International Journal of Pharmacy and Pharmaceutical Sciences
ISSN- 0975-1491 Vol 2, Issue 3, 2010

Research Article

IDENTIFICATION OF THE ANTIBACTERIAL COMPONENT OF SOME INDIAN MEDICINAL PLANTS AGAINST *KLEBSIELLA PNEUMONIAE*

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Received: 17 Feb 2010, Revised and Accepted: 17 March 2010

ABSTRACT

The antibacterial activity of traditional folk medicine used by tribals of Mahakoshal region in Central India for the treatment of infectious diseases was evaluated using disc diffusion assay. In the search of bioactive compounds; aqueous, acetone, ethanol and chloroform extract of 20 Indian folkloric medicinal plants were screened for antibacterial property. The active antibacterial compounds were analyzed by TLC, TLC-bioautography and were confirmed by high performance liquid chromatography. The ethanol extract of *Commiphora mukul* Arn. (Burseraceae) containing tannin exhibited best antibacterial activity at 5mg/ml. Ethanol extract of *Eucalyptus mannifera* R.T. Bak. (Myrtaceae) containing tannin, chloroform extract of *Azadirachta indica* Juss. (Meliaceae) containing azadirachtin and salanin, *Allium sativum* L. (Liliaceae) containing allicin, *P. granatum* containing tannin and *Pimpinella anisum* L. (Apiaceae) containing saponin showed best activity against *Klebsiella pneumoniae* with MIC of 10 mg/ml. Thus, *Commiphora mukul* plant can be used as potential source of natural antimicrobial agents against multidrug resistant *K. pneumoniae*.

Keywords: Medicinal plants, Disc diffusion assay, Phytochemical analysis, TLC- bioautography, HPLC.

INTRODUCTION

Infectious diseases are the leading cause of death worldwide. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens[1]. *Klebsiella pneumoniae* is a frequent nosocomial pathogen, being the fourth and fifth most common cause of pneumonia and bacteremia respectively, in intensive care patients[2]. Every year 1-9 million children under 5 year of age die from pneumonia[3]. Pneumonia is a global disease that is typically curable in developed countries but often fatal in developing countries. The global perceptions of pneumoniae as a public health problem are emasculated by its familiar and benign image in the industrialized world[4].

Bacterial pathogens have evolved numerous defense mechanisms against antimicrobial agents, and resistant to old and newly produced drugs is on the rise. The increasing failure of chemotherapeutic and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antibacterial activity[5]. Despite the existence of potent antibiotics and antibacterial agents, resistant or multi-resistant strains are continuously appearing. Thus, there is an urgent need to systematically evaluate the plants used in traditional medicine as green medicine, which is safe, more dependable than costly drugs and which have no adverse side effects.

In the present study, we systematically evaluated antibacterial activity of twenty plants used by tribals in Mahakoshal region of Central India for treating Infectious diseases against *Klebsiella pneumoniae* which could lead to the discovery of new plant derived drugs against this pathogen.

MATERIALS AND METHODS

Collection of plant materials

20 plant samples were collected on the basis of the information provided in the ethnobotanical survey of India and by local medicine men of tribal region of Central India (Table-1). Each specimen was labelled, numbered and annotated with the date of collection. Each specimen was identified by Prof. J.L. Shrivastava Scientist, State Forest Research Institute, Jabalpur (M.P.), India. These samples were dried in shade, grinded to fine powder and stored in airtight containers at room temperature in the dark until used[6].

Preparation of extracts

Aqueous extraction

10 g of air-dried powder was placed in distilled water and boiled for 6 hrs. At intervals of 2 hrs it was filtered through 8 layers of muslin cloth and centrifuged at 5000×g for 15 min. The supernatant was collected and concentrated to make the final volume one-fourth of the original volume[7].

Solvent extraction

Organic solvent (acetone, ethanol and chloroform) extract of the plant materials were prepared according to the method described by Nair *et al*[7] with some modifications. 10 g of plant material was air dried, crushed and blended into powder using an electric blender. The blended material was transferred to a conical flask and kept in a rotary shaker (190-220 rpm) for 30 min., then filtered through eight layers of muslin cloth and centrifuged at 5000×g for 15 min. Supernatant was collected and evaporated to make the final volume one-fourth of the original volume.

Antibacterial assay

In vitro antibacterial activities of different plant extracts were tested against nine environmental isolates of *Klebsiella pneumoniae* (BGCC#432, BGCC#436, BGCC#438, BGCC#439, BGCC#440, BGCC#448, BGCC#449, BGCC# 450 and BGCC#452) procured from the Bacterial Germplasm Culture Collection, Bacteriology Laboratory, R. D. University, Jabalpur, India and standard isolate of *Klebsiella pneumoniae* (BLKp/001), obtained from Microbial Type Culture Collection (MTCC), Chandigarh (India) and were maintained on nutrient agar slants. 0.2 ml of overnight grown cultures of each organism was dispensed into 20 ml of sterile nutrient broth and incubated for 3-5 hrs at 37^0C to standardize the culture to 10^6 CFU/ml[8]. 10 µl plant extract (40 mg/0.1 ml) was soaked by sterile filter paper discs (5 mm in diameter). The sterile filter paper discs were impregnated with plant extract placed on the surface of the medium and incubated at 37^0C for 24 hrs. The assessment of antibacterial activity was measured around the disc[9].

Minimum inhibitory concentration (MIC) of plant extracts

The MIC of the plant extracts was determined on Mueller Hinton Agar medium following the method of Collins *et al*[8] and Rios *et al*[9]. A lawn of *Klebsiella* culture was made on Mueller Hinton Agar plates and the discs impregnated with different concentration of plant extracts (5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 40 mg/ml) were placed on the medium and incubated at 37^0C for 24 hrs. The

lowest concentration inhibiting the visible growth of the organism was considered as MIC.

Minimum inhibitory concentration (MIC) of antibiotics

Minimum inhibitory concentration of antibiotics was performed by following disc diffusion method of Bauer *et al*[10] using commercially available strips (Himedia, Mumbai, India) of Ciprofloxacin, Nalidixic acid**,** Cefotaxime, Norfloxacin. The Hicomb strips were placed on the Mueller Hinton agar medium and the plates were incubated at 37^{0}C for 24 hrs. and MIC was determined.

Phytochemical screening

The plant extracts prepared were screened for the presence of flavonoids, tannins, alkaloids, saponins, terpenoids and glycosides using methods of Sofowora[11] and Harborne[12].

Table 1: Medicinal plants of Mahakoshal region of central India used against *Klebsiella Pneumoniae*

S. No.	Plant name	Family	Vernacular name	Part used
1.	*Azadirachta indica* (A. Juss)	Meliaceae	Neem	Leaves
2.	*Commiphora mukul* (Arn.)	Burseraceae	Guggul	Stem bark
3.	*Pimpinella anisum* (L.)	Apiaceae	Saunph	Seeds
4.	*Punica granatum* (L.)	Lythraceae	Anar	Leaves
5.	*Allium sativum* (L.)	Liliaceae	Lahsun	Rhizome
6.	*Eucalyptus manniferra* (RT. Bak)	Myrtaceae	Eucalyptus	Stem bark
7.	*Acacia arabica* (Lam.)	Mimosaceae	Babool	Leaves
8.	*Corriander sativum* (L.)	Umbelliferae	Dhaniya	Seeds
9.	*Curcuma longa* (L.)	Zingiberaceae	Haldi	Rhizome
10.	*Datura stramoniun* (L.)	Solanaceae	Datura	Fruits
11.	*Cinnamomum cassia* (Auct.)	Lauraceae	Dalchini	Stem bark
12.	*Ocimum sanctum* (L.)	Labiateae	Tulsi	Leaves
13.	*Phyllanthus urinaria* (L.)	Euphorbiaceae	Bhuiamla	Leaves
14.	*Bacopa monnieri* (L.)	Scrophulariaceae	Brahmi	Leaves
15.	*Piper nigrum* (L.)	Piperaceae	Kalimirch	Seeds
16.	*Calandula officinalis* (L.)	Umbelliferae	Genda	Flowers
17.	*Syzygium cumini* (L.)	Myrtaceae	Jamun	Seeds
18.	*Terminalia chebula* (Retz.)	Combretaceae	Harrah	Fruits
19.	*Trachyspermum copticum* (L.)	Umbelliferae	Ajwain	Seeds
20.	*Zingiber officinale* (Roscoe)	Zingiberaceae	Adrak	Rhizome

Thin layer chromatography and TLC-bioautography analysis

Plant extracts (30 µl) were applied at 2.5 cm from the base of prepared silica gel-G TLC plate. After drying, the TLC plates were developed with ethyl acetate: methanol: water (81: 11: 8) as the developing solvent and were run in duplicate. One set was used as the reference chromatogram and other set was used for bioautography.

The reference TLC plates were then developed using anisaldehyde/sulphuric acid spray reagent (465 ml ethanol, 5 ml glacial acetic acid, 13 ml p-anisaldehyde and 13 ml H_2SO_4 mixed in order), which were then heated at 110^{0}C for 5-10 min and visualized under visible and UV light at 254 and 366 nm[13]. Chromatogram, developed as described above, was placed in TLC plates (10×20 cm) with the inoculum of *K. pneumoniae* containing 10^6 CFU/ml in molten Mueller Hinton Agar, was distributed over the TLC plates. After the solidification of the medium, the TLC plate was incubated overnight at 37^{0}C. Subsequently, bioautogram developed was sprayed with 1% aqueous solution of 2, 3, 5, -triphenyl tetrazolium chloride (TTC) and incubated at 37^{0}C for 4 hrs. Inhibition zone indicated the presence of active compounds[14].

High-performance liquid chromatography (HPLC)

HPLC fingerprints were prepared using a Chemito LC 6600 model, equipped with isocratic pump and UV-VIS detector. Solvents were pre-filtered by using a millipore system and analysis were performed on reverse phase Lickrospher C_{18} column (250 × 4.6 mm i.d., 5 µm). For injection in HPLC system the active spots were scrapped from the reference TLC plates and dissolved in methanol. Injection volume was 20 µl for all the cases. All the extracts were detected at UV wavelength of 310 nm. The flow rate was 0.7 ml/min in all cases. Mobile phases used for different extracts were methanol and water (70: 30)[15].

RESULTS

The antimicrobial activity of the extracts and their potency was quantitatively assessed by the presence of inhibition zone and zone diameter, respectively. The plants differed significantly in their activity against the tested strains and the best antimicrobial activity was observed with six plants namely *Commiphora mukul, Eucalyptus mannifera, Punica granatum, Allium sativum, Azadirachta indica* and *Pimpinella anisum.*

The MIC values of *C. mukul* ranged from 5-15 mg/ml. The MIC value for *E. mannifera, P. granatum, A. sativum, A. indica* and *P. anisum* were found to be 10 mg/ml against *K. pneumoniae*. Regarding the efficiency of the antibiotics, MIC range for the antibiotics tested i.e. Chloramphenicol (C), Ciprofloxacin (Cf), Cefotaxime (Ce), Norfloxacin (Nx) against *K. pneumoniae*, were found to be 22-38 µg/ml, 1-6 µg/ml, 55-66 µg/ml and 11-16 µg/ml respectively (Table-2).

BGCC#432, BGCC#436, BGCC#438, BGCC#439, BGCC#440, BGCC#448, BGCC#449, BGCC#450, BGCC#452 are environmental strains *and* BLK_P/001 is the standard strain of *K. pneumoniae*; BGCC: Bacterial Germplasm Collection Centre; (-): No activity; S*: Solvent, Chloramphenicol (C), Ciprofloxacin (Cf), Cefotaxime (Ce), Norfloxacin (Nx).

Phytochemical analysis demonstrated the presence of tannins, saponins, flavonoids and alkaloids in six active extracts. The phytochemical analysis of the *A. indica* extract revealed the presence of alkaloid, while that of *P. anisum* showed the presence of saponin. Crude extracts of *C. mukul*

and *E. mannifera* indicated that both plants contain tannin, while that of *A. sativum* showed the presence of allicin.

Thin layer chromatography using ethyl acetate: methanol: water (81: 11: 8) as the developing solvent was able to separate different chemicals having different retention factor (Rf value) present in plant extracts. Single spot was observed with the extracts from *P.*

anisum (Rf 0.90), *C. mukul* (Rf 0.611), *P. granatum* (Rf 0.833) while two spots were observed in the extracts of *A. indica* (Rf 0.633, 0.866), *A. sativum* (Rf 0.31, 0.627) and *E. mannifera* (Rf 0.619 and 0.78).

Table 2: Minimum Inhibitory concentration of most active plant extracts (mg/ml) and antibiotics (µg/ml) against *K. pneumoniae*

Plant extracts (S*) and antibiotics	Environmental isolates (BGCC No.)									Standard strain
	432	436	438	439	440	448	449	450	452	BLKp/001
A. indica (Choloroform)	10	10	10	10	10	10	10	10	10	10
C. mukul (Ethanol)	10	10	5	10	15	10	10	10	10	10
P. anisum (Chloroform)	10	10	10	10	10	10	10	10	10	10
P. granatum (Chloroform)	10	10	10	10	10	10	10	10	10	10
A. sativum (Chloroform)	10	10	10	10	10	10	10	10	10	10
E. mannifera (Ethanol)	10	10	10	10	10	10	10	10	10	10
C	32	30	22	38	33	36	33	22	32	25
Cf	4	5	2	5	4	3	6	1	3	4
Ce	66	64	55	66	64	66	58	65	55	61
Nx	15	16	16	15	14	15	13	16	12	11

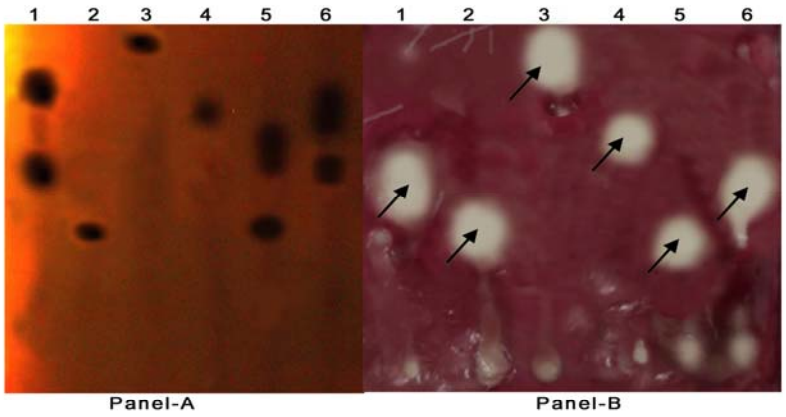

Fig. 1-Thin layer chromatography plates of 6 medicinal plants were run in duplicate and one set was visualized by anisaldehyde/sulphuric acid spray reagent (Panel-A). The other set was used for bioautography with *Klebsiella pneumoniae* (Panel-B). 1: *Azadirachta indica*; 2: *Commiphora mukul*; 3: *Pimpinella anisum*; 4: *Punica granatum*; 5: *Allium sativum*; 6: *Eucalyptus mannifera*. Arrows indicate antibacterial activity.

The HPLC analysis of the *P. anisum* extract showed two prominent peaks with retention time of 2.23 and 3.31 min. Out of these one peak has been identified as saponin with retention time 3.31 min. *A. sativum* showed four prominent peaks with retention time of 6.0, 7.3, 8.1 and 11.8 min. and two peaks were identified as saponin and allicin with retention time 3.31 and 11.68 respectively. The HPLC analysis of *C. mukul* extract showed one peak of tannin with retention time of 8.05 min. The HPLC analysis of *E. mannifera* showed three prominent peaks with retention time of 4.11, 5.20 and 8.07 min; out of these one peak was identified as tannin with retention time 8.07 min. *P. granatum* showed four peaks with retention time of 5.8, 7.2, 8.0 and 36.78 min. One peak was identified as ellagic acid with retention time 36.78 min. compared with standard of ellagic acid (retention time 36.5 min.). The HPLC analysis of *A. indica* extract showed three peaks with retention time of 5.58, 16.2 and 17.3 min. Out of these two peaks were identified as salanin and azadirachtin.

DISCUSSION AND CONCLUSION

The plant extracts tested have antibacterial properties against at least one of the ten isolates of *K. pneumoniae* tested during the present study. Among the four different solvents used for the extraction, the chloroform extract, followed by ethanol extract of several plant species showed maximum inhibitory activity than the aqueous and acetone extract. Similar reports on antibacterial activities of certain plants such as *P. granatum*, *A. indica*, *A. sativum* were also reported by other workers[16-19]. However antimicrobial activity of some Indian medicinal plants namely *C. mukul*, *E. mannifera* and *P. anisum* has been reported here for very first time against *K. pneumoniae*. Among the six plants which were selected on the basis of screening experiment, *C. mukul* showed low MIC i.e. 5 mg/ml particular in ethanol extract. The low MIC value against *K. pneumoniae* is a good indication of high efficacy of this plant and can be a good source of bioactive components with antimicrobial potency. Plants that showed high MIC value may be an indication of low efficacy of crude extract of plant or that the organism has the potential for developing resistance to the bioactive compounds[20].

To assess the major active constituent responsible for antimicrobial activity against *K. pneumoniae*, TLC-bioautography was performed. Clear inhibition zone were observed at R_f of 0.633 for *A. indica*, 0.611 for *C. mukul*, 0.900 for *P. anisum*, 0.833 for *P. granatum*, 0.310 for *A. sativum* and 0.619 for *E. mannifera* (Fig. 1). The HPLC analysis of the

plant extract showed peaks with different retention times. The peaks have been identified by using standard solutions, under similar conditions. The chromatographic fingerprint profile of antimicrobial compounds is presented in Fig. 2.

Phytochemical analysis of six active extracts demonstrated the presence of phytoconstituents like tannin, saponins, flavonoids and alkaloids. The medicinal value of the plant lies in bioactive phytochemical action on the human body[21]. Some of the most important bioactive phytochemical constituents were alkaloids, flavonoids, tannins, terpenoids, saponins and phenolic compounds as reported earlier. Antibacterial properties of several plant extracts have been attributed to some of these secondary metabolites[22-24]. The results of phytochemical analysis are in agreement with the reports of other workers[25]. Antimicrobial action of tannin may be related to their ability to inactivate microbial adhesions, enzymes, cell envelops transport proteins etc.[26]. Flavonoids activity is probably due to their ability to complex with extracellular and soluble proteins and with bacterial cell wall. More lipophilic flavonoids may also disrupt microbial membrane[27]. The presence of these phytochemical compounds was also detected by thin layer chromatography. TLC is a standard technique, which separates the organic compounds from lower molecular weight according to their polarity[28,29]. It is expected that more active compounds can be detected by TLC bioautography, if different solvent systems, microbial strains and more plant extracts are used. Bioautography of thin layer chromatographic plate showed clear zones containing substances that inhibited the growth of *K. pneumoniae* over the region containing the components with high and medium polarity. In majority of the plants tannins were observed as most active constituents. These findings are found to be correlated with the findings of Tanaka *et al*[30] and Silva *et al*[31].

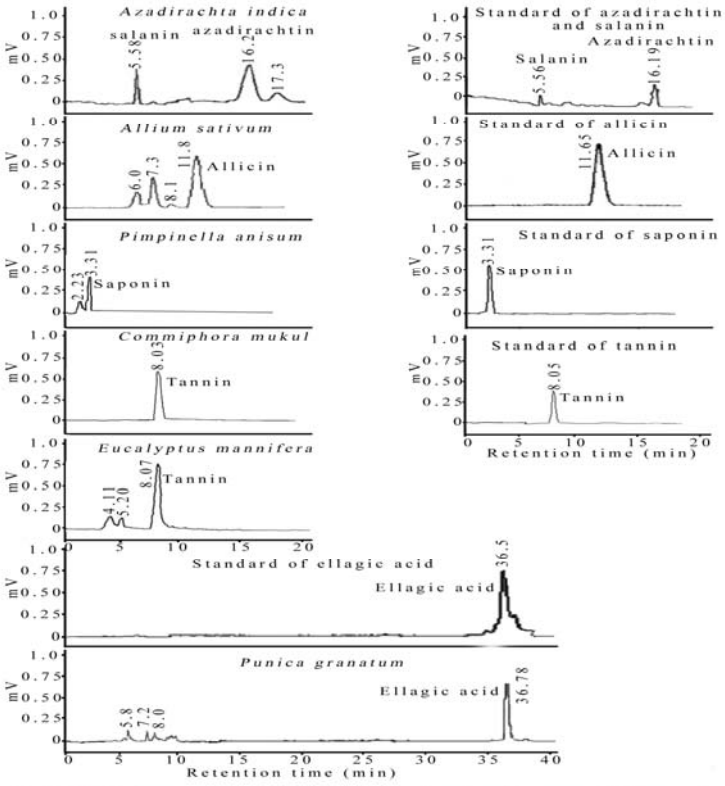

Fig.2- HPLC chromatograms of pure standard and methanolic extract of different plants obtained by using C18 column, methanol: water (70:30) as a mobile phase and UV detector set at 310 nm.

HPLC is regarded as a prime technique applied to develop fingerprint of crude herbs due to precision, sensitivity and reproducibility[32, 33]. HPLC analysis revealed slight changes in the retention times of the identified compound(s) in the extracts. It may either due to overlap of the peaks in the mixture or due to the fact that in the extracts these individual components are not in the free form but are bound as glycosides and other water soluble forms.

The findings of this study support the traditional knowledge of local users and provide a preliminary scientific validation for the use of *A. indica, C. mukul, P. anisum, P. granatum, A. sativum* and *E. mannifera* for antibacterial activity. TLC and HPLC analysis revealed the identity of bioactive constituents present in plant extracts. Characterization of active components is required and their activity has to be evaluated in further work.

ACKNOWLEDGEMENT

The authors are thankful to the Head, Department of Post Graduate Studies and Research in Biological Sciences, R. D. University, Jabalpur, India for providing laboratory facilities and SFRI, Jabalpur for identification of plant materials.

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