International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2017; 5(5): 182-191
Received: 25-07-2017
Accepted: 26-08-2017

Naynika Patel
Department of Biosciences, Vallabh Vidhyanagar, Sardar Patel University, Gujarat, India

JSS Mohan
Department of Biosciences, Vallabh Vidhyanagar, Sardar Patel University, Gujarat, India

Correspondence
Naynika Patel
Department of Biosciences, Vallabh Vidhyanagar, Sardar Patel University, Gujarat, India

Isolation and characterization of potential bioactive compounds from *Piper betle* varieties Banarasi and Bengali leaf extract

Naynika Patel and JSS Mohan

Abstract
Since many centuries Chinese and Indian folk medicine been using *Piper betle* leaves as medicine and different research works and traditional medicinal uses which signify the tremendous potential of "*Piper betle*" to come out as Green medicine. In the present study, *Piper betel* varieties Banarasi and Bengali, Calcati, Kapoori and Malbari was extracted with sequential extraction method using n-Hexane, Ethyl acetate, Methanol and Distilled water. The crude ethyl acetate extract of Banarasi and Bengali was separated using analytical thin-layer chromatography (TLC).The separated compounds were tested for their potential antioxidant capacity and antimicrobial activity against bacteria using TLC bioautography approach. Antimicrobial activity was visualized on TLC after spraying with MTT and triphenyltetrazolium chloride reagents which screen antioxidant and antimicrobial compounds, respectively.Extraction of the bioactive constituents from the leaf extract of *Piper betle* varieties Banarasi and Bengali was carried out and characterized by separation techniques like HPTLC and structure elucidation spectroscopic analysis techniques like 1H-NMR and FT-IR. Phytochemical screening of different extractions showed presence of phenols, flavanoids and the isolation purification active principles as Hydroxy Chavicol, Chavicol, Eugenol from leaves extract when subjected to physical, chemical and spectral identification.

Keywords: FT-IR, 1H-NMR, Phytochemicals, Phenols and Flavanoids

1. Introduction
The world populations depend on herbal medicines for major source of health care. Now-a-days there is a growing interest in Western countries on plant based or herbal medicines. However the mechanism of action of herbal drugs differs from that of the synthetic drugs. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [1]. Plants are a rich source of secondary metabolites with remarkable biological activities. The secondary metabolites are significant source with a variety of structural arrangements and properties [2]. Natural products which come out from medicinal plants are important for pharmaceutical research and for drug development as a source of therapeutic agents. At presents the demand for herbal or medicinal plant products has increased significantly [3]. Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest in plant chemistry has grown throughout the world. Research studies leading to extraction, isolation, identification and biological study of plant constituents have now formed the major field of the study [4].

The *Piper betel* L. belongs to family Piperaceae is widely found in South East Asia, and its leaves possess strong pungent and aromatic flavor and widely consumed as mouth freshener [5].Traditionally leaves are medicinally used for wound healing, digestive and pancreatic lipase stimulate. In Indian folkloric medicine, betel leaf is popular as an antiseptic and is commonly applied on wounds and lesions for its healing effects. This particular property has paved way for further experimental studies, which have established paan extract to have antimicrobial and antileshmian properties [6]. Fresh juice of betel leaves is also used in many ayurvedic preparations. Betel leaves have long been studied for their diverse pharmalogical actions.

Traditional healers from different remote communities in India claim that their medicine obtained from these betel leaves is cheaper and more effective than modern medicine.

Patients belonging to these communities have a reduced risk of acquiring infectious diseases from resistant pathogens than the people from urban areas who may be treated with regular antibiotics. A novel approach to the prevention of antibiotic resistance of pathogenic species is the use of new compounds that are not based on existing synthetic antimicrobial agents [7]. Reports of various researches show that betel extract and betel oil exhibit antimicrobial and antioxidant activities in model systems [8-10]. Antioxidant activity includes free radical scavenging capacity, inhibition of lipid peroxidation, metal ion chelating ability and reducing capacity. *Piper betel* leaves are also known to contain significant amount of anti-oxidants like hydroxychavicol, eugenol, ascorbic acid and b-carotene [11].

The objective of this study includes the evaluation of the phytochemical constituents of the betel leaf varieties Banarasi and Bengali ethyl acetate and methanol extract.

2. Materials and methods

2.1 Plant material

Plant material in form of leaf were collected and washed thoroughly with running tap water to remove dirt particles. All the materials were dried at room temperature and powdered with pulverizer (Sumit Mixer-Grinder, India).

2.2 Preparation of plant extract

Extract was prepared by infusion extraction method given by Houghton and Raman [12]. For sequential extraction 50 gm of dry powdered or fresh material of each sample was soaked in 250 ml ethyl acetate at room temperature for 24 hours. Extracts were filtered through Whatman filter paper no.1 and the filtrates were centrifuged at 3000 rpm for 10 minutes to remove solid debris. The supernatant was collected and concentrated by solvent recovering assembly (J-sil, India) and dried completely at room temperature and stored it in a refrigerator until further use. The extracts were filtered and the filtrate was centrifuged at 3000 rpm for 10 minutes and the supernatants were collected. All the fractions were stored in a refrigerator until further use.

2.3 Column Chromatography-Ethyl acetate extract

The silica gel (60-120 mesh, Chiti Chem, India) column was prepared in column (30x450 mm size) and dead space was packed with glass wool. Silica gel (75g) was equilibrated in mobile phase to form approximately 72cm column length and 4cm breadth. The packed column was allowed to settle for 12hr and then the column was washed twice with mobile phase. The ethyl acetate extract were loaded on the top of silica gel column and eluted with increasing polarity of toluene: ethyl acetate and chloroform: methanol (100:0, 80:20, 60:40, 20:80, 0:100). Twenty fractions, each of 25ml were collected at flow rate of 1ml/min. The collected fractions were then concentrated using rotary vacuum evaporator under reduced pressure for spectral analysis.

2.4 Bioautography

Bioautography to check the antimicrobial activity by using semiautomatic Camag Linomate 6 sample applicator (Camag, Switzerland). Twenty fractions, each of 25ml were collected at flow rate of 1ml/min. All the fractions were concentrated and evaluated for the presence of antibacterial activity by bioautography. A 10µl of aliquote from each of fraction was loaded onto 10x10cm pre-coated silica gel plate by Linomate 5 sample applicator for even and uniform distribution of tracks. The solvent systems used for ethyl acetate and methanol extracts were Toluene: Ethyl acetate (8:2) for separation of active compound/s. Nutrient agar medium seeded with selected test organisms bacteria was overlaid on previous developed silica gel plate and incubated at 37 ^{0}C for 24 hours. The plate was flooded with 0.1% 2, 3, 5 - triphenyl tetrazolium chloride to visualize inhibition area which appear as yellow in colour against pink red background (Lawn growth of living organism).

For fungus 0.6% phenol red was incorporated into potato dextrose agar seeded with selected test organisms were overlaid with the sample loaded silica gel plate and incubated at 28 ^{0}C for 48 hours. The plate was flooded with 0.1% Methylthiazoyl tetrazolium chloride (MTT) to visualize inhibition area against purple background (Lawn growth of living organism).

2.5 Phytochemical Screening

Phytochemical screening of eluted fractions was tested for the presence of various phytochemical constituents i.e. phenols and flavonoids.

2.5.1 Determination of Total Phenol estimation

Each plant extract (0.2 ml) in test tube was taken separately and added 3 ml distilled water and then added 0.5 ml FCR. After 3min. of incubation, 2 ml of 20% Na_2CO_3 solution was added into each tube and mixed thoroughly. Reaction tubes were placed in boiling water bath for exactly 1 min, cooled and the absorbance was measured at 650 nm against a reagent blank using visible spectrophotometer. A standard curve was prepared using different concentrations of catechol. Total phenol was expressed as mg phenol in terms of catechol per gram of fresh tissue [13].

2.5.2 Determination of Total Flavonoids estimation

Aluminum chloride colorimetric method was used with some modification to determine flavonoids content. 1 ml of each plant extract was mixed with 3ml of methanol, 0.2ml of 10% aluminum chloride, 0.2ml of 1M Potassium acetate and 5.6ml of distilled water and kept at room temperature for 30minutes.The absorbance was measured at 420nm using UV-Visible spectrophotometer. Quercetin was used as standard 1mg/ml. All the tests were performed in triplicates. Flavonoid content was determined from the standard curve and expressed as Quercetin equivalent mg/gm of extracted compound [14].

2.6 Phytochemical Separation by using HPTLC Method

Each fractions of the column eluted sample was subjected to TLC to find out the separation of single compound and confirmation from the fraction. Thin Layer Chromatography was performed on prepared plates with Silica gel F254 grade (Merck, Germany) as stationary phase. A one-dimensional ascending development technique was used to detect the constituents of an extract on TLC plate. Visual detection was done in daylight and under UV light at a wave length of 254 and 344 nm depending on the nature of compounds separated.

2.7 Spectroscopic analysis

The isolated compounds were dissolved in deuterated methanol CD_3OD and 1H NMR spectra was recorded using a Bruker Avance 400 spectrometer (Bruker, Illinois, USA) operating at 100 MHz. Tetramethylsilane (TMS) was used as an internal standard. The chemical shift values were reported in ppm (δ) unit and the coupling constants (J) are in Hz. FT-IR spectra of the compounds were measured using IR grade potassium bromide (KBr). The compounds were separately mixed with 200mg KBr to obtain round disc with the help of

hydraulic press. Round disc was later subjected to FT-IR in the range of 4000-400 cm^{-1} using Perkin Elmer spectrophotometer, spectrum instrument (Germany) with FT-IR paragon 1000 PC software.

3. Results and Discussion

Although large number of isolates were separated from successive column chromatography but quantitatively only three compounds were isolated, purified and characterized from ethyl acetate extract of leaves part of *Piper betle* by column chromatography. Isolated components were further purified by recrystallization. Compounds were further identified and confirmed by HPTLC, 1 H-NMR and IR, and spectra of the compounds. The compounds were characterized on the basis of spectroscopic analysis and compared with reported data in literature.

Antibacterial compounds of ethyl acetate extracts may be non polar in nature because they were found at higher Rf values and were detected at 546nm after spraying 10% antimony trichloride and aqueous iron chloride which is an indicator for the presence for terpenoids and phenols respectively. The specific compounds can be separated by suitable mobile phase in bioautography method and the zone of inhibition can be seen at specific active principles against the selected organism.

Piper betle var. banarasi ethyl acetate leaf extract was subjected to silica gel column chromatography to isolate the active compounds present in the extract. Ethyl acetate extract (2gm) was subjected to silica gel column chromatography and 20 fractions each of 25 ml were collected. These fractions were screened for the presence of antibacterial and antifungal activity by bioautography method against SA, SM, KP, EN, BC, BS test bacterial strains and AN, AP, TH, FO as test organisms (Fig.1 and 2). This method revealed with the clear zone of inhibition for the presence of antimicrobial compound in the fractions 7-11 against all the test organisms. Higher activity was found in the fraction 7 among the various fractions and other fractions showed less to no activity against the test organisms. The fraction no. 7 which showed antibacterial activity fall in the elution gradient range of toluene: ethyl acetate (60:40) which showed from dark green to light green in colour. For the 7th fraction which showed high antimicrobial activity was subjected to HPTLC-bioautography fingerprinting and obtained Rf values 0.07, 0.84, 0.88 which might be due to presence of polar nature of compounds compared to other fractions and found strong inhibitory activities against the test organisms selected for the experiment (Fig 1 & 2).

Further purification of fractions was done by pooling all the 7th fractions and reloaded on silica gel column and collected the fraction which falls in gradient of 80:20 toluene: ethyl acetate solvent system. In the final step, preparative thin layer chromatography was used for purification of antibacterial compound prior to structure elucidation. The TLC plate was developed in toluene: ethyl acetate (6:4) solvent mixture. Before the elution of compound bioautography was performed on the reference plate and compared with preparative plate. The compound matching reference plate was eluted and then used for structural elucidation.

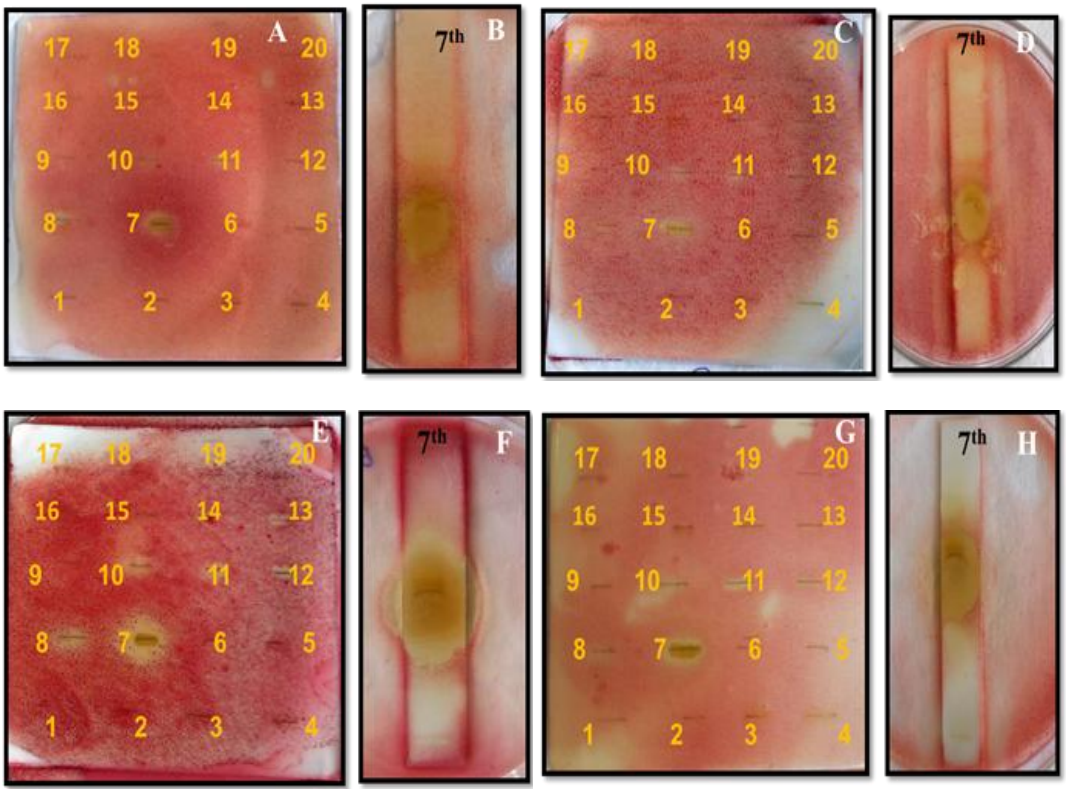


Fig 1: Bioautography for various fraction of ethyl acetate leaf extract of *Piper betle* var. banarasi against various bacteria **A.** Purified column fractions 1-20 against *Serratia marcescens* B. Purified column fraction no. 7 showing zone of inhibition against *Serratia marcescens* **C**. Purified column fractions 1-20 against *Klebsiella pneumonia* **D.** Purified column fraction no. 7 showing zone of inhibition against *Klebsiella pneumonia* E. Purified column fractions 1-20 against *Enterococcus faecalis* **F.** Purified column fraction no.7 showing zone of inhibition against *Enterococcus faecalis* **G**. Purified column fractions 1-20 against *Bacillus subtilis* **H**. Purified column fraction no. 7 showing zone of inhibition against *Bacillus subtilis*

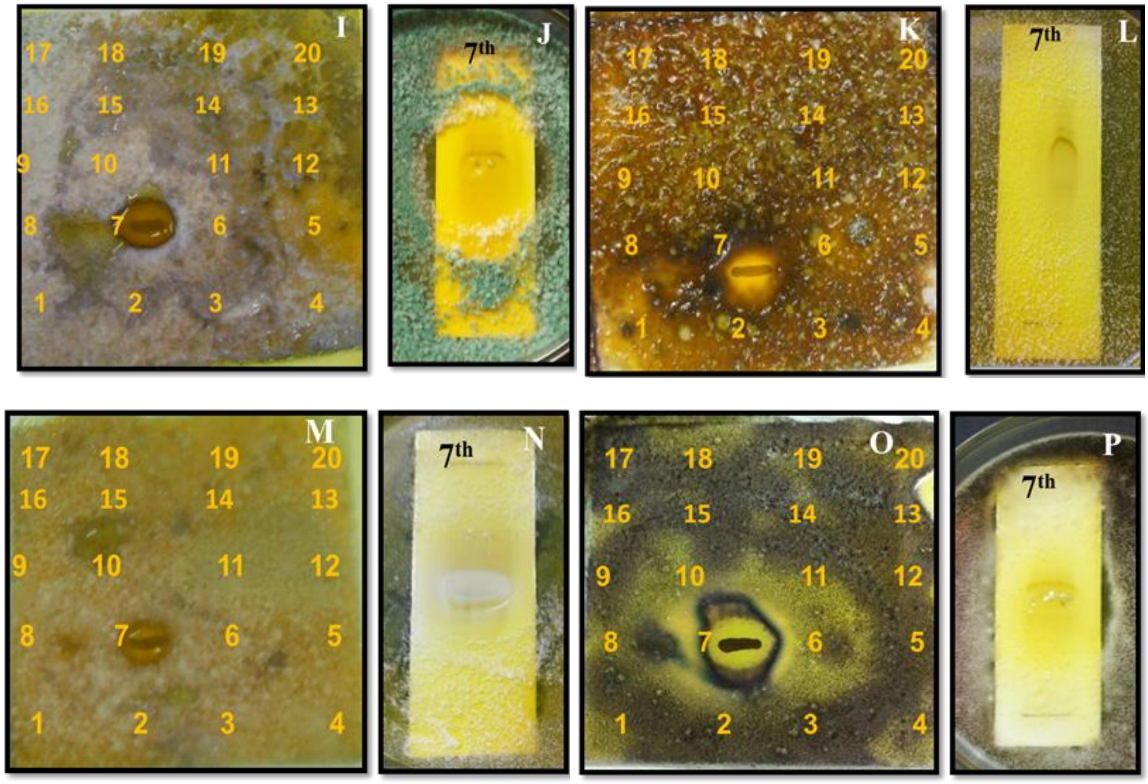


Fig 2: Bioautography for various fraction of ethyl acetate leaf extract of *Piper betle* var. banarasi against various fungi **I.** Purified column fractions 1-20 against *Trichoderma harzianum* **J.** Purified column fraction no.7 showing zone of inhibition against *Trichoderma harzianum* **K.** Purified column fractions 1-20 against *Fusarium oxysporium* **L.** Purified column fraction no. 7 showing zone of inhibition against *Fusarium oxysporium* **M.** Purified column fractions 1-20 against *Aspergillus parasiticus* **N.** Purified column fraction no.7 showing zone of inhibition against *Aspergillus parasiticus* **O.** Purified column fractions 1-20 against *Aspergillus niger* **P.** Purified column fraction no. 7 showing zone of inhibition against *Aspergillus niger*

***Piper betle* var. bengali**

P. betle variety bengali ethyl acetate leaf extract was subjected to silica gel column chromatography to isolate the active compounds present in the extract. Ethyl acetate extract (2gm) was subjected to silica gel column chromatography and 20 fractions each of 25 ml were collected. Bioautography method was done for presence of antibacterial activity against BC and SM (Fig 3). This method revealed the presence of antibacterial compound in 7th and 8th fractions. Higher activity was found in the 7th fraction of the extract. The activity gradually declined from fraction 9 to 20. The 7th fraction which showed antibacterial activity fall in the elution gradient range of toluene: ethyl acetate (80:20) which showed the colour from dark green to light green. HPTLC-bioautography fingerprinting was done for the 7th fraction and found Rf value at 0.66. Further purification of fractions was done by pooling the 7th fractions and reloaded on silica gel column and collected the fraction and tested for the antibacterial activities. The 7th fraction showed significantly high inhibitory activity (Fig. 3). In the final step, preparative thin layer chromatography was used for purification of antibacterial compound prior to structure elucidation. The TLC plate was developed in toluene: ethyl acetate (6:4) solvent mixture. Before the elution of compound, bioautography was performed on the reference plate and compared with preparative plate. The compound matching reference plate was eluted and then used for structural elucidation. The structural elucidation the fractions from both the varieties of *P. betle* were pooled together and identified the bioactive compound present in them.

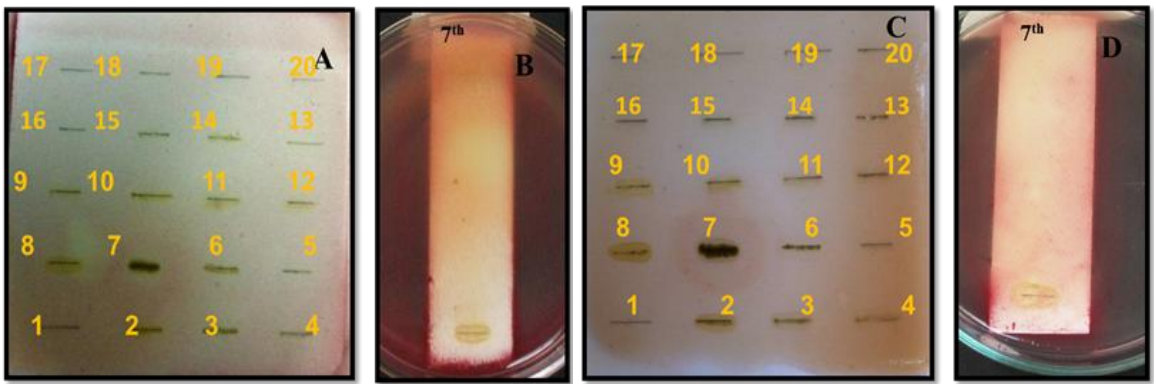


Fig 3: Bioautography of ethyl acetate leaf extract of *Piper betle* var. bengali (A-D) against various bacteria. **A.** Purified column fractions 1-20 against *Bacillus cereus* **B.** Purified column fraction no.7 showing zone of inhibition against *Bacillus cereus* **C.** Purified column fractions 1-20 against *Serritia marcescens* **D.** Purified column fraction no. 7 showing zone of inhibition against *Serritia marcescens*

Phytochemical analysis of column purified fractions indicate the presence of total phenols and flavonoids among different

varieties of *Piper betle* var. Bengali and Banarasi contained higher content in both ethyl acetate leaf extracts. Total phenol concentration was performed in purified column fractions of *P. betle* var. banarasi and bengali in ethyl acetate leaf extract shows the presence of total phenol content in purified column fractions (1-20). In fraction 7 of both banarasi and bengali ethyl acetate purified column fractions found high phenolic content i.e. 3.74mg/ml and 9.37 g/ml respectively as compared to other fractions as shown in figure 4.

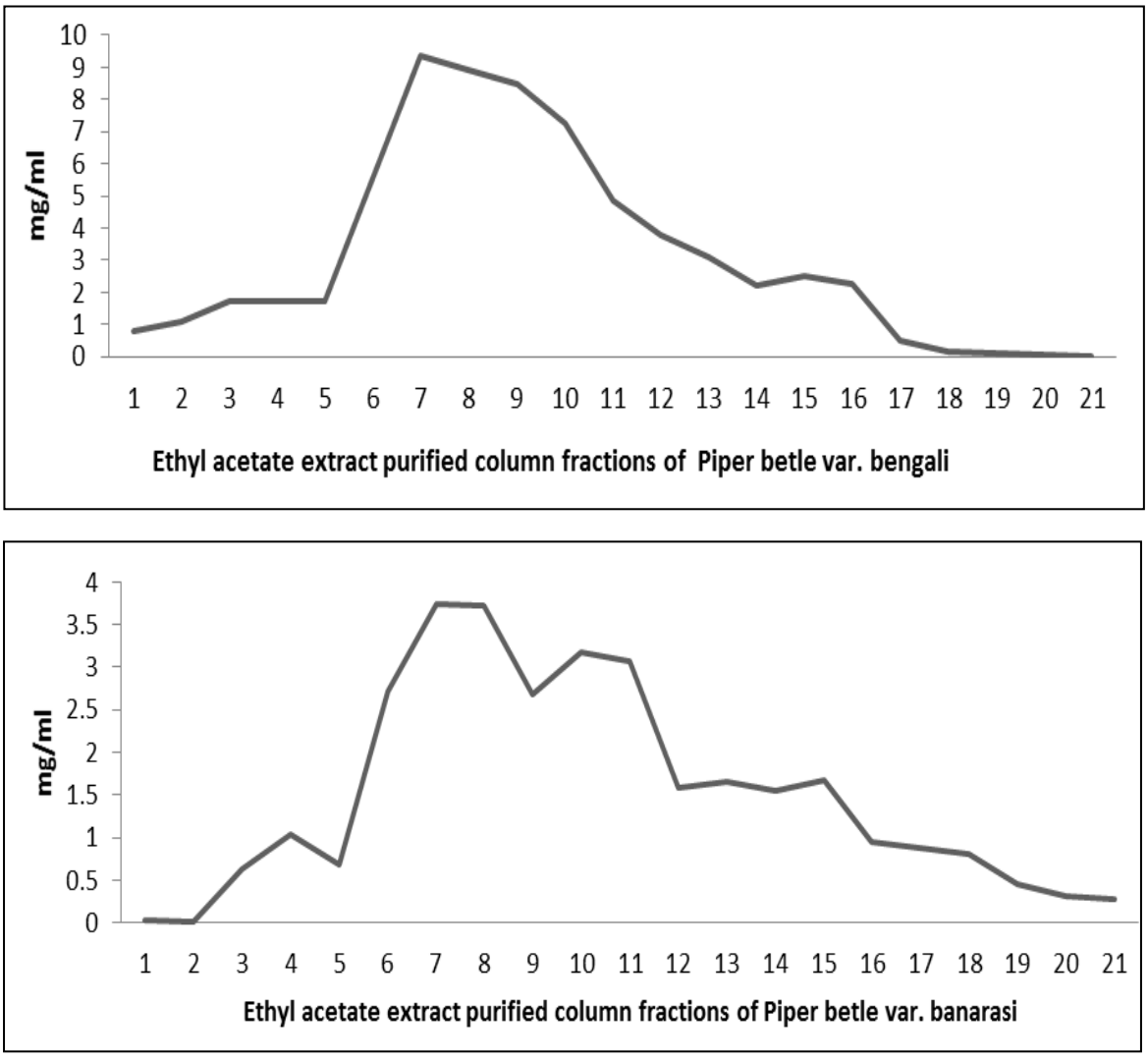
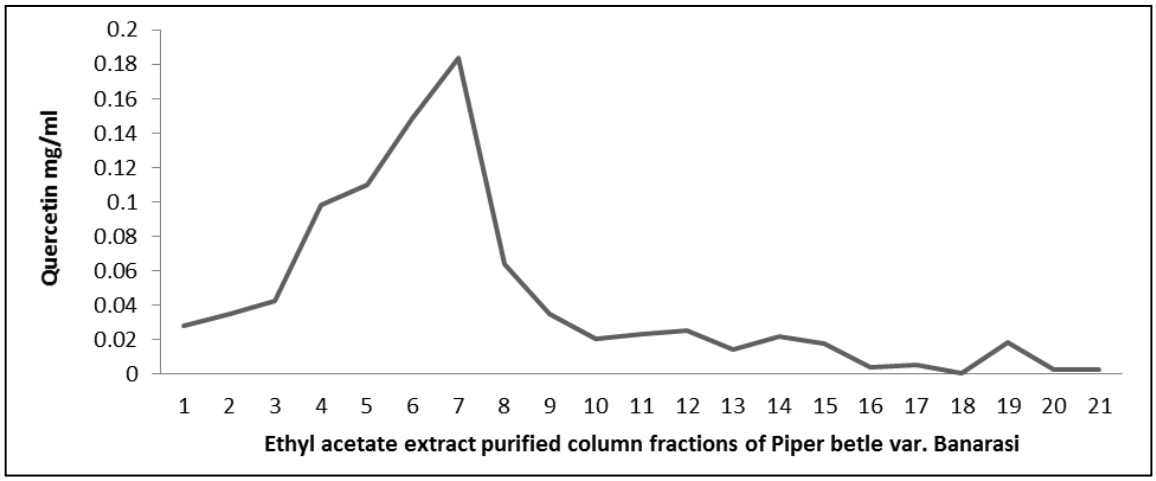


Fig 4: Total phenols concentration in ethyl acetate extract purified column fraction of *Piper betle* var. banarasi and bengali.

Total flavonoid content was estimated in purified column fractions of *P. betle* var. banarasi and bengali in ethyl acetate leaf extracts shows the presence of total flavonoid content in purified column fractions (1-20). In fraction 7 of both banarasi and bengali ethyl acetate purified column fractions showed high flavonoid content i.e. 0.18mg/ml and 0.12mg/ml respectively as compared to other fractions as shown in figure 5.



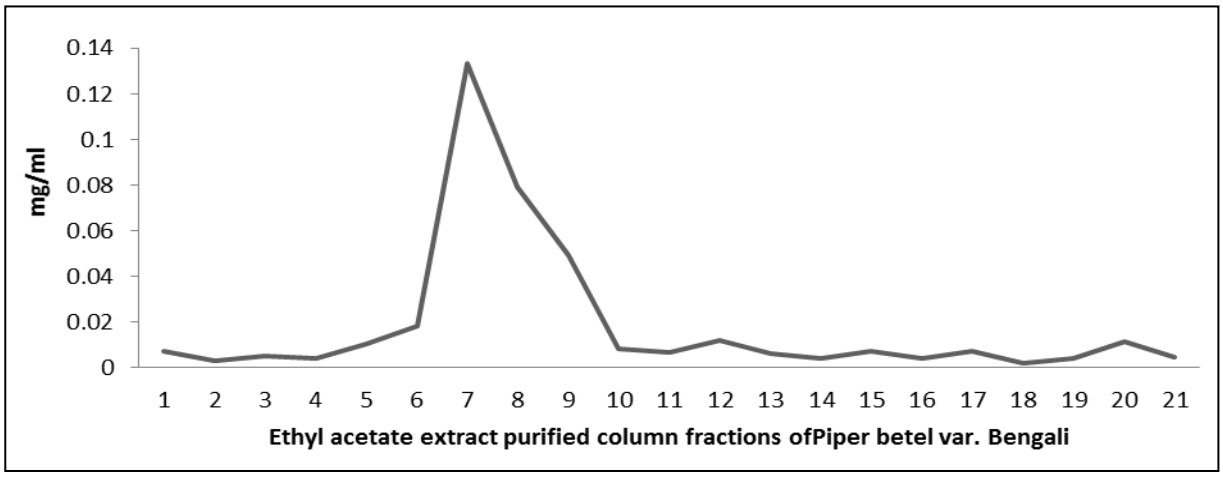


Fig 5: Total Flavanoids concentration in ethyl acetate extract purified column fraction of *Piper betle* var. banarasi and bengali**.**

The antioxidant activity is due to presence of phenols and flavonoids. The isolation of therapeutically active constituent from plant extract gives better antioxidant activity. In this study it was observed that both banarasi and bengali varieties of *P. betle* leaf extracts and their fractions have shown considerable phenol and total flavonoids.

Quantitative estimation and identification of active principles of purified column fractions of *P. betle* var. banarasi and bengali ethyl acetate leaf extracts were performed by HPTLC method (Table- 1). In the present study HPTLC separation of ethyl acetate extract revealed the presence of three different compounds by appearance of fluorescents spots when visualized under UV light (Figure 6 & 7). Among the different fractions of various plant extracts the presence of active principle was observed with Rf values 7th fraction of *P. betle* variety banarasi (0.07-1.17) (Figure 6) and bengali (0.08-1.64) leaf extract (Figure 7). Phytochemical study of the leaf extract of *piper beetle varieties* banarasi and bangali leaf extracts *showed* that leaf comprised a wide range of active chemical constituents such as flavonoids, phenols, carotenoids and tannins while alkaloids, saponins and oils were absent. HPTLC analysis also confirmed the presence of flavonoids, phenols and tannins in the studied plant.

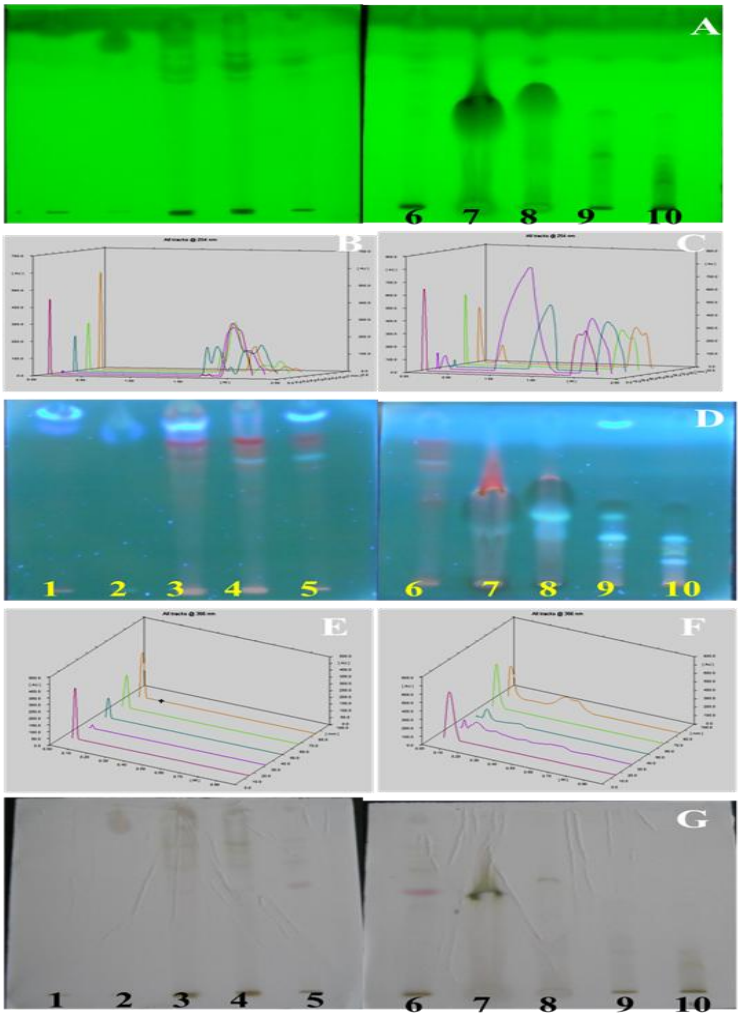


Fig 6: HPTLC finger printing of ethyl acetate leaf extract *Piper betle* var. banarasi purified column fractions.

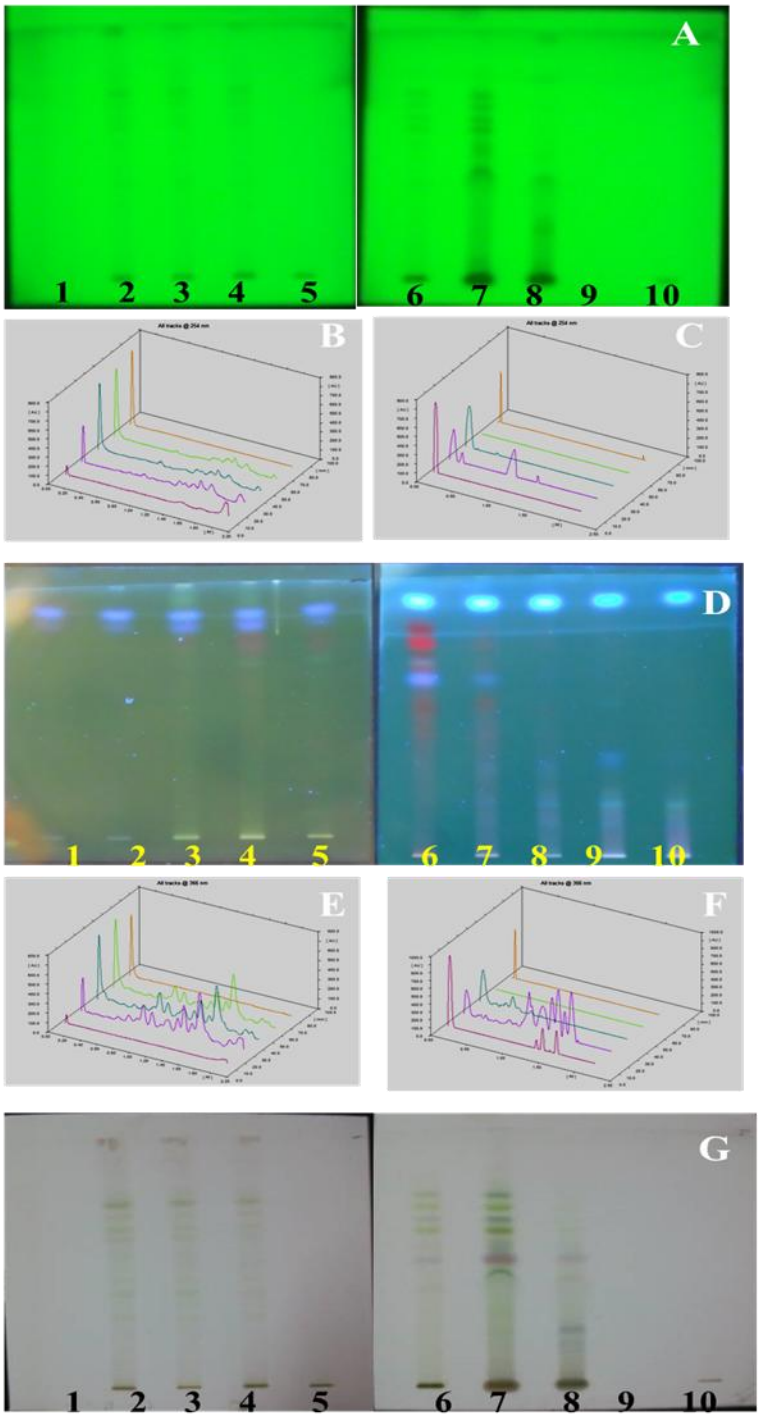


Fig 7: HPTLC finger printing of ethyl acetate leaf extract *Piper betle* var. bengali purified column fractions**.**

The spectral analysis of isolated fractions revealed the presence of Hydroxy Chavicol (Compound A), Chavibetol (Compound B) and Eugenol (Compound C) as compounds in the leaf extracts of different *Piper betle* extracts. The structures were determined by extensive 1H NMR, FT-IR as well as by comparison of their spectral data with previously reported data.

Compound A

^{1}H NMR (MeOD): δ ppm, 8.45 (S, Ar -H) 5.24 (s, OH), 5.23 (d, 2.0 Hz, H), 4.75 (d, *J* = 6.5 Hz,), 3.21 (ddt, *J*=16.9 Hz, *J*=10.4 Hz, *J*=6.5 Hz), 3.20 (m,), 3.20 (ddt, *J*=16.9 Hz, *J*=3.2 Hz, *J*=3.2 Hz). In the spectrum there are eight signals. Signal at δ 5.24 indicates the presence of hydroxyl proton of hydroxyl group. The aromatic protons resonated at δ 8.45 ppm. From the values of coupling constants it is clear that the proton which gives first signal is in ortho position to allyl substituent. The proton in ortho to the allyl and hydroxyl substituents gives second signal. Third signal from the proton in *meta* position with respect to the allyl substitution is clearly seen in the spectrum. The up field positions of these three signals are due to the influence of hydroxyl group. Two protons of methylene (CH_2) group resonate at δ 3.21 ppm. Three signals from the vinylic proton (- C= CH_2 and – CH=C) resonated at δ 4.75, 5.23 and 5.24 ppm. The methoxy methyl signal (- OCH3) is absent in 1H NMR spectrum at δ 3.75. C-H bond in the alkanes found in molecule.

In the FT-IR spectrum of compound A (figure 8), the band at 1201.02 Cm^{-1} confirms the C-O stretching of oxygen attached to aromatic ring. The appearances of the weak band at around 1632.30 and 1605.61 Cm^{-1} are due to the C=C stretching. CH_3 banding band appears at 1440.01 Cm^{-1}. The banding band at

around 968.07 cm^{-1} confirms the presence of =C-H of alkenes. The peak at 780 cm^{-1} observed due to the C-H stretching of aromatic ring. Hence the structure was identified and confirmed by comparison with the spectral data such as, FT-IR and 1 H-NMR for the isolated compound A from ethyl acetate extract of *Piper betel* leaf extract was found to be Hydroxy Chavicol as shown in figure 11 (Compound A).

Compound B

^{1}H NMR (MeOD): δ ppm, 3.21 (2H, d, *J* = 7.1 Hz, -CH_2-), 3.20 (3H, S, -OCH_3), 4.75 (2H, m, C = CH_2), 5.67 – 5.79.1 (1H, m, - C00H=C), 6.38-6.58 (3H, m, aromatic H), 5.86 (s, OH). The ^{1}H NMR showed the presence of methoxy group (OCH_3) at δ 3.20. The aromatic hydrogen resonated at δ 6.38, 6.50 and 6.58 with coupling constants *J* = 7.6 Hz, and *J* = 1.95 and *J* = 7.8 Hz. The values of coupling constants indicate the position of three protons at *ortho* and *meta* position with respect to allyl and allyl and hydroxyl substitution respectively. The two terminal alkene protons resonated at δ 5.76 and δ 5.79 and one more H of alkene resonated at δ 5.86. The two methylene proton resonated at δ 3.21 with *J*=7.1 Hz. Three signals of vinylic proton resonated at δ 4.48 -6.38.

The compound B identified from FT-IR spectrum (figure 9) revealed the presence of chavibetol. The major IR bands are as follows. The strong and broad band at 3426.74 Cm^{-1} is due to the presence of OH group. The weak peak at 3077.86 Cm^{-1} appeared due to C-H stretching of aromatic ring. The two peaks appear at around 2929.28 and 2854.97 Cm^{-1} are due to the C-H stretching of methylene (CH_2) group present in the structure. The presence of band at 1722.04 cm^{-1} is due to the C=O of OCH_3. Peak observed at 1605 cm^{-1} confirms the presence of aromatic ring present in the structure. Strong band appears at 1509 cm^{-1} is due to the C=C stretching. Two banding bands appear at around 1443.91 and 1383.82 cm^{-1} are of CH_3. The band at 1280.24 cm^{-1} is of C-O stretching. Peaks at 994.61 and 913.14 cm^{-1} are of aromatic hydrogen. Hence the structure was confirmed by comparison with the spectral data such as UV, IR, 1 H-NMR and MS of isolated compound B from ethyl acetate extract of *Piper betel* as Chavibetol as shown in figure 11 (Compound B).

Compound C

^{1}H NMR (MeOD): δ ppm 7.12 (S, Ar -H), 3.20 (2H, d, *J*=6.6 Hz, H-8), 3.20 (3H, s, OCH_3), 5.83 (1H, s, Ph-OH), 5.73 and 5.74 (1H, each d, *J*=19.7, H-10), 4.90 (1H, m, H-9), 6.35 (1H, s, H-3), 6.51 (1H, d, *J*= 8.6 Hz, H-5), 6.59 (1H, d, *J*= 8.6Hz, H-6). The NMR spectrum showed presence of 12 protons in the molecule. The aromatic protons of benzene ring resonated at δ 7.12. The singlet at δ 5.78 indicated the hydroxyl group attached to aromatic ring. The three downfield protons at δ 6.35, 6.51, 6.59 ppm indicated the presence of an aromatic ring in the molecule which is substituted at three positions. The singlet obtained for three equivalent protons at δ 3.20 suggested the presence of a methoxy (OCH_3) group on the aromatic ring. Further, the presence of doublet at δ 3.20 for two protons indicated the presence of a (-CH_2) attached to an aromatic ring. The presence of downfield doublet at δ 5.73 and δ 5.74 (1 H each) further suggested the presence of an exocyclic double bond which was supported by the presence of one proton multiplet at δ 4.90 for a methane proton.

The FT-IR spectrum (figure 10) obtained for another isolated compound revealed nearly same bands with little variation. However, from the measurement of other chemical and physical properties the compound was identified as eugenol. The FT-IR spectrum displayed strong absorption bands of hydroxyl (OH) group at 3429.50 cm^{-1}. C-H stretching around 2928.69 and 2854.53 cm^{-1} are of aliphatic alkanes CH_2 and CH. The band appear at 1730.26 cm^{-1} is due to the C=O of acetate (OCH_3). Presence of medium weak band at 1509.57 and1632.28 cm^{-1} is of C=C stretching of isolated alkenes. CH_3 banding bands are observed at 1413.49 and 1383.98 cm^{-1}. Appearance of three different bands at around 1314.84, 1274.58 and 1115.19 cm^{-1} is due to the C-O stretching. Band due to the C-O stretching is observed at 1041.27 cm^{-1}. C-H stretching of aromatic ring is confirmed from the peaks observes at 874.07 and 779.76 cm^{-1}. Hence the structure was confirmed by comparison with the spectral data such as UV, IR, 1 H-NMR of isolated compound C from ethyl acetate extract of *Piper betel* was found to be Eugenol as shown in figure 8 (Compound C).

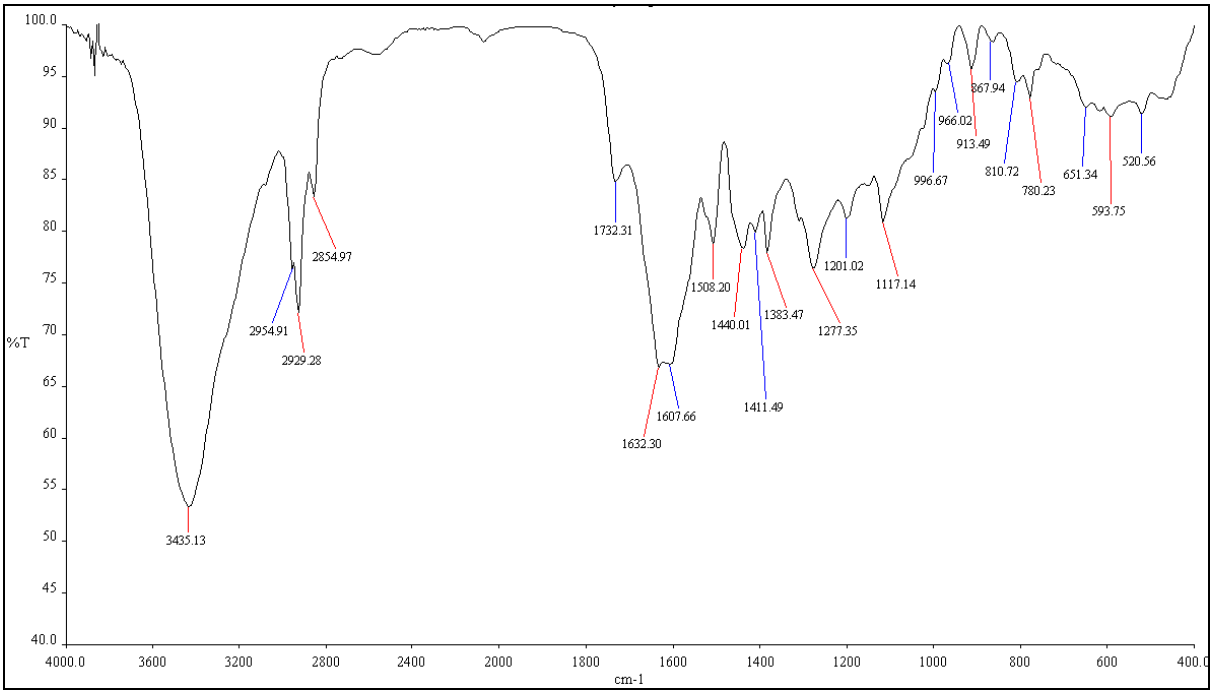


Fig 8: FT-IR spectrum of compound A.

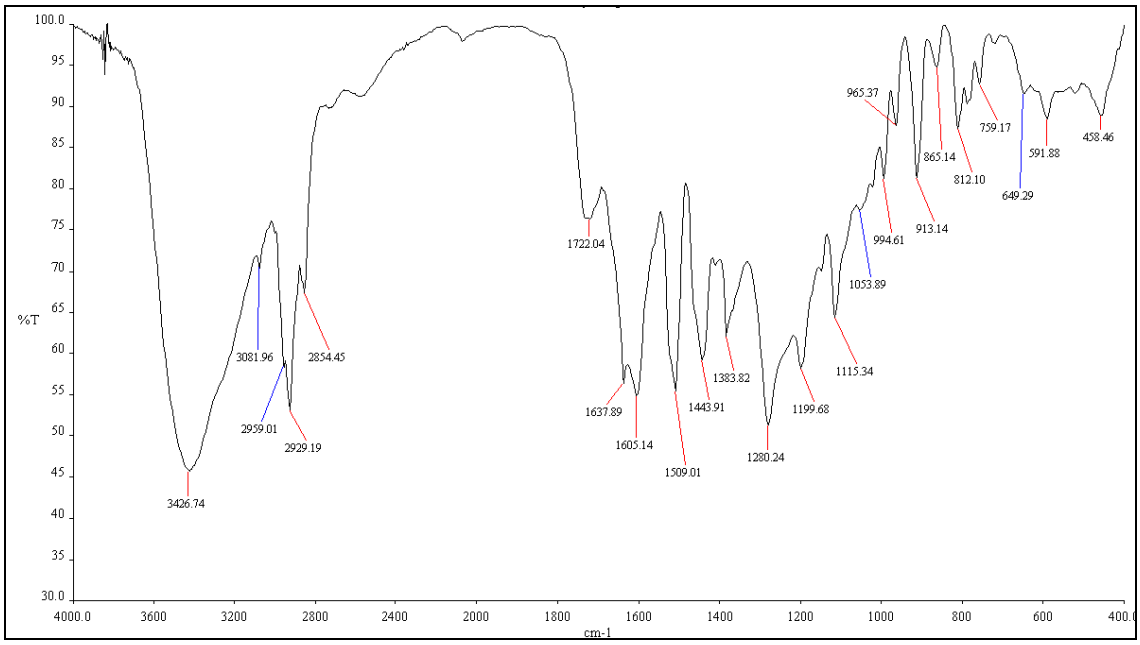


Fig 9: FT-IR spectrum of compound B.

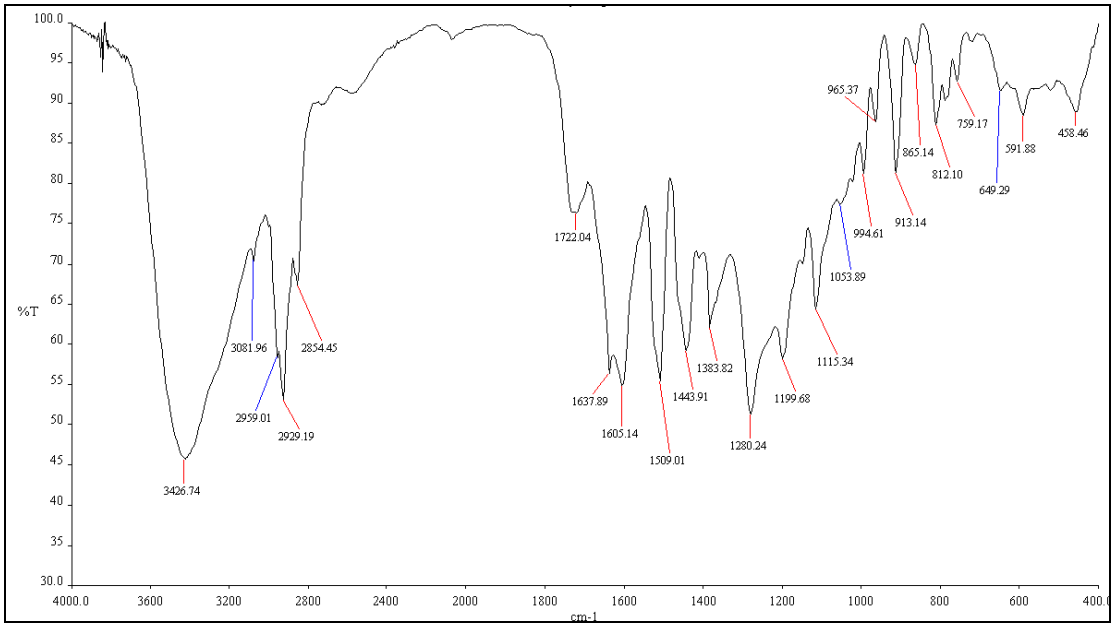
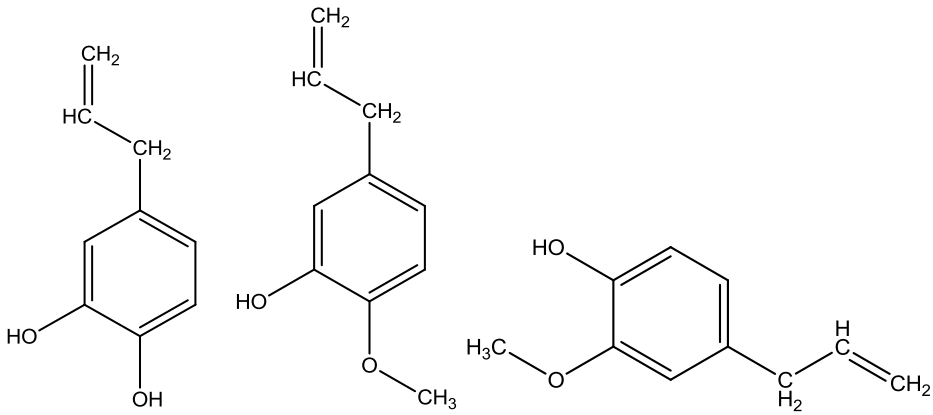


Fig 10: FT-IR spectrum of compound C.



Hydroxy Chavicol Compound A | Chavibetol Compound B | Eugenol Compound C

Fig 11: Bioactive compounds

4. Conclusion

In present investigation three bioactive compound were identified from ethyl acetate leaf extracts of *Piper beetle* varieties Banarasi and Bengali by the spectral analysis with 1H- NMR and FT-IR spectroscopic techniques. The presence of various bioactive compounds in *Piper beetle* proved that the pharmaceutical importance.

5. Acknowledgement

The authors are thankful to Dr. K.S. Rao, Head of the Department for granting the permission to perform the work and providing excellent facilities. Authors are also thankful to staff of Sophisticated Instrument Center for Applied Research & Testing (SICART), V.V. Nagar for providing data of analysis required during the research work.

6. References

1. Duraipandiyan V, Ayyanar M, Ignacimuthu S. Antimicrobial activity of some ethnomedicinal plants used by paliyar tribe from Tamil Nadu, India. BMC Compl. Alt. Med. 2006; 6:35-41.
2. Vanitha V, Umadevi KJ, Vijayalakshmi K. Determination of Bioactive Components of *Annona squamosa* L. Leaf by GC- MS Analysis. Int. J. Pharm. Sci. Drug. Res. 2011; 3(4):309-312.
3. Dhivya R, Manimegalai K. Preliminary phytochemical screening and GC- MS profiling of ethanolic flower extract of *Calotropis gigantea* Linn. (Apocynaceae). J. Pharma. Phyto. 2013; 2(3):28-32.
4. Gaikwad S, Devare S, Kale A, Deshpande NR, Salvekar JP. Isolation and characterization of a diethyl pthalate, a bioactive compound from *Cassia auriculata* L. Int. J. Pharm. Sci. Rev. Res. 2013; 19(1):56-57.
5. Santhanam G, Nagarjan S. Wound healing activity of *Curcuma aromatica* and *Piper betle*. Fitoterapia. 1990; 61:458-459.
6. Sarkar A, Sen R, Saha P, Ganguly S, Mandal G, Chatterjee M. An ethanolic extract of leaves of *Piper betle* (Paan) Linn. mediates its antileishmanial activity via apoptosis. Parasitol. Res. 2008; 102(6):1249-1255.
7. Shah PM. The need for new therapeutic agents: what is in the pipeline? Clinical Microbiol. Inf. 2005; 11:36-42.
8. Salleh MN, Runnie I, Roach PD, Mohamed S, Abeywardena Y. Inhibition of Low density lipoprotein oxidation and up regulation of low-density lipoprotein receptor in HepG2 cells by tropical plant extracts. J. Agric. Food Chem. 2002; 50:3693-3697.
9. Lei D, Chan CP, Wang YJ, Wang TM, Lin BR, Huang CR *et al*. Antioxidative and antiplatelet effects of aqueous inflorescence *Piper betle* extract. J. Agric. Food Chem. 2003; 51:2083-2088.
10. Bhattacharya S, Subramanian M, Roychowdhury S, Bauri S, Kamat JP, Chattopadhyay S, Bandyopadhyay SK. Radio protective property of the ethanolic extract of *Piper betle* leaf. J. Radiat. Res. 2005; 46:165-171.
11. Daniel, M. Method in plant chemistry and economic botany. 1st Edn., Kalyani Publishers, New Delhi, India, 1991.
12. Houghtan PJ, Raman R. Laboratory handbook for the fractionation of Natural extracts. Chapman and Hall. London. 1998, 1-28.
13. Thimmaiah SK. Standard Methods of Biochemical Analysis. Kalyani Publishers; New Delhi, India. 2004, 304-307.
14. Aiyegroro OA, Okoh AI. Preliminary phytochemical screening and *In vitro* antioxidant activities of aqueous extract of *Helichrysum longifolium* DC. BMC Compl. Alt. Med 2012; 10: 21.