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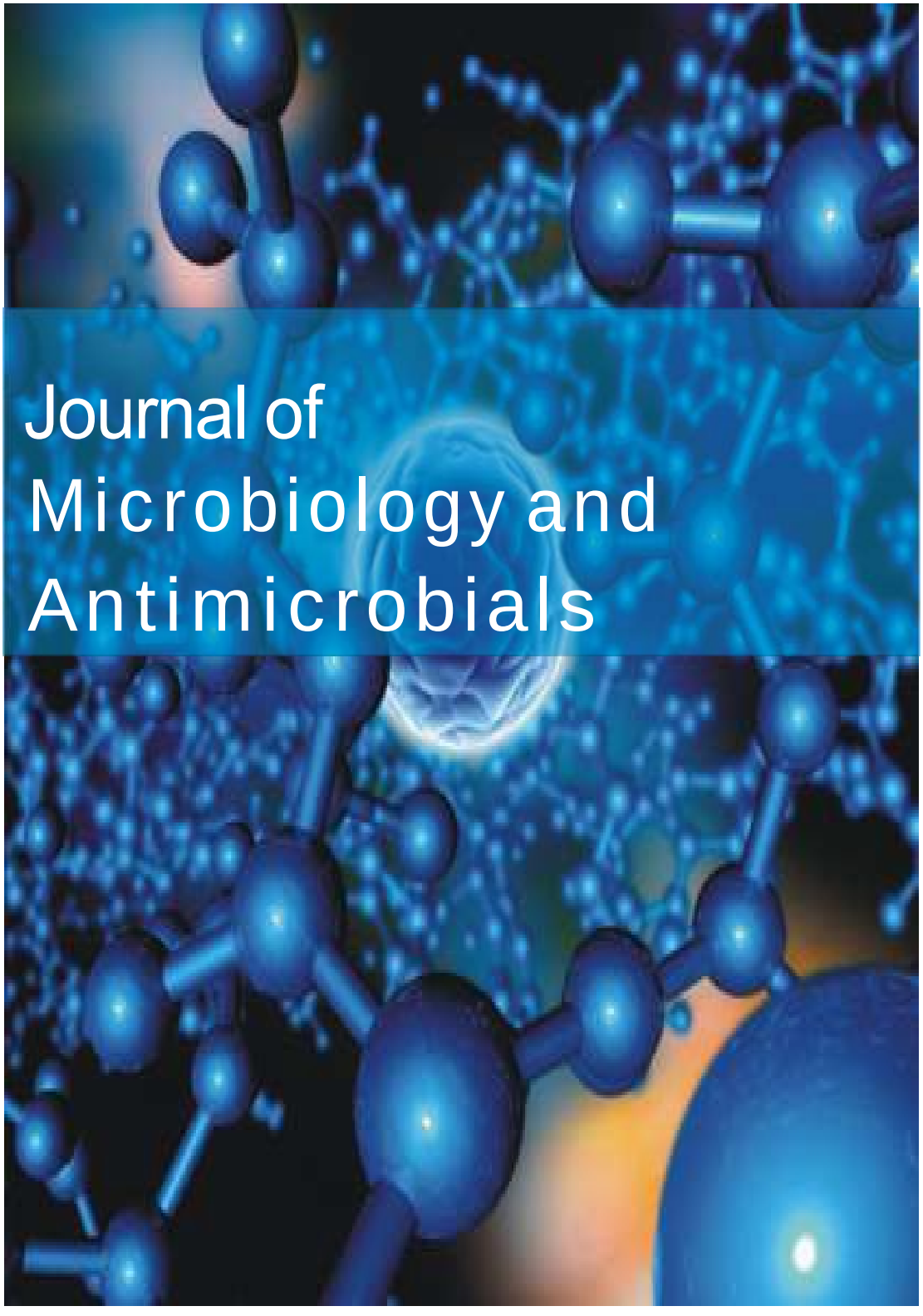

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Journal of Microbiology and Antimicrobials

Full Length Research Paper

Antimicrobial effects of *Rumex nepalensis* and *Echinop sphaerocephalus* crude extracts on selected pathogenic bacteria

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***In vitro* anti-microbial activity of *Rumex nepalensis* (leaves and root) and *Echinop sphaerocephalus* (leaves) crude extracts was tested against some clinical isolates and standard pathogenic bacteria. The crude extracts were prepared by using water, methanol and acetone. The antimicrobial effects of extracts were explained in terms of MBC and MIC. Among the extracts tested, methanol extracts of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) was found to have significant activity against *E. coli* ATCC2592 (17 mm ± 1.00), *K. pneumoniae* clinical isolate (16.67 mm ± 0.577), *E. coli* clinical isolate (15.67 mm ± 0.765) and *Staphylococcus aureus* ATCC25923 (14.67 mm ± 0.577) followed by aqueous extract of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) which showed antibacterial activity against *K. pneumoniae* clinical isolate (15 mm ± 0.00), *E. coli* clinical isolate (13.17 mm ± 0.76), *S. pneumoniae* ATCC63 (12.67 mm ± 0.577), and *S. pneumoniae* clinical isolate (12.50 mm ± 0.50). The acetone extract of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) showed activity against *E. coli* ATCC2592 (12 mm), *E. coli* clinical isolate (12 mm) and *S. aureus* ATCC25923 (10.33 mm ± 1.00). The methanol, acetone and distilled water extract of *R. nepalensis* leaves showed statistically significant bactericidal activity against *S. aureus* standard strain ($p < 0.05$). This study concludes that the tested plants may be having promising antibacterial compounds.**

Key words: Antimicrobial activity, *Echinop sphaerocephalus*, medicinal plants, *Rumex nepalensis*.

INTRODUCTION

Plants have been used directly as medicine for thousands of years by people all over the world. According to World Health Organization 80% of the population mostly in developing countries still relies on plant-based medicines (Ajiboye et al., 2015; Song, 2022). Medicinal plants research is alarmingly increased to isolate novel antibiotic compound to overcome synthetic drug (Mosaddegh and Naghibi, 2002; Marathe et al., 2013) and also to find out a solution against drug resistant bacteria (Kouadio et al., 2020).

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Many conventional drugs were obtained from plants which were initially used in crude form in traditional or folk healing practice (Lulekal et al., 1999; Idris and Abubakar, 2016). Beginning with the discovery of pure compounds from plants, many secondary metabolites were discovered and exhibited diverse bioactivities such as antioxidant, anti-inflammatory, antitumor, anti-mutagenic, anti-carcinogenic, anti-protozoan, antifungal, antibacterial or antiviral.

Some of the drugs isolated from the plants include aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), taxol (from Pacific yew tree) and morphine (from opium poppy) (Vickers and Zollman, 1999; Marathe et al., 2013). Even though, number of new antibiotics discovered, their high costs and the acquisition of resistance still lead to high morbidity and mortality of the population (Hancock, 2005; Ayandele et al., 2018; Mimura et al., 2020). Such emergence and spread of antibiotics resistance urges searching for novel herbal medicines (Idris and Abubakar, 2016; Yhiler et al., 2019). Use of herbal products as antimicrobial agents may provide best alternative to the wide and injudicious use of synthetic antibiotics (Marathe et al., 2013; Kouadio et al., 2020).

Today, natural products (their derivatives and analogs) still represent over 50% of all drugs in clinical use which are derived from higher plants (Chin et al., 2006; Ayandele et al., 2018). The discoveries of new and novel antimicrobials and antioxidants have been endless activities for pharmaceutical industries and research institutes. In line with this fact and related with the extensive and even misuse of antibiotics and their consequences in drug resistance, human pathogens become the future challenge for treating infectious diseases. In this effect, efforts need to be made to preserve drugs at hand and search for antibiotics from traditional medicinal plants (Ko and Stone, 2020; Song, 2022). So *in-vitro* antimicrobial test on natural substances can be a source of alternative to current chemical used to treat infectious diseases and contribute to overcome the resistance against it (El-Banna and Qaddoumi, 2016; Kouadio et al., 2020). Therefore, the present study was initiated to observe *in vitro* anti-microbial activity of crude extracts of *Rumex nepalensis* and *Echinop sphaerocephalus* against some clinical isolates of pathogenic bacteria and standard pathogenic bacterial isolates.

MATERIALS AND METHODS

Study area and period

The study was conducted at Microbiology laboratory, Department of Biology, College of Natural and Computational Sciences, University of Gondar, North West part of Ethiopia. The study area is located at latitude and longitude of 12°36′N 37°28′E with an elevation of 2133 m above sea level (Gondar Statistics Office, 2016). The study was conducted from September 2016 to May 2017.

Plant material collection and identification

Roots and leaves of *R. nepalensis* (local name Yeberie melase in Amharic) and leaves *of E. sphaerocephalus* (local name Bergude in Amharic) were collected from in and around road sides of Gondar and also University of Gondar garden. The voucher specimen of *R. nepalensis* (Polygonaceaes), and *E. sphaerocephalus* (Asteraceae) plants were prepared and the identity was confirmed with Botanical Herbarium Laboratory of Addis Ababa University and deposited at national herbarium of Addis Abeba University, Ethiopia.

The *R. nepalensis* is persistent herbaceous plant and categorized to Polygonaceae (buckwheat family) that produce erect, branched 50 to 180 cm tall stems and large rootstock. The plant consumed as food, medicine, and source of tannin (Vasas et al., 2015; Shaikh et al., 2018). *E. sphaerocephalus* is a fast-growing perennial shrub with spiny grey-green leaves and white flowers which are produced throughout the summer, that is, their Blooming time is from June to August. It belongs to the family of Asteraceae. These plants are traditionally used to treat ailments related to respiratory tract including cough and sore throat; and to relief inflammation, pain, and fever symptoms (Menzel et al., 2017; Veronika et al., 2020).

Plant extraction

The collected leaves and roots of *R. nepalensis* and leaves of *E. spearocephalus* were washed with tap water followed by distilled water and dried at room temperature to prevent loss of active components. The dried leaves and roots were powdered with a mechanical grinder and stored in a sterile bottle at room temperature in dark place (Bonjar, 2004). The powdered sample was successively extracted with acetone, methanol and water in increasing order of polarity. In this study, 20 g of powder was taken in to 250 ml conical flask and mixed with 100 ml of 95% methanol, acetone and water individually and kept three days in a shaker for continuous agitation to extract the bioactive compound in the plant body. The extracts were filtered by passing through Whatman's No. 1 filter paper and the solvent extracts were concentrated under reduced pressure using rotary evaporator and solvent free residue was collected and preserved at 4°C in air tight glass container for further investigation (Akinyemi et al., 2005). The extracted plant material was tested at the concentration of 50 mg/ml.

Test organism for anti-microbial assay

Standard and drug resistant clinical isolates of Gram positive and Gram negative strains such as *Escherichia coli* (ATCC2592), *Streptococcus pneumoniae* (ATCC 63), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* clinical isolate, methicillin resistance *Staphylococcus aureus* (MRSA), clinical isolate *Streptococcus pneumoniae* and clinical isolate *Klebsiella pneumoniae* were obtained from Microbiology Department, Gondar College of Medical and Health Sciences, University of Gondar. Each bacterium was separately cultured on nutrient agar for 24 h. Three to four isolated overnight cultured colonies were transferred to a tube containing sterile saline. The bacterial suspension was compared to the 0.5 McFarland standards (Bonjar, 2004).

Inoculum preparation of 0.5 McFarland turbidity standard

The McFarland 0.5 turbidity standard was prepared by adding 50 µl of 1.175% (w/v) barium chloride dehydrate ($BaCl_2.2H_2O$) solution to 9.95 ml of 1% (v/v) sulfuric acid (NCCLS, 2003). McFarland standard tube was sealed with parafilm to prevent evaporation and stored in dark at room temperature. The accuracy of prepared

McFarland standard density was checked using spectrophotometer with a 1-cm light path length. For the 0.5 McFarland standards, the absorbance was adjusted at a wavelength of 625 nm and water used as a blank. The 0.5 McFarland standards were vigorously agitated to turbidity on a vortex mixer before use. As with the barium sulfate standards, 0.5 McFarland standard was comparable to a bacterial suspension of 1.5×10^8 cells per/ml (NCCLS, 2003).

Antibacterial activity determination

Agar well diffusion

Bacterial broth culture was prepared to a density of 10^8 cells ml^{-1} of 0.5 McFarland standards. The aliquot was spread evenly onto Muller Hinton agar using sterilized cotton swab. Then, the plated medium was allowed to dry at room temperature for 30 min (Lopez et al., 2011). On each plate, 6 mm diameter, equidistant wells were prepared with the help of sterilized cork borer, 2 mm from the edge of the plate. Hundred microliters of each plant extract (50 mg/ml) was aseptically introduced into a respective agar well. Penicillin (5 µg/ml), methicillin (5 µg/ml) and amoxicillin (25 µg/ml) 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 min for pre-diffusion followed by incubation at 37°C for 24 to 48 h. The formation of clear inhibition zone of ≥7 mm diameters around the wells was regarded as significant susceptibility of the organisms to the extract (Okwori et al., 2007). The experiment was performed in triplicate. If any experiment gave contradicting results was repeated for the fourth time to minimize experimental error as per the suggestion of Bauer et al. (1966).

Determination of minimum inhibitory concentration (MIC)

MIC was determined for extracts that showed ≥7 mm diameter growth inhibition zone. The experiment was conducted using macro-tube dilution methods, extract solution (50 mg/ml) was serially diluted as 1:2, 1:4, 1:8 to bring 25, 12.5, and 6.25 mg/ml concentration, respectively (Parekh et al., 2005). MIC was determined according to the method described by Bonjar (2004). Extracts were diluted to concentrations ranging from 6.25 to 25%. To each dilution of *R. nepalensis* leaves and root and *E. spearocephalus* leaves extract, nutrient broth tubes were seeded with 100 µl of the pathogenic standard and resistant clinical bacterial inoculums. Negative control tubes were simultaneously maintained with no bacterial inoculation. Tubes were incubated aerobically at 37°C for 24 h. The lowest concentration of the extract that produced no visible growth (turbidity) was recorded as the MIC.

Determination of the minimum bactericidal concentration (MBC)

Dilutions showing no visible growth for the MIC were sub cultured into a fresh MHA plate and incubated at 37°C for 24 h for the determination of MBC. In brief, dilution showing no visible growth in the determination of MIC was streaked (sub cultured) in to MHA and incubated for 24 h. The least concentration of the extract with no visible growth after incubation was taken as the MBC (Parekh et al., 2005).

Determination of synergetic antimicrobial activities

The crude extracts of R. nepalensis leaves and root and E. *sphaerocephalus* leaves were diluted to 10% extracts of each as well as combination of extracts were diluted to 10% extract of *R. nepalensis* leaves and root plus 10% *E. sphaerocephalus* leaves to evaluate synergetic effect. Then 100 µl of each antimicrobial agent were added into the wells of 6 mm to examine inhibition zone differences.

Data analysis

The data were analyzed using SPSS version 16.0. Means and standard deviations of the triplicate analysis were calculated using analysis of variance (ANOVA). The significant differences between the means were compared using Duncan's multiple range test when the F-test demonstrated significance. The statistically significant difference was defined as $p \leq 0.05$.

RESULTS

The plant extracts yield

The crude extract obtained from 20 g of plant leaf powder of *R. nepalensis* was 19, 23 and 17% and *E. sphaerocephalus* was 18.6, 21.7 and 24.2% using acetone, methanol and water, respectively. The 20 g of *R. nepalensis* root powder yielded 12.9, 15.2 and 16.4% of the crude extract using acetone, methanol and water, respectively.

Determination of antimicrobial activity of plant extracts using agar well diffusion method

Among the organic solvents, methanol extracts of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) were found to have significant activity against *E. coli* ATCC2592 (17.00±1.00 mm), *K. pneumoniae* clinical isolate (16.67±0.57 mm), *E. coli* clinical isolate (15.17±0.76 mm), and *S. aureus* ATCC25923 (14.67±0.57 mm) followed by aqueous extract of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) which showed antibacterial activity against *K. pneumoniae* clinical isolate (15.00±0.00 mm), *E. coli* clinical isolate (13.17±0.76 mm), *S. pneumonia*e ATCC63 (12.67±0.57mm) and *S. pneumoniae* clinical isolate (12.50±0.50 mm). The acetone extract of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) showed antibacterial activity against *E. coli* ATCC2592 (12.00 mm), *E. coli* clinical isolate (12.00 mm) and *S. aureus* ATCC25923 (10 ±1.00 mm). Methanol extract of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) was found effective with the inhibition zone sizes ranging from 12.33 mm to17.00 mm. Aqueous extract of *R. nepalensis* (leaves and root) and *E. sphaerocephalus* (leaves) of showed inhibition zone in the range of 10.33 to 15 mm.

Antibacterial activity of crude extracts of *R. nepalensis* leaves prepared by methanol, acetone and distilled water extract showed statistically significant difference against *S. aureus* standard strain ($p < 0.05$). Statistically significant difference ($p < 0.05$) was also observed in *S.*

Table 1. Antibacterial activity of organic and aqueous extracts of *R. nepalensis*.

Organism	Solvent	Organic and aqueous extracts of *R. nepalensis*
		Zone of inhibition (mm) ± standard deviation
S. aureus ATCC2592	Met	14.67±1.20^{b}
	Act	10.00±1.00^{c}
	Aq	10.33±0.57^{a}
MRSA	Met	13.33±0.57^{ab}
	Act	20.83±0.76^{c}
	Aq	9.83±0.76^{c}
S. pneumoniae ATCC63	Met	13.33±1.52^{ab}
	Act	0.00±0.00^{a}
	Aq	12.67±0.57^{bc}
S. pneumoniae (clinical)	Met	12.33±1.15^{a}
	Act	0.00±0.00^{a}
	Aq	12.50 ± 0.50^{bc}
E. coli (clinical)	Met	15.17±0.76^{cd}
	Act	12.00±0.00^{d}
	Aq	13.17 ± 0.76^{c}

MRSA-Methicillin Resistant *S. aureus,* met-methanol, act-acetone, aq- aqueous, SD-standard deviation, RN - *R. nepalensis* Values are means. Values within the same row followed by different supper scripts are significantly different at ($p < 0.05$).
Source: Authors

pneumoniae standard isolates in which methanol extract showed greater inhibition zone than aqueous (Table 1).

Among the two organic extracts of *E. spearocephalus,* aqueous was found to have significant activity against *K. pneumoniae* (clinical) (15±0.45 mm), *E. coli* ATCC2592 (13.17±0.76 mm), and *E. coli* (clinical) (12.10±0.76 mm) followed by methanol extract against *S. pneumoniae* (clinical) isolates (12.67±0.57 mm), *S. pneumoniae* ATCC63 (11.67±0.57 mm), *S. aureus* ATCC25923 (11.67±0.57 mm) and MRSA (11.33±0.57 mm). The acetone extract showed activity against MRSA (23.00±0.57 mm), *K*. *pneumoniae* (clinical) (10.00 mm) and *S. aureus* ATCC25923 (13.67±0.57 mm) (Table 2).

Table 2 shows antibacterial activities of methanol, acetone and distilled water crude extracts of *E. spearocephalus* leaves. The results revealed statistically significant difference in bactericidal activity against MRSA ($p < 0.05$). Statistically significant difference ($p < 0.05$) was observed against *E. coli* ATCC2592 standard isolate.

The comparison of *E. spearocephalus* leaves crude extracts by different solvent types with positive control drugs for Gram positive was presented in Table 2. As a result, no statistically significant difference was seen for acetone extracts and penicillin positive control drug against *S. aureus* ATCC25923. Inhibition by Amoxicillin, Penicillin, and Methicillin was greater than inhibition by crude extracts of methanol, and aqueous extract which was statistically significant at ($p < 0.05$) against *S. aureus* ATCC25923. The antibacterial activity of the crude methanol extract was statistically significant ($p < 0.05$) less than the positive control drug penicillin, Amoxicillin and Methicillin no statistically significant difference was observed between positive control drug Penicillin and acetone extracts against MRSA. Inhibition by methanol, acetone and aqueous extract was less than Amoxicillin, Penicillin and Methicillin positive control drugs against *S. pneumoniae* ATCC63 standard.

Among the solvent extracts, acetone was found to have significant antimicrobial activity against *S. aureus* ATCC25923 (26.67±1.52 mm), *E. coli* ATCC2593 (27.00±4.35 mm), MRSA (25.33±1.15 mm), *K. pneumoniae* (clinical) (24.67±1.52 mm), *S. pneumoniae* ATCC 63 (24.00±1.00 mm), *E. coli* (clinical) (20.67±0.57 mm) and *S. pneumoniae* (clinical) (20.33±0.57) followed by methanol extract which shows antibacterial activity against *E. coli* ATCC2592 (25.33±0.57 mm), *S. pneumoniae* (clinical) (28.00±1.00 mm), *S. pneumoniae* ATCC63 (23.33±0.57 mm), MRSA (22.33±0.57 mm), *S. aureus* ATCC25923 (22.00±1.00 mm), *K. pneumoniae* (clinical) (21.00±1.00 mm) and *E. coli* (clinical) (19.00±1.00 mm).

The aqueous extract shows significant activity against *E. coli* ATCC2592 (25.33±0.57 mm), *S. pneumoniae* (clinical) (21.67±1.15 mm), *S. aureus* ATCC25923 (25.67±0.57 mm), *S. pneumoniae* ATCC63 (21.67±0.57 mm), *E. coli* (clinical) (19.00±1.00 mm), *K. pneumoniae* (clinical) (17.00±1.00 mm) and MRSA (17.00±1.00 mm). Acetone extract was found effective with zone sizes

Table 2. Antibacterial activities of organic and aqueous solvent extracts of *E. spearocephalus* leaves.

Organism	Solvent	Inhibition zones(mm) ± SD			
		E. spearocephalus leaves	Positive control drugs		
Gram positive			**Amoxicillin**	**Penicillin**	**Methicillin**
	Met	11.67±0.57^{bc}			
S. aureus ATCC25923	Act	13.67±0.57^{c}	21.33±1.17^{bcd}	16.33±1.35^{bc}	14.77±0.88^{b}
	Aq	10.33±0.57^{a}			
	Met	11.33±0.57^{b}			
MRSA	Act	23.00±0.57^{d}	22.33±1.00^{dc}	18.44±0.57^{bcd}	0.00± 0.00^{bc}
	Aq	11.50±1.49^{a}			
	Met	11.67±0.57^{bc}			
S. pneumoniae ATCC63	Act	9.00±1.00^{bc}	22.00±1.00^{dc}	16.44±0.86^{bc}	16.88±1.00^{bc}
	Aq	12.67±0.57^{bc}			
	Met	12.67±0.57^{d}			
S. pneumoniae (clinical)	Act	0.00±0.00^{c}	22.55±0.88^{cd}	0.00±0.00^{bc}	15.22±0.88^{ab}
	Aq	12.50±0.50^{bc}			
Gram negative					
	Met	9.67±0.57 ^{a}			
K. pneumoniae (clinical)	Act	10.00±0.04^{c}			
	Aq	15±0.45^{d}			
	Met	9.33±0.57 ^{a}			
E. coli ATCC2592	Act	7.67±0.57^{a}			
	Aq	13.17±0.76^{c}			
	Met	7.33±0.57 ^{a}			
E. coli (clinical)	Act	8.47±0.57^{a}			
	Aq	12.10±0.76^{c}			

MRSA-Methicilin Resistant *S. aureus,* met-methanol, act-acetone, aq- aqueous, SD-standard deviation, E.S – *E. spherocephalies* Values are means. Values within the same row followed by different supper scripts are significantly different at ($p < 0.05$).
Source: Authors

ranging from 26.67 to 20.33 mm, methanol extract showed inhibition zone in the range of 26 to 20.67 mm. Aqueous extract was found effective against *E. coli* ATCC2592 and MRSA with zone size of 25 and 17 mm, respectively. As shown in Table 3, the antibacterial activities of crude extracts of *R. nepalensis* root prepared by methanol, acetone and distilled water extraction showed statistically significant difference in bactericidal activity against *E. coli* ATCC2592 standard strain ($p < 0.05$). Statistically significant difference ($p < 0.05$) observed against *S. pneumoniae* (clinical) standard isolate.

Methanolic crude extracts exhibited a considerably broader antimicrobial activity compared to acetone and aqueous crude extracts (Table 4). The maximum inhibition zone was produced by methanolic crude extract against *E. coli* ATCC2592 (18.00 mm), *S. aureus* ATCC25923 (17.67±0.57 mm), *S. pneumoniae* ATCC63, (17.33±0.57 mm) and *K. pneumoniae* (clinical) (17.00±1.00 mm). The effective inhibition zone for methanolic crude extracts of *R. nepalensis* leaves and *E. spearocephalus* leaves in synergetic ranged from 18 to 13.33 mm followed by aqueous extract which showed antibacterial activity against MRSA (17.33±4.93), *K. pneumoniae* (clinical) (16.67±0.57 mm), *E. coli* ATCC2592 (16.00±1.00 mm), *S. aureus* ATCC25923 (15.33±0.57 mm) and *S. pneumoniae* (clinical) (14.00 mm). Acetone extract was found *E. coli* ATCC2592 effective with zone sizes of 15.33 mm aqueous extract showed inhibition in the range of 17.33 to 12.67 mm. As it was shown in Table 4, the antibacterial activities of crude extracts of *R. nepalensis* leaves and *E. spearocephalus* leaves in synergetic prepared by methanol, acetone and distilled water extraction showed statistically significant difference in bactericidal activity against *S. aureus* standard strain ($p < 0.05$). Statistically significant

Table 3. Antibacterial activities of organic and aqueous solvent extracts of *R. nepalensis* root using agar well diffusion method and comparison by inhibition zone of crude extracts of *R. nepalensis* root with positive control drugs for gram positive bacteria by their inhibition zones.

Organism	Solvent	Inhibition zones ± SD			
		***R. nepalensis* root**	**Positive control drugs**		
Gram positive			**Amoxicillin**	**Penicillin**	**Methicillin**
	Met	22.00±1.00^{abc}			
S. aureus ATCC25923	Act	26.67±1.52^{c}	24.00 ± 0.00^{ad}	22.33±0.00^{abc}	22.00± 0.00^{ab}
	Aq	25.67±0.57^{cd}			
	Met	22.33±0.57^{abc}			
MRSA	Act	25.33±1.15^{bc}	19.11±0.57^{ab}	19.00±0.71^{ab}	0.00± 0.00^{abc}
	Aq	17.00±1.00^{a}			
	Met	23.33±0.57^{abc}			
S. pneumoniae ATCC63	Act	24.00±1.00^{b}	20.44±0.52^{abc}	22.10±0.96^{bc}	18.78±0.86^{ac}
	Aq	21.67±0.57^{bc}			
	Met	28.00±1.00^{d}			
S. pneumoniae (clinical)	Act	20.33±0.57^{a}	24.44±1.00^{bc}	0.00^{b}± 0.00	20.11±1.00^{abc}
	Aq	21.67±1.15^{d}			
Gram negative					
	Met	21.00±1.00^{ab}			
K. pneumoniae (clinical)	Act	24.67±1.52^{bc}			
	Aq	17.00±1.00^{a}			
	Met	25.33±0.57^{e}			
E .coli ATCC2592	Act	27.00±4.35^{d}			
	Aq	25.33±0.57^{e}			
	Met	19.00±1.00^{b}			
E. coli (clinical)	Act	20.67±0.57^{a}			
	Aq	19.00±1.00^{b}			

MRSA-Methicillin Resistant *S. aureus,* met-methanol, act-acetone, aq- aqueous, SD-standard deviation, R.N – *R. nepalensis* root Values are means of triplicate determinations. Values within the same row followed by different supper scripts are significantly different at ($p < 0.05$).
Source: Authors

Table 4. Antibacterial activities of organic and aqueous solvent extracts of *R. nepalensis* leaves with *E. spearocephalus* leave in synergetic using agar well diffusion method.

Organism	Zone of inhibition (mm) ± standard deviation		
	***R. nepalensis* and *E. spearocephalus* in synergetic**		
Gram positive	**Water**	**Acetone**	**Methanol**
S. aureus ATCC25923	15.33±0.57^{a}	13.67±0.57^{b}	17.67±0.57^{c}
MRSA	17.33±4.93^{a}	14.00±1.73^{bc}	14.67±1.52^{a}
S. pneumoniae ATCC63	14.00±0.00^{a}	14.33±0.57^{bc}	17.33±0.57^{c}
S. pneumoniae (clinical)	14.00±0.00^{a}	14.33±0.57^{bc}	17.33±0.57^{c}
Gram negative			
K. pneumoniae (clinical)	16.67±0.57^{a}	13.67±0.57^{b}	17.00±1.00^{c}
E. coli ATCC2592	16.00±1.00^{b}	15.33±0.57^{d}	18.00±0.00^{c}
E. coli (clinical)	12.67±0.57^{a}	12.00±0.00^{a}	13.33±0.57^{a}

Source: Authors

Table 5. Antibacterial activities of organic and aqueous extracts of *Rumex nepalensis* root with *E. spearocephalus* leave in synergetic using agar well diffusion method.

Organism	*Rumex nepalensis*		
	Zone of inhibition (mm) ± standard deviation		
Gram positive	**Water**	**Acetone**	**Methanol**
S. aureus ATCC25923	24.00±1.00^{bc}	16.00±0.00^{a}	25.00±0.00^{cd}
MRSA	21.33±5.50^{ab}	22.00±3.46^{b}	24.00±2.64^{bc}
S. pneumoniae ATCC63	21.00±0.00^{b}	15.67±0.57^{a}	23.67±0.57^{c}
S. pneumoniae (clinical)	26.00±1.00^{c}	18.00±0.00^{a}	21.33±0.57^{a}
Gram negative			
K. pneumonia (clinical)	21.33±5.50^{ab}	22.00±3.46^{b}	24.00±2.64^{bc}
E. coli ATCC2592	21.00±1.00^{b}	17.00±1.00^{a}	27.00±0.00^{d}
E. coli (clinical)	18.67±0.57^{a}	18.33±0.57^{a}	24.00±0.00^{a}

Source: Authors

difference ($p < 0.05$) was observed in *K. pneumoniae* (clinical) isolates in which methanol extract showed greater inhibition zone compared to other extracts.

From the organic extracts methanol was found to have significant activity against *E. coli* ATCC 2592 (27.00 mm), *S. aureus* ATCC25923 (25 mm), *K. pneumoniae* (clinical) (24.00±2.64 mm), *E. coli* (clinical) (24 mm), MRSA (24.00±2.64 mm) and *S. pneumoniae* ATCC63 (23.67±0.57 mm) followed by aqueous extract which show antibacterial activity against *S. pneumoniae* (clinical) (26±1.00 mm), *S. aureus* ATCC25923 (24.00±1.00 mm), *K. pneumoniae* (clinical) (21.33±5.50 mm), MRSA (21.33±5.50 mm), *E. coli* ATCC 2592 (21.00±1.00 mm) and *S. pneumoniae* ATCC63 (21 mm). The acetone extract shows significant activity against *K. pneumoniae* (clinical) (22.00±3.46 mm), MRSA (22.00±3.46 mm), *E. coli* (clinical) (18.67±0.57 mm), *S. pneumoniae* (clinical) (18 mm), *E. coli* ATCC2592 (17.00±1.00 mm), *S. aureus* ATCC25923, (16 mm) and *S. pneumoniae* ATCC63 (15.67±0.57 mm), methanol extract was found effective with zone sizes ranging from 27 to 21.33 mm, aqueous extract showed inhibition in the range of 26 to 18.67 mm. Acetone extract was found effective against *K. pneumoniae* (clinical) and *S. pneumoniae* ATCC63 with zone size of 22 and 15.67 mm, respectively. As it was shown in Table 5, the antibacterial activities of crude extracts of *R. nepalensis* root and *E. spearocephalus* leaves prepared by acetone methanol and distilled water extraction showed no statistically significant difference in bactericidal activity against *E. coli* (clinical).

The antibacterial activities of crude extracts *R. nepalensis root* and *E. spearocephalus* leaves prepared by methanol, acetone and distilled water extraction was showed statistically significant difference in bactericidal activity against *S. aureus* standard strain ($p < 0.05$). Statistically significant difference ($p < 0.05$) was observed against *E. coli* ATCC2592 standard isolate.

The extracts that showed activity on anti-microbial activity determinations were further tested for MIC and MBC and the results are as shown in Figure 1. Methanol extract of *R. nepalensis* leaves showed MIC and MBC of 6.25% against *S. aureus* ATCC25923, 6.25 and 12% MIC and MBC was shown against *S. pneumoniae* ATCC63, respectively. The MIC and MBC against *E. coli* and MRSA, ATCC2592 was 12.5% and also *S. pneumoniae* (clinical) and *E. coli* (clinical). Acetone extract of *R. nepalensis* leaves showed MIC and MBC of 6.25% against *E. coli* ATCC2592, 6.25 and 12.5% MIC and MBC were shown against *S. aureus* ATCC25923, respectively. MIC and MBC were shown against *S. pneumonia,* ATCC was 12.5%, whereas MIC and MBC of 25% was shown against *E. coli* (clinical). 25 and 50% of MIC and MBC, respectively were shown against MRSA. 12.5 and 25% of MIC and MBC were observed against *S. pneumoniae* (clinical) and *K. pneumoniae* (clinical), respectively.

Aqueous extract of *R. nepalensis* showed MIC and MBC of 6.25% against *S. pneumoniae* ATCC63 and *E. coli* clinical whereas MIC and MBC of 12.5% against *K. pneumoniae* (clinical). 6.25 and 12.5% of MIC and MBC were shown against MRSA and *E. coli* ATCC2592, respectively, *S. pneumoniae* (clinical) and *S. aureus* ATCC25923 showed 12.5 and 25% MIC and MBC, respectively

The quantitative measurements of the *in vitro* activity for MIC and MBC are as shown in Figure 2. The study showed that methanol's extract of *E. sphaerocephalus* leave showed MIC and MBC of 12.5% against *S. aureus* ATCC25923, *S. pneumoniae* clinical and MRSA. 12.5 and 25% MIC and MBC were shown against *E. coli* ATCC2592 and *E. coli* (clinical), whereas MIC and MBC of 25 and 50% were shown against, *K. pneumoniae* clinical. *S. pneumoniae* ATCC63 showed 6.25 and12.5% of MIC and MBC, respectively. Acetone extract of *E. sphaerocephalus* leaves showed MIC and MBC of 12.5%

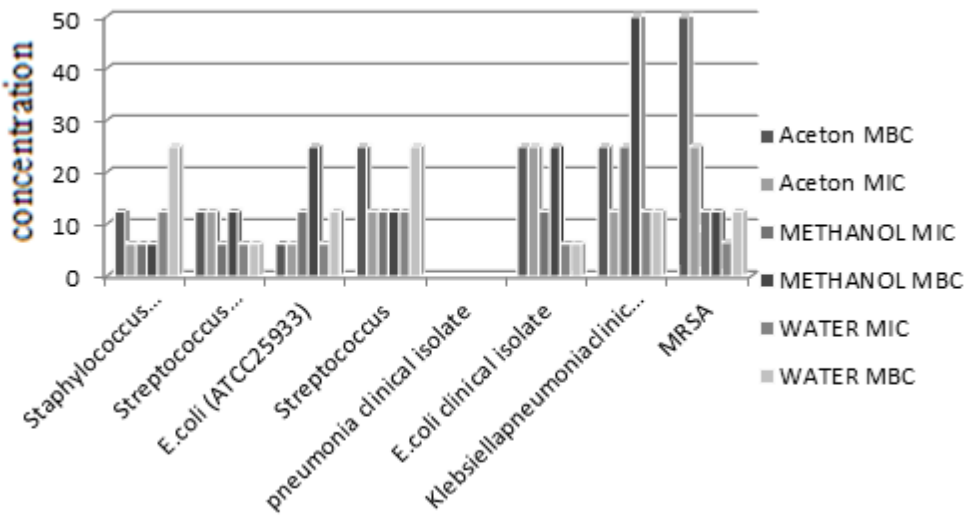

Figure 1. MIC and MBC value of leave extract of *R. nepalensis*.
Source: Authors

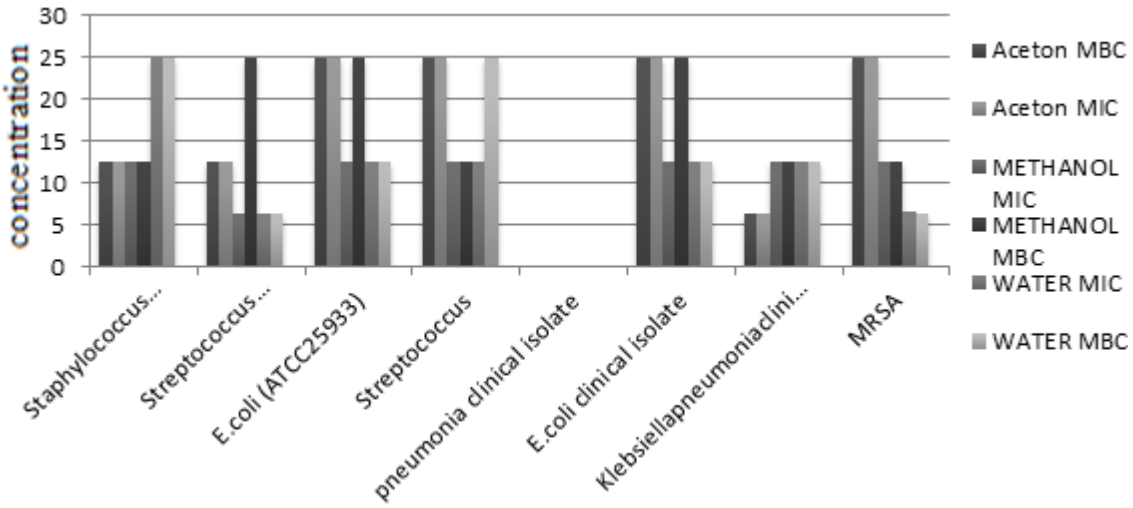

Figure 2. MIC and MBC value of leave extract of *E. sphaerocephalus.*
Source: Authors

against *S. aureus* ATCC25923, *S. pneumoniae* ATCC63. *E. coli* ATCC and *E. coli* (clinical) showed MIC and MBC of 25%. MRSA and *K. pneumoniae* (clinical) showed MIC and MBC of 25 and 50%, respectively. *S. pneumoniae* (clinical) showed 12.5 and 25% of MIC and MBC, respectively.

Aqueous extract of *E. sphaerocephalus* showed MIC and MBC of 12.5% against *E. coli* ATCC2592, *S. pneumoniae* (clinical) and *K. pneumoniae* (clinical), whereas *S. pneumoniae* ATCC63 and *E. coli* (clinical) showed MIC and MBC of 6.25%, respectively. *S. aureus* ATCC 25923 showed 25% of MIC and MBC, respectively, whereas MIC and MBC of 6.25 and 12.5% were shown against MRSA.

The extracts that showed activity on microbial activity determinations were further tested for MIC and MBC and the results are as shown in Figure 3. Methanol's extract of *R. nepalensis* root showed MIC and MBC of 6.25 and 25% against *S. pneumoniae* ATCC63, respectively. *S. aureus* ATCC, 25923 *S. pneumoniae* (clinical), *K. pneumoniae* (clinical) and MRSA showed 12.5% of MIC and MBC. Whereas MIC and MBC of 12.5 and 25% against, *E. coli* ATCC 2592 and *E. coli* (clinical), respectively.

Acetone extract of *R. nepalensis* root showed MIC and MBC of 12.5% against *S. aureus* ATCC 25923 and *S.*

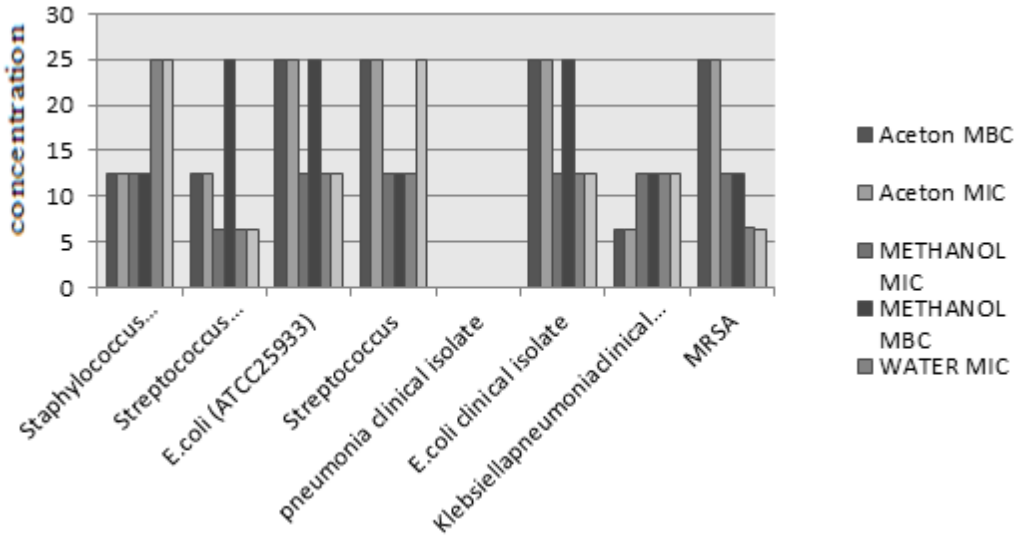

Figure 3. MIC and MBC value of *R. nepalensis* root with *E. spearocephalus* leave in synergy.
Source: Authors

pneumoniae ATCC63. Whereas MIC and MBC of 25% were shown against *E. coli* ATCC2592, MRSA, *E. coli* (clinical) and *S. pneumoniae* (clinical). *K. pneumoniae* (clinical) showed 6.25% of MIC and MBC.

Aqueous extract of *R. nepalensis* root showed MIC and MBC of 6.25% against *S. pneumonia*e ATCC63 and MRSA whereas MIC and MBC of 12.5% against *K. pneumoniae* (clinical), *E. coli* (clinical) and *E. coli* ATCC2592. *S. pneumoniae* (clinical) showed MIC and MBC of 12.5 and 25%, respectively. *S. aureus* ATCC25923 showed MIC and MBC of 25%.

DISCUSSION

Testing of antimicrobial properties of plants and chemicals are encouraged to be done with agar diffusion method (Bauer et al., 1966 as cited in de Oliveira Santos et al., 2016), because it is simple, reproducible, in expensive and reliable method (de Santos et al., 2016). The development of multi drug resistance to many antibiotics by microorganisms has initiated the search of new and effective antimicrobial constituents from medicinal plants. Bioactive components in plants may provide potential sources of new drugs for the safe and effective treatment of microbial diseases (de Santos et al., 2016; Ayandele et al., 2018). These compounds, alkaloids, flavonoids, coumarins, saponins and steroids are the compounds of plant origin, have significant therapeutic application against human pathogens including bacteria. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds (Guleria and Kumar, 2006; Marathe et al., 2013; Ajiboye et al., 2015). Therefore, medicinal plants are finding their way into pharmaceuticals (Ajiboye et al., 2015; Yhiler et al., 2019).

The determination of the minimum concentration of leaves extract which can kill pathogens bacteria with minimum effect has a great importance in determining antibiotic potential of herbal medicine (Idris and Abubakar, 2016; Kouadio et al., 2020). The data in this experiments showed that methanol and aqueous extracts of *R. nepalensis* leaves were active against the locally isolated human pathogens like *E. coli, S. aureus*, *K. pnemonia* and standard strain of *S. pneumonia* and *E. coli*. The acetone extracts of *R. nepalensis* leaves showed significant antimicrobial activity against standard strain of *E. coli*. Though, the mechanism of the action of these plant constituents is not yet fully known, it is clear that the effectiveness of the extracts largely depends on the type of solvent used. The methanol extracts provided more powerful antimicrobial activity as compared to aqueous extracts. This observation clearly indicates that the existence of non-polar residues in the extracts which have higher both bactericidal and bacteriostatic abilities. The antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily solubilized in organic solvents.

Antibacterial activity difference between extracts may be attributed to the fact that different compounds from the plant material get extracted in solvents of different polarities (Marathe et al., 2013; Ajiboye et al., 2015). In this experiment, methanol extracts of *R. nepalensis* leaves inhibited *E. coli* ATCC better than others (Table 1). On the other hand, aqueous extracts inhibited *K. pneumonia* (clinical) better than other microbes. Ahmad et al. (2010) reported that methanol extracts of different

Rumex species such as *R. persicaria*, *R. hastatus* and *R. dentatus* had antibacterial activities but their inhibitory effects varied against Gram negative and Gram positive bacteria. Therefore, maximum inhibition was shown by aqueous extract against Gram positive bacteria than the Gram negative bacteria (Yhiler et al., 2019). This may be due to the presence of outer membrane which acts as effective barrier in Gram negative bacteria. It is evident that methanol and acetone extracts of *R. nepalensis* leave showed significant activity against Gram positive bacteria. The other possible explanation is the presence of the terpenoid in the extract might be responsible for the extract to higher effect against Gram-positive than Gram-negative bacterium (Lima et al., 2006 as cited in de Santos et al., 2016).

In this study, the methanol extract of *R. nepalensis* leaves have great anti-microbial effect against *S. aureus* (clinical isolate) as similar to the result reported by Ghosh et al. (2001). As part of this study the anti-microbial effect of methanol and aqueous extracts of *R. nepalensis* were greater than of *R. nepalensis* extracts reported by Ghosh et al. (2001) against *K. pneumonia* (clinical isolate). In the present study, *in vitro* antimicrobial activity of crude extracts of *R. nepalensis* root were assessed by the agar well diffusion method showed good antibacterial activity against Gram-positive bacteria such as: *S. aureus* (ATCC 25923), *S. pneumonia* (ATCC 63), MRSA (clinical isolate), *S. pneumoniae* (Clinical isolate) and Gram-negative bacteria such as: *K. pneumoniae* (clinical isolate), *E. coli* (ATCC2592) and *E. coli* (clinical isolate) (Table 5).

In the present study inhibition zone of methanol extracts of *R. nepalensis* root against *S. pneumoniae* (clinical) was (26 mm) greater than the inhibition zone of methanol extracts of *R. nepalensis* root (17 mm) reported by Carral et al. (1987). Acetone extract of *R. nepalensis* root showed maximum growth inhibition (28 mm) against *E. coli* (clinical) which was reported by Coolins (1998) in contrast to the present study which was 20 mm growth inhibition zone.

In this study, the *in vitro* anti-microbial activity of aqueous crude extracts of *E. spearocephalus* leave was higher against *K. pneumoniae* (clinical), *E. coli* ATCC2592, and *E. coli* (clinical) as compared to crud extracts of *E. spearocephalus* leaves reported by Debela (2002). Li and Nikaido (2004) claim that, treatment with antibacterial combination, using two or more extract with other than the individual antimicrobial agents alone indicated synergetic effect against the tested microorganism; hence, it will be the promising area where new effective drugs will come into discovery towards resistant pathogens. Like the rest plant extracts, synergetic effect were also noted with *R. nepalensis* leave with *E. sphaerocephalus* leave extracts in combination against Gram negative and Gram positive bacteria. The result of this study showed that all the standard and drug resistance isolates of bacteria were susceptible to all solvent extracts of tested plants with the least inhibition zone 12 mm to largest inhibition zone 18 mm diameter in the agar well diffusion assay.

The overall synergetic activities of the plant extracts to both Gram positive and Gram negative bacterial strains, especially to those drug resistant clinical isolates, reveals that the therapeutic potentials of *R. nepalensis* plants when associated with *E. spearocephalus* of control continually emerging resistant bacteria, which are becoming a threat to human health.

Determination of MIC and MBC is crucial in antibacterial experiment, respectively (Idris and Abubakar, 2016; Ko and Stone, 2020). The quantitative measurements of the *in vitro* activity for MIC are as shown in Figures 1, 2, and 3. The study showed that MIC of the extract of *R. nepalensis* leaves, root and *E. spearocephalus* leaves was higher against *K. pneumonia* (clinical isolate), *S. pneumonia* (clinical isolate) and *E. coli* (clinical isolate), and MRSA where higher concentration of the crude extract of *R. nepalensis* is required to inhibit the growth of these bacteria. In contrast, the MIC of the methanol aqueous and acetone extract of *R. nepalensis* leave, root and *E. spearocephalus* were lower 6.25% indicating less concentration to inhibit the growth of pathogenic bacteria.

The MBC of the methanol, acetone and aqueous extracts against *K. pneumonia* (clinical isolate), *S. pneumonia* (clinical isolate), *E. coli* (clinical isolate), and MRSA was found to be greater than other test bacteria in the present study. For these two known medicinal plants, even though there is no any *in vivo* work to analyze the efficacy to treat infectious diseases, it is known that our community use these remedy to treat different human alignments (diseases). In addition, qualitative determination of concentration is also critical. The indigenous usage of these local medicinal plants must be determined scientifically. Especially, as plant antimicrobial extracts contain high impurities, various methods must be applied to separate these antimicrobial extracts.

Conclusion

The results of present investigation clearly indicate that the antibacterial activity vary with the species of the plants and plant material used. Thus, this study revealed that *R. nepalensis* leaves and root and *E. sphaerocephalus* leaves contain potential antimicrobial components that may be of great use for the development of pharmaceutical industries as a therapy against various diseases. The use of plants to heal diseases, including infectious one, has been extensively applied by people. Data from the literature as well as these results reveal the great potential of plants for therapeutic treatment, in spite of the fact that they have not been completely investigated.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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