

**IDENTIFICATION OF CLASSES OF COMPOUNDS AND ESTIMATION OF FLAVANOID AND PHENOL FROM SEEDS OF *PIPER LONGUM* AND *PIPER NIGRUM*****PRIYA N. C* AND P. SARAVANA KUMARI¹****Research and Development centre, Bharathiar University, Coimbatore, India.**¹Department of Microbiology, Sree Narayana Guru College, Coimbatore, India.***ABSTRACT**

The present investigation deals with the identification of phytochemical compounds present in chloroform and methanol extract of *Piper longum* and *P. nigrum*. It showed the presence of phytochemical constituents like tannins, terpenoids, and phenolic compounds, alkaloids and flavonoids. The total phenolic content was determined using the Foin-Ciocalteu assay and total flavonoids by the Aluminium chloride colorimetric assay. Percentage of phenol in mg equivalence of gallic acid of both the peppers in chloroform and methanol extract was same and the percentage of flavonoids in mg equivalence of Quercetine was high in both pepper species. In order to find out the chemical compounds GC-MS analysis was done. It showed the presence of 10-15 volatile compounds and it gave an integrated percentage of many compounds. The black pepper in chloroform contains 15 components, out of that highest integrated percentage of Aspartic acid were present. In the case of long pepper in chloroform extracts gave 14 components, out of that the highest integrated percentage of Micosporine and Graveoline were present. However further studies are focussed towards the importance of these compounds to prevent diseases.

KEYWORDS: *Piper longum*, *Piper nigrum*, Phenol, Flavonoid, GCMS**PRIYA N. C**

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INTRODUCTION

The term spices refer to aromatic or pungent vegetable substances used for flavouring foods and have several commercial uses. Since ancient times people have used spices for preventing food deterioration and pathogenic diseases. Spices have become today as an integral part of our daily diet and many of the spices are widely used to flavour food beverages, for food preservations, medicinal preparations, as masalas and also included in the prescriptions of cosmetics, perfumery, bakery goods and various products. Even today spices are used as an ingredient in drug preparations in Unani, Homeopathy and Ayurveda systems of medicine^{1,2,3,4}. One of the commonly used spices is pepper which has the characteristics aroma and flavour due to their chemical substances especially the volatile oil. *Piper nigrum* is famous as the spices king due to its pungent quality⁵. Phytochemicals are compounds present in plants that are used as food and medicine to protect against illness and to maintain human health⁶. Phytochemicals have antioxidant or hormone-like effect which helps in fighting against many diseases including cancer, heart disease, diabetes, high blood pressure and preventing the formation of carcinogens on their target tissues⁷. Black pepper (*Piper nigrum*) is used to treat asthma, chronic indigestion, colon toxins, obesity, sinus, congestion and fever⁸. It has been shown that Piper has antimicrobial activity⁹ and some have already produced compounds, effective against antibiotic resistant strains of bacteria¹⁰. *Piper longum* L. is popularly known as "pippali" or "long pepper", It is an important medicinal plant in Indian traditional medicine. It is distributed throughout the Indian subcontinent¹¹. It has been reported that the fruits extract of the plant had antidepressant, antinociceptive, anti-inflammatory, antioxidant, anticancer, antidiabetic, antibacterial, antifungal, antitumor, anti-allergic, antiasthmatic, antifertility, antiulcer, antihypertensive, antiplatelet, antithyroid, immunomodulatory, antiamebic, hepatoprotective, vasodilating, insecticidal and mosquito larvicidal activities^{12,13,14,15}. The dried fruit of *Piper nigrum* and *Piper longum* (belonging to the family Piperaceae), commonly used as a spice and seasoning is known to possess a multitude of pharmacological activities. These two plants are extensively used in Ayurvedic system of medicine. The present study aimed at phytochemical analysis of solvent extract/fractions of two medicinal plants; *Piper nigrum* and *Piper longum* to detect the presence of various phytochemical constituents which are essential for its medicinal properties.

MATERIALS AND METHODS

1) Collection and drying

The dried seeds of *Piper nigrum* and *P. longum* fruit were collected from local market. The materials were sun dried for 3 days and powdered. The powdered samples were hermetically sealed in separate polythene bags until the time of the extraction.

2) Extraction of pepper using Methanol and Chloroform¹⁶

One gram of powdered *Piper nigrum* and *P. longum* were extracted successively with 20 ml of Methanol and chloroform using reflux method. The extracts were then filtered with Whatman filter paper and stored in a sterile container.

3) Procedure for Phytochemical analysis¹⁷

a. Test for Alkaloids

Mayer's Test: To 1 ml of the filtrate 2 drops of Mayer's reagent added along the sides of the test tube. Presence of precipitate indicates presence of alkaloids

b. Test for Tannin

Ferric chloride test: About 50 mg of extract was dissolved in distilled water and to this 0.5 ml of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

c. Test for Terpenoids

To 2 ml of the extract, 2ml of chloroform and then concentrated sulphuric acid was added to form a layer. A reddish brown precipitate indicates presence of terpenoids.

d. Test for Saponin

Foam test: About 100 mg of extract was diluted with distilled water to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. Formation of 2 cm layer of foam indicated the presence of saponins.

e. Test for Glycosides

Molich's test: Two ml of filtrate was added with two drops of alcoholic solution of α -naphthol. The mixture was shaken well and one ml of concentrated sulphuric acid was added along the sides of the test tube and allowed to stand. A violet or purple ring indicated the presence of carbohydrates

f. Test for Phenolic compounds

Lead acetate test: About 50 mg of extract was dissolved in distilled water and to this three ml of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

g. Test for flavonoids

Shinoda's test: About 50 mg of extract was dissolved in alcohol, few magnesium turnings and concentrated hydrochloric acid (drop wise) were added. The appearance of magenta colour showed the presence of flavonoids.

4) Estimation of Phenol

Phenol was estimated by using the method of Bray and Thorpe(1954). Two ml of sample(80mg/ml) was introduced into test tubes followed by 1 ml of Folin-Ciocalteu's reagent. The tubes were allowed to stand for 3 min. At the end of this period, 4 ml of sodium carbonate (20%) was mixed thoroughly.

Kept it in boiling water for 1 minute and then cooled. The absorbance was measured at 650 nm. The total phenolic contents of the methanolic and chloroform extract of black pepper and long pepper were calculated with the help of a standard calibration curve and are reported as gallic acid equivalent (mg/g of dry weight)¹⁸.

5) Estimation of Flavonoid

The total flavonoids were estimated by the Aluminium chloride method. Two millilitre solution (80mg/ml) of the extract in methanol and chloroform was separately mixed with 1 ml of 10 % aluminium chloride, 1 ml of 1 N potassium acetate and mixed well. The absorbance of the reaction mixture was measured at 650 nm. The quantity of the flavonoids present in the extracts were calculated as % mg equivalence of Quercetin¹⁹.

6) GC-MS Analysis

Gas chromatography-mass spectrometry (GC/MS) was used to determine the composition of the solvent extracts of two pepper varieties. For the gas chromatographic and mass spectroscopic analysis the equipment used was Agilent network GC. The detector was flame ionisation detector with a fuel gas of hydrogen and the carrier gas was nitrogen and the flow rate was maintained at 2.5ml/min. The column used in the analysis is HP-

5MS (5%phenyl methyl siloxane) with a dimension of (30mx320umx0.25um). The injection temperature was 280°C and the detector temperature is 250°C. The oven temperature was maintained at 90°C for the first 2 minutes and 90°C to 200°C increased @2°C/min. After that the temperature was increased to 250°C @3 °C /min and 250°C was maintained till the end. The split ratio was 50:1 and the split flow was 60ml/min. The total run time was 60 minutes²⁰.

RESULTS

Phytochemical analysis

The black pepper and long pepper samples were extracted with methanol and chloroform and subjected to phytochemical analysis. Qualitative analysis results confirmed the absence of saponins and glycosides. Tannins, Terpenoids, alkaloids, flavonoids and phenolic compounds were present in the organic extracts from *Piper nigrum*. But in *P.longum*, saponins, flavonoids and glycosides were absent and tannins, terpenoids and phenolic compounds were present in the organic extracts. Concentration of phenolic compounds and terpenoids were high in both extracts which is shown in Table 1 and 2 (fig. 2.).

Table 1
Phytochemical analysis of Piper nigrum
extracts of methanol and chloroform

Phytochemicals	Methanol	Chloroform
Alkaloids (Mayer's Test)	+	-
Tannins (Ferric chloride test)	+	-
Terpenoids	+	+++
Saponins (Foam test)	-	-
Glycosides (Molich's test)	-	-
Phenolic compounds (Lead acetate test)	+++	-
Flavonoids (Shinoda's test)	++	-

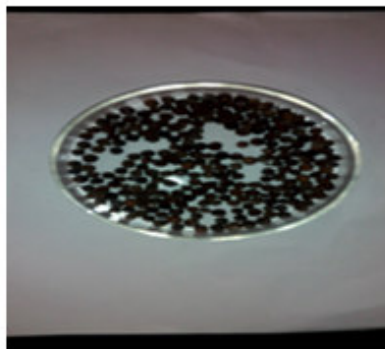
Keys: - Negative; + Mild positive; ++ Average positive; +++ Large positive

Table 2
Phytochemical analysis of Piper longum extracted by methanol and chloroform

Phytochemicals	Methanol	Chloroform
Alkaloids (Mayer's Test)	-	-
Tannins (Ferric chloride test)	+	-
Terpenoids	+	+++
Saponins (Foam test)	-	-
Glycosides (Molich's test)	-	-
Phenolic compounds (Lead acetate test)	+++	+
Flavonoids (Shinoda's test)	-	-

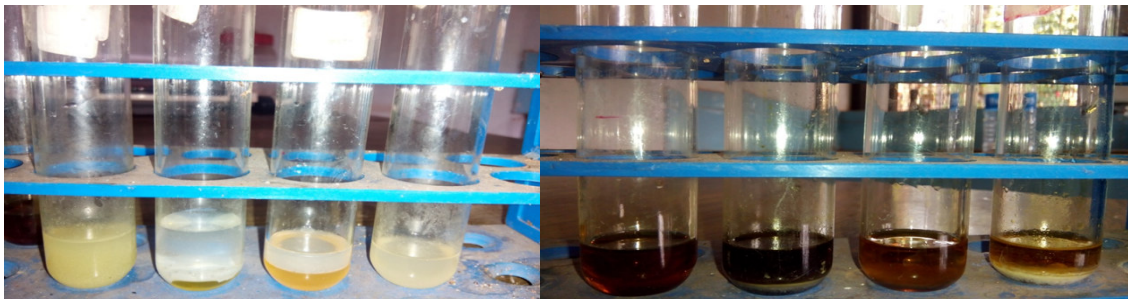
Keys: - Negative; + Mild positive; ++ Average positive; +++ Large positive

Figure 1
Samples collected



Long pepper, Black pepper and tubes showing the Black pepper in chloroform, methanol and Long pepper in chloroform and methanol extract respectively

Figure 2
Phytochemical analysis of pepper samples



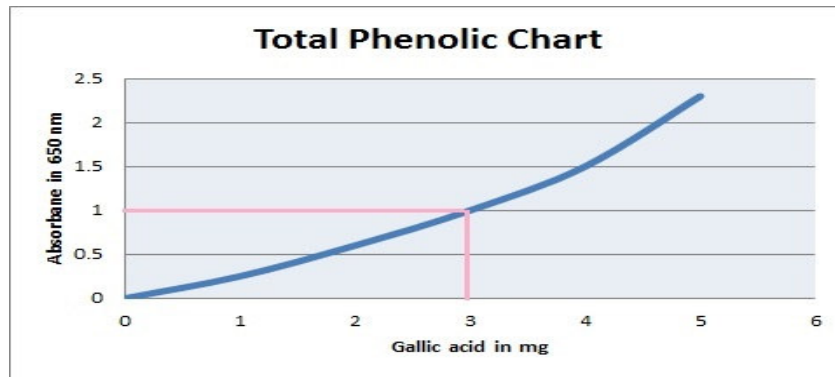
Test tube showing results of Phenolic compounds (Lead acetate test) and terpenoids. The tubes are black pepper methanol, black pepper chloroform, long pepper methanol, long pepper chloroform respectively.

2. Phenol Estimation

The amount of total phenolics was investigated by the Folin-Ciocalteu method. The total phenolic content was expressed as gallic acid equivalents (mg gallic acid/ml of extract). The total phenolic contents of the methanolic and chloroform extract

of black pepper and long pepper were calculated with the help of a standard calibration curve (Graph: 2) and are reported as gallic acid equivalent (mg/g of dry weight). Quantitative assay revealed that concentration of phenol in both extracts are 3 mg/ml.

Graph 2
Standard graph of Gallic acid



3. Flavonoid estimation

The total flavonoid content in spice extracts was also analysed using Aluminium chloride method using Quercetin as standard. The quantity of the flavonoids present in the extracts were calculated as % mg equivalence of Quercetin. Quantitative assay revealed that concentration of and flavonoids ranges between 10 – 90 µg/ml of the extract. The results for total phenolic and total flavonoid percentage and the ratio of total

flavonoids/phenolics in the sample are presented in the table 3. Phenol % mg equivalence of Gallic acid of both the pepper in chloroform and methanol extract is same and the flavonoids % mg equivalence of Quercetin is high in the case of chloroform extract of both the pepper species. The flavonoid phenol ratio of black pepper and long pepper in chloroform extract is high compared to methanol extract.

Graph 2
Standard graph for estimation of flavonoids

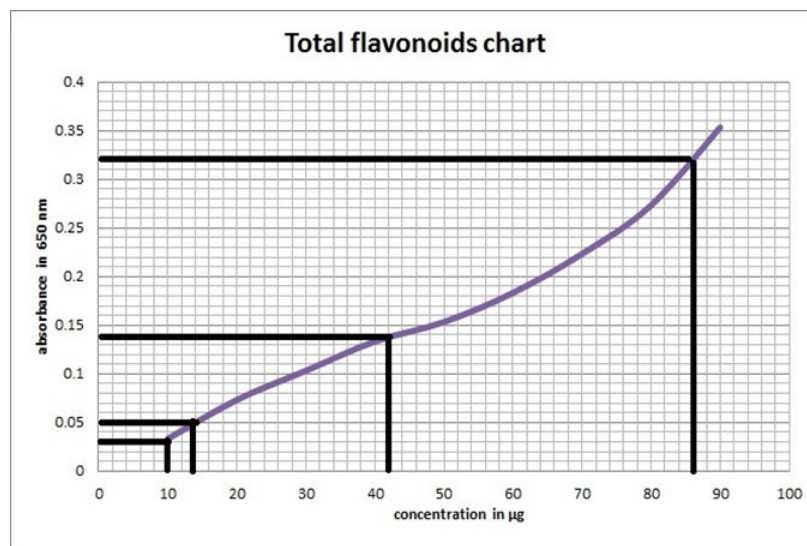


Table 3
Total Phenolics and flavonoids acid content in the pepper extracts

Sample	Phenol mg equivalence Gallic acid	% of Flavonoids equivalence of Quercetin	% mg Flavonoids/ Phenol ratio
Black pepper+methanol	3.75%	0.0175%	0.0047
Black pepper+chloroform	3.75%	0.108%	0.0288
Long pepper +methanol	3.75%	0.0125%	0.003
Long pepper+chloroform	3.75%	0.0525%	0.014

4. GC-MS Analysis

GC-MS analysis of the chloroform extracts of *Piper nigrum* and *P. longum* showed the presence of 10-15 volatile compounds. The GC-MS of black pepper and long pepper in chloroform extracts gave an integrated percentage of many compounds. The black pepper in chloroform contains 15 components out of that highest integrated percentage of Aspartic acid is present and

moderate amount of Limonene, Betasetosterol, and Asarinin (table 4). In the case of long pepper in chloroform extracts showed 14 components out of that the highest integrated percentage of Micosporine and Graveoline was present and moderate amount of Dihydroxymethyl anthraquinone, Caryophyllene, Damnacanthal and Coumaric acid (table 5). The report is given as integrated peak in figure 2 and 3

Table 4
Components of black pepper in chloroform extract

Serial Number	Name Of The Compound	Molecular Mass	Integrated Percentage
1.	Linoleic Acid	280.45	0.23
2.	Lucidin	287.32	0.01
3.	Scopoletin	192.17	0.54
4.	Arginine	174.21	2.65
5.	Aspartic Acid	133.11	26.02
6.	Beta Setosterol	414.72	3.02
7.	Asarinin	354.36	2.56
8.	Limonene	136.24	8.25
9.	Caryophyllene	204.36	0.02
10.	Damnacanthal	282.25	0.45
11.	Rubichloric Acid	414.37	0.001
12.	Rubiadin	254.24	0.65
13.	Physcion	284.27	0.56
14.	Alizarin	240.22	5.25
15.	Ricinoleic Acid	298.47	6.25

Figure 2
GCMS data report of black pepper in chloroform extract

GCMS DATA REPORT AGILENT NETWORK GC

User : Admin
Sample : Priya-1
Inj. Volume : 10 ul.
Data Name : C:\GCMSsolution\FID
Method Name : C:\GCMSsolution\Uwin

GCMS Spectrum

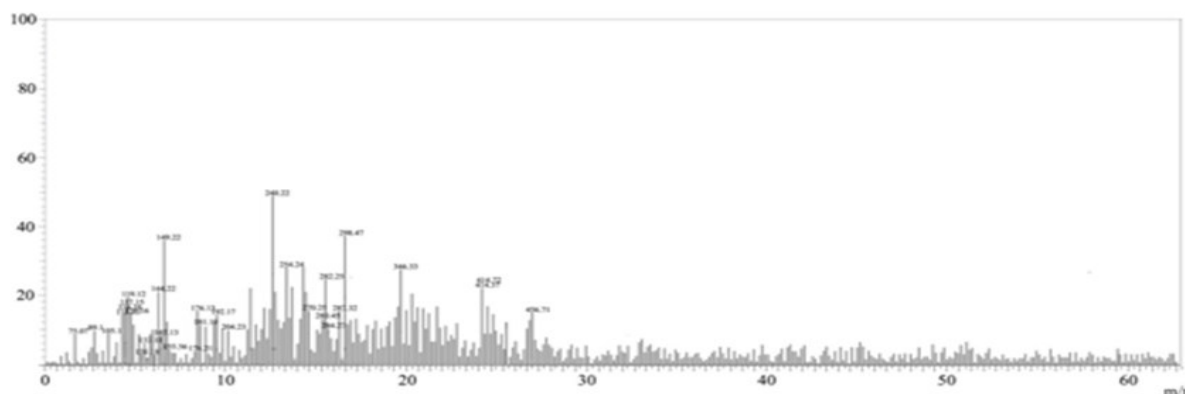


Table 5
Components of long pepper in chloroform extract

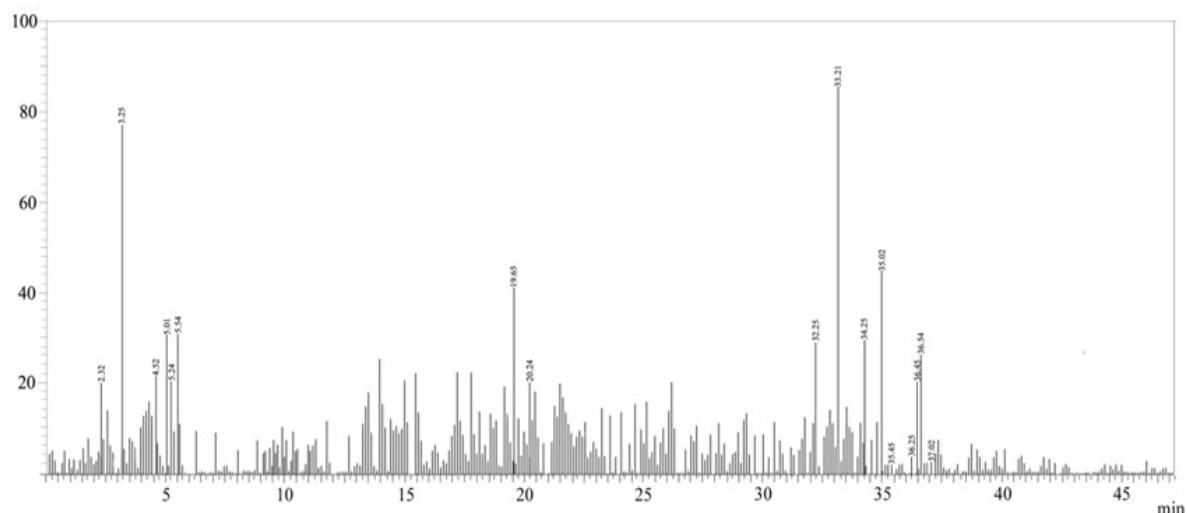
Serial Number	Name Of The Compound	Molecular Mass	Integrated Percentage
1.	Piperitone	152.24	0.002
2.	Citronellole	156.27	0.03
3.	Obotenic Acid	158.12	0.05
4.	Coumaric Acid	163.16	2.32
5.	Dihydroxy Methylanthraquinone	254.25	5.24
6.	Anantine	253.31	0.08
7.	Micosporine	261.28	10.25
8.	Erythroidine	273.34	2.65
9.	Graveoline	279.30	8.01
10.	Acrophylline	283.33	0.54
11.	Piperine	285.34	0.04
12.	Limonene	136.24	0.08
13.	Caryophyllene	204.36	5.26
14.	Damnacanthal	282.25	3.25

Figure 3
GCMS data report of long pepper in chloroform extract

GCMS DATA REPORT AGILENT NETWORK GC

User : Admin
 Sample : Priya-2
 Inj. Volume : 10 ul.
 Data Name : C:\GCMSsolution\FID
 Method Name : C:\GCMSsolution\Uwin

GCMS Spectrum



DISCUSSION

Spices are one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives

to enhance aroma and flavour²¹. Phytochemicals are natural bioactive compounds present in the plants which are responsible for the therapeutic action of the plants in many disease conditions. Phytochemicals having solubility in ethanol include tannins, polyphenols, polyacetylenes, flavonol, sterols and alkaloids²². Pepper varieties showed

the presence of phenolic compounds, flavonoids, alkaloids and terpenoids. In a previous study the fruit extract of long pepper shows all the solvent extract gives positive result for alkaloids, tannin, phenol and sterol, flavonoids present in methanol extract while not present in Aqueous and hexane extract²³. Studies have reported that spicy tang of pepper is due to the presence of piperamides which are the pungent bioactive alkaloids accumulate in the skin and seeds of the fruit. Among them piperine is the major chemical constituent responsible for the bitter taste of the black pepper²⁴. In the present study also qualitative estimation of alkaloids were found to be high in both varieties of pepper. In previous studies it was reported that the total phenolic and flavonoid contents in the methanolic extract of *P. nigrum* fruits were found to be (1.728 \pm 0.049 0) mg/g and (1.087 \pm 0.002) μ g/g respectively. These results showed that the *P. nigrum* fruits extract contains significant quantity of phenolic and flavonoid compounds. The results showed significant activity of piperine and suggesting its use as natural antimicrobial agent²⁵. Previous reports on mass spectrometry analysis of pepper varieties identified compounds aliphatic esters, alcohols, terpenoids and acids were most representative chemical classes of the volatile fraction. The aliphatic esters with fruity odor type were most abundant with twenty-six compounds identified. Hexyl isopentanoate (9.17%), Pentyl 3-methylbutanoate (4.21%), Hexylpentanoate (5.62%) were the major one. Hexylisopentanoate and hexyl isobutanoate (0.19%) were reported to have a powerful fruity odor note by Forero *et al.*, 2009²⁶. The seventy volatile compounds were identified among them esters, alcohols, acid and terpenoids were the major classes which is confirmed by the

previous study. Previous study on GC-MS analysis revealed that five different chemical components were identified in the essential oil of Long pepper, including eugenol (98.979%), trans-caryophyllene (0.643%), 1, 3, 6-Octatriene, 3,7-dimethyl (0.084%), Preg-4-ene-3-one (0.149%), and Phenol, 2-methoxy-4-(2-propenyl)-, acetate (0.145%). Eugenol comprises 98.979% of the essential oil extracted from Long pepper and is the compound most responsible for the Long pepper's aroma/fragrant odor²⁷

CONCLUSION

We conclude from the present scientific investigation that the organic solvent chloroform able to extract more components than the methanol in the case of the two pepper species which are taken for the study. Both the long pepper and the black papper contains promising class of compounds which reveals the biological efficacy of the species. Both the plants are being used in the Indian System of Medicines so the current work support the scientific part for the usage of the both as drugs in Indian System of Medicines. In the current analytical work we listed all, the volatile constituents in both the black pepper and long pepper. The list showed the presence of highly promising constituents in the plants. The percentage content is also calculated so that the method which we developed using the Gas Chromatography analysis can be further used for the routine quality assessment of the plant.

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