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## **Antioxidant and Antimicrobial Activities of the Hot Pungent Chabbarin are Responsible for the Medicinal Properties of *Piper chaba*, Hunter**

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### **ABSTRACT**

There is great incentive to discover biologically active natural products from higher plants that are better than synthetic chemicals and are much safer, from a health and environmental point-of-view. Now a day, major research emphasis has been given to discover biologically active natural products from plants due to their potent medicinal effects, lower cost and fewer side-effects. *Piper chaba*, Hunter (Piperaceae) a less well-known spice, is very much effective against cold and cough. The aim of present study is to isolate and identify the bioactive compound which is responsible for its medicinal properties and also evaluate its antioxidant and antimicrobial activity. Molecular characterization was done by chromatographic and spectral analysis. Free radical scavenging potential was assessed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) test and antimicrobial activity by inhibition zone test. A strong bioactive compound with hot pungent in nature have been isolated and purified from the acetone extract of *Piper chaba* stem (20-22% of the dry weight with 99.9% purity). Chromatographic and spectral analysis revealed the compound to be 5-Benzo (1,3) dioxol-5-yl-1-piperidin-1-yl-penta-2,4-dien-1-one or designated as chabbarin. This fraction showed significant DPPH radical scavenging activity with IC<sub>50</sub> values of 3.0 µg mL<sup>-1</sup>. It was highly effective on gram negative bacteria than gram positive. In case of *Escherichia coli* and *Pseudomonas aeruginosa* (gram negative), maximum inhibitory activity was noticed at 1500 ppm. The Minimum Inhibitory Concentration (MIC) for *Escherichia coli* was found at 0.01562 mg mL<sup>-1</sup> whereas in *Pseudomonas aeruginosa*, it is at 0.00391 mg mL<sup>-1</sup>. In *Staphylococcus aureus* and *Sarcina lutea* (gram positive) MIC was detected at 0.00781 mg mL<sup>-1</sup>. Chabbarin also exhibited a remarkable antifungal activity. *Aspergillus tamarii* is very much sensitive to chabbarin (MIC at 0.01562 mg mL<sup>-1</sup>) than *Penicillium chrysogenum*, *Aspergillus niger* and *Aspergillus fumigatus* (MIC at 0.03125 mg mL<sup>-1</sup>). So Chabbarin is responsible for the medicinal properties of the plant.

**Key words:** *Piper chaba*, Hunter, hot pungent compound, chabbarin, antioxidant activity, antifungal, antibacterial activity

## INTRODUCTION

The knowledge and use of traditional medicinal plants with potential curative properties have now been investigated for the development of common drugs and herbal remedies (Rahalison *et al.*, 1991). Now a day, it is emerging as an important research area because of their potent pharmacological activities, low toxicity and economic viability (Auddy *et al.*, 2003). Prevention of fungal diseases by synthetic antifungal chemicals causes high mortality despite of treatment due to their unavoidable harmful side effects (Lorthoraly *et al.*, 1999; Vincent, 1999). Identification of strong bioactive allelochemicals are a useful source for the development of biological herbicides and fungicides and their implication in control of diseases would be environmentally friendly as natural chemicals are renewable and easily degradable.

*Piper chaba*, Hunter (Piperaceae) is a relatively less well-known spice. This was not even mentioned by Pruthi (1998). Some spices possess phenolic compound and antioxidant properties (Muchuweti *et al.*, 2007). This plant bears both medicinal and spicy properties. The edible part of the plant is the swollen stem whose taste is better than that of *Piper nigrum*, according to some consumers. The stem of the plant is very much effective against cold and cough and also enhances immunity against this disease. We are therefore interested to isolate and identify the compound responsible for its medicinal properties.

There is currently immense interest in natural anti-oxidant and their role in health and diseases, considerable data has been generated around the globe (Aruoma, 1994), however traditionally used medicinal plants awaits such screening and *Piper chaba* is one such plant. Vaghasiya and others (Vaghasiya *et al.*, 2007) studied Anti-Bacterial and Anti-Inflammatory properties of some Piper species on 15 clinically important bacterial strains. In recent time, major research emphasis has been on the plants with anti-oxidant and antimicrobial properties (Arora *et al.*, 2011; Uddin *et al.*, 2008; Aisha *et al.*, 2011; Gupta and Sharma, 2010; Duru and Onyedineke, 2010; Jimenez *et al.*, 2009). The literature studies have shown very scanty information on this plant and thus we are prompted to work on anti-oxidant and antimicrobial properties of *Piper chaba*.

## MATERIALS AND METHODS

**Isolation and characterization of Active hot pungent compound of *Piper chaba*:** Samples were collected from Habra, Bongoan and Dumdum (North 24-parganas, West Bengal) in the month of January, 2010 and present study continued for 2-years. Two hundred grams of chai (*Piper chaba*), stem dust was dried and powdered using Sample Miller Machine (Cyclotec 1093. Sample Mill, TECATOR) and then soaked in 500 mL of methanol for 7 to 10 days. The entire mixture was then vortexed in high speed (3000 rpm) using Mechanical Stirrer (Model No. DC Stirrer NZ-1000s AC220V, EYELA) for 1 h and then filtered through sintered disc funnel. The brown colored extract was collected and concentrated in a rotary vacuum evaporator (EYELA, Model No. N1-NW) and subsequently extracted with hexane, ethyl acetate, acetone and methanol, respectively. Finally the compounds were purified by column chromatography and thin layer chromatography. Here we put emphasis on Acetone fraction of *Piper chaba* (henceforth referred to as PCAF or *Piper chaba* acetone fraction or Fraction-3) because highly potent bioactive compound was obtained in this fraction (Fig. 1).

**Thin layer chromatography (TLC):** TLC plates (20×20 cm) were used for this work. Silica gel G of TLC grade was used as a coating material and the plates were coated uniformly with 0.5 mm

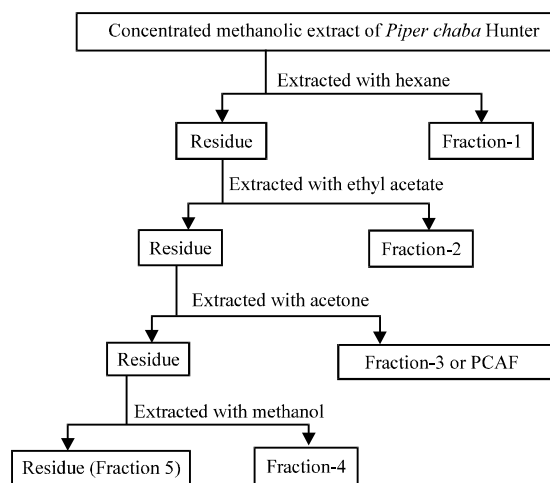


Fig. 1: Schematic representation of extraction of compound from the concentrated mathanolic extract of *Piper chaba* Hunter

thick layer of silica gel. A solvent mixture in the ratio of 20: 80:: Ethyl Acetate: Hexane was used as solvent system (Stahl, 1969). Plates were loaded with 20  $\mu$ L solution (500 ppm of PCAF) and developed up to a height of 18 cm within the glass chamber, pre-saturated with desired solvent system. TLC plates were then taken out and dried under a stream of hot air. Finally compounds were detected by exposing the plates under iodine vapor or under UV light (365 nm).

**Spectral analysis:** Mass Spectrometric analysis was done at Indian Institute of Chemical Biology (IICB), Jadavpur, Kolkata, 700032. Fraction-3 of *Piper chaba*, Hunter was subjected to MS analysis for detecting the exact molecular weight of the compound. Mass Spectra (ESI) was recorded on a Micro mass Q-TOF Micro TM Spectrometer using positive ion mode.

$^1\text{H}$ NMR (300 MHz,  $\text{CDCl}_3$ ) available at Indian Institute of Chemical Biology, Jadavpur, Kolkata 700032, was used for analysis of extra-purified PCAF of *Piper chaba*. For  $^1\text{H}$ , Pulse Programme is Zg and number of scan is 64.  $^1\text{H}$ NMR spectra were observed on  $\delta$  ppm (0-10) scale with end sweep at 0 ppm. Samples were analyzed at ambient temperature.  $\text{CDCl}_3$  was used as solvent for PCAF compound.

$^{13}\text{C}$ NMR (150 MHz,  $\text{CDCl}_3$ ) available at Indian Institute of Chemical Biology, Jadavpur, Kolkata 700032, was used for analysis of extra-purified PCAF of *Piper chaba*. For  $^{13}\text{C}$ NMR, Pulse Programme is Zgdc and number of scan is IK (1024).  $^{13}\text{C}$ NMR spectra was observed on  $\delta$  ppm (0-10) scale with end sweep at 0 ppm. Samples were analyzed at ambient temperature.  $\text{CDCl}_3$  was used as solvent for Fraction-3.

FTIR analysis was conducted to confirm the important functional group in the extracted and purified PCAF of *Piper chaba* with the help of FTIR Spectrometer (Model No. QC/FTIR/006 available at Chembiotek, Kolkata 700054).

**Antioxidant activity of PCAF of *Piper chaba*:** The antioxidant activity (free radicle scavenging activity) of the extracts [as shown in Flow-diagram i.e., Hexane fraction (PCHF), ethyl acetate fraction (PCEtoAcF), Acetone Fraction (PCAF) and methanol fraction (PCMF)] (Ravishankara *et al.*, 2002) on the stable radicle 1,1-diphenyl-2-picrylhydrazyl (DPPH) was

determined by the method of Brand-Williams (Brand-Williams *et al.*, 1995). In the experiment, 2.0 mg of pure PCAF compound was dissolved in methanol. Solution of varying concentrations such as 500, 250, 125, 62.50, 31.25, 15.62, 7.8125, 3.91, 1.95 and 0.98  $\mu\text{g mL}^{-1}$  were obtained from serial dilution technique. Two milliliter of the methanol solution of PCAF of each concentration was mixed with 3 mL of a DPPH-methanol solution (20  $\mu\text{g mL}^{-1}$ ) and was allowed to stand for 20 min for the reaction to occur. Then the absorbance was determined at 517 nm and from these values the corresponding percentage of inhibition were calculated by using the following formula:

$$\text{Inhibition (\%)} = 1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Then percentage inhibitions were plotted against respective concentrations used and from the graph  $\text{IC}_{50}$  was calculated by using tert-butyl-1-hydroxytoluene (BHT), ascorbic acid, potential antioxidant, were used as positive control.

**Effects of PCAF of *Piper chaba* at different concentrations on fungi:** Inhibition zone test technique was performed for testing the impact of extracted PCAF against four different fungal species viz., *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus tamaris* and *Penicillium chrysogenum*. Few fungal spores of test fungi were transferred to PDA (Potato Dextrose Agar Media) slants and incubated for one week for colony growth. After one week, one loop full of fungal spore of each species was added separately to the sterile sahne water and mixed well. Fungal spore suspension (1 mL) in water was then poured in a sterile Petri dish containing molten PDA and allowed to solidify the plates. Four cups (25 mm<sup>2</sup> size) were cut at equidistant positions and in these cups 0.5 mL solution of PCAF at 500, 1000, 1500 and 2000 ppm was added. Treated plates were incubated at 28±1°C for 24-48 h. After 48 h plates were taken out and observations were recorded for colony growth inhibition. Area of inhibition zone was calculated as: Area of inhibition at x ppm = 3.14 (TR<sub>x</sub><sup>2</sup>-r<sup>2</sup>) where x = concentration used; r = radius, TR = Total radius of the inhibition zone at specific concentration (Biswas *et al.*, 2009).

**Effects of PCAF of *Piper chaba* at different concentrations on bacteria:** Effects of PCAF against four different bacterial species viz., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Sarcina lutea* was measured by using inhibition zone test. Firstly the strains of test bacteria were transferred to NA (Nutrient Agar Media) slants and incubated for 24 h. After 24 h, 1 loop full of bacterial spore of each test species was added separately to sterile nutrient broth and mixed well and incubated at 37°C for 2.5 h. Bacterial spore suspension (1 mL) in sterile nutrient broth was then added to sterile Petri dish containing molten NA medium and allowed to solidify. After complete solidification four cups (25 mm<sup>2</sup> size) were cut at equidistant positions and in these cups 0.5 mL solution of PCAF at 500, 1000, 1500 and 2000 ppm was added. Treated plates were incubated at 37±1°C for 24 h. After this period, plates were taken out and observations of inhibition zone were recorded, using the formula described for fungal effects.

## RESULTS

**Isolation and characterization of active allelochemical:** Fraction-3 of Piper Chaba was purified by column chromatography and thin layer chromatography. By repeatedly following the proposed scheme of fractionation we could extract the compound of interest (Acetone fraction of



Fig. 2: Crystals of the Fraction-3 (PCAF) of *Piper chaba*

*Piper chaba*, henceforth referred to as Fraction-3 or PCAF) as a single pure compound (purity of >95%). At least eight repetitions of the procedure were necessary to achieve this goal.

**TLC analysis:** After purification PCAF (*Piper chaba* acetone fraction) was run on TLC in the solvent system (20: 80 :: Ethyl Acetate: Hexane) showed single bright yellow spot in iodine vapor and violet color quenching spot under UV light with Rf value 0.81. Purified PCAF was off-white in color with hot pungent flavor and physical state of compound was crystalline at room temperature (Fig. 2).

**Spectral analysis:** Mass spectra (Fig. 3a, b) of purified PCAF revealed the molecular weight, i.e.,  $M^+$  285. The conclusion is drawn based on the appearance of the (M+H) and (M+Na) peaks at the spectrum. Though the molecular ion i.e.,  $M^+$  peak is not visible in the spectrum but 100% abundance molecular ion peak at 286 (M+H)<sup>+</sup> and low abundance peak at 308 corresponding to (M+Na)<sup>+</sup> molecular ion is answering for the difference of 23 (molecular weight of sodium), strongly suggesting our conclusion. Fragmentation of the original compound plus hydrogen is 286. Hence molecular weight of the given compound is (286-1) = 285.

The <sup>1</sup>H NMR spectrum (Fig. 4a, b) of this compound exhibited signals at  $\delta$  5.97 (s, 2 h),  $\delta$  3.62 (s, 4 h),  $\delta$  1.58 (brs, 2 h),  $\delta$  1.64 (brs, 2 h),  $\delta$  1.79 (brs, 2 h),  $\delta$  7.39 (q, J = 9.8 Hz, 1 h), w 7.26 (s, 1 h),  $\delta$  6.97 (d, J = 8.2 Hz, 1 h), 6.75-6.90 (m, 3 h), 6.43 (d, 1 h).

<sup>13</sup>CNMR spectrum of PCAF (Fig. 5a) showed signals for 17 carbons. In this compound there are six CH<sub>2</sub> at  $\delta$  101.19, 46.82, 43.16, 26.65, 25.57, 24.58 ppm as evident clearly from the Dept-135 Spectra (5b). Dept-90 spectra indicated the presence of seven CH at  $\delta$  105.58, 108.39, 120.00, 122.41, 125.28, 138.11, 142.38 ppm (5c). Peak at  $\delta$  165.34 ppm supported the presence of >C = O. The triplet signal at  $\delta$  (77.42, 77.00, 76.58 ppm) occurred for the CHCl<sub>3</sub> (solvent used for dissolving the compound). Benzene carbon atom peak observed at  $\delta$  130.94 ppm. Two more peaks at  $\delta$  148.11 and 148.30 ppm were due to the other carbons of the aromatic ring.

Figure 6 showed the IR Spectra of purified PCAF of *Piper chaba*. Peak at 2930 and 2857 cm<sup>-1</sup> are due to the asymmetrical stretching ( $\nu_{as}$ CH<sub>2</sub>) and symmetrical stretching ( $\nu_s$ CH<sub>2</sub>), respectively.

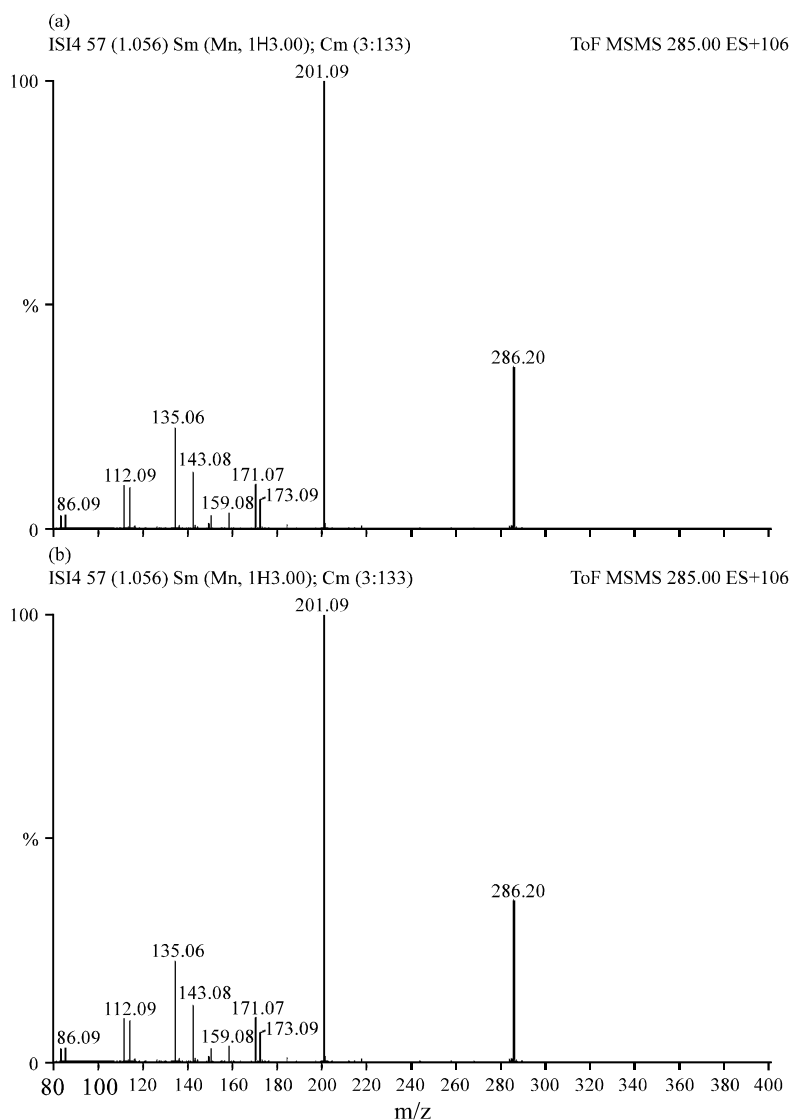


Fig. 3(a-b): (a) MS and (b) MS/MS spectra of purified PCAF of *Piper chaba* hunter

Peak at  $1443\text{ cm}^{-1}$  suggested  $\text{CH}_2$  bending. Peak at  $803\text{ cm}^{-1}$  strongly supported the benzene ring within the compound. Peak at  $1252\text{ cm}^{-1}$  indicated the C-O stretching vibration (Aromatic methylene dioxy compounds). Stretching of -CO-N observed at  $1632\text{ cm}^{-1}$ . Peak at  $3435\text{ cm}^{-1}$  indicated the Intermolecular hydrogen bonding.

Chromatographic and Spectral analysis of PCAF revealed the compound to be 5-Benzo (1,3) dioxol -5-yl-1-piperidin-1-yl-penta-2,4-dien-1-one or designated as chabbarin (Fig. 7). About 20-22% (of dry weight with 99.9% purity) of chabbarin have been isolated and purified from the stem of *Piper chaba*.

**Antioxidant activity of PCAF of *Piper chaba*:** In case of antioxidant screening (Table 1), the  $\text{IC}_{50}$  value of BHT and ascorbic acid was obtained 26.0 and  $5.0\ \mu\text{g mL}^{-1}$ , respectively. In this investigation, the acetone extract (PCAF) of the plant showed the highest antioxidant activity with

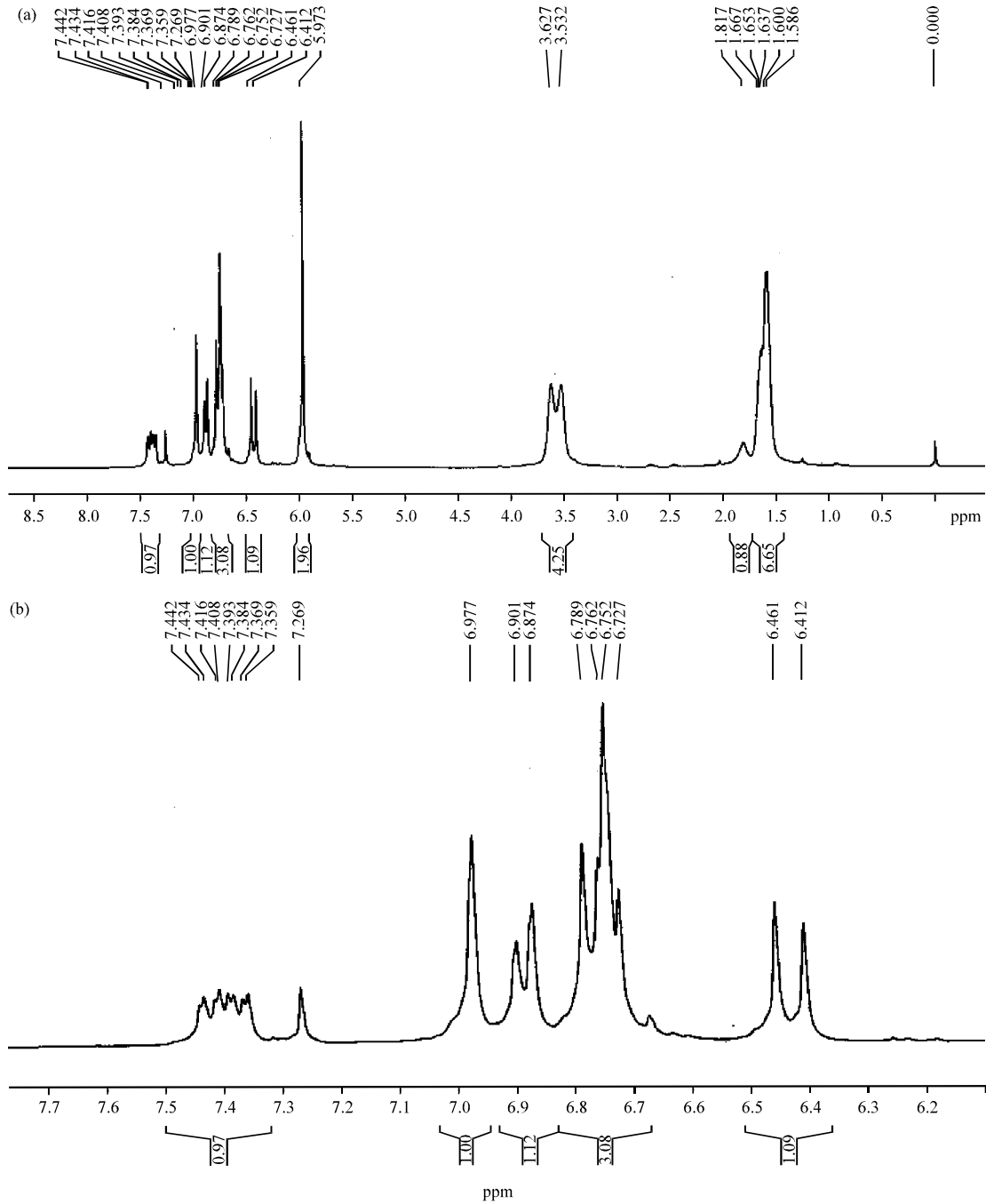


Fig. 4(a-b): (a) <sup>1</sup>H NMR and (b) Expanded <sup>1</sup>H NMR spectra of purified PCAF of *Piper chaba*

IC<sub>50</sub> value of 3.00 µg m<sup>-1</sup>. Methanol soluble fraction (PCMF) of the methanol extract also revealed potent antioxidant activity (IC<sub>50</sub> = 8 µg mL<sup>-1</sup>). On the other hand the ethyl acetate (PCEtoAcF) and n-hexane soluble fraction (PCHF) showed moderate and low antioxidant activity with the IC<sub>50</sub> of 126 and 132 µg mL<sup>-1</sup>, respectively. So we find the Acetone Fraction of *Piper chaba* with the maximum antioxidant activity than the other three fractions.



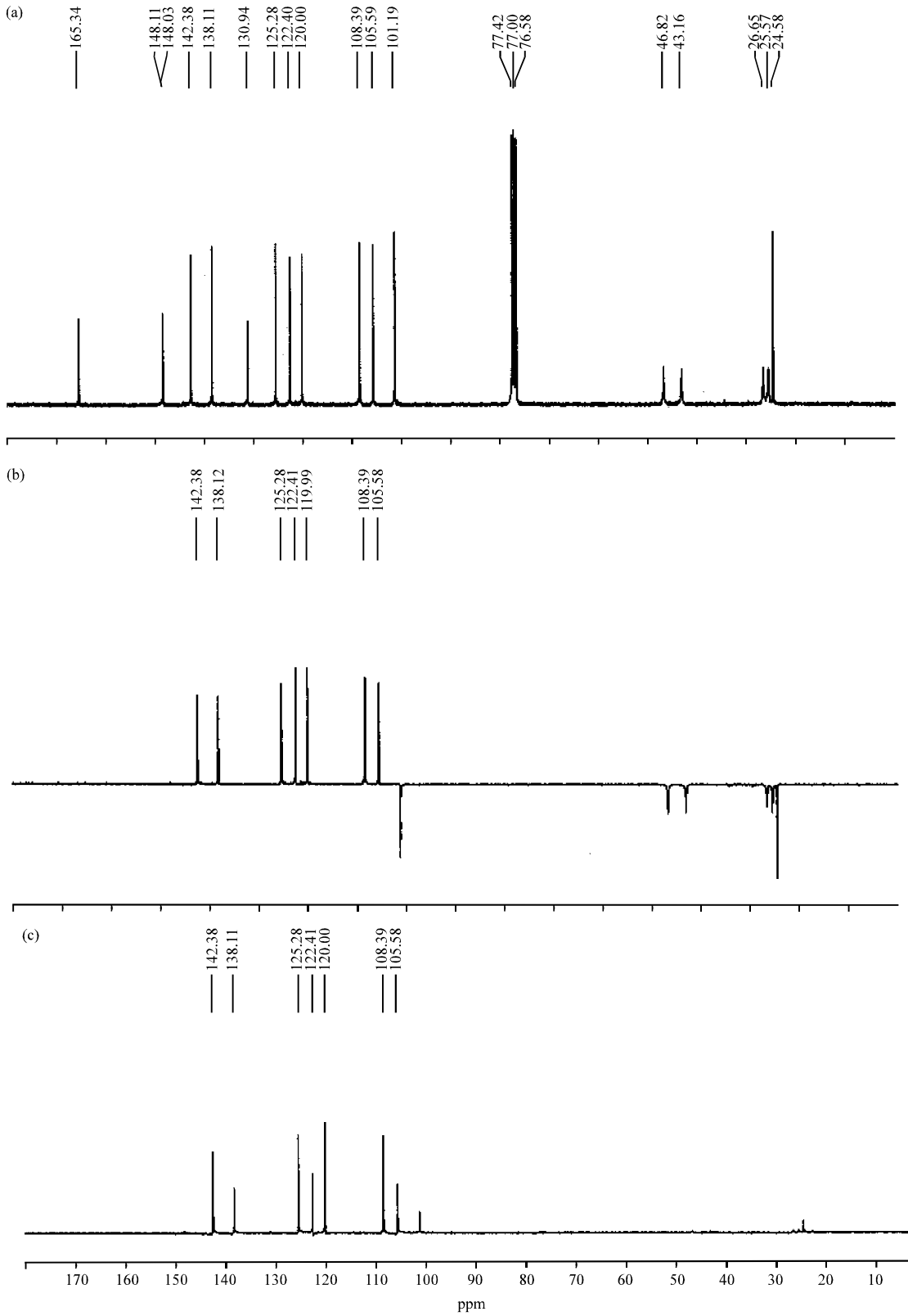


Fig. 5(a-c): (a)  $^{13}\text{C}$ NMR, (b) DEPT-135 and (c) DEPT-90 spectra of purified PCAF of *Piper chaba*

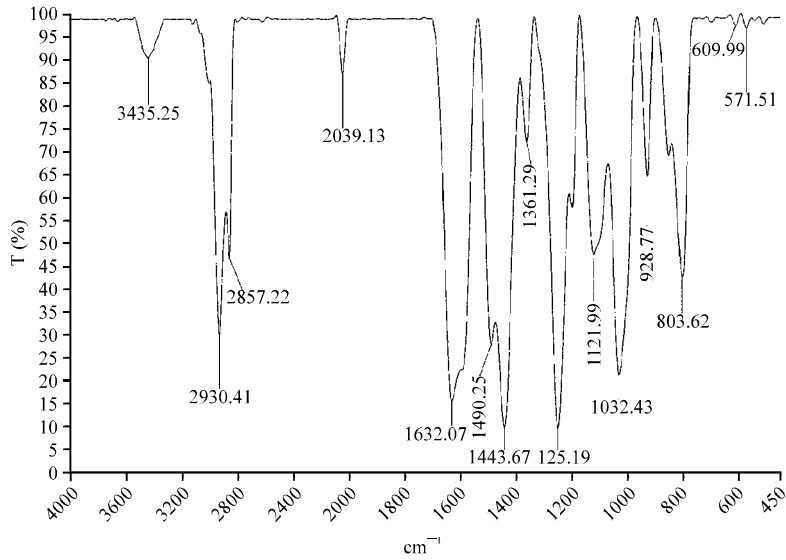


Fig. 6: IR spectra of purified PEA compound of *Piper chaba*, Hunter spectra name: 78.sp, Accumulations: 16, Resolution: 4.00 cm<sup>-1</sup>

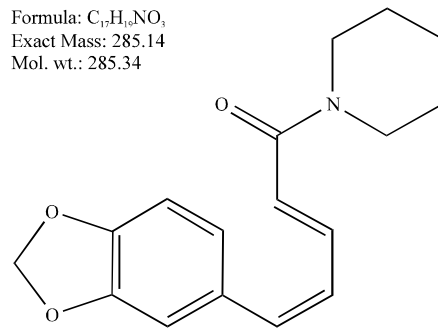


Fig. 7: Molecular structure of Fraction-3 (PCAF) of *Piper chaba*

Table 1: IC<sub>50</sub> values of different fractions of *Piper chaba* along with tert-butyl-1-hydroxytoluene and ascorbic acid

Samples	IC <sub>50</sub> (µg mL <sup>-1</sup> )
BHT	26.0
AS	5.0
PCHF	132.0
PCEtoAcF	126.0
*PCAF	*3.0
PCMF	8.0

\*PCAF showed highest antioxidant activity

**Effects of PCAF of *Piper chaba* at different concentrations on fungi:** Chabbarin showed strong antifungal activity against *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus tamarii*, *Penicillium chrysogenum* (Fig. 8). *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus tamarii* showed maximum inhibitory activity at 2000 ppm, whereas in *Penicillium chrysogenum* maximum inhibitory activity was noticed at 500 ppm. In case of *Aspergillus tamarii*, MIC is 0.01562 mg mL<sup>-1</sup> whereas in other three fungal species, it is 0.03125 mg mL<sup>-1</sup>.

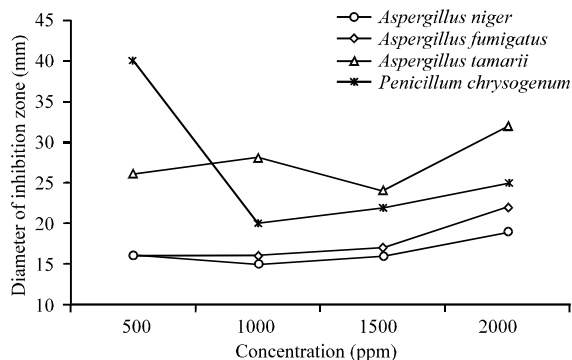


Fig. 8: Effects of Fraction-3 of *Piper chaba* (PCAF) at different concentration on *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus tamarii*, and *Penicillium chrysogenum*. Correlation is significant at 0.01 level (Pearson 2-tailed)

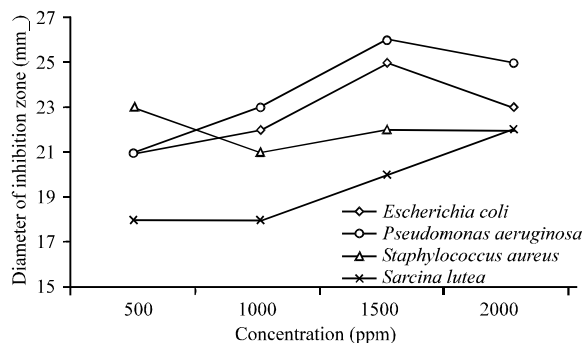


Fig. 9: Effects of Fraction-3 *Piper chaba* (PCAF) at different concentration on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Sarcina lutea*. Correlation is significant at 0.01 level (Pearson 2-tailed)

**Effects of PCAF of *Piper chaba* at different concentrations on bacteria:** Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) are much more effective to Chabbarin than gram positive bacteria (*Sarcina lutea* and *Staphylococcus aureus*). Gram negative bacteria exhibited maximum inhibitory activity at 1500 ppm. For *Escherichia coli*, MIC was found at 0.01562 mg mL<sup>-1</sup> and that of *Pseudomonas aeruginosa* at 0.00391 mg mL<sup>-1</sup>. In case of *Staphylococcus aureus*, maximum inhibitory activity noticed at 500 ppm and *Sarcina lutea* inhibitory activity increased with concentration. In both gram positive bacteria, MIC was detected at 0.00781 mg mL<sup>-1</sup> (Fig. 9).

## DISCUSSION

Natural products and their derivatives have historically been invaluable as a source of therapeutic agents. *Piper chaba* has been traditionally used as a prime medicament for cold and cough (Rukachaisirikul *et al.*, 2002) but the compound responsible for this medicinal property has never been studied before (Bhandari *et al.*, 1998). We have isolated and purified the hot pungent compound from the stem of *Piper chaba*. About 20-22% (dry weight) of the compound has been recovered with up to 99.9% purity and was identified as 5-Benzo [1, 3] dioxol -5-y1-1-piperidin-1-

yl-penta-2, 4-dien-1-one or designated as Chabbarin. This compound possesses strong antimicrobial and antioxidant activity. It is very much effective both on gram positive and gram negative bacteria with minimum MIC ranging from 0.00391 mg mL<sup>-1</sup> to 0.01562 mg mL<sup>-1</sup>. It also exerted strong antifungal activity with minimum MIC ranging from 0.01562 to 0.03125 mg mL<sup>-1</sup>. Significant antioxidant activity has also been detected in this compound. The acetone extract (PCAF) of the plant showed the maximum antioxidant activity with IC<sub>50</sub> value of 3.00 µg mL<sup>-1</sup> as compared to the other fractions, with the minimum being in n-hexane (132 µg mL<sup>-1</sup>). Hence it is a potent bioactive compound responsible for the medicinal properties of the plant (Jayashree *et al.*, 2003).

Numerous drugs have entered the international pharmacopoeia via the study of ethno-pharmacology and traditional medicine (Sunilson *et al.*, 2009; Joseph and Sujatha, 2011; Zongo *et al.*, 2009; Kolawole, 2007). For traditional medicines, newer guidelines of standardization, manufacture and quality control and scientifically rigorous research on the scientific basis for traditional treatments will be required (Chopra *et al.*, 1992). Traditional knowledge can serve as powerful search engine which will greatly facilitate intentional, focused and safe natural product drug discovery and help to rediscover the drug discovery process.

Ayurvedic, Indian and traditional Chinese systems are living great traditions. These traditions have relatively organized database and more exhaustive description of botanical material that is available and can be tested using modern scientific methods. Both systems of medicine thus have an important role in bio prospecting of new medicines. Good botanical practices which can improve the quality control procedures of monitoring impurities and other toxins in the raw material can make the ethno pharmacology research more meaning full (Aqil and Ahmad, 2007).

Natural pharmaceuticals, nutraceuticals and cosmeceuticals (Sanjib, 2009) are of great importance as a reservoir of chemical diversity aimed at new drug discovery and can be explored as potential antimicrobial, cardiovascular, immunosuppressive and anticancer drugs.

A golden triangle consisting of Ayurveda-Modern medicine- Modern Science will converge to form a real discovery engine that can result in newer, safer, cheaper and effective therapies.

## CONCLUSION

The hot pungent compound, chabbarin, extracted from the acetone fraction of the stem of *Piper chaba*, is the key reason for the medicinal property of the plant. The compound exhibits strong antifungal (MIC range 0.01562 to 0.03125 mg mL<sup>-1</sup>) and antibacterial (both on gram positive and gram negative; MIC range 0.00391 to 0.01562 mg mL<sup>-1</sup>) activity. It also has a high range of antioxidant activity of IC<sub>50</sub> value 3.00 µg mL<sup>-1</sup>. Thus we conclude that our study on the isolation and identification of the active compound supports the fact that *Piper chaba*, Hunter is one of the potential sources of natural pharmaceuticals.

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