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Isolation, identification and antimycobacterial evaluation of piperine from *Piper longum*

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ABSTRACT

Piperine, an alkaloid responsible for the pungency of black pepper & long pepper. Systemic pharmacological studies on Piperine have revealed that this compound elicited diverse pharmacological activities, analgesic, anti-pyretic, anti-inflammatory, anti-convulsant, CNS-depressant activities & anti-mycobacterial activity. Piperine was isolated from Piper Longum Linn. (Piperaceae). The identity of the compound was confirmed by qualitative analysis and TLC. The purity of the compound was ascertained by UV, FTIR studies and by differential scanning calorimetry (DSC). The anti-mycobacterial activity of the isolated piperine was tested against M. tuberculosis H37Rv strains with standard of Rifampicin by Resazurin Microplate Assay (REMA).

Key words – *Piper Longum*, Piperine, Resazurin Microplate Assay(REMA), Micobacterium Tuberculosis

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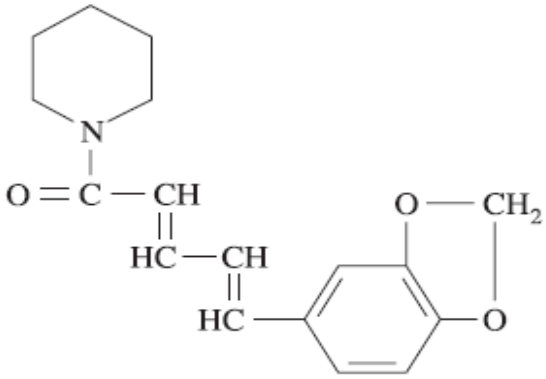
INTRODUCTION

Piper Longum (*Piperacae*), is found both wild as well as cultivated, throughout the hotter parts of India from central to the north-eastern Himalayas. The herb also grows wild in Malaysia, Singapore, Bhutan, Myanmar and elsewhere. The plant is a slender, aromatic and perennial climber with woody roots, numerous wide ovate and cordate leaves. The inflorescence is a cylindrical, pedunculate spike; the female flower is up to 2.5 mm long and 4-5 mm in diameter, the male flowers being larger and more slender. The fruits are small, ovoid berries, shiny blackish green, embedded in fleshy spikes.

Fig. No: 1 Structure of Piperine



Piperine is the principle pungent agent which is a constituent of piper species. It is a Piperine derivative with various traditional uses such as analgesic, antipyretic, CNS depressant, anti-inflammatory, antitumor and hepatoprotective activities.

Tuberculosis is a major health problem throughout the world, infecting more than 8 million individuals each year. It is the world's leading cause of death from infectious disease. Tuberculosis is an infectious disease caused by the ***Mycobacterium tuberculosis*** bacillus. Isoniazid, Rifampicin, Ethambutol and Pyrazinamide are the most effective oral anti-TB drugsPatient non-compliance to the long term therapy, emergence of Multi Drug Resistant (MDR) and Extensively Drug Resistant(XDR) tuberculosis, adverse effects (toxic, idiosyncratic and hypersensitivity reactions) anti tubercular agents and degradation of the drug before reaching their target are the main disadvantages of conventional therapy.

Herbs have been used for many years to treat respiratory problems like tuberculosis. Some can inhibit bacterial growth and others can help strengthen the immune system so the body can fight off the infection. Garlic, Onions, Astragualus, Indian gooseberry etc are some of the drugs used for the treatment of tuberculosis. Considering the potential and bioadversity of natural flora, it is important to explore it for new drug prototypes and bio-enhancers.
The aim of the present study is to isolate, identify the alkaloid piperine from *Piper Longum* and to ascertain its purity by various methods such as FTIR, UV and DSC. Anti-mycobacterial property of the drug is also evaluated.

MATERIALS AND METHODS

COLLECTION AND AUTHENTIFICATION OF THE PLANT

Fruits of *Piper longum* were collected from an ayurvedic shop located in Muvattupuzha, Ernakulam district Kerala in the month of august 2010. The plant was identified and authenticated by Dr. A.K. Pradeep, Curator, Department of botany, University of Calicut and a voucher specimen was deposited at the university Calicut herbarium.

PRELIMINARY TREATMENT

The foreign, earthy matter and residual materials were removed carefully from the fruits and then cleaned and dried in the shade. It was then mechanically reduced to coarse powder form.

PREPARATION OF THE EXTRACT

Placed 15g of ground long pepper in a 250 ml Soxhelt apparatus, added 150 ml of 95% ethanol and 5 boiling chips, and heated at reflux for 2h. Filtered the mixture by suction filtration and then concentrated the filtrate to a volume of 10-15ml by simple distillation or by use of a rotary evaporator.

QUALITATIVE PHYTOCHEMICAL ANALYSIS

The 95% ethanolic extract of dried fruits of *Piper longum* were subjected to the following chemical test separately for identification of alkaloids.

Detection of Alkaloids

a. **Mayer's Test:** To 1ml of the extract added to 2ml of Mayer's reagent, a dull white precipitate indicates presence of alkaloid
b. **Dragendroff's Reagent:** To 1ml of the extract added 1ml Dragendroff's reagent, an orange red precipitate indicates presence of alkaloid
c. **Hager's Test:** To 1ml of the extract added 3ml of Hager's reagent, a yellow precipitate indicates presence of alkaloid
d. **Wagner's Test:** To 1ml of the extract added to 2ml of Wagner's reagent, a reddish brown precipitate indicates presence of alkaloid

ISOLATION OF PIPERINE

To 10mL of a 10% solution of KOH in 95% ethanol contained in a 125mL Erlenmeyer flask added the concentrated pepper extract. Heated the resulting solution and add water drop wise. A yellow precipitate formed. Added water until no more solid appears to form and then allowed the mixture to stand at least overnight collect the solid by suction filtration and recrystallized it with 10-20mL of acetone.

IDENTIFICATION OF ISOLATED PIPERINE

A. THIN LAYER CHROMATOGRAPHY

The Piperine (in µL) was subjected on to the precoated and activated (kept the plates in oven for 1hr at 70^0C) silica gel TLC plates. The mobile phase is Toluene: Ethyl acetate in 70:3 ratios and the detecting agent is Vanillin-

Sulphuric acid reagent. After the TLC run and spraying the detecting agent the yellow spots of Piperine were identified visually. Rf value was calculated.

CHARACTERISATION OF PURE PIPERINE

B. FTIR SPECTROSCOPY STUDIES

15mg of isolated Piperine and 300 mg of KBr (Potassium Bromide) was taken in a mortar and mixed. The mixture was placed into an evacuable die on a hydraulic laboratory press and compressed under 10-ton pressure to form a transparent pellet. Placed the KBr pellet in a pellet holder and put it into the sample beam of an IR spectrophotometer. Spectrum of the pellet is taken from 4000 cm^{-1} to 400 cm^{-1}

C. UV SPECTROSCOPY STUDIES

Dissolved 100mg of the isolated Piperine in 100ml volumetric flask made the volume up to the mark with ethanolic HCl (1:1) (100µg/ml). From this solution 5ml is taken to 100ml volumetric flask and made up the volume (50µg/ml). The sample is scanned in UV Visible spectrophotometer. The peaks obtained in each spectrum were compared.

D. DIFFERENTIAL SCANNING COLORIMETRY

Samples (3-5 mg) were placed in aluminium pans and lids at constant heating range of 15°C/min, covering temperature range to 300°C. Nitrogen was used as purge gas through DSC cell. The instrument measures the difference in the heat flow between the sample and the reference.

ANTI-MYCOBACTERIAL STUDY OF PIPERINE

METHOD

The anti-TB activity of the compounds was tested by Resazurin Microplate Assay (REMA) as per Martin et al (2003) with slight modification. Resazurin, a redox dye, is blue in its oxidized state. In the presence of viable cells it is reduced into resorufin, which is pink in color.

TEST STRAINS OF Mycobacterium Tuberculosis

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth (Difco BBL, Sparks, MD, USA) supplemented with 10 % OADC (Becton Dickinson, Sparks, MD, USA) and 0.5% glycerol. The optical density of the bacterial culture was adjusted to McFarland 1.0 unit and 50 µl from this suspension was used as the inoculum.

PREPARATION OF SAMPLE SOLUTION

Stock solutions of the test compounds were prepared in Dimethyl Formamide (DMF) and were added to fresh medium in the wells of a 96-well microplate to which 50 µl inoculum was added making the total assay volume 200 µl. The final concentrations of the test molecules were 1, 10 and 100 µg/ml.

PROCEDURE

Stock solutions of the test compounds prepared in Dimethyl Formamide (DMF) were added to fresh medium in the wells of a 96-well microplate to which 50 µl inoculum was added. The final concentrations of the test molecules were 1, 10 and 100 µg/ml. Growth control wells contained medium and *M. tuberculosis* H37Rv alone. Rifampicin (1.0 µg/ml) served as positive control for inhibition of growth. Negative control wells contained the highest volume of DMF used in test wells without any compound. After incubation at 37^0C for 7 days, 15 µl of 0.01% resazurin (Sigma, St. Louis. MO, USA) solution in sterile water was added to the first growth control wells and incubated for 24 hours. Once the first set of growth controls turned pink, the dye solution was added to the second set of growth controls and the test wells, and incubated for 24 hours at 37^0C. Blue color in the wells containing the test compounds would indicate inhibition of growth and pink would indicate lack of inhibition of growth of *M. tuberculosis.*

RESULTS AND DISCUSSION

EXTRACTION

The research work was initiated with the extraction of powdered *Piper longum* fruits using 95% of ethanol and the percentage yield obtained was found to be 5.3%

PHYTOCHEMICAL EVALUATION FOR ALKALOIDS

S.No	TESTS	ETHANOL EXTRACT
a.	Dragendroff's Test	+ve
b.	Wagner's test	+ve
c.	Hager's test	+ve
d.	Mayer's Test	+ve

ISOLATION

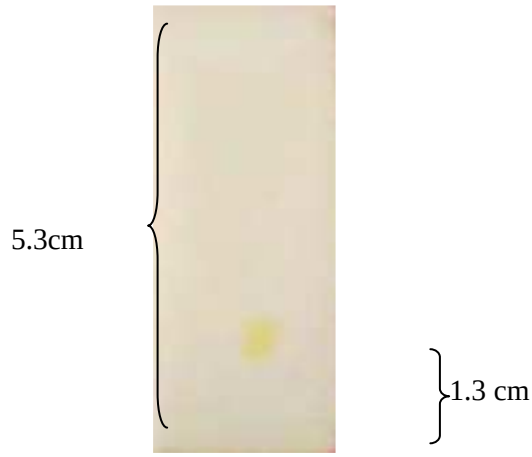
The Piperine was isolated from the long pepper fruits and the percentage yield of Piperine from pepper powder was found to be 2.5%.

IDENTIFICATION OF ISOLATED PIPERINE

THIN LAYER CHROMATOGRAPHY

After isolation, Piperine is identified by TLC. The standard Rf- value of Piperine from the literature was 0.25. The Rf- value of purified Piperine from TLC was found to be 0.245. Data is given in fig:1

Fig.No: 2 Thin layer chromatography of isolated Piperine

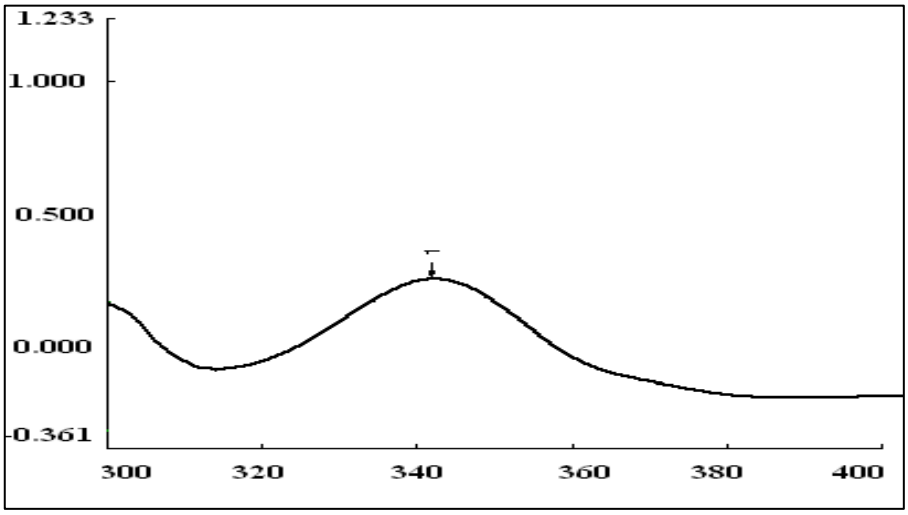


Rf value of isolated Piperine: 0.245

DETERMINATION OF UV MAXIMA OF ISOLATED PIPERINE

UV maxima of isolated Piperine was taken in 30 parts of ethanol and UV maxima obtained at 343nm

Fig. No: 3 Determination of UV maxima of isolated Piperine



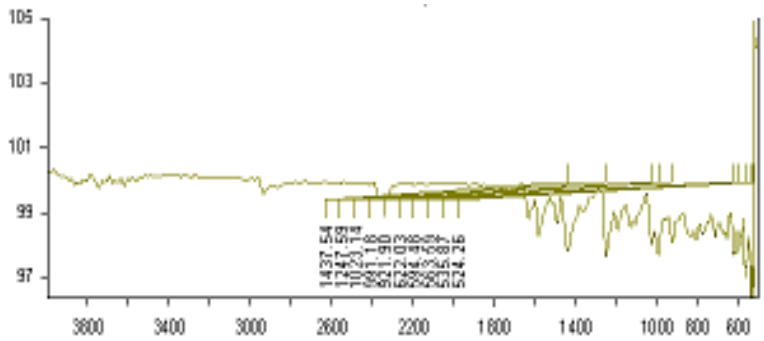
FTIR STUDIES

The peaks obtained after taking the FTIR spectra of pure Piperine was shown in

Table. No1: Types of peaks obtained for isolated Piperine

S.No	Types of phenomenon	Wave number cm^{-1}
1	Symmetric and asymmetric stretching of C=C (diene) 1	1635; 1608
2	Aromatic stretching of C=C (benzene ring) 1	1608; 1580; 1495
3	Stretching of -CO-N	1635
4	Asymmetric and symmetric CH_2 stretching, aliphatic C-H stretching	2925; 2840
5	CH2 bending	1450
6	Asymmetrical stretching =C-O-C	1250; 1190
7	C-O stretching (most characteristic)	930
8	Out-of-plane C-H bending 1,2,4-trisubstituted phenyl (two adjacent hydrogen atoms)	850; 830; 805

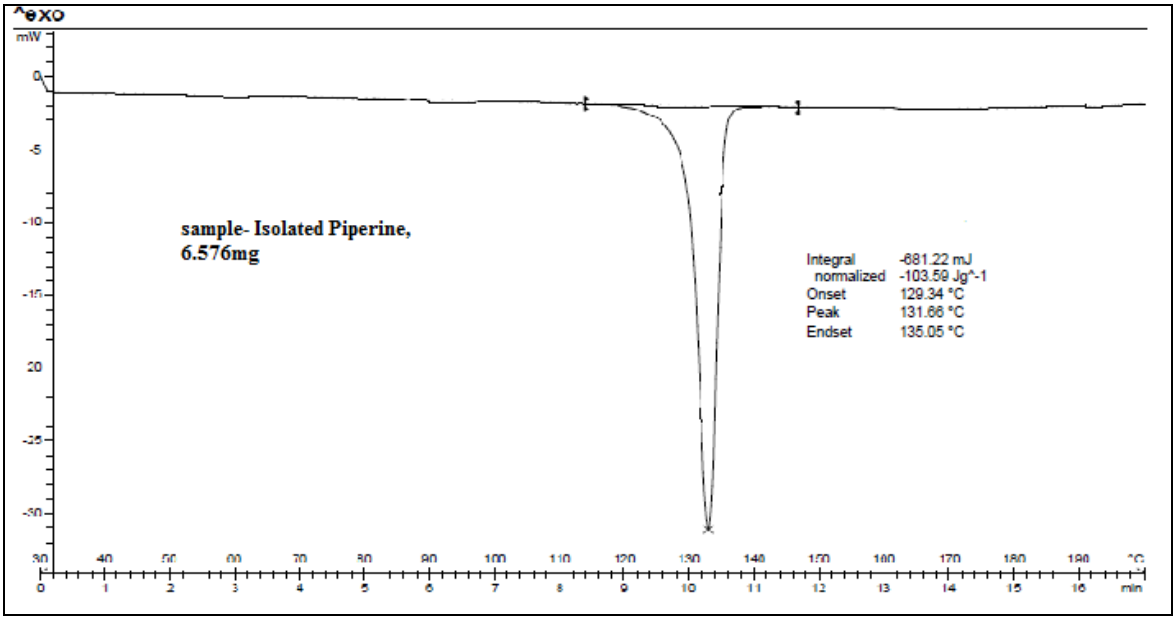
Fig. No: 4 FTIR spectra of isolated Piperine



DIFFERENTIAL SCANNING COLORIMETRY

Thermal analysis of the isolated Piperine was done by using Differential Scanning Colorimeter and the result obtained shown below.

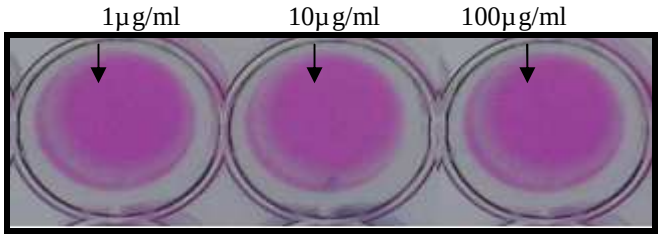
Fig. No: 5 DSC Thermogram of Isolated Piperine



ANTI-MYCOBACTERIAL SCREENING OF PIPERINE MICROSPHERES

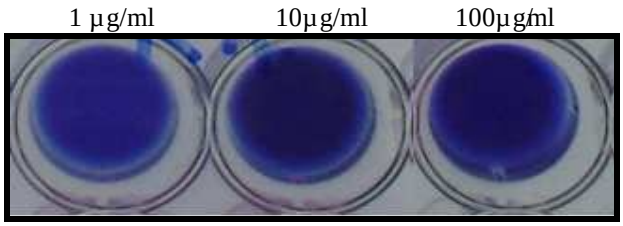
When 15 µl of 0.01% resazurin in sterile water was added to the plate after incubation of the microplate and incubated for 24 hrs at 37^0C negative control showed pink colour. The colour of the culture changed to blue at 100µg/ml which showed the inhibition of growth. Comparison of the growth inhibition of Rifampicin was also performed and the results were shown in figure 8

Fig.No: 6 Anti-Mycobacterial screening of Negative control



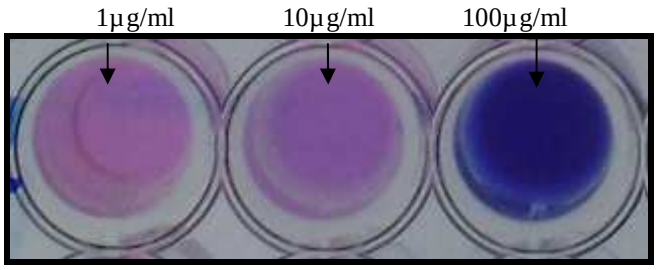
Pink colour of the micro plates indicated that there is no inhibition of growth as the control containing the highest volume of DMF used in test wells without any compound.

Fig. No: 7 Anti-Mycobacterial screening of positive control Rifampicin



Blue colour of the microplates indicated that there is an inhibition of growth of *M. tuberculosis* H37Rv strains in all the Rifampicin concentrations (1µg/ml, 10µg/ml, 100µg/ml).

Fig.No: 8. Anti-Mycobacterial screening of isolated Piperine



Blue colour of the microplates indicated that there is an inhibition of growth of *M. tuberculosis* H37Rv strains at a concentration of 100µg/ml.

CONCLUSION

Alkaloid Piperine was successfully isolated from the Ethanolic (95%) extract of dried fruits of Piper longum. Isolated Piperine was identified by various methods such as Thin Layer Chromatography (TLC) and UV-Visible spectroscopy. The purity of isolated piperine was confirmed by FTIR and DSC studies. From the Anti-mycobacterial studies it is concluded that isolated piperine has better anti-mycobacterial activity when compared to Rifampicin.

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