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Research Article

LEAF EXTRACT OF *CENTRATHERUM PUNCTATUM* EXHIBITS ANTIMICROBIAL, ANTIOXIDANT AND ANTI PROLIFERATIVE PROPERTIES

NAVEEN KUMAR PAWAR AND NEELAKANTAN ARUMUGAM*[1]

Department of Biotechnology, School of Life Sciences, Pondicherry University, R Venkatarama Nagar, Kalapet, Puducherry, India
E mail: n_arumugam@hotmail.com

ABSTRACT

Centratherum punctatum, the Brazilian button flower, is very closely related to its counterpart *C. anthelmethicum* - a plant known for its high medicinal value. To validate if *C. punctatum* would also be of any such value, the leaf extract of the plant was evaluated for anti-microbial, antioxidant, human cell toxicity properties and analyzed for the presence of phytochemical constituents. Powdered leaf of the plant was extracted with different organic solvents and tested for anti-microbial activity by the agar well-diffusion method. The antioxidant activity was analyzed by Ascorbic acid method. The toxicity of the extract was tested by the MTT assay using human peripheral blood mononuclear cells (PBMCs). Extracts were then subjected to bioautography and the phyto-chemical constituents isolated and tested for antimicrobial activity. TLC fractions that tested positive for anti-microbial activity were partially characterized for functional group identification by KBr method using Fourier Transform Infrared Spectroscopy. Acetone, methanol and ethyl acetate extracts of leaf showed inhibitory activity against four out of five pathogenic bacteria including the multi drug resistant (MDR) *Acinetobacter baumanii* and *Staphylococcus aureus* tested. Anti-fungal activity was exhibited by acetone and ethyl acetate extracts. Phytochemical analyses revealed the presence of flavonoids, tannins and cardiac glycosides, of which flavonoids showed anti-bacterial activity. The IC_{50} value for the acetone extract was found to be 10.63 µg/ml. FTIR analysis revealed the presence of alkene, alkane, aliphatic amine and aromatic functional groups among others. We conclude that the present study adds credence to the ethno-medicinal properties of *C. punctatum*. Further characterization of phytochemical compounds from this prolific herb may yield potential antimicrobial agents.

Keywords: *Centratherum punctatum*, *Acinetobacter baumanii, Staphylococcus aureus,* multi drug resistance, antioxidant, flavanoids, tannins, cardiac glycosides.

INTRODUCTION

Nature has been a valuable source of medicine and has helped human in the maintenance of his health since time immemorial[1]. According to the World Health Organization (WHO), almost 80% of the world's population relies on traditional medicines for their health needs due to better cultural acceptibility, fewer side effects and better compatibility with the human body[2, 3].

Indiscriminate use of antibiotics appears to promote development of pathogens showing resistance to multiple drugs and this scenario continues to become grave day by day. Exhibition of multi-drug resistance by pathogenic bacteria conveys a great challenge ahead for the society thereby causing a renewal of interest in ethno-botanic medicines[4, 5]. Of late there have been several attempts made for discovery of new antimicrobial substances from natural sources [6–8].

Centratherum punctutam Cass. (*Asteraceae*), the Brazilian bachelor button, is one among 33 species of the type genus *Centratherum* and is a perennial bushy plant of 45–60 cm height (Fig. 1). It has a well-branched stem with refreshing scented foliage and purple flower heads. Recently an essential oil containing nearly 59 different compounds has been isolated from leaves of this plant[9].

Centratherin, a sesquiterpene lactone, has been isolated from *C. punctatum* but its medicinal properties have not yet been established conclusively[10]. A related species *C. anthelminticum* is known for antifilarial[11] and antihyperglycemic properties[12]. Therefore in this study we attempted to seek a scientific justification for the use of *C. punctatum* as a source of herbal medicine by testing the extract on some clinically important human and plant pathogens for anti-microbial activity. Phytochemical analysis was performed to detect the presence of bioactive constituents and an attempt was also made to characterize the bioactive TLC fractions by FTIR.

MATERIALS AND METHODS

Plant materials

Centratherum punctutam Cass. (*Asteraceae*) (Fig.1) was obtained from herbal collections of Indian Council of Agricultural Rearch - Krishi Vigyan Kendra, Puducherry, and maintained in the experimental garden of the Department of Biotechnology, Pondicherry University. The identity of the plant was authenticated by Prof. N. Parthasarathy, a taxonomist of our University, and a voucher specimen of the plant bearing the number DBTAZ002 has been deposited in the Herbaria maintained by him in the Department of Ecology and Environmental Sciences of our University.

Chemicals

Solvent and other chemicals used for this study were of AR grade quality and were procured from Merck (India) and Hi-Media, Mumbai. Pre-coated silica gel 60 F_{254} plates used for thin layer chromatography (TLC) were obtained from Merck, Germany.

Bacterial cultures

The test organisms (*Pseudomonas aeruginosa* (ATCC 2706), *Acinetobacter baumanii* (ATCC 19606), *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Providencia rettgeri*) were obtained from the culture collections of our Department. Fungal plant pathogens, *Fusarium spp.* and *Curvularia spp*., were obtained from IMTECH, Chandigarh, India. Except for *B. subtilis* the bacterial cultures were grown and maintained on Mueller-Hinton agar slants. *B. subtilis* was maintained as a spore suspension in sterile distilled water. Fungal cultures were maintained on potato dextrose agar (PDA) slants.

Culture media

The culture media used for testing anti-microbial activity were - Mueller-Hinton agar, Davies Synthetic Minimal medium (only for *B. subtilis*) and Potato dextrose agar for fungal strains. The culture media were prepared and sterilized following the manufacturer's (Himedia, India) instructions.

Preparation of plant extracts

Ten grams of air-dried leaf samples were powdered and soaked in 100 ml of hexane or ethyl acetate or acetone or methanol and incubated at room temperature for 48 hours with intermittent shaking. The solvent-leaf mixtures were then centrifuged at 6,000 rpm at 4°C for 10 mins to obtain the supernatant and the supernatant was concentrated using a Rotavapour (Buchi-R-Switzerland) at 50°C. The residue obtained after drying was weighed

and reconstituted into suspension by dissolving in the respective solvents with which they were extracted.

Test for anti-microbial activity by agar-well diffusion method

The efficacy of the plant extracts for anti-microbial activity was evaluated in terms of zone of inhibition by the agar well-diffusion method[3]. The target bacteria (except *B.subtilis*) were cultured separately by inoculation on Mueller-Hinton agar. For *B. subtilis,* the spore suspension was mixed with Davies Synthetic Minimal Agar medium prior to plating for solidification and the plates were subjected to agar-well diffusion assay.

Anti-fungal activity was determined by culturing the spores of test fungi on PDA plates. Wells with 6 mm diameter and 5 mm depth were cut out from the agar plates using a sterile cork borer. 30 µl plant extracts from each of the solvent extracts adjusted to 1, 5, 10, 15 and 20 mg/ml was injected into the wells. Pure solvents served as negative controls. The culture plates were incubated at 37°C and after 48 hours the diameter of the inhibition zone was measured using millimeter scale. The tests were carried out at least twice.

Phytochemical analysis

Analysis for the presence of alkaloids, tannins, saponins, flavonoids and cardiac glycosides in the leaf extract was done following the methods described in Harborne[13].

Bioautography of phytochemical compounds

The metabolites tested positive for the phytochemical analysis were isolated and tested for anti-bacterial activity by the agar well-diffusion method. Flavonoids were isolated by heating 0.5 g of leaf sample with 5 ml methanol on water bath at 40°C for 10 min.

The filtrate was concentrated by evaporation to 1/4th of its original volume. Tannins were isolated by heating 0.5 g of the sample with 10 ml 2M HCl in a boiling water bath for 30 min. The filtrate was mixed thoroughly with 1 ml ethyl acetate, and ethyl acetate layer was then discarded. Five drops of amyl alcohol were added to aqueous phase and shaken thoroughly.

The alcoholic layer was retained for antibacterial assay. Cardiac glycosides were isolated by heating 0.5 g of the sample with 5 ml of 50% (v/v) methanol and 10 ml of 10% (w/v) lead (II) acetate solution in a water bath at 40°C for 10 min. The filtrate was cooled and extracted twice with 10 ml dichloromethane/isopropanol (3:2). The lower organic or aqueous phases were combined, filtered over anhydrous sodium sulphate and evaporated to dryness. The residue was dissolved in 1 ml dichloromethane/isopropanol (3:2) and this solution was used for anti-bacterial assays. The test organisms used for testing anti-bacterial activity were *A.baumanii, S.aureus* and *B.subtilis.*

Anti-oxidant assay

Antioxidant assay was performed following the protocol of Prieto *et al* [14]. Ascorbic acid in the range of 10–100 µg was used as standard to make a standard curve for anti-oxidant activity. 300 µl of the standard and test samples (leaf extracts) were mixed with 3 ml of the reagent solution (0.6M H_2SO_4, 28mM Sodium Phosphate, 4mM of Ammonium molybdate) taken in a test tube. The test tubes were covered with aluminium foil and incubated in a thermal block at 95°C for 90 min.

The reaction mix was allowed to cool to room temperature and the absorbance of the solution was measured at 695 nm against a blank containing 3 ml of reagent solution and the appropriate volume of the corresponding solvent and incubated under the same conditions as the test samples. Butylated hydroxytoluene (BHT), a well known antioxidant used as food additive, was used a positive control (dilutions prepared from 1 mg/ml BHT stock solution in ethanol).

Standard graph for anti-oxidant activity was drawn by plotting concentration in µg on x-axis and O.D. at 695 nm on y-axis was plotted. The antioxidant activity of the plant extracts and positive control was determined using the standard graph.

Anti-proliferative assay (MTT assay)

MTT assay was performed following Alley *et al*[15], by plating phytohemagglutinin (PHA; 1 µg/ml) induced human peripheral blood mononuclear cells (PBMCs) in 96 well plates containing 200 µl of RPMI-1640 medium per well along with concentrations of each of the crude leaf extracts adjusted to 0.1, 1, 10, 25, 50 and 100 µg/ml and incubated at 37°C for 24 hours.

Following the completion of incubation, 10 µl of MTT from the stock solution (5 mg/ml MTT dye in PBS) was added to each well, and the plate was incubated at 37°C in a 5% CO_2 atmosphere for 4 hours. After 4 hours, the supernatant was removed without disturbing the cell pellet, 50 µl DMSO was added to each well and after thorough mixing (to dissolve the dye crystals), the absorbance was measured using an ELISA plate reader set at 570 nm. The controls for this assay consisted of untreated cells (positive control), mitogen-induced cells, mitogen-induced cells treated with DMSO and mitogen-induced cells treated with Triton-X-100 (negative control).

Thin-Layer Chromatography and Bioautography

The crude leaf extracts obtained using different solvents were subjected to thin-layer chromatography (TLC) by loading on precoated TLC silica gel 60 F_{254} plates (8 cm x 6 cm; Merck™). Ethyl acetate-hexane (1:9 v/v) mixture was used as the mobile phase. TLC chromatograms were scanned under UV light at 254 nm and the fluorescent bands were marked. The bands from TLC performed on acetone extract were scraped separately, dissolved in 1 ml of acetone, vortexed, and centrifuged at 6,500 x g for 15 min. The supernatants were collected and used to evaluate anti-bacterial activity by agar well-diffusion assay.

FTIR analysis of bioactive TLC fractions

TLC fractions that showed anti-bacterial activity were further subjected to spectroscopic analysis for identification of the functional groups in the bioactive compounds. Sample was prepared as described in Naumann *et al*[16].

A known weight of TLC-purified fractions of the acetone leaf extract (1 mg) was taken in a mortar and pestle and ground with 2.5 mg of dry potassium bromide (KBr). The powder so obtained was filled in a 2 mm internal diameter micro-cup and loaded onto FTIR set at 26°C ± 1°C. The samples were scanned using infrared in the range of 4000–400 cm^{-1} using Fourier Transform Infrared Spectrometer (Thermo Nicolet Model-6700). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

Statistical analysis

The data obtained were analyzed statistically and the results are presented as Mean ± Standard deviation.

RESULTS

Anti-microbial spectrum of leaf extracts

The organic solvent extract of leaf when used at a minimal concentration of 1 mg/ml inhibited four out of five bacterial strains tested (Table 1). The hexane extract was poor in exhibiting anti-microbial activity which may be attributed to polar nature of the anti-bacterial substances. *Bacillus subtilis* in particular was inhibited by all four extracts.

Acetone and ethyl acetate extracts in addition showed anti-fungal activity against *Fusarium* spp. P*seudomonas aeruginosa* and *Curvularia* sp. showed resistance to all the extracts. It was also observed that the diameter of zone of inhibition increased with increase in concentration of the extract (Fig. 2). This has led us to the conclusion that the inhibition is concentration-dependent.

Phytochemical analysis and bioautography

Phytochemical analysis of the powdered leaf samples of *C. punctatum* revealed the presence of three out of five secondary metabolites tested (Table 2). Of the three phytochemicals detected, flavonoids inhibited the growth of both pathogenic and non-

pathogenic bacteria. It is significant to note that the flavonoid from *C. punctatum* was able to inhibit even the multi-drug resistant (MDR) *Acinetobacter baumanii* and *Staphylococcus aureus* (Table 3). The activity of flavonoids is possbily due to their ability to complex with extracellular and soluble proteins, often leading to inactivation of the protein and loss of function. The probable targets for flavonoids are the proteins present in the bacterial cell wall with some of the lipophilic flavonoids disrupting even the bacterial membranes[16]. The other two phytochemicals, namely, tannins and cardiac glycosides, inhibited only *Bacillus subtilis* (Table 3).

Anti-oxidant Assay

The residues of leaves obtained by extraction procedure described in this paper exhibited anti-oxidant activities that ranged from 210 µg/ml to 353 µg/ml (Table 4). Among the four solvents the acetone extract showed the maximum activity of 353.36 µg/ml. The anti-oxidant activity was however found to be lower than the strandard anti-oxidant butylated hydroxyl toluene (BHT).

Anti-proliferative Assay

Anti-proliferative effect of the leaf extracts at different concentrations (0.1, 1, 10, 25, 50 and 100 µg/ml) was tested using PBMCs. After 24 hours exposure, the PBMCs were subjected to MTT assay and the results are presented in Figure 3. Untreated cells and cells treated with DMSO served as positive controls, while cells treated with Triton-X-100 served as negative control. A concentration-dependent increase in inhibition of cell proliferation was observed for all the four extracts. IC_{50} value was lowest for the acetone extract at 10.63µg/ml, while the hexane extract showed a higher IC_{50} value of 95.54 µg/ml (Fig. 3).

TLC and bioautography

In order to resolve different components from the crude extract and test their efficacy for bioactivity, TLC of the leaf extract was performed. Depending on the solvent used for extraction, the residues resolved into 7, 8, 10 and 13 different bands for ethyl acetate, methanol, hexane and acetone respectively. Since acetone extract of the leaf gave the maximum number of bands it was taken for further investigation. The bands of acetone extract resolved on TLC were eluted individually and tested for anti-bacterial activity. Of the 13 TLC bands the fractions eluted from bands 1, 2, 3, 5 and 6 were observed to show anti-bacterial activity against the three pathogenic bacteria *B.subtilis*, *A.baumanii* and *S.aureus* and the data is presented in Table 5.

Fourier Transformed Infrared Spectroscopy (FTIR)

Spectroscopic analysis was performed for the five TLC fractions that showed anti-bacterial activity. A comparison of the FTIR spectrum obtained with that of the reference chart revealed the presence of functional groups such as alkanes, amides, amines aromatics aliphatic amines, alkyl halides etc in the leaf extract (Fig. 4; Table 6).

DISCUSSION

One of the common exercise done to label a plant as a new source of drug is to test an extract of the plant for antimicrobial activity. The antimicrobial extract is then subjected to biophysical and biochemical analysis to identify the active principle that might have been responsible for such biological activity. Though a few cytotoxic compounds have been isolated and identified from *Centratherum punctatum*, it has not been proven to be a medicinal plant so far [10, 17].

In the present study an attempt was made to find out if the unexplored *C. punctatum* plant has any medicinal properties. The study revealed that the leaf extract obtained by treatment with certain organic solvents indeed possessed antimicrobial properties.

The efficacy of extract in terms of the number of different bacteria strains that it can inhibit differed with the type of solvent used. The acetone extract of the leaf was found to be maximally efficient in inhibiting five of the six bacteria and one of the two pathogenic fungi tested. This is in line with observations on antibacterial activities of other medicinal plants reported earlier[18–20].

One of the criteria for confirming the antimicrobial activity is to determine if the Minimum Inhibitory Concentration (MIC) is in reasonable ranges i.e., from 0.1 mg/ml to 1 mg/ml, with an effective zone of inhibition around 15–22 mm on the target pathogen[20]. The observation made in this study indicates that MIC obtained with the extracts falls short of this range. This may be improved by refining the isolation procedures.

Analysis of phyto-constituents of *C. punctatum* showed the presence of flavonoids, tannins and cardiac glycosides in the extract. Bioautography of these chemicals indicate that they might be the agents of antimicrobial activity as reported in several of such systems[3, 7].

In general seed or roots are used as souces of ethnomedicine. This study showed that the leaves of *C. punctatum* can be a potential source of ethnomedicine as is evident from our observation on MTT assay for anti-proliferative activity of the extract with an IC_{50} value of 10.63µg/ml for the optimal sample obtained with acetone. An IC_{50} value of less that 50 µg/ml is considered to be a good indicator for considering a plant to be of medicinal importance.

CONCLUSION

Of the four different leaf extracts tested, the acetone extract was found to exhibit the highest anti-microbial, anti-oxidant and anti-proliferative capabilities. Presence of flavonoids might be responsible for the anti-microbial properties of *C. punctatum.* These compounds need further evaluation before they can be declared as potential medicinal agents. We are now attempting to purify the antimicrobial compounds identified in large quantities for further characterization and testing.

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Fig. 1: A plant of C*entratherum punctatum* at flowering stage in the experimental garden of the Department of Biotechnlogy, Pondichery University

Table 2: Phytochemical constituents present in the leaf extracts of *c. Punctatum*

S. No.	Phytochemical	Occurrence
1.	Alkaloids	-
2.	Flavonoids	+
3.	Saponins	-
4.	Tannins	+
5.	Cardiac Glycosides	+

Table1: **Data on zone of inhibition by extracts of leaf of *centratherum punctatum* obtained using different organic solvents.**

Organism		Zone of Inhibition (mm)			
Name	**Characteristic**	**Hexane**	**Acetone**	**Ethyl Acetate**	**Methanol**
Pseudomonas aeruginosa	Gram negative	0	0	0	0
Acinetobacter baumanii	Gram negative	0	0	2 ± 2	0
Escherichia coli	Gram negative	4.67 ± 0.58	3.33 ± 0.58	1.67 ± 1.53	2.67 ± 0.58
Providencia rettgeri	Gram negative	0	0	3.34 ± 0.58	0
Staphylococcus aureus	Gram positive	0	3.667 ± 0.58	5 ± 1	0
Bacillus subtilis	Gram positive	0	7.334 ± 0.58	7 ± 1	4.67 ± 1.53
Fusarium spp.	Plant pathogen	0	2 ± 1	0	0
Curvularia	Plant pathogen	0	0	0	0

The leaf extracts were used at 1mg/ml by dry weight.

Table 3: Antibacterial activity of phytochemicals isolated from *c. Punctatum*

Organism	Zone of Inhibition (mm)		
	Flavanoids	**Tannins**	**Cardiac Glycosides**
Bacillus subtilis	21	8	7
Acinetobater baumanii	9	-	-
Staphylococcus aureus	10	-	-

Table 4: Anti-Oxidant Activity of Leaf Extracts Of *C.Punctatum*

S. no.	Sample	Concentration of anti-oxidant (µg/ml)
1.	Acetone extract	353.36
2.	Methanol extract	287.46
3.	Ethyl Acetate extract	258.50
4.	Hexane extract	210.69
5.	Butylated Hydroxytoluene (BHT)	889.91

Table 5: Data on zone of bacterial growth inhibition by fractions (1-5) eluted from TLC of leaf extract of *c. Pucntatum*

Organism	Zone of Inhibition (mm)				
	1	**2**	**3**	**4**	**5**
B. subtilis	7	5	4	4	5
A. baumanii	3	4	-	4	5
S. aureus	-	-	-	-	5

Table 6: Functional groups identified by FTIR spectroscopy in the fraction five of the tlc of leaf extract of *c. Pucntatum*

Wave number	Bond	Functional group (cm^{-1})
2927.8	C–H stretch	Alkanes
1639.4	NH out of plane	Amides
1564.3	NH_2 in plane bend	Amines
1426.2	C–C stretch (in–ring)	Aromatics
1098.6	C–N stretch	Aliphatic amines
798.8	CH out of plane bending	1,2,3,4-tetra-substituted benzene
466.3	C-I stretch	Alkyl Halide

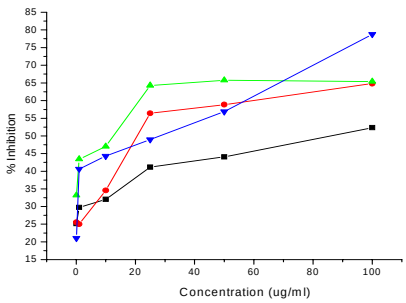

Fig. 3: Anti-proliferative activity of hexane (black), methanol (blue), ethyl acetate (red) and acetone (green) extracts of C*entratherum punctatum* leaf extracts on human PBMCs

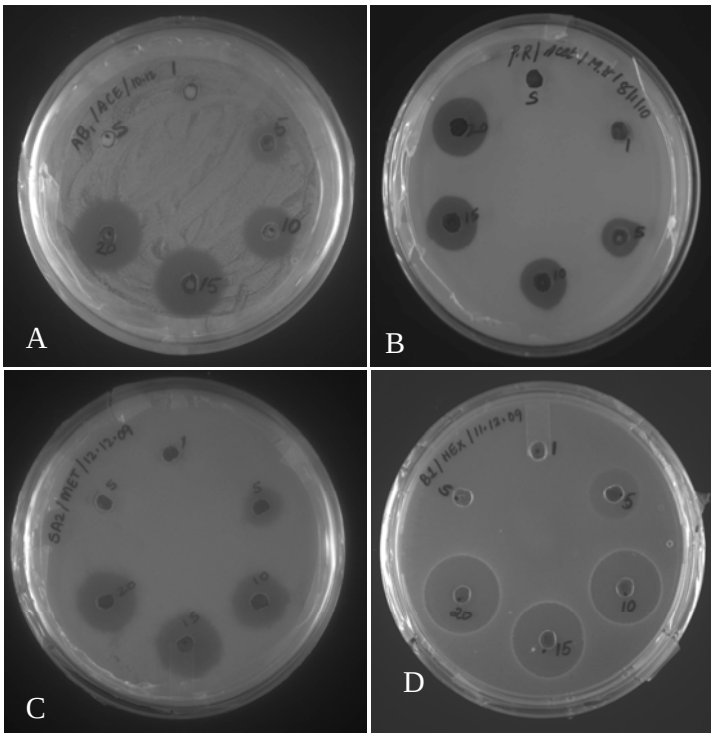

Fig. 2: Zone of Inhibition caused by acetone (A, B), methanol (C) and hexane (D) extracts of C*entratherum punctatum* on the pathogenic microbes *Acinetobacter baumanii* (A), *Providencia rettgeri* (B), *Staphylococcus aureus* (C) and *Bacillus subtilis* (D)

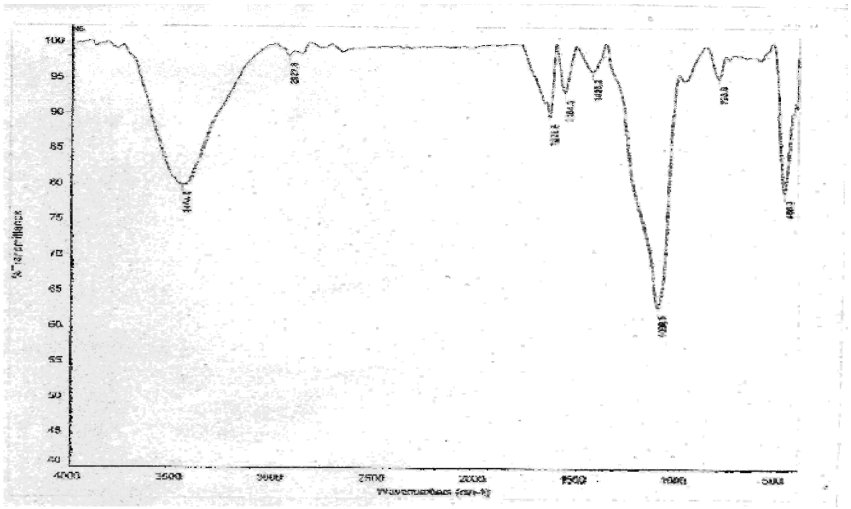

Fig. 4: FTIR (Fourier Transformed Infrared) Spectrum of TLC Fraction 5 of leaf extract of *Centratherum punctatum* (for details please refer to Table 6)

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