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Antimicrobial efficacy of *Althaea officinalis* Linn. seed extracts and essential oil against respiratory tract pathogens

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ABSTRACT

In present study, *Althaea officinalis* seed extracts and essential oil were screened for antimicrobial activity against five bacteria and one fungi responsible for dominant, lethal or opportunistic infection of respiratory regions. The maximum inhibition was noted by essential oil against *Streptococcus pyogenes* (21.3±0.28 mm) and *Haemophilus influenzae* (19.0±0.50 mm) at 200 mg/ml. The minimum inhibitory concentration values for methanol extract was 3.12-12.5 mg/ml. The antifungal activity noted highest with 41.28% by essential oil and 36.27% inhibition by aqueous extract represented by dosage-response curve.

INTRODUCTION

Plants are the pavement bricks of all the living organisms on the earth. They produce a wide range of secondary metabolites such as alkaloids, unsaturated fatty acids, flavonoids, phenols, tannins and terpenes that can be used to treat various chronic and infectious diseases (Reichling, 2010). In recent years, increasing strains of microorganisms throughout the world have developed resistance to large number of antibiotics that has created immense clinical problem and made the management of infectious diseases quite complicated (Davis, 1994). The way to avoid antibiotic resistance of pathogenic species is by using plant based compounds rather than existing synthetic antimicrobial agents (Shah, 2005). *Althaea officinalis* Linn. (Malvaceae) is commonly known as Khatmi in Hindi and Marshmallow in English. It is native of British Isles and found in temperate regions of India, currently it is distributed throughout Europe and some parts of America (Ross, 2001). *A. officinalis* is a perennial herb 60-120 cm high. Stem is erect, woody and unbranched. Leaves are short-petioled with an ovate and acute leaf blade. The reddish-white flowers are usually in axillary or terminal cluster. Compressed dark brown kidney-shaped seeds are glabrous (Ozkan and Uzunhisarcikli, 2009). *A. officinalis* is used in irritation of oral, pharyngeal mucosa and associated dry cough, mild gastritis, skin burns, insect bites, catarrh of the mouth, throat, gastrointestinal tract and urinary tract, inflammation, ulcers, abscesses, burns, constipation and diarrhoea. Seeds are diuretic and febrifuge (Shah, 2011).

It has been used as an aid in promoting coughing up of phlegm and respiratory problems. Due to high contents of polysaccharides, it is used in relieving dryness and chest and throat irritation happened by colds and persistent coughing (Sutovska *et al*., 2007). *A. officinalis* has a broader spectrum of antibacterial as well as antifungal activity. The antibacterial activity of *A. officinalis* roots were tested against anaerobic and facultative aerobic periodontal bacteria (Lauk *et al*., 2003). Many researches showed that *A. officinalis* possessed antimicrobial, anti-inflammatory, immunomodulatory, demulcent and soothing, antittusive and many other pharmacological effects (Naovi *et al*., 1991; Rouhi and Ganji, 2007). Therefore, in present study the antibacterial and antifungal screening of *A. officinalis* seed extracts and essential oil has been aimed against selected respiratory disease microorganisms.

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MATERIAL AND METHODS

Plant Material

A. officinalis was collected from Herbal Automation, Haridwar, Uttarakhand. The seeds were washed in fresh running water, dried under shade at room temperature, crushed by using pestle and mortar and powdered in an electric grinder.

Preparation of Extract

Plant extracts were prepared by immersing 200 g of powdered seeds in 600 ml of four different solvents including petroleum ether (PET), acetone (ACE), methanol (MeOH) and aqueous (H_2O) loaded in Soxhlet assembly and extracted for 72 h through successive method (Ahmad *et al*., 1998). Plant extracts were filtered through Whatman No. 1 filter paper and crude extracts obtained by removing solvent in vacuum evaporator at 30°C. Residues were stored at 4°C until further use. Extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 200 mg/ml for agar well diffusion method.

Essential oil extraction

The air dried plant seeds (400 g) were subjected to hydrodistillation for 6 h using Clevenger type apparatus. Material was loaded in 2.0 L round bottom flask containing 1.5 L distilled water. Due to heating of oil collecting tube, distillate is allowed to collect in a conical flask placed over ice to minimize the loss of volatile components. 1000 ml of aqueous phase containing essential oil was collected and divided into four (4×250 ml) fractions. Each fraction (250 ml) was fractionated (2×150 ml) with chloroform ($CHCl_3$) using separating funnel, aqueous layer was discarded. $CHCl_3$ layer containing essential oil was concentrated in rotary vacuum evaporator under reduced pressure at 20°C. After complete removal of chloroform, essential oil was collected and moisture content was removed by passing it over anhydrous Na_2SO_4 and stored at 4°C prior to use. The oil yield was 0.09% (w/w).

Antimicrobial Assay

Microorganisms used

The microbial strains causing respiratory infections used in this study were *Haemophilus influenzae* (MTCC 3826), *Pseudomonas aeruginosa* (MTCC 2474), *Staphylococcus aureus* (MTCC 1144), *Streptococcus pneumoniae* (MTCC 655), *Streptococcus pyogenes* (MTCC 442) and *Aspergillus niger* (MTCC 921). Microorganisms were procured from Institute of Microbial Technology (IMTECH), Chandigarh.

Antibacterial testing

Antibacterial activity of different extracts was determined by agar well-diffusion method (Ahmad *et al*., 1998). In vitro antibacterial activity was screened by using Mueller-Hinton Agar (MHA) medium no. 173 (Hi media Pvt. Ltd., Mumbai, India). 0.1 ml of 12-16 h incubated cultures of bacterial species were mixed in molten medium and poured in pre-sterilized petri plates. Plates were allowed to solidify for 5-10 minutes. A cork borer (6 mm diameter) used to punch wells in medium and filled with extracts of 45 µl of 200 mg/ml final concentration of extracts. DMSO was used as negative control. Efficacy of extracts against bacteria was compared with broad spectrum antibiotic erythromycin (positive control). Erythromycin was dissolved into double distilled water. Plates were incubated at 37°C for 24 h in BOD incubator. At the end of incubation, inhibition zones formed around the well were measured with transparent ruler in millimetre. Each sample was assayed in triplicate and mean values were observed. The antibacterial activity was interpreted from size of diameter of zone of inhibition measured to the nearest millimetre (mm) as observed from clear zones surrounding the wells.

Determination of Minimum Inhibitory Concentrations (MICs)

Two-fold serial dilution method was used to determine the minimum inhibitory concentrations (MICs) against selected bacterial organisms (Aboaba *et al*., 2006). MeOH extract was diluted double fold (2:2) with nutrient broth in a series of six test tubes. Concentration of 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml of crude MeOH extract were prepared separately and dissolved in 1 ml of DMSO. An aliquot of 1 ml of bacterial suspension (1.5×10^6) was inoculated into each tube (Fig. 2). Control tubes were inoculated with same quantity of sterile distilled water. All tubes were incubated at 37°C for 24 h. The lowest concentration that did not permit any visible growth when compared with control was considered as the minimum inhibitory concentration. The MICs was considered as the lowest concentration that could not produce a single bacterial colony. The contents of all tubes that showed no visible growth were cultured on Mueller-Hinton agar, incubated at 37 °C for 24 h.

Antifungal testing

The antifungal activity of different extracts and essential oil was determined by poisoned food technique (Grover and Moore, 1962; Nene and Thapliyal, 2002). 250 mg/ml concentration of different plant extracts and 100 mg/ml essential oil were aseptically poured into Petri plates followed by addition of 19 ml of melted SDA medium and swirled gently to achieve through mixing of the contents. 6 mm mycelial discs from the margins of two day old culture of *A. niger* was punched aseptically with a sterile cork borer and then put in the centre of agar plates. In the control set, no extract was used. Percentage inhibition of mycelial growth was evaluated by measuring the relative growth of fungus in treatment and control and calculated by using the following formula.

$$I = (C-T)/C \times 100$$

Where I is the percentage inhibition, C the mean growth rate of control, and T that of the treatment. The efficacy of extracts and essential oil against fungi was compared with erythromycin as the reference drug. The plates were incubated at 25°C for 48-72 h in BOD incubator. Each sample was assayed in triplicate and the mean values were observed.

Table 1: Antibacterial properties of *A. officinalis* extracts, essential oil and reference antibiotic (Erythromycin) against respiratory bacterial strains

Microorganisms	*Diameters of the inhibition zone (mm)					Positive Control
	PET	ACE	MeOH	H_2O	EO	(Erythromycin)
H. influenzae	7.3±0.28	15.3±0.76	14.0±0.50	14.0±0.50	19.0±0.50	21.3±0.76
P. aeruginosa	8.6±0.28	16.3±0.76	24.3±0.76	19.0±0.50	18.0±0.50	16.6±0.76
S. aureus	8.0±0.50	15.0±0.50	11.3±0.76	14.3±0.76	15.0±0.50	30.3±0.76
S. pneumoniae	9.3±0.28	20.0±0.50	14.0±0.50	18.0±0.50	17.3±0.57	19.3±0.57
S. pyogenes	9.0±0.50	17.3±0.76	16.0±0.50	15.3±0.57	21.3±0.28	25.6±0.28

*Values are mean± Standard Error (SE), Cork borer diameter: 6 mm;

Table 2: Effect of *A. officinalis* extracts and essential oil and reference drug (erythromycin) on the mycelial growth of *Aspergillus niger*

Fungicide	Mycelial growth (mm)	Control	% inhibition
PET	37.3±0.57	37.6±0.28	0.87
ACE	33.3±0.28	37.6±0.28	11.49
MeOH	28.6±0.76	37.6±0.28	23.89
H_2O	24.0±1.00	37.6±0.28	36.27
EO	21.3±0.57	36.3±0.57	41.28
Erythromycin	12.3±0.57	33.6±0.57	63.45

*Values are mean± Standard Error (SE)

Table 3: Phytochemical analysis of *A. officinalis* extracts

S. No.	Solvents	Phytoconstituents						
		Alkaloids	Flavonoids	Glycosides	Steroids	Terpenes	Saponins	Tannins
1.	PET	+	-	-	+	-	-	-
2.	ACE	+	-	-	-	+	+	+
3.	MeOH	+	+	+	-	+	+	+
4.	H_2O	+	+	+	-	+	+	+

+ = present, - = Absent

Determination of Effective Dose for 50% inhibition (ED_{50})

To represent the activity/dose relationship between extracts and fungal organism, a linear dosage-response (DR) curve for most effective extract i.e. H_2O extract of *A. officinalis*, those act as fungicides, was drawn by plotting percentage inhibition. The effective dose for 50% inhibition (ED_{50}) were obtained and dose-response curve for initial 72 h of incubation was drawn (Fig. 3).

Phytochemical Screening

The plant extracts were subjected to phytochemical examination for alkaloids, flavonoids, glycosides, steroids, terpenes, saponins and tannins as described by using standard procedures (Evans, 1996; Scalbert, 1991).

RESULTS AND DISCUSSION

The present study showed that *A. officinalis* possess good antimicrobial activity against selected respiratory tract pathogens. The antibacterial efficacy of extracts and essential oil is summarized in Table 1. The results revealed that the essential oil was more potent in comparison to plant extracts. The zone of inhibition above 7 mm in diameter is considered positive result. The maximum inhibition by essential oil was noted against *S. pyogenes* (21.3±0.28 mm) and *H. influenzae* (19.0±0.50 mm) and by MeOH extract against *P. aeruginosa* (23.3±0.76 mm) and lowest against *S. aureus* (11.3±0.76 mm) in comparison to other extracts. The ACE and H_2O extracts were moderately active against *H. influenzae*, *S. pneumoniae* and *S. pyogenes* respectively. In comparison with erythromycin, the plant extracts and essential oil were found less effective. There was no inhibition of growth with the negative control (DMSO). In a study, crude MeOH and H_2O extracts of aerial parts of *A. officinalis* were tested against 137 strains belonging to 52 bacterial species and found that MeOH extract was most active especially against *Acidovorax facilis*, *Bacillus* sp., *Enterobacter hormachei* and *Kocuria rosea*. The H_2O extract had no antibacterial effect (Ozturk and Ercisli, 2007). MeOH extract of *A. officinalis* root had been reported to possess an inhibitory activity against periodontal pathogens including *Porphyromonas gingivalis*, *Prevotella* spp., *Actinomyces odontolyticus*, *Veilonella parvula*, *Eikenella corrodens*, *Fusobacterium nucleatum*, and *Peptostreptococcus* spp. respectively.

In a recent report by Rezaei *et al*. (2015), *A. officinalis* had reported wound healing properties with antimicrobial role against *S. aureus*, *P aeruginosa*, *E. coli* and *L. monocytogenes*. The hydroalcoholic extract of *A. officinalis* flowers screened for antimicrobial activity against *P. aeruginosa*, *S. aureus*, *L. monocytogenes* and *C. albicans* (Shakib *et al*., 2013).

The MIC values for *A. officinalis* MeOH extract was ranged between 3.12 to 12.5 mg/ml (Fig. 1). The inhibition was noted at 3.12 mg/ml against *H. influenzae*, similar inhibition at 12.5 mg/ml against *S. aureus* and *S. pyogenes*, and 6.25 mg/ml against *P. aeruginosa* and *S. pneumoniae*. The MIC values of *A. officinalis* were reported for 80% ethanolic extract at 50-100 mg/ml concentration (Al-Snafi, 2013).

The antifungal activity of plant extracts and essential oil were represented in Table 2. The percentage inhibition was noted highest with 41.28% by essential oil against *A. niger*, H_2O extract with 36.27% and MeOH extract with 23.89% of inhibition respectively. The control mycelial growth diameter was 33.6±0.57- 37.6±0.28 mm. The potency of plants were compared with reference antibiotic (erythromycin) showed 63.45% inhibition

at similar (250 mg/ml) concentration. The ED_{50} value for H_2O extract was observed at 320 mg/ml concentration represented by dose response (DR) curve (Fig. 2).

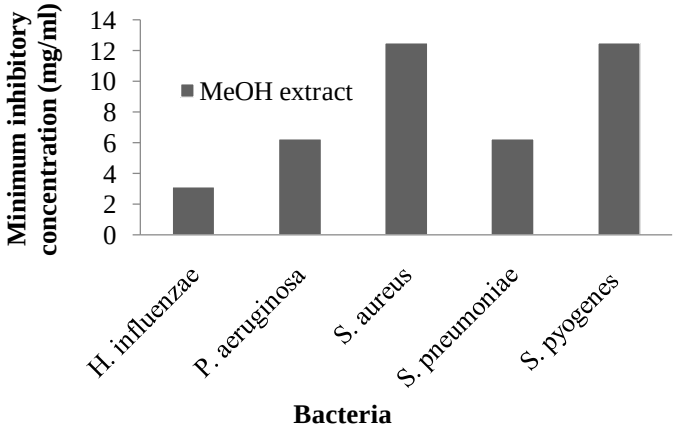

Fig. 1: Minimum inhibitory concentrations (MICs) of MeOH extract of *A. officinalis*. The inhibition is noted at (a) 3.12 mg/ml against *H. influenzae* (b) 12.5 mg/ml against *S. aureus* and *S. pyogenes*; and (c) 6.25 mg/ml against *P. aeruginosa* and *S. pneumoniae*

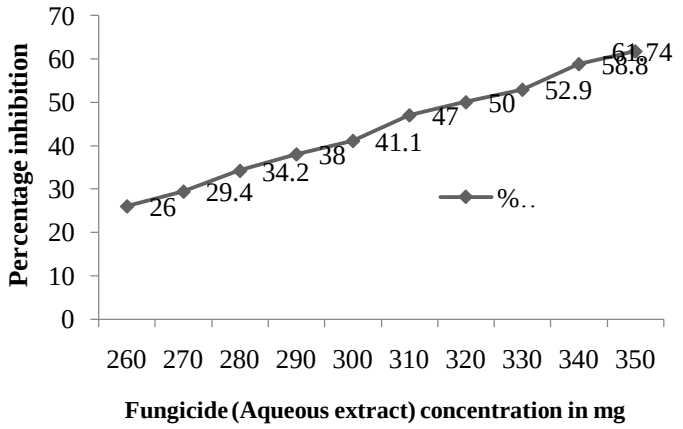

Fig. 2: Effective dose for 50% inhibition (ED_{50}) and Dosage-response (DR) curve from *A. officinalis* aqueous extract against *Aspergillus niger*.

The phytochemical screening of *A. officinalis* extracts showed the presence of alkaloids, flavonoids, glycosides, terpenes, saponins and tannins in MeOH extract, alkaloids and steroids in PET extract, alkaloids, terpenes, saponins and tannins in ACE extract and alkaloids, flavonoids, glycosides, terpenes, saponins and tannins in H_2O extract respectively (Table 3). *A. officinalis* has wide range of curative properties with lots of studied phytoconstituents including pectins 11%, starch 25-35%, mono-, and di-saccharide saccharose 10%, mucilage 5%, flavonoids (Hypolaetin-8-glucoside, isoquercitrin, kaempferol, caffeic, pcoumaric acid), coumarins, scopoletin, phytosterols, tannins, asparagines and many amino acids (Al-Snafi, 2013). Two new phenolic compounds and one new acid ester characterized as 3,4-dihydroxy benzyl octadecane, 24β, 28β-dihydroxyocta tetracont-36-en-1-oic acid and 5β, 13β-dihydroxynacosanylgodoleate had been isolated from roots of *A. officinalis* along with known compounds n-triacotanic acid, n-tetracosane, n-pentatriacontane and althaealanostenoic acid glucoside (Zoobi and Mohd, 2011).

The GC/MS profiling of hexane extracts of flowers of *A. officinalis* showed the major components were palmitic acid (13.0 %), heptacosane (9.3 %) and nonacosane (11.2 %) and roots extract with palmitic acid (16.8 %), linoleic acid (omega-6) (28.0 %) and naphthalene decahydro 2,6-dimethyl (16.4 %) respectively (Naovi *et al*., 1991).

Therefore, *A. officinalis* exhibited a basis for use of extracts and essential oil in cure of respiratory ailments which could be caused by *H. influenzae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, *S. pyogenes* and *A. niger*.

CONCLUSIONS

It can be concluded that *A. officinalis* have good antimicrobial potential against tested microorganisms. It can be used in the treatment of respiratory diseases caused by themselves. The synergistic effect between the antibiotics, plant extracts and essential oil against selected pathogens leads to new choice of treatment. It is recommended that further research should be carried out to investigate the bioactive component of this plant.

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