

Antimicrobial Potentiality of *Elsholtzia densa* against Pathogenic Bacterial and Fungal Strains

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The present study was proposed to evaluate the antimicrobial efficacy of *Elsholtzia densa*, a rare annual herb of Kashmir valley. Five bacterial strains *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Shigella flexneri* and *Salmonella typhi* and three fungal strains *Penicillium notatum*, *Acremonium alternatum* and *Aspergillus niger* were tested for their susceptibility to *Elsholtzia densa* extracts. All the extracts of *Elsholtzia densa* with a concentration of 10% (methanol, ethanol, butanol, ethyl acetate and aqueous) were tested for antimicrobial activity by the disc diffusion assay. The total phenolics concentration was found to be 62.5mg% and 77.5mg% in case of absolute ethanolic and 50% ethanolic extracts respectively. However, only ethyl acetate extract showed antimicrobial activity which was visible as the zones of inhibition formed in the different cultures of Gram positive and Gram negative bacteria as well as in case of fungal cultures. The maximum activity was seen for *Staphylococcus aureus* with inhibition zone diameter of 23.66 ± 2.08 mm followed by *Escherichia coli* with inhibition zone diameter of 20.33 ± 2.51 mm. The extract had the highest MIC and MBC (4mg/ml) for *Staphylococcus aureus* and *Salmonella typhi* while a lowest MIC of 2 mg/ml and MBC of 1.8 mg/ml was shown for *Shigella flexneri*. In case of antifungal activity, the maximum inhibition zone diameter of 8.00 ± 2.00 mm was observed for *Acremonium alternatum* followed by 17.33 ± 1.15 mm for *Aspergillus niger* and 16.33 ± 1.15 mm for *Penicillium notatum*. The MICs and MBCs for the fungal strains ranged between 2.0 – 4.0 mg/ml. The purpose of screening was to justify, authenticate and validate the use of this herb as a traditional medicine in the treatment of infectious diseases.

Key words: *Elsholtzia densa* extracts, Antimicrobial activity, Bacterial & Fungal strains.

The increasing failure and side effects of popularly used chemotherapeutics and appearance of multiple drug resistance phenotypes in pathogenic bacteria has led to the search for new compounds with antibacterial activity. Worldwide as well as in the developing countries, most human deaths are due to infectious bacterial

diseases (Nathan, 2004). Bacterial organisms including Gram positive and Gram negative, like the different species of *Bacillus*, *Staphylococcus*, *Salmonella* and *Pseudomonas*, are the main source of severe infections in humans. These organisms have the ability to survive in harsh conditions due to their multiple environmental habitats (Ahameethunisa and Hoper 2010). Synthetic antibiotics, due to drug resistance, with the passage of time lose efficacy are not effective against microbes (Walsh and Amyes, 2004; Alder, 2005). In recent years, antimicrobial properties of medicinal plants are being increasingly reported

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from different parts of the world (Grosvenor *et al.*, 1995; Ratnakar *et al.*, 1995; Silva *et al.*, 1996; Saxena *et al.*, 1997; Saxena *et al.*, 1999; Nimri *et al.*, 1999; David, 1997). It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. From ancient times, plants have provided tremendous support in traditional medicine systems as well as for the development of new potential drugs in modern pharmaceutical industries. To consider the immense importance of medicinal plants for therapeutic target, intensive studies have been performed on different plant extracts to isolate biologically active compounds (Rios and Recio, 2005). More than 25% of the drugs used during the last 20 years are directly derived from plants (Amin *et al.*, 2009). Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of bacterial infections. The major groups of antimicrobial secondary metabolites include alkaloids, flavonoids (flavones and flavonols), quinones, essential oils, lectins, phenolics, polyphenols, tannins and terpenoids (Samy and Gopalakrishnakone, 2008). Although plants produce these chemicals to protect themselves, research provides evidence that they can treat humans against infections (Cseke *et al.*, 2006). The genus *Elsholtzia* belongs to the Lamiaceae family and has approximately 40 species worldwide, which are mainly distributed in Asia. Among these, some are used as medicines, some are taken as food and some are source of honey manufacture. The genus *Elsholtzia* generally possesses plentiful volatile oil, which exerts strong inhibition of central nervous system and takes on definite analgesic effect. It shows antibiotic and anti-inflammatory effects as well. *Elsholtzia fruticosa*, a plant belonging to this genus has been found to be effective against *Klebsiella species* (Himal *et al.*, 2008)

Elsholtzia densa is an annual erect herb with a pubescent stout 4-angled stem and is commonly known as *Sanik*, widely distributed in the open slopes in countries like Korea, China and also in Indian Himalayan regions. It has been mainly used as a traditional Chinese folk drug for the treatment of influenza, sore throat, measles, headache, dermatitis, ostealgia, furunculosis and bloody stools. However, at the best of our knowledge, the active principles of this plant are

unknown except for few constituents which are identified as hexacosanol, oleanolic acid, β -sitosterol, kaempferol and saussurenos (Ding *et al.*, 2004). The crude extracts of the plant have shown a potent radical scavenging activity as has been previously estimated by *in vitro* antioxidant assays (Khan *et al.*, 2012). In this paper the antimicrobial activity of the crude extracts of the plant has been evaluated.

MATERIALS AND METHODS

Chemicals/ Test organisms

Chemicals used in this study were of analytical grade and of highest purity procured from standard commercial sources in India. Three specific human pathogenic bacterial strains, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were obtained from Department of Microbiology, SKIMS whereas two bacterial strains *Shigella flexneri* and *Salmonella typhi* and three fungal isolates *Penicillium notatum*, *Acremonium alternatum* and *Aspergillus niger* were obtained from the Microbiology Lab, CORD, University of Kashmir.

Plant material

The plant *Elsholtzia densa* was collected from higher reaches of Naranag in the month of July-August and was identified and authenticated courtesy Centre of Plant Taxonomy, Department of Botany, University of Kashmir. The voucher specimen was retained in the herbarium of Taxonomy, Department of Botany, University of Kashmir for future reference under herbarium number (KASH-610).

Preparation of plant extracts

The plant material (whole) was shade dried under room temperature at $30 \pm 2^\circ\text{C}$, cut into small pieces and powdered using mortar and pestle and sieved with a sieve of 0.3mm aperture size. The powdered plant material was successively extracted in each of hexane, ethyl acetate, butanol, absolute ethanol, 50% ethanol, methanol and distilled water by using soxhlet extractor ($60-80^\circ\text{C}$). The plant material was suspended in the main chamber of soxhlet extractor which was then placed onto a flask containing the extraction solvent. The soxhlet was then equipped with a condenser. The flask was heated, the solvent evaporated and moved up into the condenser where it was

converted into a liquid that trickled into the extraction chamber containing the sample. The chamber containing the solid material was slowly filled with warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied, with the solvent running back down to the distillation flask. This cycle was repeated many times, over hours or a few days. After many cycles the desired compound was concentrated in the distillation flask. At the end of hot extraction each extract was filtered. The filtrate was concentrated on a hot water bath and the solvent was recovered using distillation unit. The extracts were then kept in a desiccator to remove any moisture if present and finally stored at 4°C for further use.

Determination of total phenolic content

The total phenolics in methanolic, ethanolic and 50% ethanolic extracts of *Elsholtzia densa* was determined by using Folin- Ciocalteu reagent. One hundred mg of extracts was dissolved in 200 ml of methanolic/water (60:40, v/v, 0.3% HCl) and filtered through Whatman's filter paper. To 100µl filtrate, 100 µl of 50% Folin- Ciocalteu reagent, and 2 ml of 2.5% sodium carbonate were added and mixed completely. After two hours incubation at room temperature, the absorbance of the solution at 750nm was measured with spectrophotometer. Quantitation was based on the standard curve of gallic acid (50 mg%), which was dissolved in methanol/water (60:40, v/v, 0.3% HCl) (Kyoung *et al.*, 2005).

Antimicrobial Activity

Bacterial culture medium preparation

All the glassware and prepared medium were sterilized by autoclaving at 121°C and 15lbs for 15 – 20 min. The Nutrient agar was used to culture the test bacteria. Thirty seven grams of Nutrient agar (containing ingredients agar, peptone, beef extract) were mixed in 1 liter of distilled water in an Erlenmeyer flask and mixture was heated in order to dissolve the agar completely. The Agar from the flask was poured in the boiling tubes, each containing 20-25 ml of agar. The agar tubes were tightly cotton plugged and sterilized by autoclaving at 15 lbs pressure for 20-25 min. The tubes were then stored at room temperature for further use.

Fungal culture medium preparation

The Potato Dextrose Agar (PDA) was used to culture the test fungi. Thirty nine grams of PDA

(containing ingredients Sucrose, Peptone, Agar) were mixed in 1 litre of distilled water in an Erlenmeyer flask and the mixture was heated in order to dissolve the agar completely. The agar from the flask was poured in the boiling tubes, each boiling tube containing 20-25 ml of PDA. The agar tubes were tightly cotton plugged and sterilized by autoclaving at 15 lbs pressure for 20-25 min. The tubes were then stored at room temperature for further use.

Anti-Microbial Assay

The disc diffusion method of Iennette (1985) was used with some modification to determine rate of inhibition of growth of bacteria by plant extract. Before doing anti-microbial assay, the working surface of the laminar hood was sterilized using ethanol and metal instruments like, inoculating loop and forceps were sterilized by red hot flaming. Then the U.V of laminar hood was kept on for 30 min, the air flow was allowed to run and then inoculation was performed in the laminar hood by the pour plate method. Sterile paper discs (6 mm in diameter) were impregnated with 50 µl and 70 µl of known extract concentrations. Discs (6 mm diameter) of chloramphenicol (30 µg) were used as positive controls. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the discs (mm). The growth inhibition diameter was an average of three measurements, taken at three different directions. The plates were then incubated for 18 hrs at 37°C for test bacteria and the fungal plates were incubated for 3-4 days at 30°C. For each extract three replicate trials were conducted against each organism.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The Minimum Inhibitory Concentrations (MICs) of test samples found to be active by the diffusion test were determined based on the macrodilution method (Berghe and Vlietinck, 1991) with some modifications. The extract was dissolved in ethyl acetate to give a stock concentration of 100mg/ml and serially diluted (two-fold) in a series of test tubes to a working concentration ranging from 1.560 to 100mg/ml using nutrient broth supplemented with 10% glucose and 0.05% phenol red (colour indicator). These were later inoculated with 0.2ml suspension of the test organisms. A tube containing nutrient broth only was seeded

with the test organisms as described above to serve as control. Microbial growth was determined by observing for color change in the tube (red to yellow when there is growth). The lowest concentration that showed no change of color was considered as the MIC.

To determine the Minimum Bactericidal Concentration (MBC), for each set of test tubes in the MIC determination, a loopful of broth was collected from those tubes which did not show any growth and inoculated on sterile nutrient agar (for bacteria) and potato dextrose agar (for fungi) by streaking. Nutrient agar and PDA only were streaked with the test organisms respectively to serve as control. Plates inoculated with bacteria were then incubated at 37°C for 24 hours while those inoculated with fungi were incubated at room temperature (30 – 32°C) for 48 h. After incubation the concentration at which no visible growth was seen was noted as the minimum bactericidal concentration.

Statistical analysis

The values are expressed as mean \pm standard deviation (SD). The results were evaluated by using the Primer software and evaluated by one way ANOVA followed by Bonferroni t-test. Statistical significance was considered when value of p was < 0.05 .

RESULTS AND DISCUSSION

Many reports are available on the antiviral, antibacterial, antifungal, antihelminthic, antimolluscal and anti-inflammatory properties of plants (Samy *et al.*, 2000). The extent of antimicrobial activity of the extracts based on inhibition zone diameter has been described as low (12-18 mm), moderate (19-22 mm) and strong activity (23-38 mm) by Ahmad *et al.* (1999). In order to identify the herb (*Elsholtzia densa*) with having antibiotic properties against various bacterial and fungal strains, using the disc-diffusion method, it was established that the plant possesses the potential to inhibit the growth of various pathogens. All the extracts of *Elsholtzia densa* with a concentration of 10% (methanol, ethanol, butanol, ethyl acetate and aqueous) were tested for antimicrobial activity, however, only the ethyl acetate extract showed anti-microbial activity which was visible as the zones of inhibition formed in the

different cultures of Gram positive and Gram negative bacteria as well as in case of fungal cultures. Five bacterial strains *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Shigella flexneri* and *Salmonella typhi* and three fungal strains *Penicillium notatum*, *Acremonium alternatum* and *Aspergillus niger* were tested for their susceptibility to *Elsholtzia densa* extracts. Out of the five solvents used for extraction, the ethyl acetate extracts showed the highest activity against the test organisms, followed by the ethanol extracts and water extracts (data not shown). Different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent (Doughari, 2006). Ethyl acetate extracts in this study might have had higher solubility for more phytoconstituents, consequently the highest antibacterial activity.

Total Phenolic Content

Total phenolic concentration was found to be highest for 50% ethanolic extract (77.5 mg%) followed by ethanolic extract (62.5 mg%) and lowest for methanolic extract (35mg%). Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and herbivores (Lutterodt *et al.*, 1999; Marjorie *et al.*, 1999). Phenols were found to be a component of this plant and could be one of the components responsible for the antibacterial activity since it was reported by Al Genaidy (1993). This may therefore explain the demonstration of antimicrobial activity by the crude extracts of *Elsholtzia densa*.

Antibacterial activity

As mentioned above, the ethylacetate extract showed a high antimicrobial activity against the pathogenic bacteria like *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Shigella flexneri* and *Salmonella typhi*. The maximum activity was seen for *Staphylococcus aureus* with inhibition zone diameter of 23.66 ± 2.08 mm followed by *Escherichia coli* with inhibition zone diameter of 20.33 ± 2.51 mm as is depicted in Table 1 and clearly shown in Fig.1(a-e).

Antifungal activity

In case of antifungal activity, the ethyl acetate extract was found to be effective against

Table 1. Anti-bacterial activity of 10% (w/v) ethyl acetate extract of *Elsholtzia densa* against different bacterial cultures

Test Bacteria	Volume of ethyl acetate extract used (μ l)	Diameter of zone of inhibition (mm)	Diameter of zone of inhibition (mm) *Chloramphenicol disc
<i>Escherichia coli</i>	50	8.66 \pm 1.52	25.66 \pm 4.04
	70	20.33 \pm 2.51	
<i>Staphylococcus aureus</i>	50	10.33 \pm 1.52	30.66 \pm 4.04
	70	23.66 \pm 2.08	
<i>Klebsiella pneumoniae</i>	50	10.66 \pm 1.15	30.00 \pm 5.00
	70	19.66 \pm 2.51	
<i>Shigella flexneri</i>	50	8.00 \pm 1.00	18.66 \pm 0.55
	70	10.00 \pm 2.00	
<i>Salmonella typhi</i>	50	8.00 \pm 2.00	17.33 \pm 1.15
	70	11.00 \pm 3.60	

*The concentration of the Chloramphenicol used was 30 μ g/disc

Table 2. Anti-fungal activity of 10% (w/v) ethyl acetate extract of *Elsholtzia densa* against different fungal cultures

Test Fungi	Volume of ethyl acetate extract used (μ l)	Diameter of zone of inhibition (mm)	Diameter of zone of inhibition (mm) *Amphotericin B disc
<i>Penicillium notatum</i>	70	16.33 \pm 1.15	19.00 \pm 1.00
	50	9.00 \pm 2.64	
<i>Aspergillus niger</i>	70	17.33 \pm 1.15	20.67 \pm 1.52
	50	9.33 \pm 1.15	
<i>Acremonium alternatum</i>	70	18.00 \pm 2.00	21.33 \pm 1.15
	50	10.33 \pm 1.52	

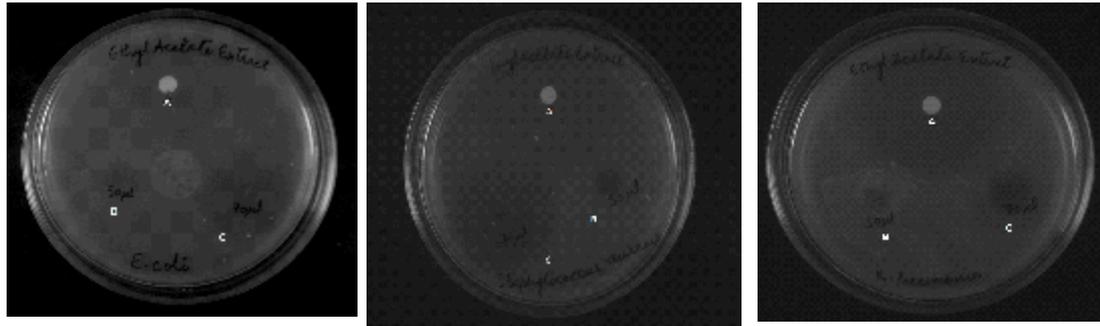
*The concentration of the Amphotericin B used was 30 μ g/disc

Table 3. Results of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the ethyl acetate extract of *Elsholtzia densa*

Microorganism	Ethyl acetate extract (mg/ml)	
	MIC	MBC
Bacterial Strains		
<i>Escherichia coli</i>	3.0	2.7
<i>Staphylococcus aureus</i>	4.0	4.0
<i>Klebsiella pneumoniae</i>	3.0	3.0
<i>Salmonella typhi</i>	4.0	4.0
<i>Shigella flexneri</i>	2.0	1.8
Fungal Strains		
<i>Penicillium notatum</i>	3.0	3.2
<i>Aspergillus niger</i>	4.0	4.0
<i>Acremonium alternatum</i>	3.0	3.0

the inhibition of growth of three fungal strains. The maximum inhibition zone diameter of 8.00 \pm 2.00 mm was observed for *Acremonium alternatum* followed by 17.33 \pm 1.15 mm for *Aspergillus niger* and 16.33 \pm 1.15 mm for *Penicillium notatum* as can be seen in Table 2 and clearly depicted in Figs. 2-4.

The inhibition zones produced by the extracts indicated that plant shows effective antimicrobial and antifungal activities, although the ethyl acetate extract showed higher activity, based on inhibition zone sizes (Tables 1, 2). The demonstration of antibacterial activity against both gram positive and gram negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds (Srinivasan *et al.*, 2001). This will be of immense advantage in fighting the menace



(a) *Escherichia coli*

(b) *Staphylococcus aureus*

(c) *Klebsiella pneumoniae*



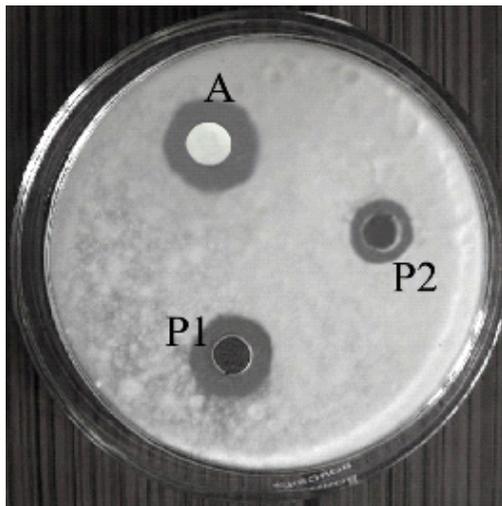
(d) *Shigella flexneri*



(e) *Salmonella typhi*

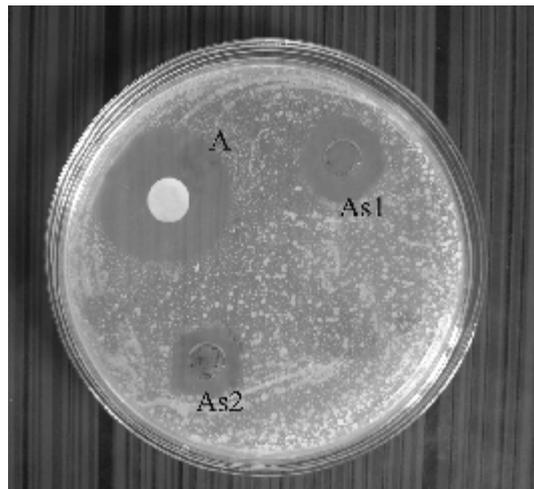
A - known antibiotic, Chloramphenicol discs (30µg/disc);
B and C - 50 µl and 70 µl contain 5mg and 7mg of the plant extract respectively.

Fig. 1. Antibacterial activity of 10% ethyl acetate extract of *Elsholtzia densa*



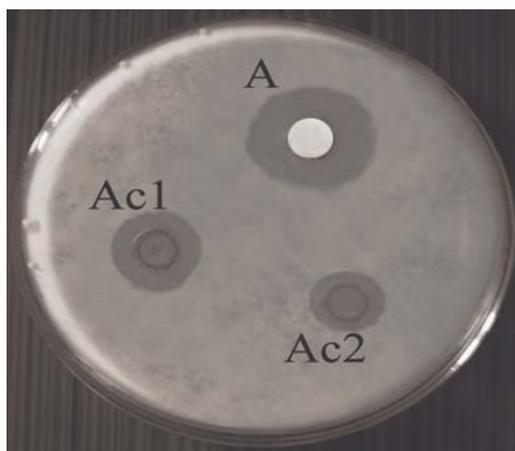
A represents the known antibiotic
i.e Amphotericin B disc (30µg/disc);
P1 represents 70 µl containing 7 mg of the plant extract
P2 represents 50 µl containing 5mg of the plant extract

Fig. 2. Antifungal activity of 10% ethylacetate extract of *Elsholtzia densa* against *Penicillium notatum*



A represents the known antibiotic
i.e Amphotericin B disc (30µg/disc);
As1 represents 70 µl containing 7 mg of the plant extract;
As2 represents 50 µl containing 5mg of the plant extract.

Fig. 3. Antifungal activity of 10% ethylacetate extract of *Elsholtzia densa* against *Aspergillus niger*



A represents the known antibiotic i.e Amphotericin B disc (30 μ g/disc); Ac1 represents 70 μ l containing 7mg of the plant extract; Ac2 represents 50 μ l contain 5mg of the plant extract.

Fig. 4. Antifungal activity of 10% ethylacetate extract of *Elsholtzia densa* against *Acremonium alternatum*

of antibiotic refractive pathogens that are so prevalent in recent times.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MICs and MBCs of the ethyl acetate extract against eight microorganisms were tested and the results are tabulated in Table 3. It was observed that all tested bacterial (gram positive as well as gram negative) and fungal species were susceptible to ethyl acetate extract and exhibited the MICs ranging from 2.0-4.0 mg/ml and MBCs from 1.8-4.0 mg/ml. The results showed that the extract had the highest MIC and MBC (4mg/ml) for *Staphylococcus aureus* and *Salmonella typhi* while a lowest MIC of 2 mg/ml and MBC of 1.8 mg/ml was shown for *Shigella flexneri*. In case of fungal strains the values of MICs and MBCs ranged between 3.0-4.0 mg/ml.

The highest MIC and MBC values for *Staphylococcus aureus* and *Salmonella typhi* is an indication that either the plant extract is less effective on some bacteria or that the organisms have the potential of developing antibiotic resistance, while the lower MIC and MBC values for other bacteria is an indication of the efficacy of the plant extract.

CONCLUSION

The screening of antimicrobial activity performed on *Elsholtzia densa* crude extracts which is traditionally used as herb, shows that the plant is endowed with potentially utilizable antimicrobial activity. Accordingly, this implies the inhibition of microbial pathogenesis that is linked to pathological incidents such as heart disease, aging and cancer. It was seen that the ethyl acetate extract showed the maximum inhibitory effects against both bacterial and fungal growths. This may be due to the presence of such ingredients in the said extract like flavonoids, terpenes, tannins, polyphenolic compounds, alkaloids, etc. Thus, it can be used as antibacterial supplement towards the development of new therapeutic agents. The demonstration of broad spectrum of antibacterial activity by the plant extract may also help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control. This investigation has opened up the possibility of the use of this plant in drug development for human consumption possibly for the treatment of gastrointestinal, urinary tract, skin and wound infections. However, the components responsible for antimicrobial activity of these extracts are currently unclear. The effect of this plant on more pathogenic organisms and toxicological investigations and further purification therefore, needs to be carried out. *In vivo* evaluation of antimicrobial activity along with toxicity studies of the extracts from *Elsholtzia densa* are suggested for further studies.

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