Pharmacophore 2010, Vol. 1 (3), 184-195 ISSN 2229 – 5402

Pharmacophore

(An International Research Journal)

Available online at http://www.pharmacophorejournal.com/

Original Research Paper

ANTIBACTERIAL ACTIVITY AND QUANTITATIVE DETERMINATION OF PROTEIN FROM LEAF OF *DATURA STRAMONIUM* AND *PIPER BETLE* PLANTS

A. Kumar[1]*, B.R. Garg[2], G. Rajput[3], D. Chandel[4], A. Muwalia[4], I. Bala[4], Sumeer Singh[5]

[1]* Dept of Biotechnology, HNBGU (A Central University), Srinagar, (Garhwal)-246174 (UK), India

[2] Principal, Himachal Institute of Pharmacy, Paonta Sahib-173025 (HP), India

[3] Dept of Biotechnology, Beehive College of Advance Studies, Selaqui, Dehradun, Uttarakhand, India

[4] Dept of Biotechnology, Himachal Institute of Life Sciences, Paonta Sahib-173025 (HP), India

[5] Dept of Microbiology, Himachal Institute of Life Sciences, Paonta Sahib-173025 (HP), India

ABSTRACT

All plants containing active compounds are important. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant. *Datura* and *Piper betle*, medicinal plants were tested against three standard microorganisms *E-coli* DH5 (MTCC 2804), *Bacillus amyloliquefaciens* (MTCC 4012) and *Pseudomonas aeruginosa* (MTCC 1265) collected form MTCC Chandigarh for antibacterial activity. Among the medicinal plants tested in this study, *Piper betle* showed valuable antibacterial activities. The result showed that *Piper betle* didn't show any antibacterial activity against *Pseudomonas aeuroginosa* whereas *Datura stramonium* didn't show any antibacterial activity against *Bacillus amyloliquefaciens and E-coli*.

Keywords: Secondary metabolites, Alkaloids, Antibacterial activity, Ethanolic extract, MTCC

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Various medicinal plants have been used for years in daily life to treat disease all over the world. They have been used as a source of medicine. Medicinal plants are a source of great economic value in the Indian subcontinent**.**

All the plants have different compounds of biological significance, these compounds are more complex and specific and are found in certain taxa such as family, genus and species, but heterogeneity of secondary compounds is found in wild species. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct. The plants secondary products may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules or neurotransmitters and thus have beneficial medicinal effects on humans due to similarities in their potential target sites. Therefore, random screening of plants for active chemicals is as important as the screening of ethno botanically targeted species (Essman, 1984).

Interest in a large number of traditional natural products has increased. It has been suggested that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antiviral, antitumor and antibacterial agents. Medicinal plants have a long history of use and their use is widespread in both developing and developed countries. Among the nearly 15,000 flowering plants documented, many of them are used as sources of medicine. According to the report of the world health organization, 80% of the worlds population rely mainly on traditional therapies which involve the use of plant extracts or their active substances because of their easy availability and low cost of treatment.

The modern allopathic system of medicine is known to produce serious side-effects and moreover the microorganisms have developed resistance against many antibiotics due to the indiscriminate use of antibacterial drugs. This creates problems in the treatment of infectious diseases. Furthermore, antibiotics are sometimes associated with side effects, whereas there are some advantages of using antibacterial compounds of medicinal plants, such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature. All these data highlights the need for new alternative drug regimens **(**Julseth and Deibel, 1974).

Datura stramonium, known by the common names jimson weed, "poor man's acid", ditch weed, stink weed, loco weed, Korean morning glory, Jamestown weed, thorn apple, angel's trumpet, devil's trumpet, devil's snare, devil's seed, mad hatter, crazy tea, malpitte, and, along with *datura betel*, zombie cucumber is a common weed in the Nightshade Family. It contains tropane alkaloids that are sometimes used as a hallucinogen. The active ingredients are atropine, hyoscyamine and scopolamine which are classified as deliriants, or anticholinergics. Due to the elevated risk of overdose in uninformed users, many hospitalizations, and some deaths, are reported from recreational use, though this is not always the case (Saldanha, 1984).

The leaves of *Piper betel* are used as a stimulant, an antiseptic and a breath-freshener Paan. In Ayurvedic medicine, they are used as an aphrodisiac. In Malaysia they are used to treat *headache*, *arthritis* and *joint pain*. In the Philippines, Thailand, Indonesia and China

they are used to relieve *toothache*. In the Philippines, they are used specifically as a stimulant. In Indonesia they are drunk as an infusion and used as an antibiotic (Kurian, 2001). They are also used in an infusion to cure indigestion, as a topical cure for constipation, as a decongestant and as an aid to lactation. In India, *betel* is used to cast out (cure) worms (Saldanha, 1984; Saldahna and Nicolson, 1978).

The antibacterial properties of the medicinal plants are reported from all over the world and used in the treatment of many diseases such as, *Malaria*, *AIDS* and *sexually transmitted diseases*. In the present study, *in vitro* antibacterial activities of *Piper betel* and *Datura stramonium* were investigated against some major pathogens. In microbiology, it is the lowest concentration of an antibacterial that will inhibit the visible growth of a microorganism after overnight incubation and this is known as minimum inhibitory concentration (MIC). Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antibacterial agent and also to monitor the activity of new antibacterial agents. Agar diffusion techniques are used widely to assay plant extracts for antibacterial activity MICs can be determined by broth dilution methods usually following the guidelines of a reference body such as the BSAC.

MATERIAL AND METHODS

Preparation of Plant Extract

Plant material (leaves) of *Piper betle* and *Datura stramonium* were selected and kept for drying. The dried plant material was macerated with 80% ethanol (sample/ethanol in 1:30 ratio) for five days with a constant shaking and crude was filtered. The filtrate obtained was again kept for drying for 4-5 days and was evaporated to a thick residue at 40^0 C. These residues were suspended/dissolved in 30 ml of Tris Buffer Saline (pH 7.6) and used for its antibacterial activity and MIC experiments **(**Kumar *et al.*, 2009; Obi *et al.*, 2002).

Bacterial Strain and Culture Medium

The three bacterial strains *E-coli DH5* (MTCC 2804), *Bacillus amyloliquefaciens* (MTCC 4012), *Pseudomonas aeruginosa* (MTCC 1265) were selected. They were collected form MTCC Chandigarh. The selected microorganisms (*Bacillus sp, E-coli and Pseudomonas sp*) were streaked on nutrient agar plates and were kept overnight incubation at 37^0 C for 24-48 hrs and the colonies were obtained. From the microbial colonies obtained through overnight incubation a small amount of the organisms were scraped off from their respective petri-plates and were inoculated into the prepared nutrient broth in separate containers. After 48 hrs the growth of specific microorganism was observed (Atlas, 1993; Aneja, 2002).

Preparation of Agar Plates for MIC Determination

For the preparation of Agar plate's Nutrient agar was used. Prepared nutrient agar was autoclaved at 121^0 C with 15 psi for 15-20 minutes. The autoclaved agar solution was then kept in laminar air flow and allowed to cool. Under aseptic condition of laminar air flow the inoculum added nutrient agar was then poured into respective plates in equal volume and then plates were kept for solidification process for 20-30 min. After solidification of the inoculum added nutrient agar the wells were punched in the solidified gel by using borer (Nair *et al.*, 2005).

Antibacterial Activity Assay

The agar diffusion method was used for antibacterial evaluations. The standard concentration (50, 75 and 100 µg/µl) of the

plant sample extracts (*Piper betle* and *Datura stramonium*) was added into the wells of *Bacillus sp*, *E-coli* and *Pseudomonas sp* plates respectively. The plates were incubated for 24 hrs at 37^0 C. In order to get the standard reference values the Ampicillin, Tetracycline and Amoxicillin drugs were chosen. Different concentrations (10, 20 and 30 µg/µl) of these drugs were also poured into the wells of separate *Bacillus sp*, *E-coli* and *Pseudomonas sp* plates respectively (Kumar *et al.*, 2009; Eftekhar *et al.*, 2005; Ahmad and Beg, 2001).

Sample Extract Preparation for Quantitative Determination of Protein

1 gm samples (dried leaves) from the two plants were weighed, macerated in pestle mortar in 5 ml phosphate buffer and transferred to centrifuge tubes respectively. The homogenates were centrifuged at 8000 rpm for 20 min, supernatants were collected and the extraction was repeated 4-5 times for each plant sample separately. The supernatant for the two plants were then combined separately and the volume was set to 50 ml with phosphate buffer for each extract (Harborne *et al.*, 1998**)**. The composition of protein extraction buffer is listed in Table 1.

Bradford Assay

Took 0.1 ml of sample solution and the volume was made to 1 ml with 0.1 M phosphate buffer (pH 7.5). Appropriate aliquots of bovine serum albumin (BSA prepared by dissolving 5 mg of BSA in 50 ml of 0.1 M phosphate buffer) containing 0-100 µg protein were pipette out and the volume was made to 1 ml with 0.1 M phosphate buffer (pH 7.5) in all the tubes. Bradford reagent was added into all the tubes and absorbance was recorded at 595 nm against the reagent blank. The protein concentrations of unknown sample were determined by BSA standard curve (Sawhney and Singh, 2001).

Partial Purification of Protein by Acidified Acetone/ Methanol Precipitation

Acidified acetone (10 µl HCl mixed with 120 ml acetone and final concentration was made 1mM) and precipitation reagent (acidified acetone was mixed with equal volume of methanol and stored at -20^o C) were prepared. Protein solution and cold precipitation reagent were mixed in the ratio of 1:4 and kept overnight at -20^o C. The following day, the mixture was spined in microcentrifuge at 15000 rpm for 15 minutes at 4^o C, the supernatant was discarded and pellet was retained. This sample was dry under vacuum to eliminate any acetone methanol residue.

Partial Purification of Protein by TCA-Ethanol Precipitation

To the protein sample 5% TCA, double the amount of protein sample was added and the mixture was kept on ice for 20 min. Then spinning was done at 12000 rpm at 4^o C for 15 minutes in micro centrifuge the supernatant discarded, the retained pellet was washed with 70% ice cold ethanol (300 µl) and again centrifuged at 12000 rpm for 10 min, supernatant discarded, pellet dried and finally resuspended in TE buffer (50-100 µl).

Table 1: Composition of Protein extraction buffer (for 50 ml)

Stock Solution	Required Concentration
1M Tris-Cl (pH8.0)	2.5 ml, 50mM
100 mM $MgCl_2$	5 ml, 50mM
0.5M- EDTA	10 µl, 0.1mM
5 mM Isoascorbate	44 mg, 5mM
Final volume	50 ml with ddw

Table 2: Composition for Resolving Gel

Reagent	Volume (30 ml)	Volume (10 ml)
40% Acrylamide stock	9.4	3.1
Distilled Water	12.3	3.8
1.5 M Tris-HCl pH 8.8	7.5	2.5
10% SDS	0.3	0.1
10 %Ammonium per sulphate	0.5	0.5
TEMED	10 µl	4 µl

Table 3: Composition for Stacking Gel

Reagent	Volume (30 ml)	Volume (10 ml)
40% Acrylamide stock	1.7	1.1
Water (distilled)	10.8	7.1
1 M Tris-HCl pH 6.8	1.9	1.25
10% SDS	0.15	0.1
10 % Ammonium persulphate	0.5	0.5
TEMED	20 µl	5 µl

Denaturing Gel Electrophoresis (SDS-PAGE)

12% running gel (Table 2) was poured in glass slabs and the gel was overlaid by saturated iso-butanol. The gel was allowed to be solidified for half an hour. After solidification the gel was rinsed from top with double distilled water to remove iso-butanol and the slabs were wiped out with blotting paper. 4% stacking gel (Table 3) was poured over the running gel and the comb was inserted in the space between the two glass slabs. Then the glass slabs placed in the running tank and filled with running buffer. The samples were pipetted down in the wells and run at 50V. As the bands crossed the stacking gel the voltage was increased to 120V. The samples were prepared by boiling the cell pellet with cracking dye for 5 minutes at 1000C. After boiling the pellet was centrifuged at 8000 rpm for 10 minutes at 40 C, immediately the supernatant was aliquoted and stored at –200C. The samples were loaded in the wells along with protein marker. The electrophoresed gel was stained with coomassie brilliant blue (R 250) for one hour with shaking at 25- 30 rpm or on rotor. Then the gel was destained with destaining solution for 3-4 hrs and the destaining solution was changed several times.

RESULTS AND DISCUSSION

The antibacterial activity of *Piper betle* and *Datura stramonium* was tested against three selected standard stains collected form MTCC Chandigarh INDIA. The measurements of zone of inhibition are listed in Table 4. From the observations, *Datura stramonium* showed antibacterial activity against *Pseudomonas sp*. It didn't show any antibacterial activity against *E-coli* and *Bacillus sp. Piper betle* showed antibacterial activity against *Bacillus sp* and *E-coli.* It didn't show any antibacterial activity against *Pseudomonas sp*. Ampicillin showed antibacterial activity against *Pseudomonas sp, E-coli* and *Bacillus sp.* Tetracycline showed antibacterial activity against *Pseudomonas sp* and *E-coli*.

Amoxicillin showed antibacterial activity against only *Pseudomonas sp*. According to the above data *Piper betle* showed maximum Zone of Inhibition against *Bacillus sp* and *Datura* showed against *Pseudomonas sp*. Ampicillin showed maximum activity against *Bacillus sp*. Tetracycline showed maximum activity against *E-coli* (Figure 1 a-j). The crude protein of these plants were also tested for antibacterial activity and it was observed that the crude protein extract of *Piper betle* showed the antibacterial activity against *Bacillus sp* and *Pseudomonas sp* (Figure 2 a and b) only not against *E. coli* but the crude protein of *Datura stramonium* did not show the significant zone of inhibition against all the three selected strains (Table 5).

Table 4: Zone of Inhibition of Standard Antibiotics and Ethanolic Extract of Plants

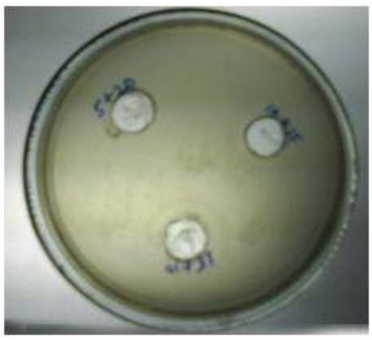
Antibiotics and Ethanolic Extract	Zone of Inhibition in mm		
	25 µl (5 µl+20 µl)	**25 µl (10 µl+15 µl)**	**25 µl (15 µl+10 µl)**
Ampicillin against *E. coli*	4 mm	7 mm	8 mm
Ampicillin against *P. aeruginosa*	6 mm	20 mm	21 mm
Ampicillin against *B. amyloliquefaciens*	18 mm	21 mm	23 mm
Tetracycline against *E. coli*	9 mm	16 mm	18 mm
Tetracycline against *B. amyloliquefaciens*	-	-	-
Tetracycline against *P. aeruginosa*	11 mm	15 mm	17 mm
Amoxicillin against *E. coli*	-	-	-
Amoxicillin against *B. amyloliquefaciens*	-	-	-
Amoxicillin against *P. aeruginosa*	14 mm	16 mm	19 mm
Piper betle against *E. coli*	4 mm	5 mm	7 mm
Piper betle against *B. amyloliquefaciens*	9 mm	11 mm	12 mm
Piper betle against *P. aeruginosa*	-	-	-
Datura stramonium against *E. coli*	-	-	-
Datura stramonium against *B. amyloliquefaciens*	-	-	-
Datura stramonium against *P. aeruginosa*	15 mm	20 mm	25 mm

Note: There is – for zone of inhibition not detected.

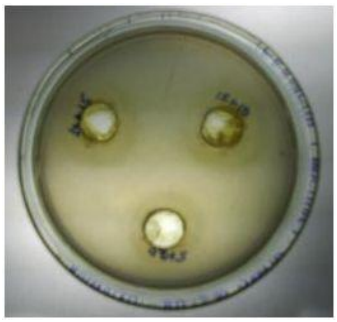
Table 5: Zone Of Inhibition of Crude Protein Extract

Plants/Strains	***Piper betle*** **(**245 µg/ml **)**	***Datura stramonium*** **(**183.5µg/ml**)**
E. coli	-	-
Pseudomonas aeurogenosa	11 mm	-
Bacillus	8 mm	-

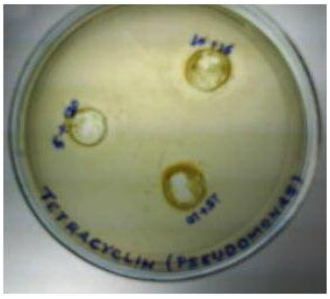
Note: There is – for zone of inhibition not detected.



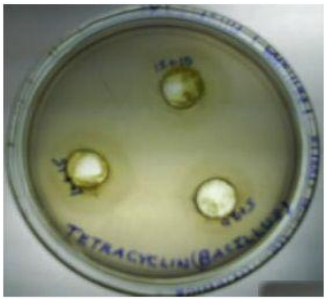
a- *Piper betle* against *Bacillus*



b- *Piper betle* against *E coli*

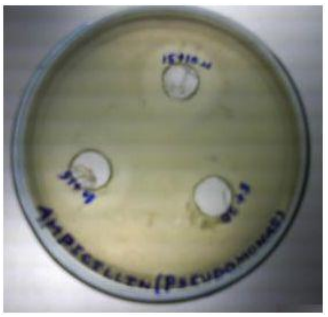


c-Tetracycline against *Bacillus*

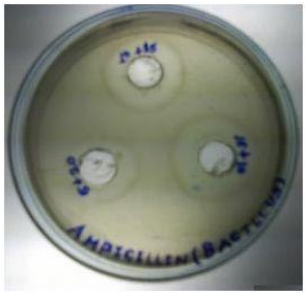


d-Tetracycline against *Pseudomonas*

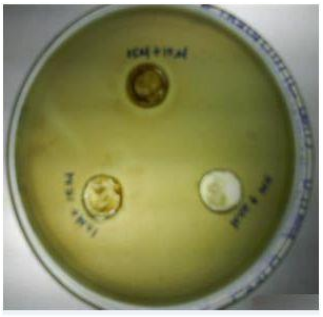
e- Ampicillin against *E coli*



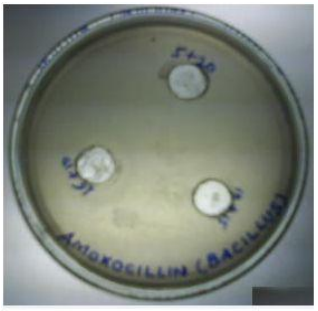
f- Ampicillin against *Pseudomonas*



g- Ampicillin against *Bacillus*



h- *Datura stramonium* against *Pseudomonas*

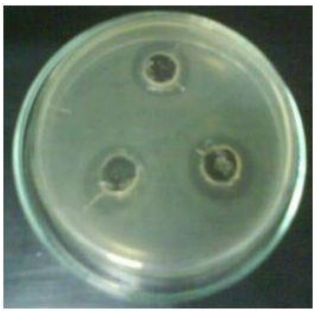


i-Amoxicillin against *Bacillus*

j- Amoxicillin against *Pseudomonas*

Figures 1 (a-j): Zone of inhibition of Ethanolic Extract against selected strains

a- Crude protein extract of *Piper betle* against *Bacillus sp*



b- Crude protein of *Piper betle* against *Pseudomonas sp*

Figures 2 (a-b): Zone of Inhibition of Crude Protein against selected strains

Bradford Assay

Unlike Lowry's method, metal ions such as $NH4^+$, Na^+ , K^+ , phenols and carbohydrates such as sucrose do not interfere in this assay. The procedure is based on interaction of a dye, Coomassie Brillinat Blue, with proteins. The unbound dye has absorbance maxima at 465 nm. However, on interaction with protein the dye turns blue and their absorbance maximum is displaced to 595 nm. Thus from the absorbance at 595 nm the amount of protein in a sample solution can be quantitatively estimated (Table 6). The standard curve was prepared on the basis of optical density at 595 nm of the sample (Figure 3). From the standard curve, the concentration of the *Pipre betle* according to the absorbance was 2.8 µg/ml and the concentration of *Datura stramonium* was found 23 µg/ml.

Table 6: Readings from Bradford Assay

S. No.	BSA(µl)	ddw (µl)	Final Conc. (µg/ml)	Bradford Reagent (ml)	OD at 595 nm
Blank	0	500	0	2	0.00
01	50	450	2.5	2	0.024
02	100	400	5.0	2	0.077
03	150	350	7.5	2	0.079
04	200	300	10	2	0.178
05	250	250	12.5	2	0.242
06	300	200	15	2	0.272
07	350	150	17.5	2	0.237
08	400	100	20	2	0.343
09	450	50	22.5	2	0.348
10	500	0	25	2	0.364

Piper betle	10 ul of Protein sample + 490 ul of ddw + 2 ml of B.R.	0.057
Datura stramonium	10 ul of Protein Sample + 490 ul of ddw + 2 ml of B.R.	0.467

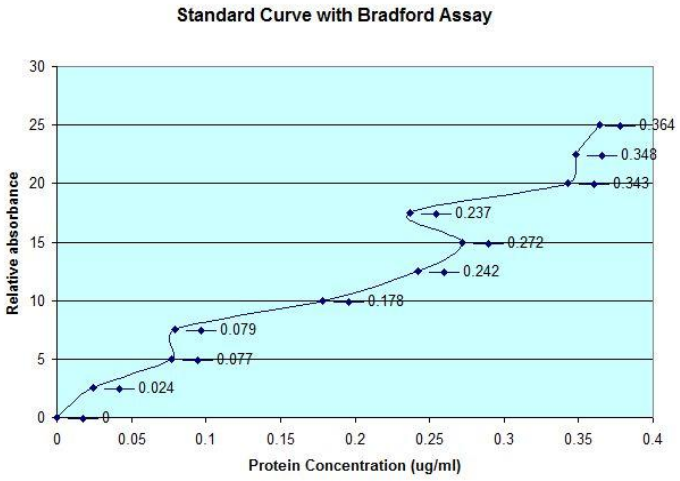


Figure 3: Standard curve determined with Bradford assay

Electrophoresis:

The electrophoretic result obtained and presented in figure 4, which show the protein present in the cell free extracts of the selected plants (*Datura stramonium* and *Piper betle*) ranging from 12 to 68 Kda.

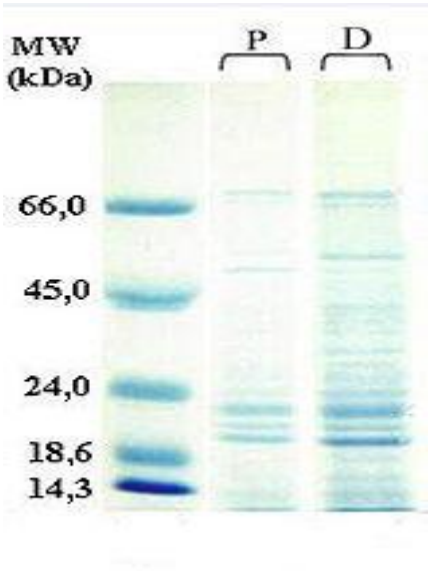


Figure 4: SDS PAGE profile of total proteins of cell free extracts of *Datura stramonium* and *Piper betle*.

M: Molecular weight marker

P: Total protein profile of ***Piper betle***

D: Total protein profile of ***Datura stramonium***

Plant originated antibacterial drugs are of interest because in part many human and animal pathogens show multi-drug resistance and in part certain antibiotics have undesirable side effects. In this study Antibacterial activity test of the crude ethanolic extracts of two, selected, domestic, medicinal plants *Piper betle* and *Datura stramonium* was carried and determined against test microorganisms. It was found that *Piper betle* showed the antibacterial activity against *Bacillus amyloliquefaciens and E. coli* whereas *Datura stramonium* showed antibacterial activity against *Pseudomonas aeuroginosa* only.

CONCLUSION

Among the medicinal plants tested in this study, *Piper betle* showed valuable antibacterial activities. Hence leading to the conclusion that, these plants would serve as sources of novel antibacterial agents. Further studies are needed to find out the active compounds of these plants. It is possible to find the better therapies for many infectious diseases from the plants. The *Piper betle*

would helpful in the treatment of bacterial diseases.

ACKNOWLEDGEMENTS

Authors are grateful to Mrs. K.P. Rathoure for her co-operation, valuable suggestions and technical help.

REFERENCES

1. Ahmad, I and AZ, Beg (2001), "Antibacterial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens", ***J. Ethnopharmacol***, 74, 113-123.
2. Aneja, KR (2002), "***Experiments in Microbiology***", New Age International Publications, New Delhi, India.
3. Atlas, RM (1993), "***Hand Book of Microbiological Media***", LRC Press, London.
4. Bayoud, B; Djilani, SE; Legseir, B; Ouahrani, MR *et al.* (2007), "Antibacterial activity of ethanol extracts and total alkaloids of *Datura stramonium* and *Ruta graveolens*", ***Icfai Journal of Life Sciences,*** 1(1), 78-81.
5. Eftekhar, F; Yousefzadi, M and Tafakori, V (2005), "Antibacterial activity of *Datura innoxia* and *Datura stramonium*", ***Fitoterapia***, 78(1), 118-120.
6. Essman, E J (1984), "The medical uses of herbs", ***Fitoterapia***, 55, 279-289.
7. Harborne, JB (1998), "***Phytochemical methods: A guide to modern techniques of plant analysis***", 3rd Ed., Chapman and Hall, London, 235.
8. Julseth,RM and Deibel, RH (1974), "Microbial profile of selected spices and herbs at import", ***J. Milk Food Technol.***, 37,414-419.
9. Kumar, Ashok; Rajput, Gaurav; Srivastava, Gaurav *et al.* (2009), "Phytocontent screening of mucuna seeds and exploit in opposition to pathogenic microbes", ***J. Biol. Environ. Sci.***, 3 (9), 71-76.
10. Kurian, JC (2001), "***Plants That Heal***", Ed. 2, Oriental Watchman Publishing House, 264,300.
11. Nair, R; Kalariya, T and Chanda S. (2005), "Antibacterial activity of some selected Indian medicinal flora", ***Turk J Biol***, 29, 41-7.
12. Obi, CL; Potgieter, N and Randima, LP (2002), "Antibacterial activities of five plants against some medically significant human bacteria", ***South African Journal of Science***, 98 (1-2), 25-28.
13. Saldanha, CJ and Nicolson DH (1978), "***Flora of Hassan District***", Amerind Publishing Company Private Limited, New Delhi, 336-339.
14. Saldanha, CJ (1984), ***Flora of Karnataka***", Vol.-I & II, Oxford and IBH publishing company, New Delhi.
15. Sawhney, SK and Singh, R (2001), "***Introductory practical Biochemistry***", Narosa publishing house, India.