

Bioactivity of Diterpens from the Ethyl Acetate Extract of *Kingiodendron pinnatum* Rox. Hams

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ABSTRACT

Background: *Kingiodendron pinnatum* Rox. Hams. is an endangered medicinal plant used in gonorrhoe, catarrhal conditions of genito-urinary and respiratory tracts. The scientific and pharmacological formulation of *K. pinnatum* has not been established so far though it is being traditionally used by tribes of the region. **Objective:** P hytochemical screening and identification of the bioactive compounds from the ethyl acetate extract of *Kingiodendron pinnatum* Rox. Hams.

Materials and Methods: Chromatographic separation was carried out by thin layer chromatography and column chromatography. Bio-autography of the column fractioned extract and TLC chromatogram were evaluated *in vitro* for antibacterial activity. The PTLC, HP TLC were used for crude extract and HPLC, LCMS, FTIR, ¹HNMR and ¹³CNMR were employed for the isolated compound in the ethyl acetate extract of *K. pinnatum*.

Results: Evaluation of solvent system for chromatographic separation revealed that ethyl acetate: petroleum ether in the ratio of 7:2.5 ml was the most appropriate one for the separation of diterpene compounds. The antibacterial bio-autography screening of TLC separated compound showed positive activity with *Staphylococcus aureus* and negative activity with *Escherichia coli*. Spectroscopic analysis of the isolated compound from the ethyl acetate extract of *K. pinnatum* revealed the presence of diterpene compound. **Conclusion:** It is evident from the present study that the ethyl acetate extract of *K. pinnatum* is rich in diterpene compounds and having potential antibacterial activity.

Key words: Bio-autography, HPLC, HPTLC, LC-MS, NMR

SUMMARY

- Novel extraction method for phytochemicals from *Kingiodendron pinnatum* at RT
- Antibacterial property of diterpens extracted from *Kingiodendron pinnatum* Rox. Hams against *S. aureus*



Abbreviations Used: TLC: Thin Layer Chromatography, PTLC: Preparatory Thin Layer Chromatography, HPTLC: High performance Thin Layer chromatography, HPLC: High Performance Liquid Chromatography, LC-MS: Liquid chromatography Mass Spectra, FTIR: Fourier Transform Infrared Chromatography, NMR: Nuclear Magnetic Resonance

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INTRODUCTION

Higher plants represent a rich source of bioactive compounds, with an almost infinite molecular diversity. These biomolecules often have specific functions, but many of them possess pharmacological properties which can be of use to humans. They also may provide lead compounds for the development of new drugs or they may act as indispensable tools in biomedical research. There is a potential need to test large numbers of plant extracts or compounds in a wide variety of biological assays. Since plants are synthesisers able to produce an unpredictable range of skeletal types and novel substances. It is prime importance to evaluate as many medicinal plants as possible in order to find sources of new drugs or lead compounds Kurt and Christian^[1] moreover, a crude extract of plant may contain several thousand different secondary metabolites and any phytochemical investigation of a given plant will reveal a spectrum of its constituents. The plant kingdom thus represents an enormous reservoir of pharmacologically valuable molecules to be discovered.^[2,3] *Kingiodendron pinnatum* is a threatened medicinal plant belonging to the family Fabaceae confined to the Western Ghats of India. The oleo-gum-resin of this plant species is used in gonorrhoea, catarrhal conditions of genito-urinary and respiratory tracts IUCN.^[4]

The scientific and pharmacological formulation of *K. pinnatum* has not been established so far though it is being traditionally used by tribes of the region. Bioautographic assays are usually employed to screen antimicrobial activity by absorbing chemicals onto the surface of chromatographic plates and placing them directly in contact with a medium that is inoculated with bacterial or fungal cultures. These assays detect clear zones where microbial growth is inhibited.^[5] Isolation of biologically active constituents from the medicinal plant extracts remains a long and tedious process. The traditional methodology of

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studying natural products includes the fractionation of a complex mixture, separation and isolation of the individual components using chromatographic techniques and structure elucidation using various spectroscopic methods.^[6,7]

In the present study, attempt has been made to standardize the suitable solvent system for the separation of diterpens and antibacterial activity from the ethyl acetate extract of *K. pinnatum*.

MATERIALS AND METHODS

Collection of the plant material

Evergreen forest of the Western Ghats region of Hassan district, Karnataka, India was explored to locate the population of *K. ingiodendron pinnatum*. The leaves of this plant were carefully exercised from this plant, shade dried and placed in polythene bags. Herbarium of this plant is deposited in the Biodiversity laboratory, Department of Environmental Science, University of Mysore.

Preparation of crude ethyl acetate extract

The leaves were shade dried at room temperature in a clean environment to avoid contamination for 14 days and powdered in a domestic grinder. Extract of this plant was made by maceration method using ethyl acetate as a solvent. Two hundred grams of shade dried leaf powder was extracted with ethyl acetate for 72 hrs at room temperature. The extract was first filtered through cheese cloth then through Whatman filter paper No. 1. First the filtrate was evaporated to 50 ml at room temperature and then in a vacuum concentrator. The dried powder was stored in sterile glass bottle and used for different bioassays.

Anti-bacterial activity of *Kingiodendron pinnatum* by agar well diffusion method

The efficacy of the leaf extract was tested against bacteria, namely *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 7410, *Shigella flexneri* MTCC 731, *Staphylococcus aureus* MTCC 7443 and *Xanthomonas campestris* MTCC7908 by the agar-well diffusion method.^[8] The bacterial cultures were obtained from IMTECH-MT Chandigarh, India. In this method, 24 h-old nutrient broth cultures of the test bacteria were swabbed uniformly on solidified sterile nutrient agar plates using a sterile cotton swab. Well of 6 mm diameter were bored aseptically in the inoculated plates with the help of a gel puncher and the extracts (2.5, 5.0 and 10.0 mg/ml of 10% DMSO), standard (Gentamicin, mg/ml) and control (10% DMSO) were added separately into the respectively labelled wells. The plates were incubated at 37°C for 24 h in an upright position and the zone of inhibition formed around the well was recorded. The experiment was carried out in triplicates and mean values were recorded.

Column chromatography and thin layer chromatography

The crude ethyl acetate extract of the *K. pinnatum* leaves was reconstituted in ethyl acetate and spotted on analytical TLC (silica gel G600, 0.25 mm thickness, Merck). Different solvent systems with different ratios were tried as mobile phase to determine the eluent with optimum performance. After separation of the compounds the TLC plate was exposed to iodine fumes and UV light.

Column separation

Column separation of the ethyl acetate extracts of *Kingiodendron pinnatum* was carried out with a glass column of internal diameter 80 mm and length 100 cm (Raghu chemicals, Mysore, India). Sufficient quantity of a column grade silica gel (100-200 mesh size) was wet-packed in to the column using ethyl acetate.

Petroleum ether solvent system

40 g amount of the crude extract was first dissolved in 20 ml of ethyl acetate, and passed through the column and continuously eluted with the mobile phase (ethyl acetate/petroleum ether: 7:2.5 ml ratio).

Bioautography

A thin layer chromatography (TLC) bioautographic agar-overlay method^[9] was used to analyse most active components in the ethyl acetate crude extract of *K. pinnatum* (as antibacterial agent). About 10 µl of ethyl acetate extract of *K. pinnatum* was applied on pre-coated aluminium silica gel Merck plates. The plates were developed with petroleum ether and ethyl acetate (7:2.5 mL v/v). TLC bio-autography was carried out against *Escherichia coli* and *Staphylococcus aureus*. The developed TLC plates were thinly overlaid with molten nutrient agar inoculated with an overnight culture of bacteria. The plates were incubated in a dark and humid chamber overnight at 37°C. Subsequently, the bioautogram was sprayed with an aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride and further incubated at 37°C for 4 h. inhibited microbial growth and appeared as clear zones in a pink background. The Rf values of the spots showing inhibition were determined.

High performance thin layer chromatography and preparatory thin layer chromatography

Chromatographic separation of ethyl acetate extract of the leaves was conducted using HPTLC purchased from CAMAG, Switzerland. Plant extract was applied with 100 µl syringe on pre coated silica gel 60F254 HP TLC plates (10 × 10 cm) with band length of 8 mm and track separation of 12 mm using Linomat V applicating device. The chromatograph was developed in twin trough chamber using solvent system of ethyl acetate: petroleum ether 2.5:7 and UV Detectors Wavelength selection: 366 nm. The peaks, graph and spectra obtained were given in. For preparative thin layer chromatography, slurry of 40 g of silica gel in 84 ml of distilled water was applied to a hundred glass plates totally (20.3 cm square), with a thin-layer spreader (Research Specialties Co.,) producing a gel layer of 250 - micron thickness. The plates were allowed to stand for 10 minutes at room temperature for 1 hour at 105°C in hot air oven and then in a desiccator for 2 hours. Later ethyl acetate extract of leaves was spotted on the plates (10 µl each spot). Development of the plates was carried out using petroleum ether and ethyl acetate in the ratio of 2.5 ml: 7 ml by the ascending method. Formation of the bands was observed under the UV light.

High performance liquid chromatography

High Performance Liquid Chromatography (HPLC) analysis was performed using different gradients of mobile phase in different run times. Analysis of the crude extract and separated compound was performed using 515 HP LC pumps and 2489 UV/VIS detectors of Waters company, USA, having reverse phase water guard Column: Symmetry C18 (5 µm, 4.6 × 250 mm) and Hamilton microliter syringe using an injection volume of 20 µl. The data analysis was done using Empower 2 software. Detection was made at 254 nm and 28°C. The HPLC mobile phase consisted of methanol and water (97: 3 ml). The mobile was filtered and degassed prior to use.^[10]

Liquid chromatography-mass spectra of isolated compound

Analyses of isolated diterpene from *Kingiodendron pinnatum* was performed with Agilent (Waldbronn, Germany) RR 1200 SL system (binary pump S L, diode array detector G1315C Starlight and automatic injector G1367C SL) connected to a micrOTof -Q mass spectrometer model from Bruker Daltonics (Bremen, Germany). Analyses were carried

out using Zorbax Eclipse XDB-C18 columns (Agilent) with a size and granulation of 2.1×100 mm 2 and $1.8 \mu\text{m}$, respectively. Chromatographic separation was performed at a 0.5 ml/min flow rate using mixtures of solvents: A B (99.5% acetonitrile/0.5% water v/v) with a 3:2 split of the column effluent, so 0.2 ml/min was delivered to the ESI ion source. The elution steps were as follows: 0–5 min linear gradient from 10 to 30% of B, 5–12 min isocratic at 30% of B, 12–13 min linear gradient from 30–95% of B, and 13–15 min isocratic at 95% of B. After returning back to the initial conditions, the equilibration was achieved after 4 min. The microTOF-Q mass spectrometer consisted of an ESI source operating at a voltage of $\pm 4.5 \text{ kV}$, nebulization with nitrogen at 1.2 bar, and dry gas flow of 8.0 l/min at a temperature of 220°C . The instrument was operated using the program MIC ROTOF control ver. 2.3, and data were using analyzed using the Bruker data analysis ver. 4 package.^[11]

Fourier transforms infrared spectroscopy

FTIR spectra were recorded with a FTIR 460 plus Jasco. The powdered leaf sample of fractionated and separated compound of *K. pinnatum* was mixed with dried potassium bromide and prepared as pellets, scanned at room temperature ($25 \pm 2^\circ\text{C}$) at $4000\text{--}400 \text{ cm}^{-1}$ spectral range. To improve the signal to noise ratio for each spectrum, 100 interferograms with a spectral resolution of $\pm 4 \text{ cm}^{-1}$ were averaged.^[12]

^1H and ^{13}C nuclear magnetic resonance

^1H and ^{13}C NMR spectra and HMQC and RO ESy of isolated diterpene from ethyl acetate extract of *K. pinnatum* were recorded on broker DRX 600 and Bruker Avance 800 instruments (Bruker, Karlsruhe, Germany) using CDCl_3 as solvent. Chemical shifts are reported in ppm.

RESULTS

Extraction of phytochemicals from the leaves of *K. pinnatum* was carried out in dark condition using maceration method. The crude extract did not inhibit the growth of *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 7410, *Shigella flexneri* MTCC 731, *Staphylococcus aureus* MTCC 7443 and *Xanthomonas campestris* MTCC7908. This may, possibly due to the antagonistic effects of the compounds present in the crude extracts, however, when the leaves extract was subjected [Figure 1] to PTLC and Bio-autography, the separated components, when tested for anti-microbial activity, showed inhibition for *Staphylococcus aureus*. Among the fourteen bands observed in ethyl acetate extract [Figure 1] seven bands were found to be active against *Staphylococcus aureus* and band one showed a high significant activity and hence further study was concentrated on this band. The areas of inhibition (colored white/light yellow on a purple/pink background) of the compounds from *K. pinnatum* against *staphylococcus aureus* were found at varying R_f values (0.09 to 0.9). With band one shows significant activity against *Staphylococcus aureus* [Figure 2]. Investigations were carried out on this band. The band was re- extracted and further analyzed using HPTLC, HP LC, FTIR, LCMS, 1DNMR to assess the composition and functional groups present in this. HPTLC analysis showed different R_f values of the separated compounds and HPLC of the isolated compound showed a single peak with [Figure 3] retention time 50. The FTIR analysis indicated that significant peaks were found at: 1659 cm^{-1} is attributed to aldehyde, 1710 cm^{-1} is a carboxylic acid function and 1682 cm^{-1} attributed to a ketone. The presence of diterpene compound was further proven with the absorption band of hydroxyl ($3500 - 3480 \text{ cm}^{-1}$), ester carbonyl ($1270 - 1150 \text{ cm}^{-1}$) and phenyl [Figure 4]. ($1600, 1420 \text{ cm}^{-1}$). LCMS studies of the isolated compound was carried out, ESI was used with negative mode. Major fragment peaks was found at m/z 79.03, 156.78, 204.94, 494.20. Here 332.10 is a parent ion and 528.22 is a daughter. The daughter ion is very stable showing maximum peak. M/Z 332 (M^+), and 204 are attributed to ($\text{C}_{14}\text{H}_{20}\text{O}$) and the molecular weight of the compound is 332.10 [Figure 5]. Proton NMR of isolated compound having two vinyl protons ($-\text{CH}=\text{CH}-$), attached to CH_2 groups and methyl

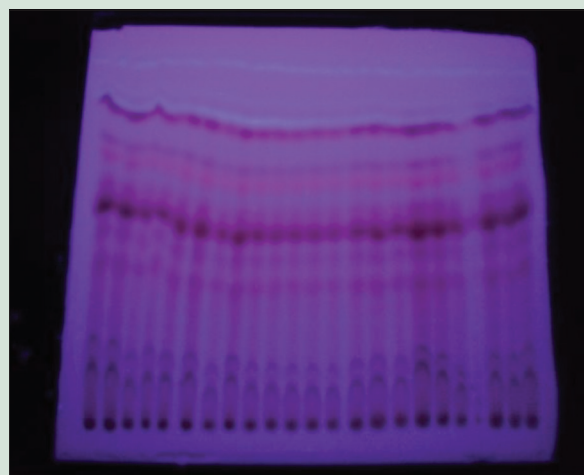


Figure 1: PTLC separation of phytochemicals from *K.pinnatum*

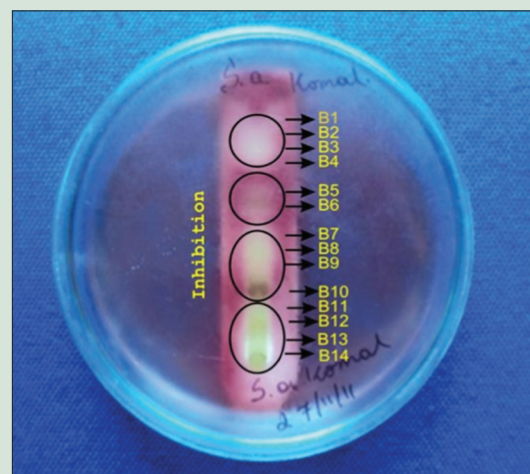


Figure 2: Bioutographic study of *Kingiodendron pinnatum*

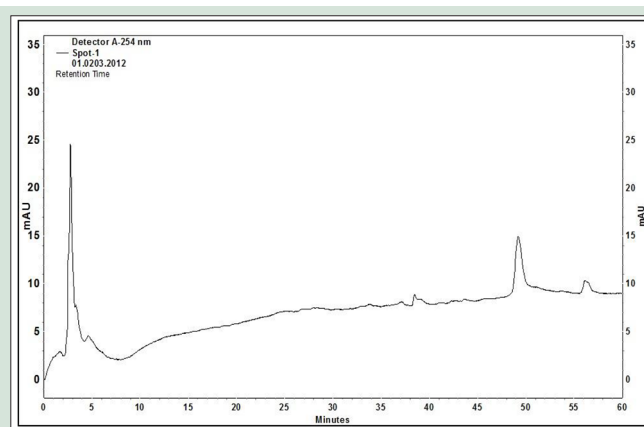


Figure 3: HPLC of Diterpene isolated from *K.pinnatum*

groups and methylene groups. Also it is having oxygenated methylene group ($-\text{CH}_2-\text{O}-$). In the carbon NMR we have 20 carbons: 2 quaternary, three methines, one oxygenated methylene, about 11 methylenes, and [Figures 6-9] three methyls. This compound could be a diterpene containing three methyl groups, one oxygenated methylene group, two vinylic protons, and two quaternary carbons, one of them could be CO or CO-NH.

DISCUSSION

Plants produce a diverse range of biologically active molecules making them rich source of different types of medicines. Various techniques are employed for their investigation which includes bio-logical assays for phytochemical screening and their evaluation for presence of bioactivity. Isolation of pure bio-active constituents from plants remains a long tedious process. This procedure enables recognition of known metabolites in extracts in the earliest stages of separation and thus economically very important. To characterize the bioactive compounds several techniques were used among which chromatographic techniques were extensively used.^[13] Success in isolating compounds, which correspond to bands from the plant material, was largely dependent on the type of solvent combination used in the extraction process. This implies that solvent with intermediary polarity separates more active components.^[14] TLC is the simplest method for separating plants constituents because the method is easy to run and reproducible and requires little time. However, for efficient separation and identification of metabolites, good selectivity and sensitivity of detection, together with capability of providing structural information by hyphenated techniques such as HPLC, HP TLC, LC-MS, FTIR, 1D NMR such as ¹³C NMR and ¹H NMR techniques. Bio-autography agar overlay method is considered as one of the most efficient methods for the detection of antimicrobial compounds.^[6] The main focus of the present study was to investigate the suitable solvent system for the separation and to find out active components from the ethyl acetate extract of *K. pinnatum* using chromatographic and Bio-autographic technique. An interesting finding from the present study is that the crude extract did not showed the anti-bacterial activity but in the Bio-autographic

method, the separated compounds from the same extract by thin layer chromatography (TLC) showed the activity against *staphylococcus aureus* [Figure 3]. However, negative results do not indicate the absence of bioactive constituents, nor that the plant is inactive. Active compound (s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed.^[15] Lack of activity can thus only be proven by using large doses Farnsworth.^[16] Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents.^[17] With no anti-bacterial activity, extracts may be active against other bacterial species which were not tested Shale *et al.*^[18]

CONCLUSION

It is inferred that the ethyl acetate extract of *K ingiodendron pinnatum* contained diterpens with antibacterial properties against *S. aureus*. The compounds were active at low concentration. These active compounds were successfully concentrated using a series of techniques, including solvent extraction, TLC, preparative TLC, column chromatography. This facilitated for the isolation of pure diterpene with activity against

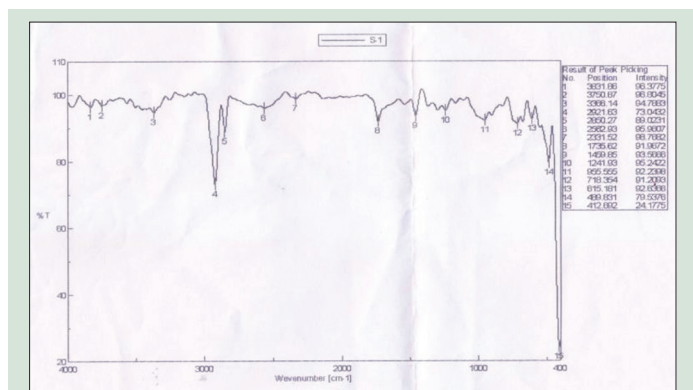


Figure 4: FTIR spectra of diterpene isolated from *Kingiodendron Pinnatum*

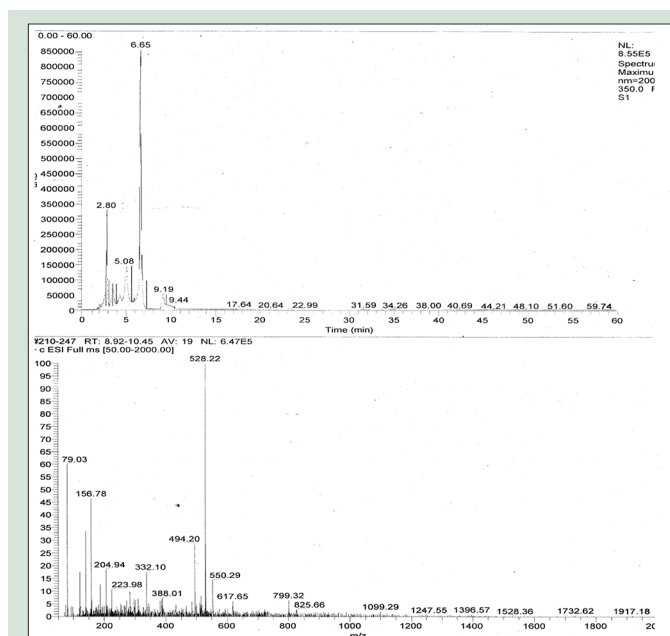


Figure 5: LC-MS spectra of Diterpene isolated from *Kingiodendron pinnatum*

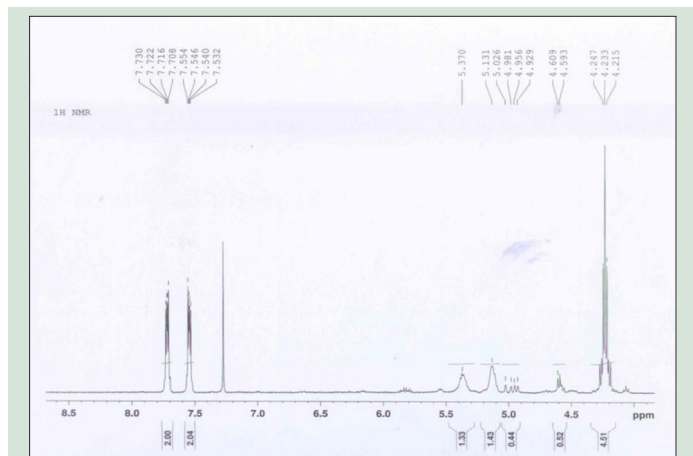


Figure 6: ¹H NMR Spectra of Diterpene isolated from the ethyl acetate extract of *Kingiodendron pinnatum*

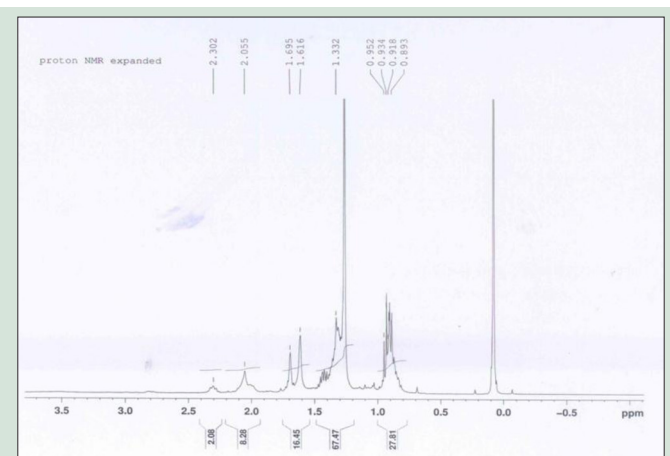


Figure 7: ¹H NMR (Expanded) spectra of diterpene isolated from the ethyl acetate extract of *Kingiodendron pinnatum*

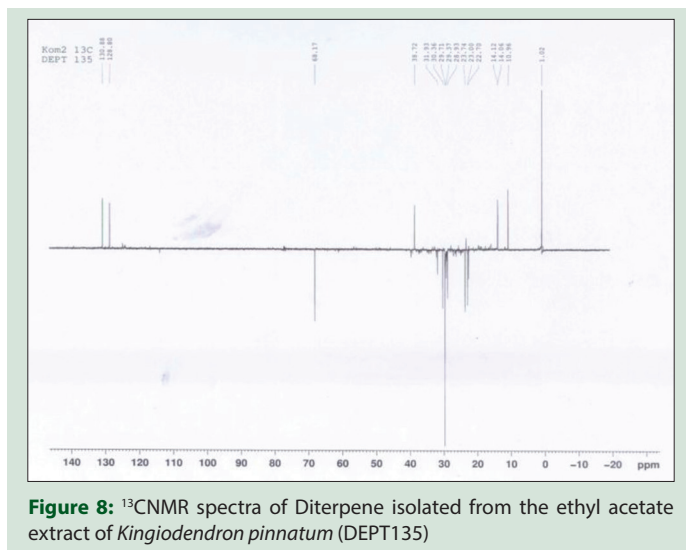


Figure 8: ^{13}C NMR spectra of Diterpene isolated from the ethyl acetate extract of *Kingiodendron pinnatum* (DEPT135)

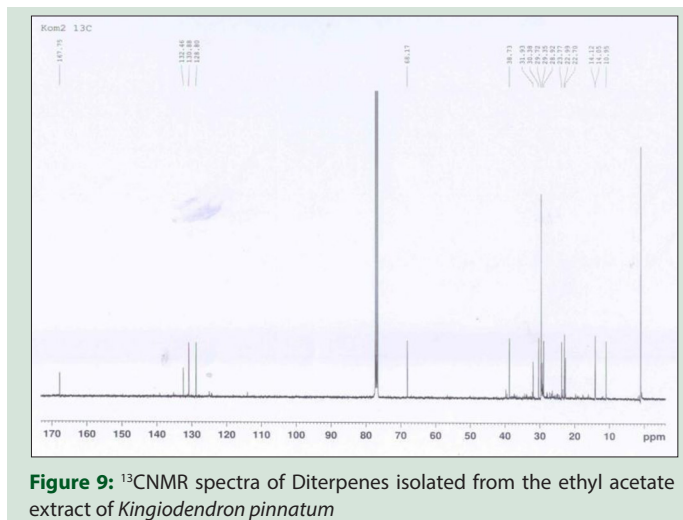


Figure 9: ^{13}C NMR spectra of Diterpenes isolated from the ethyl acetate extract of *Kingiodendron pinnatum*

S. aureus at relatively low concentration. The standardization of solvent system for the separation of bioactive compounds from the medicinal plant *K. pinnatum* is the first report which serves as a fingerprint for future research work. The basic information related to the chemical constituents of *K. pinnatum* which offers scope for further purification and identification of bioactive compounds. Bio-autography was found to be the most suitable method for assessing anti-bacterial activity in the crude extract of *K. pinnatum*. Our research investigation shows that extraction of phytochemicals at room temperature is a beneficial approach for purification of potent anti-microbial compounds from this plant. Purification of bio-active compounds is in process.

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Conflicts of interest

There are no conflicts of interest.

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