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Evaluation of antibacterial and phytochemical analysis of root extracts of *Alysicarpus vaginalis* DC. Against Respiratory Tract Pathogens

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Abstract

The present study was to evaluate the antibacterial activity and phytochemical analysis of various roots extracts of *Alysicarpus vaginalis* (Chukalai) against selected common respiratory tract pathogens i.e. *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Results showed that methanol extract was more efficient in comparison to other extracts. The zone of inmibition ranged between 10 ± 0.57 to 13 ± 0.32 mm examined at 200 mg/ml, respectively. Minimum inhibitory concentration was recorded for MeOH extracts between 3.12 mg/ml to 25 mg/ml for all the organisms. The Phytochemical analysis of extracts showed the presence of flavonoids, glycosides, alkaloids, steroids, terpenes, saponins and tannins. This investigation supports a good response to the use of *A.vaginalis* in herbal medicine and as a base for the development of new drugs and phytomedicine in foundation for its use in treatment of respiratory infectious diseases.

Keywords- Alysicarpus vaginalis, antibacterial activity, minimum inhibitory concentration, respiratory tract pathogens

Introduction

Our earth is full of medicinal plants. These medicinal plants having therapeutical properties are very useful in curing various diseases. Almost all our present medicines exhaled from medicinal plants. Approximately, 8000 species of medicinal plants are used as different systems of medicines in India. India is blessed with huge biodiversity due to different climatic zones, in which numerous medicinal plants were reported. The Indian state of Uttarakhand, located in central Himalayan region, is richly gifted with a large variety of plant species, many of which have medicinal properties. Medicinal plants play an important role in the lives of people in Uttarakhand by providing basic health care and employment to the farmers (Alam and Kop, 2005). The Central Himalayan Region covers the new state of India, provides excellent opportunities for studying the Traditional Knowledge Systems (TKS). The Indian Himalayan region alone supports about 18,440 species of plants (Angiosperms: 8000 spp., Gymnosperm: 44 spp., Pteridophytes: 600 spp., Bryophytes: 1736 spp., Lichens: 1159 spp. and Fungi: 6900 spp.) of which about 45% are having medicinal properties. According to (Samant et al., 1998) out of the total species of vascular plants, 1748 spp. species are medicinal. Uttarakhand is a store house of a rich variety herbs and medicinal and aromatic plant species. Medicinal plants produce a wide variety of compounds which in addition to give them characteristic pigments, odour and flavor characteristics may also have antimicrobial properties (Cowan, 1999; Sanjay et al., 2010; Kumar et al., 2014). For thousands of years, traditional plant derived medicines have been used in most parts of the world and their use in fighting microbial disease is becoming the focus on study (Bhavnani and Ballow, 2000). Intensive studies on extracts and biologically-active compounds isolated from medicinal plants have played an essential role in drug discovery in last decade. Various parts of such plants like root, tubers, bark, flowers, leaves and seeds are used for medicinal purposes. More so, many of these plants have been known to synthesize active secondary metabolites such as phenolic compound found in essential oils with established potent insecticidal (Kambu et al., 1982) and antimicrobial activities, which really has formed the basis for their applications in some

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pharmaceuticals, alternative medicines and natural therapies (R´ıos and Recio, 2005). As if a comparable study is made between antibiotics and pathogen resistance it is concluded that antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. But, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms (Harbottle et al., 2006). Thus, in the light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance.

Respiratory tract infections are a major cause of illness and death and are common in intensive care unit (ICU) (Cardoso et al., 2007). Respiratory diseases, including allergies, asthma and chronic obstructive pulmonary disease (COPD) are a major public health burden worldwide. The prevalence of these diseases is increasing and there is a continued need for new and improved therapies. The most frequent causal agents of these infections are *Streptococcus pneumoniae, Klebsiella pneumoniae, Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa, Acinetobacter baumannii* and *Stenotrophomonas maltophilia*s (Ibrahim et al., 2000; Gautam et al., 2012).

Alysicarpus vaginalis

The genus *Alysicarpus* belongs to family Fabaceae. Commonly called as Chukalai, there are approximately 78 species in their genus with 20 species are reported in India (Khare, 2007). They commonly found in open grass land, crop fields, and way sides, ranging an altitude 560 to 1000m Srinagar, Pauri, Chauras and Kirtinagar in Garhwal Himalaya, almost throughout India, ascending to 1000m Afghanistan, Pakistan and tropical America. The genus comprises annual Prostrate Herbs, perennial. Glabrous or a line of hairs on stem, erect or diffuse at 30cm long, branched Leaves, flowers small, mostly callus 5 mm, usually binate at each node of rachis. Flowering period started from September to November and fruiting period is October to February (Gaur, 1999). Several species of *Alysicarpus* has been used in indigenous system of medicine an anti-inflammatory in stomachache, and also an antidote to snake bite. It is also used in skin diseases and as a diuretic. The leaves are used in fever, jaundice and leaf paste is applied externally on skin allergy (Shankarnarayan, 1988). The root of *A. vaginalis* traditionally use for treatment of asthma cold cough and other Respiratory tract infection in Garhwal (Adhikari et al., 2010).

Materials and Method

Plant Material: Plant was collected from two different districts of Uttarakhand, Srinagar and Srikot (Pauri Garhwal) Chauras and Kirtinagar (Tehri Garhwal) Uttarakhand and authenticated at Botanical Survey of India (BSI), Northern Regional Center Dehradun Accession No.115354. Collected plant Root material was washed jet properly, dried under shade at room temperature and crushed to small pieces by using pestle and motor.

Preparation of Extract: Plant extracts were prepared by immersing 200g of powdered plant material in 600 ml of four different solvents according to polarity low to high i.e. petroleum ether (PET), Chloroform($CHCl_3$), methanol (MeOH) and water (H_2O), loaded in Soxhlet assembly and extracted for 72 h through successive method (Ahmed 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 200mg/ml for agar well diffusion method.

Test Microorganisms: The five bacterial strains causing respiratory infections used in this study were *Klebsiella pneumoniae* MTCC 4030, *Pseudomonas aeruginosa* MTCC 2474, *Staphylococcus aureus* MTCC 1144, *Streptococcus pneumoniae* MTCC 655, *Streptococcus pyogenes* MTCC 442. These Bacterial strains were procured from Institute of Microbial Technology (IMTECH), Chandigarh.

Preparation of Inoculums:Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiment were prepared by transferring a loopful of cells from stock cultures to test tubes of Mueller-Hinton broth (MHB) for bacteria that were incubated without agitation for 24 h at 37°C.

Antibacterial testing : The antibacterial activity of different extracts was determined by agar well-diffusion method (Ahmed et al., 1998). 0.1 ml of

12-16 h incubated cultures of bacterial species were mixed in molten Mueller Hinton Agar medium no. 173 (Hi media Pvt. Ltd., Mumbai, India) and poured in pre-sterilized petri plates. A cork borer (6 mm diameter) used to punch wells in solidified medium and filled with extracts of 45 µl of 200 mg/ml final concentration of extracts. DMSO was used as negative control. The efficacy of extracts against bacteria was compared with the broad spectrum antibiotic erythromycin (positive control). The plates were incubated at 37°C for 24 h in BOD incubator and the diameter of the zone of inhibition was measured in millimeter. Each sample was assayed in triplicate and the mean ± SD values were observed. The antibacterial activity was interpreted from the size of the diameter of zone of inhibition measured to the nearest millimeter (mm) as observed from the 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). Methanol 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 Methanol 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. 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.

Phytochemical screening

Major Phytocompound, in the crude Root extracts of *A. vaginalis* were subjected to Phytochemical screening to decide the presence of bioactive components by using standard qualitative methods (Trease and Evans, 1996).

Test for alkaloids: Test solution was acidified with acetic acid and a drop of Mayer's reagent was added. The formation of precipitate with colour change indicated the presence of alkaloids (Moffat et al., 1986).

Test for flavonoids: Methanolic extract of plant material to the test solution a mixture of zinc dust and concentrated hydrochloric acid was added drop wise. After few minutes change in colour of the sample was observed it indicated the presence of flavonoids.

Test for glycosides : Plant extract was filtered and sugar was removed by fermentation with baker's yeast. The acid was removed by precipitation with $Ba(OH)_2$. The remaining extract contained the glycosides. The hydrolysis of solution was done with conc. H_2SO_4 and after hydrolysis the presence of sugars was determined with help of Fehling's solution.

Test for Steroids : The extract mixed with 3 ml $CHCl_3$ (Chloroform) and 2 ml conc. H_2SO_4 (Sulphuric acid) was poured from side of test tube and colour of the ring at junction of two layers was noted. A red colour showed the presence of steroids.

Test for Sugars : The extract was taken and a mixture of equal parts of Fehling's solutions I and II previously mixed was added and heated. The colour of precipitate of cuprous oxide showed the presence of reducing sugar.

Test for Saponins : The extracts (0.5 mg) were boiled with water (10 ml) for two Minutes in a test tube and cooled. The mixture was vigorously shaken and then left for 3 minutes. The Formation of 1 cm layer of foam indicates the presence of saponins.

Test for Tannins : Extract was added in 1% ferric chloride and observed the colour. Bluish black colour appeared which disappeared on addition of dilute H_2SO_4 follow a yellow brown precipitate indicates the presence of tannins.

Results and Discussion

This study investigated *in vitro* antibacterial activity of crude root extract of *A. vaginalis* from different solvent. The data characterizing the antibacterial activity of crude extract of *Alysicarpus vaginalis* root are presented in Table 1. The study indicated that the Methanol extracts exhibited a higher degree of antibacterial activity as compared to water, chloroform and petroleum ether extracts. The maximum inhibition by MeOH extract was

Table 1: The inhibition zones diameters of various root extracts of *Alysicarpus vaginalis*.

Microorganism	Diameter of the inhibition zone (mm)				Positive control	Negative Control
	PET	$CHCl_3$	MeOH	H_2O	(Erythromycin)	DMSO
Staphylococcus aureus	11±0.52	9±0.86	11±0.86	8±0.73	14±0.51	0
Streptococcus pneumoniae	10±0.75	12±0.50	13±0.32	8±0.97	13±0.91	0
Streptococcus pyogenes	11±0.86	10±0.76	10±0.52	6±0.57	15±0.72	0
Klebsiella pneumoniae	10±0.57	11±0.50	12±0.50	10±0.45	12±0.12	0
Pseudomonas aeruginosa	9±0.41	8±0.86	12±0.84	8±0.97	11±0.52	0

Values are Mean±SD of three replicates; Cork borer diameter: 6 mm;
PET- Petroleum Ether. **$CHCl_3$** - Chloroform. **MeOH**- Methanol. H_2O- Water.

found against *Streptococcus pneumoniae* (13±0.32 mm), *Klebsiella pneumoniae* (12±0.50 mm) and *Staphylococcus aureus* (11±0.86 mm), respectively. The minimum activity was found against *Streptococcus pyogenes* (10±0.52 mm) followed by H_2O, $CHCl_3$ and PET. In case of *S. pneumoniae* crude methanolic extract of *A. vaginalis* give 13±0.32 mm zone of inhibition its same as commercial use broad spectrum antibiotic erythromycin. But in other case the positive control (erythromycin) was found little more effective as compared to *A. vaginalis* extracts. Erythromycin is a macrolide antibiotic with wide spectrum antimicrobial nature. For respiratory tract infections, it has better coverage of microorganisms especially for atypical organisms including mycoplasma and legionellosis. DMSO (Di methylsulphooxide) use as a negative control, they give no zone of inhibition against test organism. Peer reviewed published reports on *A. vaginalis* focus only on taxonomy, diversity and ethanobotanical aspects (Jain et al., 2009). Although some research communications are available on antibacterial activity of *A. vaginalis* but it is limited in number. Further, most of the studies have been conducted just to find out the zone of inhibition against some common bacterial pathogens, but have not investigated the minimum inhibition concentration (MICs) and zone of inhibition. Many of the present findings on these extracts are in agreement with previous workers (Khan et al., 2011). reported that whole plant extract of *A. vaginalis* use to performed antibacterial activity using Kirby Bauer disk diffusion test and broth micro dilution against clinically important bacterial pathogens two gram positive *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778) and three gram negative *Pseudomonas aeruginosa* (ATCC 9027), *E. coli* (ATCC 35218), *S. typhimurium* (ATCC 13311) but they could not found antibacterial activity of extract of *A. vaginalis* (Silva et al., 2015) The results of MICs showed that they ranged from 3.12 to 25 mg/ml (Figure-1). Root extract of *A. vaginalis* presented similar MICs against *S. aureus* and *K. pneumoniae* (6.25 mg/ml) respectively. Moreover, methanolic extract of this plant manifested a better MIC against *S. pneumoniae* (3.12 mg/ml) and maximum MIC value recorded against *S pyogenes* (25 mg/ml). Therefore, the activity observed for *A. vaginalis* provides a rationale for its use in treatment of respiratory infectious diseases. Moreover, *A. vaginalis* displayed a basis for use of extract in treatment of respiratory diseases in human beings which could be caused by *K. pneumoniae, P. aeruginosa, S. aureus, S. pneumoniae* and *S. pyogenes.*

The Phytochemical screening of root of *A. vaginalis* methanolic extracts has shown that plant contains flavonoids, glycosides, alkaloids, steroids, terpenes, sugars, saponins and tannins which are very important constituents when looking for

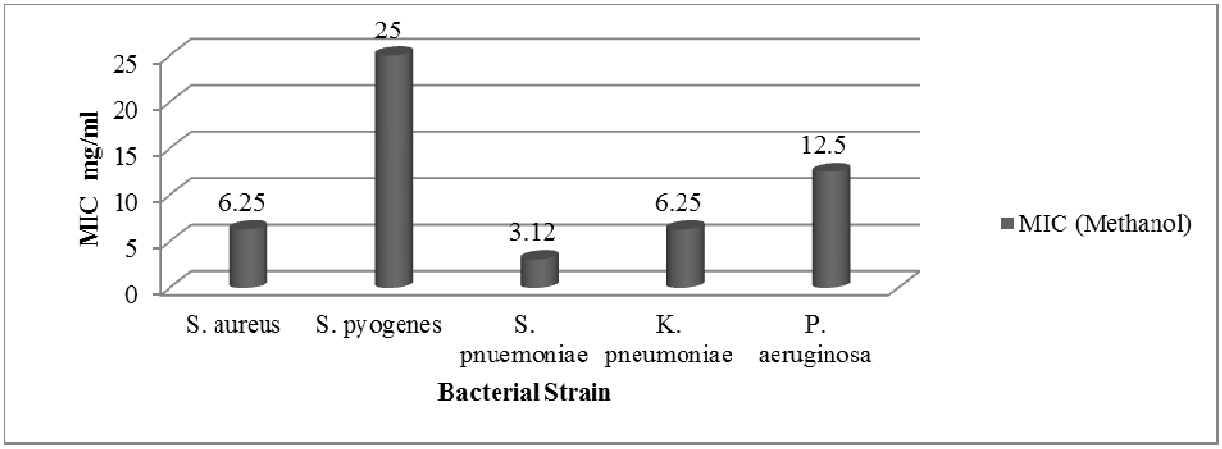

Figure-1: Minimum inhibitory concentrations (MICs) mg/ml of methanol extract of *A. vaginalis*. The inhibition is noted at 3.12 mg/ml against *S. pnuemoniae*, 6.25 mg/ml against *S. aureus* and *K. pneumoniae*, 12.5 mg/ml against *P. aeruginosa* and 25 mg/ml against *S. pyogenes*.

Table 2: Phytochemical screening of *A. vaginalis* root crude extracts.

Phytoconstituents	**Solvent**			
	Petroleum Ether (PET)	**Chloroform ($CHCl_3$)**	**Methanol (MeOH)**	**Water (H_2O)**
Alkaloids	+	+	+	+
Flavonoids	_	+	+	_
Glycosides	+	_	_	+
Steroids/ Terpenes	_	_	+	_
Sugars	_	_	_	+
Saponins	+	+	+	+
Tannins	+	+	+	+

+ = Present - = Absent

pharmacologically active Phytochemical in the plant. These secondary metabolites and compounds could be responsible for its antibacterial property against tested microorganisms. Previous pharmacological studies exposed the role of *A. vaginalis* in some Herbal drugs for treatment of common cold, asthma, and cough moreover; *A. vaginalis* displayed a basis for use of extract in treatment of respiratory diseases in human beings which could be caused *by P. aeruginosa, S. aureus, S. pneumoniae, S. pyogenes and K. pneumoniae.* Some preview report on study of *A. vaginalis* showed performed antioxidant and in vitro antibacterial activity against the test organism. (Rattanata et al., 2014) But it did not inhibit growth of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and Shigella spp (Chah et al., *2006*). *A. vaginalis* was reported to have antioxidant and antiproliferative activity (Rathi et al., 2010).Some other worker reported the Chemical Constituents of *A. vaginalis* is Caffeic acid, Catechol, Cumaric acid, Gentisic acid, Gallic acid, P-hydroxybenzoic acid Syringic acid, Vanillic acid, Salicyclic acid, Acetic Acid, Ethyl acetate. (Shahin and Ahmad, 2012). This study good supports the traditional use of *A. vaginalis* and indicated that it contains some major bioactive compounds inhibiting the growth of microorganisms there by proving very effective source of derived drugs. It is recommended that further research should be carried out to investigate the bioactive component

of this plant. The need for establishment of standard dosage cannot be over emphasized. This is necessary to investigate the toxicity level of extract resulting from over dosage or from any of phytochemical component present in plant material.

Conclusion

Based on these results, it is concluded that this study demonstrate the root of *A. vaginalis* (methanolic extract) have potent antibacterial activity against selected highly pathogenic respiratory tract pathogens which might be due to the Phytochemical present in these parts.There is future aspects of this study the identification and purification of active compound(s) those responsible for antibacterial activity of *A. vaginalis*. The conclusion indicates that scientific studies carried out on medicinal plants having traditional claims of effectiveness might warrant fruitful results. Root of *A. vaginalis* could be a source of new antibiotic compounds.

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