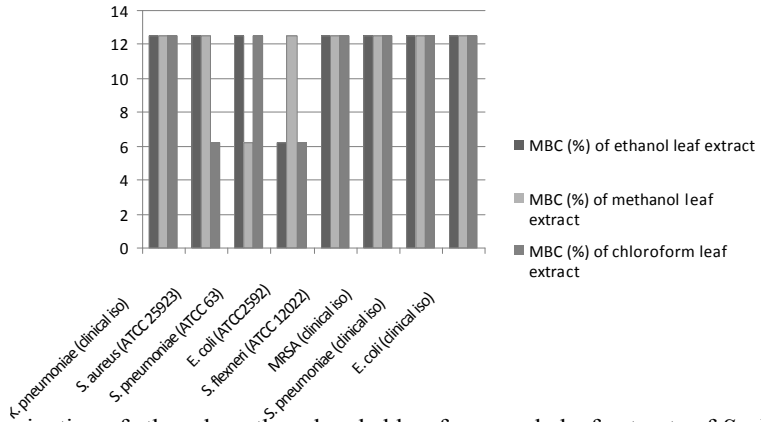

Fig. 1b: MBC determination of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* against standard and drug resistant clinical isolated pathogenic bacteria. Key: iso = isolate), *MRSA* = Methicillin resistant *Staphylococcus aureus*

and methanol crude leaf extracts of *S. rhombifolia* against *K. pneumoniae* (clinical isolate) and *S. pneumoniae* (clinical isolate) were 12.5%. At the same time, MIC of *S. rhombifolia* crude leaf extracts with ethanol, methanol and chloroform solvents against *E. coli* (clinical isolate) was 12.5%.

The minimum bactericidal concentration of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* against all drug resistant clinical isolated pathogenic bacteria including *S. flexneri* (ATCC 12022) were 12.5%. In the same manner, MBC of ethanol and methanol crude leaf extracts of *S. rhombifolia* against *S. aureus* (ATCC 25923) was 12.5% but MBC of chloroform crude leaf extract against this test organism was 6.25%. Minimum bactericidal concentration of ethanol and chloroform crude leaf extracts of *S. rhombifolia* against *S. pneumoniae* (ATCC 63) was 12.5% but MBC of methanol crude leaf extract against the same organism was 6.25%. The MBC of ethanol and chloroform crude leaf extracts of *S. rhombifolia* against *E. coli* (ATCC 2592) was 6.25% but the MBC of chloroform crude leaf extract against the same type of organism was 12.5% (Fig. 1b).

As shown in Table 4 below, the minimum inhibitory concentration of different concentrations of honey against MRSA (clinical isolate) *S. pneumoniae* (clinical isolate) and *E. coli* (clinical isolate) including *S. aureus* (ATCC 25923) and *E. coli* (ATCC 2592) were 12.5%; Whereas, MIC of different concentrations of honey against the rest pathogenic bacteria were 6.25%. Minimum bactericidal concentration of different concentrations of honey against *S. flexneri* (ATCC 12022) was 6.25% but all the rest tested pathogenic bacteria MBC of honey was 12.5% (Table 4).

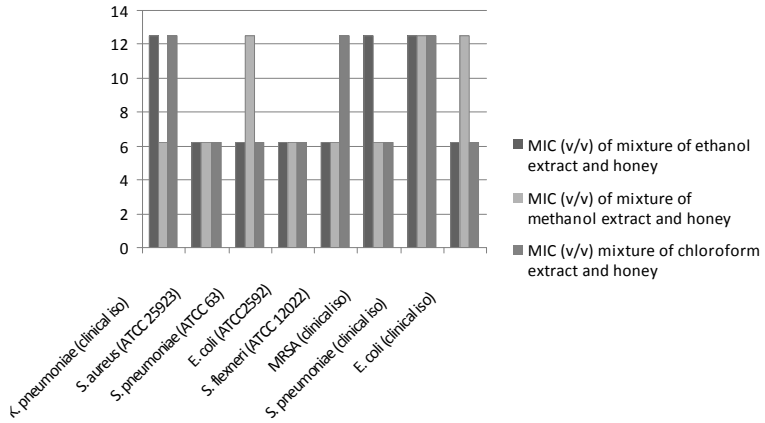

Fig. 2a: MIC (v/v) determination of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* and honey mixture.

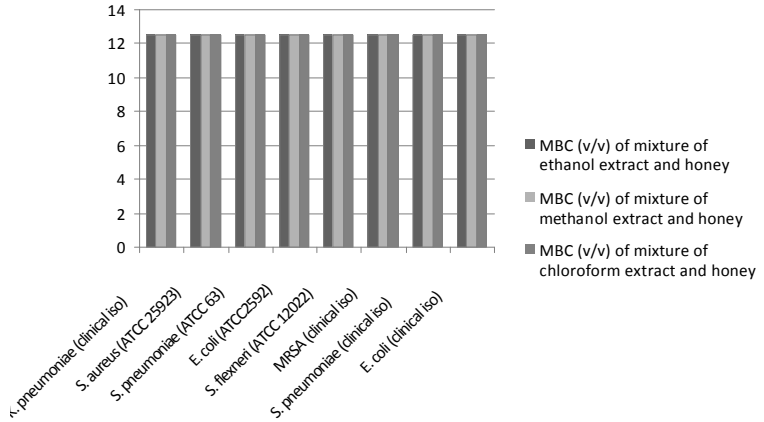

Fig. 2b: MBC (v/v) determination of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* and honey mixture.

Table 4: MIC and MBC determination of different concentrations of honey against standard and drug resistant clinical isolated pathogenic bacteria

Pathogenic bacteria	MIC of honey	MBC of honey
*K. pneumoniae (*clinical isolate)	6.25	12.5
S. aureus (ATCC 25923)	12.5	12.5
S. pneumoniae (ATCC 63)	6.25	12.5
E. coli (ATCC2592)	12.5	12.5
S. flexneri (ATCC 12022)	6.25	6.25
MRSA (clinical isolate)	12.5	12.5
S. pneumoniae (clinical isolate)	12.5	12.5
E. coli (clinical isolate)	12.5	12.4

Minimum inhibitory concentration of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* in combination with honey against pathogenic bacteria was presented on Fig. 2a and b. Minimum inhibitory concentration of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* in combination with honey against *S. pneumoniae* (clinical isolate) was 12.5%; whereas the MIC of ethanol and chloroform crude leaf extracts of *S. rhombifolia* and honey mixture against *S. pneumoniae* (ATCC 63) and *E. coli* (clinical isolate) was 6.25% but MIC for methanol crude leaf extract and honey mixture against the same test organism was 12.5%. Minimum inhibitory concentration of ethanol, methanol and chloroform crude leaf extracts of *S. rhombifolia* in combination with honey against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 2592) was 6.25%. But the MIC of ethanol and chloroform crude leaf extracts of *S. rhombifolia* and honey mixture against *K. pneumoniae* (clinical isolate) was 12.5% whereas the MIC of methanol crude leaf extract and honey in combination against the same test pathogen was 6.25% (Fig. 2a). On the other hand, the MBC of ethanol, methanol and chloroform crude leaf extracts and honey mixture against all tested pathogenic bacteria was 12.5% (Fig. 2b).

DISCUSSION

Currently, a large number of pathogenic bacterial strains have become multidrug resistant to commonly prescribed antibacterial drugs and causing a number of infectious diseases. Hence, search new drugs which are effective against the current multidrug resistant pathogenic bacteria are very important in recent times and *S. rhombifolia* have been proven as a potential source of bioactive compounds and richest source of secondary metabolites [26]. *Sida rhombifolia* and honey are one of the most commonly used natural antimicrobial agents and have been used traditionally for controlling many different pathogenic bacterial infections for a century. Natural plant product and honey based antibacterial drug discovery attained paramount importance as newly discovered drugs are likely to be effective against different pathogenic bacteria. The antibacterial potency of *S. rhombifolia* is believed to be due to ephedrine found in the leaf [26] whereas the antibacterial action of honey is believed to be due to acidity, osmolarity, hydrogen peroxide generation and phytochemical components [27]. The *in-vitro* studies of the antibacterial activity of mixture of *S. rhombifolia* crude extracts and honey, *S. rhombifolia* crude extracts and honey were separately showed good antibacterial activities against the tested pathogenic bacterial infections. It is interesting to note that mixture of *S. rhombifolia* leaf crude extracts and honey, as well as *S. rhombifolia* and honey separately were shown significant antibacterial activity against clinical drug resistant pathogenic bacteria namely, *K. pneumoniae* (clinical isolate), MRSA (clinical isolate), *S. pneumoniae* (clinical isolate) and *E. coli* (clinical isolate) where modern antibiotic therapy has limited effect. In the present study, the *in-vitro* antibacterial activity of crude leaf extracts of *S. rhombifolia*, honey and mixture of *S. rhombifolia* and honey were assessed by the agar well diffusion method and also the ethanol and methanol crude leaf extracts of *S. rhombifolia* were shown important antibacterial activity against *S. aureus* (ATCC 25923), *K. pneumoniae* (clinical isolate), *E. coli* (ATCC2592), *E. coli* (clinical isolate) and *S. flexneri* (ATCC 12022).Therefore, this result was strongly in agreement with the earlier report [28].

The inhibition zones of crude leaf extracts of *S. rhombifolia* against most test pathogenic bacteria were significantly ($p \leq 0.05$) greater than the inhibition zone of honey, but less than the inhibition zone of their synergism. On the other hand, the inhibition zone of the synergistic antibacterial effect of mixture of *S. rhombifolia* crude leaf extracts and honey against all tested pathogenic bacteria was significantly ($p = 0.05$) far greater than the mean inhibition zone of the antibacterial agents of *S. rhombifolia* and honey alone. This might be the basic reason why the local community widely used the mixture of *S. rhombifolia* and honey to treat different pathogenic bacterial infections. As a result, mixture of *S. rhombifolia* leaf crude extracts and honey may be effective to treat both Gram-positive and Gram-negative bacterial infections at low concentrations. Even though mixture of *S. rhombifolia* leaf crude extracts and honey has high antibacterial activity, *S. rhombifolia* leaf crude extracts and honey alone have also significant potential of a broad-spectrum of activity against both Gram-positive and Gram-negative bacteria. But for those Gram-negative bacteria, the crude extracts of the plant and honey in mixture or individually showed less antibacterial activity than Gram-positive bacteria. This might be due to the presence of an extra outer membrane in Gram-negative bacteria, which consists of lipopolysaccharide and makes them impermeable to lipophilic extracts; whereas the Gram positive bacteria were more susceptible because of having only an outer peptidoglycan layer which is not an effective permeability barrier. So this was a valuable reason and agreed with the previous report [29].

In this study, the results of crude leaf extracts of *S. rhombifolia* were compared with the common commercial antibiotic discs (Amoxicillin 25µg, Tetracycline 30µg and Vancomycin 30 Mcg). The mean inhibition zones of *S. rhombifolia* crude leaf extracts against all tested pathogenic bacteria were significantly ($P \leq 0.05$) greater than the inhibition zones of the commercial antibiotic discs (Amoxycillin and Tetracycline); while the mean inhibition zones of the antibiotic disc (Vancomycin 30 Mcg) against the tested organisms *S. aureus* (ATCC 25923), *S. pneumoniae* (ATCC63) and *E. coli* (ATCC2592) were significantly ($p \leq 0.05$) greater than the inhibition zones of *S. rhombifolia* crude leaf extracts. And also the mean inhibition zones of commercial antibiotic discs against all clinical isolated pathogenic bacteria except that vancomycin for MRSA were 0.00 mm. That means they were resistant against commonly prescribed commercial antibiotics. Therefore, *S. rhombifolia* crude leaf extract and honey alone or in mixture were effective to treat patients infected and encountered with such resistant pathogenic bacteria.

According to this study, MIC and MBC of *S. rhombifolia* crude leaf extracts, honey and mixture of them against most clinical isolated pathogenic bacteria were 12.5% but for those standard pathogenic bacteria, the

MIC and MBC were reduced by half (6.25%). These was because the clinical drug resistant pathogenic bacteria have the ability to resist and were not easily killed at the lowest concentration (highest dilution) by using mixture of *S. rhombifolia* crude extracts and honey, *S. rhombifolia* and honey separately when compared with standard pathogenic bacteria. But the standard pathogenic bacteria were easily killed at the lowest concentration (highest dilution) due to their sensitive for any antibiotic agents. This result was in agreement with the earlier reports [30-31].

Traditionally, *S. rhombifolia* is well known to act synergistically with honey for the treatment of many different clinical isolated pathogenic bacteria that are difficult to eradicate. The traditional medical practitioners using their indigenous knowledge opens a way to use the medicinal plant, *S. rhombifolia* and honey in combination for the treatment of different bacterial ailments. Thus, this finding was largely favor the claim of the local society or community to use the combination of *S. rhombifolia* and honey rather than using *S. rhombifolia* and honey separately for the treatment of different pathogenic bacterial infections and it opens a door to consider and acknowledge the traditional medical practices for the treatment of different infectious ailments using natural resources such as *S. rhombifolia* and honey.

CONCLUSION

In the present study it can be concluded that, the mean inhibition zones of the synergistic antibacterial activity of *S. rhombifolia* crude extracts and honey against all tested pathogenic bacteria were far greater than the mean inhibition zones of *S. rhombifolia* crude extracts and honey separately. The antibacterial activity of *S. rhombifolia* leaf crude extracts was greater than the activity of currently used antibiotics against the selected organisms. As a result, the growth of all pathogenic bacteria was completely inhibited.

Besides this, all clinical isolated pathogenic bacteria used in this study did not show any inhibition zones against the commonly prescribed commercial antibiotic discs used with the exception of vancomycin for MRSA. This was believed to be due to their resistance for these commercial antibiotic discs. Therefore, the mixture of substances under investigation has potential application against drug resistant pathogenic bacteria. Current research is focused to produce antimicrobial agents from different sources to solve the problem encountered by multidrug resistant pathogenic bacteria. Further *in vivo* studies should be conducted to evaluate the efficacy of antimicrobial activity of *S. rhombifolia* to treat microbial infections. There is a need for detailed scientific study of traditional medical practices to ensure that valuable therapeutic knowledge of the society on some plants and thereby to preserve such important natural antimicrobial resources.

ACKNOWLEDGEMENT

We are thankful to the University of Gondar for financial assistance (UOG/Budget/no.6215) for the successful completion of this research work. We would like to express our appreciation to the Department of Biotechnology for the facilities provided during this investigation.

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