*Available on line* www.jocpr.com

# *Journal of Chemical and Pharmaceutical Research*

*J. Chem. Pharm. Res., 2010, 2(4):612-622*

**ISSN No: 0975-7384**
**CODEN(USA): JCPRC5**

## Comparison and bioevaluation of *Piper longum* fruit extracts

***Beena Joy, C P Sandhya and K R Remitha**

*Agro Processing & Natural Product Division, National Institute for Interdisciplinary Science & Technoloy, Industrial Estate P.O, Thiruvanathapuram, India*

**ABSTRACT**
*Solvent extracts were prepared from dried fruits of Piper longum and their antioxidant activities were evaluated. Out of the different extracts tested, the hot ethyl acetate extract and cold hexane: water extracts (1:1) showed better antioxidant activity. The screening of antimicrobial and anti tumor effects of piper longum showed the better efficacy of these two extracts. The anti tumor activity studies showed a dose dependant behaviuor of the extracts towards the leukaemic cell lines K562 .Among the three bacteria tested, hot ethyl acetate extract showed more potency against E coli than Bacillus subtilis and was found to be less active for Staphylococcos aureus. The antioxidant, antimicrobial and anti tumor effects were found to be higher for hot extracts than cold extracts. The chemical fingerprinting of the most active extract was also carried out using HPTLC. The present study paved way for the extraction procedure for better biologically active constituents of Piper longum.*

**Key Words:** *Piper longum*; Antioxidant activity; Anti tumor activity; Antimicrobial activity; HPTLC profiling.

## INTRODUCTION

*Piper longum* (Indian long pepper), a well-known traditional medicinal plant indigenous to North-Eastern and southern parts of India and Srilanka, is used for the treatment of respiratory tract diseases like cough, bronchitis, asthma, cold, as counter-irritant and analgesic. It is applied locally for muscular pain, inflammation and internally used as a carminative in conditions such as loss of appetite and sleeplessness [1]. In the Western part of India aqueous extract of the roots

of *Piper longum* Linn. are used as food material [2]. In addition to this there is a major role for *Piper longum* Linn. in preventing the cancer development in the experimental glioma model[3]. The extract of the root of *Piper longum* and its major compound, piperine exert anti-oxidant activity and are protective in the myocardial ischemic condition [4]. The alcoholic extract of the fruits of the plant *Piper longum* and its component piperine showed significant immunomodulatory and antitumor activity [5]. Pipernonaline, a piperidine alkaloid derived from long pepper, possess a mosquito larvicidal activity [6].

Piperine was the first amide isolated from piper species and was reported to display central nervous system depression, antipyretic, and anti-inflammatory activity [7]. Moreover piperine possesses anti-inflammatory and anti-arthritic effects [8]. The isolated constituents from the n-hexane extract of *piper longum* were found to show better activity profile than the n-hexane extract, which indicates that the isolated constituents might be responsible for the antibacterial activity[9]. Ethyl acetate extracts of *Piper longum* root, stem and leaves showed relatively better anti-microbial effect against most of the tested organisms [10]. The *Piper longum* dried fruit's oil showed significant anti-inflammatory activity on carrageenan-induced rat paw edema [11]. Isolates from *Piper longum* fruit extracts showed antimicrobial activity against Gram-positive bacteria and Gram-negative bacteria [12].However, information pertaining to the systematic studies on the comparison between the hot and cold methods of extraction is lacking. Here, we wish to report the anti oxidant, antimicrobial and antitumor effects of hot and cold extracts of *piper longum* dried fruit and to make a comparison of these two extracts in terms of its activity and the chemical fingerprinting by HPTLC analysis.

## EXPERIMENTAL SECTION

2.1. Materials

*Piper longum* fruits were procured locally, authenticated by Department of Botany, University of Kerala.Chemicals and reagents used were Gallic acid, sodium carbonate, 2,2-diphenyl-2-picryl hydrazyl (DPPH), 2,2’-Azino-bis-ethyl benzothiazoline-6-sulfonic acid(ABTS), Trolox, hexane, ethyl acetate, methanol, Folin-Ciocalteu reagent, concentrated sulphuric acid, 3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyl Tetrazolium Bromide and Phosphate buffer saline(PBS). All the reagents were of analytical grade and were used without purification. HPLC grade solvents were used for HPTLC fingerprinting.

2.2. Preparation of hot extracts

About 270g of the powdered *piper longum* fruits were extracted in a soxhlet apparatus with hexane, ethyl acetate and methanol sequentially for a minimum of 8 hours. These extracts were concentrated at below $50^0$C under reduced pressure and each of these extracts were made up to a definite volume with methanol and kept as stock solution for further analysis.

2.3. Preparation of cold extracts

About 20g of the powdered *piper longum* fruits were extracted with hexane: water, ethyl acetate: water, methanol: water in 1:1 ratio sequentially by means of mechanical stirrer for a minimum of 12 hours. These extracts were also preserved in the same manner as that of hot extracts.

2.4. Antioxidant studies

2.4.1. Total Phenolic Content (TPC)

Total phenols were recorded by Folin-Ciocalteu reagent [13].Various extracts and standard Gallic acid, made up to 3.5 ml using distilled water in a series of test tubes, were treated with 0.5 ml 2N Folin-Ciocalteu reagent and incubated for 3 minutes in room temperature. The reaction was then neutralized with the addition of 1mL 20% $Na_2CO_3$ ,incubated at room temperature for 90 minutes and the absorbance of the blue color developed was read at 760nm using a Spectrophotometer (Shimadzu UV- Vis - Spectrophotometer, model 2450). The Total Phenolic Contents was expressed as:

% TPC= (Observed concentration ⁄ Actual concentration) × 100

2.4.2. DPPH radical Scavenging Capacity

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of [14]. A methanol solution of the extracts at various concentrations (12.5–50 lg) was added to 5 ml of a 0.1 mM methanolic solution of DPPH and allowed to stand for 20 min at $27^0$C. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula

% DPPH radical scavenging activity

= (control OD - sample OD/control OD) X 100

% of radical scavenging activity was plotted against the corresponding concentration of the extract to obtain IC $_{50}$ value. The $IC_{50}$ values are inversely proportional to the antioxidant activity.

2.4.3. ABTS Cation Decolorisation Capacity

The free radical scavenging activity of the extract was determined by ABTS (Sigma-Aldrich) radical cation decolourisation assay[15]. The ABTS decolorisation assay involves the generation of $ABTS^+$ chromophore by the oxidization of ABTS with potassium persulphate. The ABTS radical cation ($ABTS^+$) was produced by reacting 7mM stock solution ABTS with 2.45mM potassium persulphate and allow the mixture to stand in the dark for at least 6 hours at room temperature before use. The $ABTS^+$ solution diluted to an absorbance 0.7 ± 0.05 in the UV-visible spectrophotometer (Shimadzu UV-2450) at 736nm. Absorbance was measured 7 minutes after the initial mixing of different concentrations of the extracts. The $ABTS^+$ decolorisation capacity of the extracts were compared with the standard Trolox.The percentage of scavenging activity was calculated as:

% scavenging activity=
[(absorbance of control – absorbance of sample) ⁄ absorbance of control] ×100

2.5. Anti tumor screening studies

Cytotoxicity analysis by MTT Assay

MTT (3-(4,5 Dimethylthiazol-2yl)-2,5- Diphenyl Tetrazolium Bromide) assay, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely

impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Addition of a detergent results in the liberation of the crystals, which are solubilised. The colour can be spectrophotometrically measured. The level of the coloured formazan products is directly proportional to the number of surviving cells.

Cells harvested in the log phase of growth were counted and seeded ($5\times10^3$ cells/well in 100 µl) in 96 well titre plates. PBS was added to the outer wells (200µl/well).To allow cell attachment (in the case of adherent cells), cultures were treated with varying concentrations of the extract (two of the extract namely hot ethyl acetate extract and cold hexane: water at various concentrations like 200µg/mL, 400µg/mL and 800µg/mL) in medium and were incubated at 37°C in 5% $CO_2$ for 24 hours .Each treatment was carried out in triplicates. Untreated cells served as negative control and the plates were incubated for 48 and 72 hours. On completion of incubation, the media were removed from wells without disturbing the cells. To each well 100µl of 1mg/ml solution of MTT were added and plates were incubated for 2 hours in dark at 37ºC in a $CO_2$ incubator. 100 µl of lysis buffer was added to each well and the plates were further incubated for 4 hours in dark in a $CO_2$ incubator. After the incubation period, absorbance was read at 570 nm using a multi well plate reader and % of cytotoxicity was calculated as,

% of Cytotoxicity =
100 – [(O D of the treated cells/O D of the control cells) x100]

2.6. Antibacterial studies
The study investigates the antibacterial activity of *piper longum* fruit using broth dilution technique.*Staphylococcos aureus, Escherichia coli, Bacillus subtilis* are the organisms used for the study and Ampicillin is used as the positive control. Previous studies show that, Piperine which is one of the major constituent of piper longum show significant antitumor and antibacterial activities [16]

Nutrient broth preparation:
A media of composition Tryptone 10 Grams/L, Sodium chloride10 Grams/L, Yeast extract 5 Grams/L and Distilled water 1000mL was prepared and adjusted to a $P^H$ of 7 to 7.2.

Broth dilution technique
The tubes with nutrient broth were sterilized in an autoclave and mixed well. The flask was then cooled and cultures were inoculated and kept in a rotary shaker for 2h at 37ºC at 120 rpm. The organisms were selected for the experiment once the OD reaches 0.3 at 650 nm.10 mg of hot and cold extract of *piper longum* were dissolved in 1ml DMSO. Plated 100 µl of cells into all the wells in a 96 well plate. Then add 100 µl of different concentrations of the serially diluted extracts. Positive (Ampicillin) and negative control medium and cells were treated with 10% DMSO diluted in Nutrient broth and added in respective wells. The plate was incubated at 37ºC for 48 hours. Absorbance was taken at 620 nm in an ELISA reader and the % inhibition was calculated as follows:

% inhibition =
100- [(absorbance of the test sample / absorbance of control) ×100]

2.7. HPTLC analysis of most active extracts

HPTLC analysis was performed on the active extracts such as hot ethyl acetate and cold Hexane: water (1:1) extracts. Several amides are isolated from *Piper longum* L. by upright counter-current chromatography and reversed-phase liquid chromatography [17]. In the present study the mobile phase consisting of ethyl acetate: methanol (80:20, v/v) was selected. After development, the plates were heated for 3min at $110^0$C using TLC plate heater-III (Camag) and analysed at 300 nm. TLC Scanner-III controlled by winCATS software (Camag) was used for the analysis.

## RESULTS AND DISCUSSION

3.1. Total Phenolic Content (TPC)

It was found from the studies that ethyl acetate extract (hot extraction) of *piper longum* fruit exhibit high TPC (12.2 mg/g equivalent of gallic acid) and the phenolic contents were decreased in other extracts like methanol and hexane (7.0 and 5.5 mg/g equivalent of gallic acid). While considering the cold extraction, hexane: water [1:1] ratio exhibits high TPC (10.14mg/g equivalent of gallic acid) and the same were decreased in other extracts like ethyl acetate: water [1:1] and methanol: water [1:1] (4.20 and 3.78 mg/g equivalent of gallic acid).

Among the hot and cold extracts of *piper longum*, the total phenolic contents were found to be greater for hot extracts rather than cold ones. However, the cold extraction of *piper longum* with hexane and water in the ratio 1:1 gave better yield of the phenolics than with polar solvents like ethyl acetate, methanol and water in the ratio 1:1. While in hot extraction the ethyl acetate is suitable for total phenolic extraction.

3.2. DPPH Scavenging Activity

DPPH free radical method is a sensitive way to determine the antioxidant activity of plant extracts [18]. Because of the free radicals, disorders like neurodegenerative diseases, cancer and AIDS may arise. Antioxidants due to their scavenging activity are useful for the management of those diseases. The DPPH radical scavenging activities of all the extracts were dose dependent and the results are shown in Fig.1.The amount of DPPH scavenging activity appeared to depend on the phenolic concentration of the extracts. The highest DPPH radical scavenging activities were shown by hot extracts rather than cold ones. The ethyl acetate extract shows high radical scavenging activity ($IC_{50}$ 123.60µg/mL) compared to other hot extracts methanol and hexane ($IC_{50}$ 350.17 µg/mL and 160.01 µg/mL respectively).In the case of cold extracts, the radical scavenging activities were high in hexane: water (1:1) ($IC_{50}$ 379.38µg/mL) than in other cold extracts like ethyl acetate: water (1:1) and methanol: water (1:1) ($IC_{50}$ 726.79µg/mL and 822.401µg/mL respecticely).The $IC_{50}$ value for the standard Gallic acid was found to be 1.4 µg/mL. On comparing both the cold and hot extracts, the hot extracts of piper longum are more powerful radical scavengers.

b) ABTS radical Cation Decolorisation Capacity

The ABTS radical cation scavenging activities of all the extracts are presented in the fig. 2. Among all the extracts studied the hot ethyl acetate extract showed the higher ABTS radical cation scavenging activity ($IC_{50}$ 13.97µg/mL).The other extracts, hexane and methanol showed comparatively low decolourisation capacity ($IC_{50}$ 38.98 µg/mL and17.04 µg/mL respectively). In cold extracts, hexane: water (1:1) possesses comparatively high ABTS decolourisation capacity

($IC_{50}$ 80.38 µg/m), while the $IC_{50}$ values for other extracts like ethyl acetate: water (1:1) and methanol: water (1:1) were found to be high ($IC_{50}$ 112.35µg/mL and 123.49µg/mL respecticely) and possesses low decolourisation capacity. The $IC_{50}$ value for the standard Trolox was found to be 3.24 µg/mL. Thus the results indicated that the hot extracts of *piper longum* are good cation scavengers than the cold extracts

In all the antioxidant assays performed, the hot ethyl acetate extract possess high antioxidant activity when compared with the cold hexane: water (1:1) extract. Hot Ethyl acetate extract is the most active antioxidant extract compared to other hot and cold extracts of *Piper longum* fruits. The extraction procedure for the active components from *piper longum* is best through hot sequential extraction with solvents of increasing polarity.

3.3. Anti-tumor activity

In the case of hot ethyl acetate extract the activity towards the leukaemic cell lines K562 is 10% at 200µg/mL, 13%at 400µg/mL, 21% at 800µg/mL and for the cold hexane: water (1:1) the % cytotoxicity is 2% at 200 µg/mL, 5% at 400 µg/mL, and 9% at 800 µg/mL.The results indicated that the anti-tumor activities of hot ethyl acetate extract is higher than the cold hexane: water (1:1) extract. There was a concentration dependent increase in the percentage of cytotoxicity of all the extracts i.e. in a dose dependent manner. The low activity may be due to the less percentage of the active compounds present in the extract. The bioactivity directed fractionation can be made to increase the effectiveness of the extract towards the cancerous cell lines by enriching the active chemical components.

3.4. Antimicrobial activity

Higher antimicrobial activities were obtained in the hot ethyl acetate extract compared to the cold hexane: water (1:1) extract and the results are presented in Figs.3 and 4. Antimicrobial activity of ethyl acetate (hot extract) and hexane: water (1:1) extract of *Piper longum* were tested in three bacteria. Among the three bacteria tested, hot ethyl acetate extract showed antibacterial activity against *Escherichia coli, Bacillus subtilis* and was found to be less active for *Staphylococcos aureus.* As the concentration of the drug increases, the % cytotoxicity also increases. Since the % cytotoxicity is less than 50%, we cannot found out the $IC_{50}$ values in the case *Staphylococcos aureus* as the activity is very low. From the studies it is observed that the cold extracts showed less antibacterial activity against the tested bacteria.

3.5. HPTLC profiling

The HPTLC pattern of both the hot ethyl acetate and cold Hexane: water (1:1) extracts shows the presence of several interesting compounds (Fig 5 and 6).It is observed that the major component of hot and cold extracts of *Piper longum* fruits is piperine (with an $R_f$ value of 0.52 to 0.76).But the antioxidant effects of piperine were observed to be high compared with the extracts (22.66 µg/mL for 50% inhibition of the radicals in terms of DPPH assay[4]. Thus the results indicated that the activity directed fractionation and isolation can improve the efficacy.

**Acknowledgements**

The authors would like to thank Director, Head of AGP&NP Division, National Institute for Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram-695019, India for providing necessary facilities and funds to carry out the study.

______________________________________________________________________________

**Fig.1. DPPH Evaluation of hot and cold extracts of *Piper longum* fruit.**

*Values are means of triplicate determinations (n=3) ± standard deviation. EtOAc–ethyl acetate, MeOH–methanol*

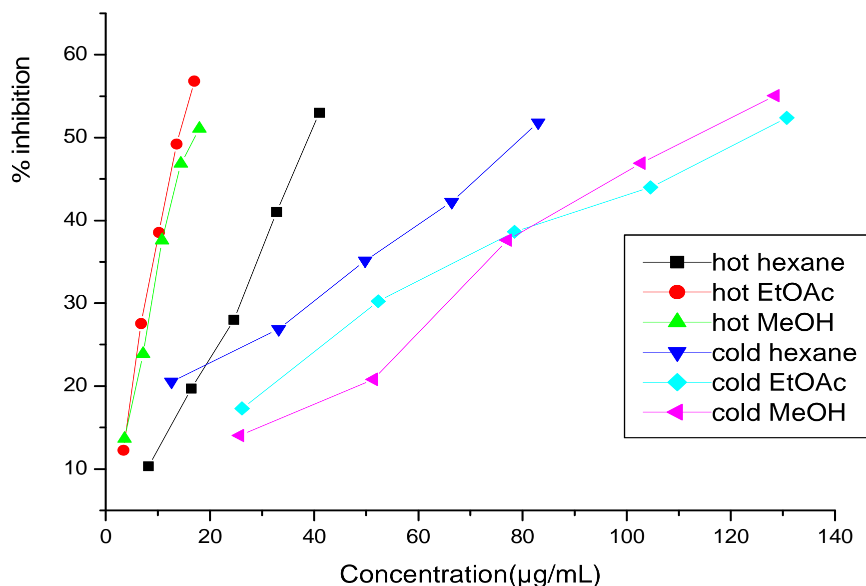



**Fig.2. ABTS Evaluation of hot and cold extracts of *Piper longum* Fruit.**

*Values are means of triplicate determinations (n=3) ± standard deviation. EtOAc–ethyl acetate, MeOH–methanol*

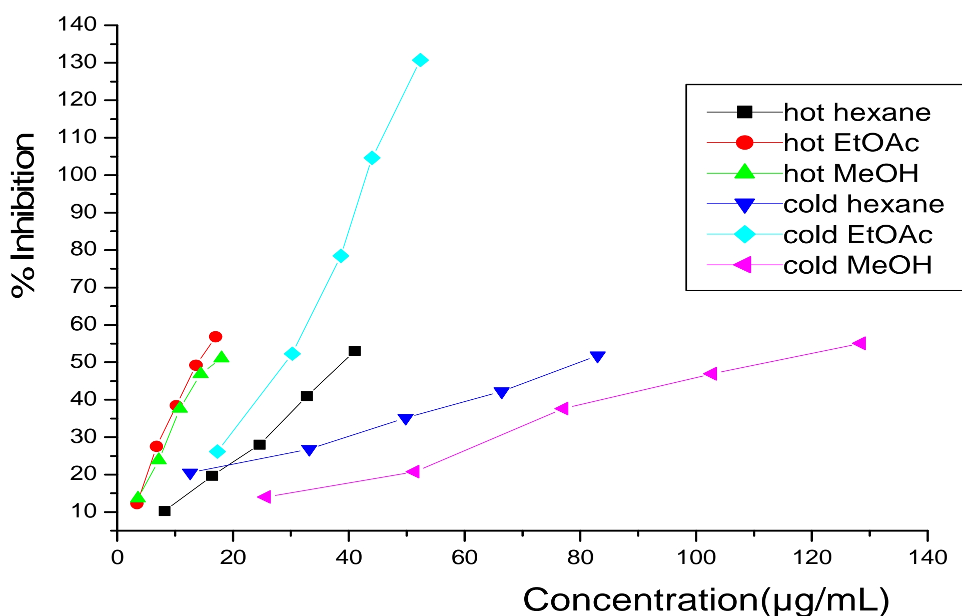


**Fig.3. Anti bacterial activity of hot extract of *Piper longum* fruit**

*Values are means of triplicate determinations (n=3) ± standard deviation. ECOLI - Escherichia coli, STAPH - Staphylococcos aureus, BACILLUS - Bacillus subtilis*

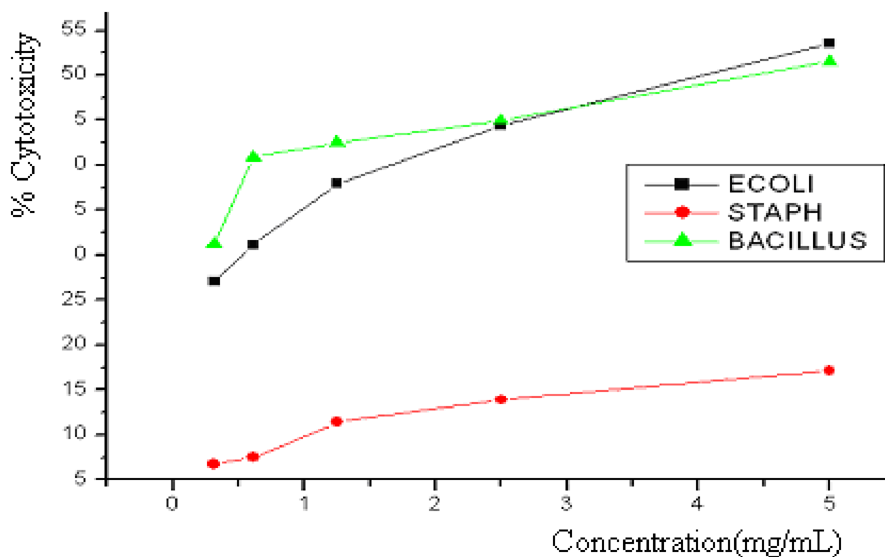



**Fig.4.Anti bacterial activity of cold extracts (1:1) of *Piper longum* fruit**

*Values are means of triplicate determinations (n=3) ± standard deviation. ECOLI - Escherichia coli, STAPH - Staphylococcos aureus, BACILLUS - Bacillus subtilis*

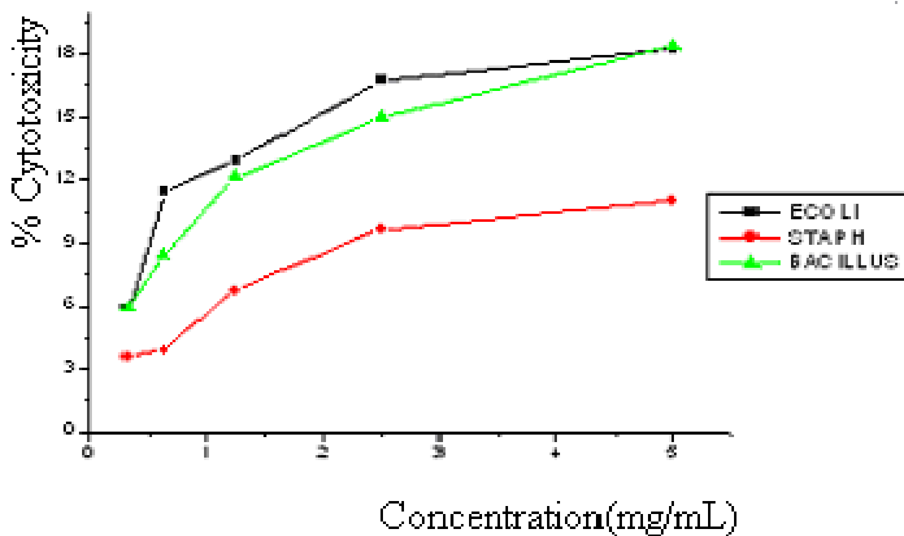


**Fig.5. HPTLC pattern of the hot Ethyl acetate extract**

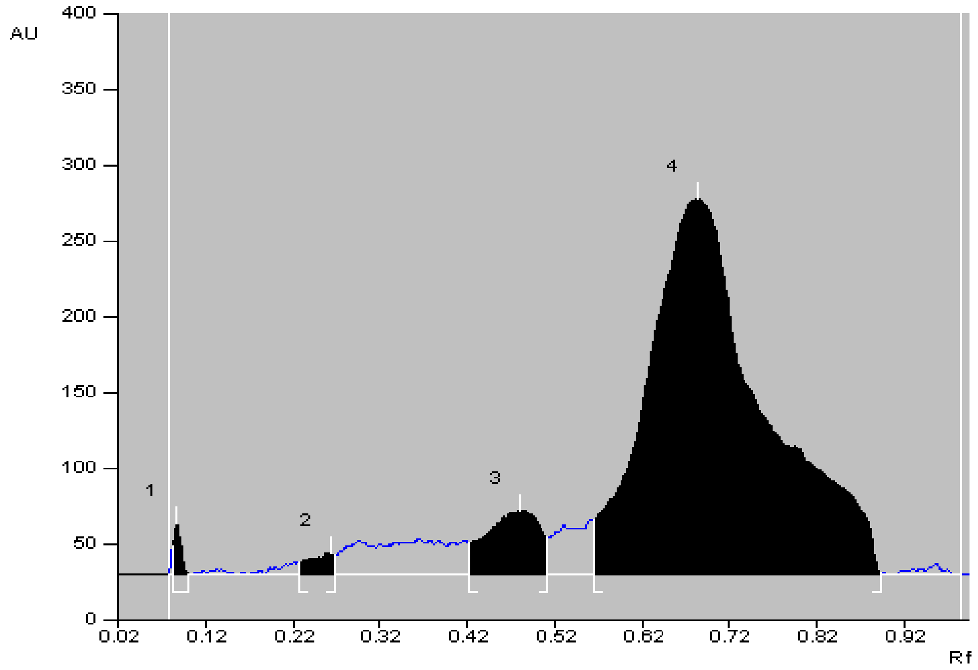
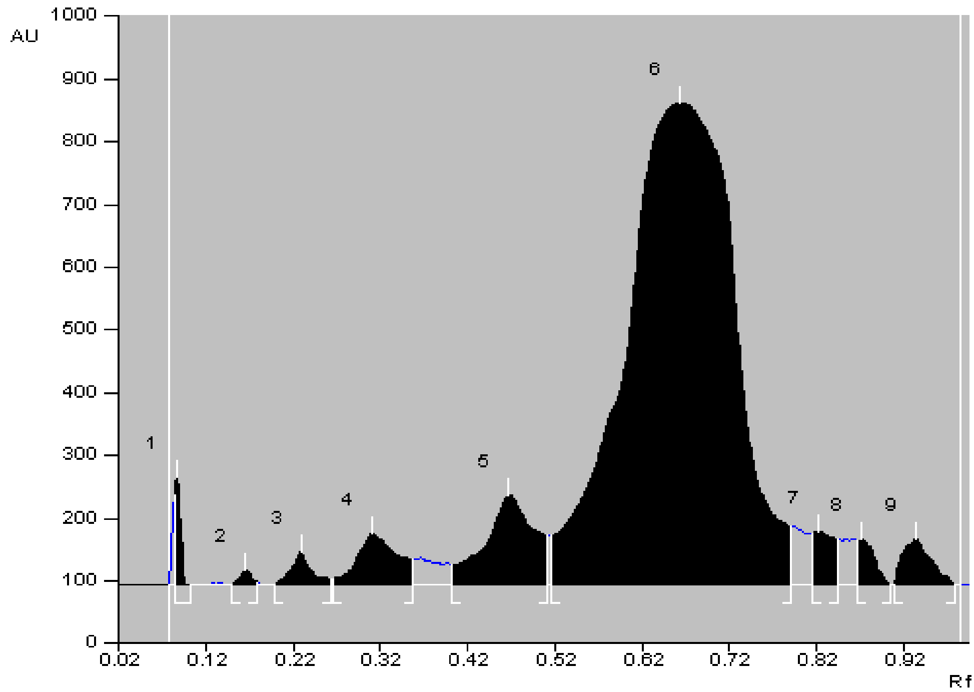



**Fig.6. HPTLC pattern of the cold Hexane: water (1:1) extract**



## CONCLUSION

The study established that the method of extraction of the active principles of *Piper longum* has a profound effect on its biological effects. We proposed hot sequential extraction with ethyl acetate after hexane for *piper longum* to have potent bioactive extracts in terms of its antioxidant, antimicrobial and anti tumor effects. The chemical finger printing of the most active extracts namely hot ethyl acetate and cold hexane: water in the ratio 1:1 indicated the presence of different types of compounds in varying composition. Hot extract of *Piper longum* fruits showed better biological efficacy than the cold extract. The bioactivity directed fractionation procedures increases the effectiveness of activity by enriching the active chemical components.

## REFERENCES

[1] PK Varrier; VPK Wambia; C Ramankutty; Vasudevan Nair R.*Indian Medicinal Plant-a compendium of 500 spices*., **1993; 3,444**.
[2] KR Kirtikar; BD Basu. *Indian medicinal plants*, International book distributors, Dehradun, India.**1987**; 3,2128-2129.
[3] Umadevi Subramanian; Sharmila Poongavanam; A J Vanisree.Studies on the neuroprotective role of Piper longumin C6 glioma induced rats, *Invest New Drugs*., **2009**; DOI 10.1007/s10637-009-9301-1.
[4] SC Jagdale; BS Kuchekar; AR Chabukswar; PD Lokhande; CG Raut. Anti-oxidant activity of *Piper longum* Linn. , *Int. J. Biol. Chem*., **2009**, 3, 119-125.
[5] ES Sunila;G Kuttan. Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine, *Journal of Ethnopharmacology* ., **2004**; 90(2-3),339-346.
[6] Sung-Eun Lee. Mosquito larvicidal activity of pipernonaline, a piperidine alkaloid derived from long pepper, piper longum, *Journal of the American Mosquito Control Association* ., **2000**, 16(3), 245-247.
[7] SP Virinder; CJ Subash; SB Kirpal; J Rajani. Phytochemistry of genus Piper, *Phytochemistry.* , **1997**, 46, 597-673.
[8] Jun Soo Bang;Da Hee Oh; Hyun Mi Choi; Bong-Jun Sur; Sung-Jig Lim; Jung Yeon Kim; Hyung-In Yang; Myung Chul Yoo; Dae-Hyun Hahm; Kyoung Soo Kim. Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1β-stimulated fibroblast-like synoviocytes and in rat arthritis models, *Arthritis Research & Therapy* ., **2009**, 11:R4989.
[9] PD Lokhande: KR Gawai; KM Kodam; BS Kuchekar. Antibacterial activity of extracts of piper longum, *Journal of Pharmacology and Toxicology*., **2007**,2(6): 574-579.
[10] M Abbas Ali; Noor Mahbub Alam; Sarmina Yeasmin; Astaq Mohal Khan; Abu Sayeed M.Antimicrobial Screening of Different Extracts of *Piper longum* Linn., *Research Journal of Agriculture and Biological Sciences*., **2007**,3(6),852-857.
[11] A Kumar; S Panghal ; SS Mallapur ; M Kumar ; Veerma Ram; BK Singh . Anti-inflammatory activity of *Piper longum* fruit oil, *Indian Journal of Pharmaceutical Sciences*., **2009**,71(4),454-456.
[12] P Srinivasa Reddy; Kaiser Jamil; P Madhusudhan; G Anjani; B Das. Antibacterial activity of Isolates from Piper longum and Taxus baccata, *Pharmaceutical biology*., **2001**, 39(3),236-238.
[13] S Mc Donald; PD Prenzler; M Autolovich; K Robards.Phenolic content and antioxidant activity of olive extracts, *Food Chem.,* **2001**, 73,73-84.

[14] MS Blois. Antioxidant determination by the use of a stable free radical, *Nature*, **1958**,181,1199-1200.
[15] R Re; N Pellegrini; A Proteggente; A Pannala; M Yang; C Rice-Evans. Antioxidant activity applying an improved ABTS radical cation decolorizing assay, *Free radicals in biology and Medicine*., **1999**, 26,1231-1237.
[16] Banibrata Das; Juthika Kundu; Sitesh Chandra Bachar; Mohammad Aftab Uddin; Joydeb Kumar Kundu. Antitumor and antibacterial activity of ethyl acetate extract of *ludwigia hyssopifolia* linn and its active principle piperine, *Pak. J. Pharm. Sci.*, **2007**, 20(2), 128-131.
[17] Shihua Wu; Cuirong Sun; Saifeng Pei; Yanbin Lu; Yuanjiang Pan. Preparative isolation and purification of amides from the fruits of *Piper longum* L. by upright counter-current chromatography and reversed-phase liquid chromatography, *Journal of Chromatography A*., **2004**, 1040(2), 193-204.
[18] PK Suresh; S Sucheta; VD Sudarshan; P Selvamani; S Latha. Antioxidant activity in some selected Indian medicinal plants, *Afr. J.Biotechnol*., **2008**, 7,1826-1828.