RESEARCH ARTICLE

Antitussive Activity of Alcoholic Extract of *Piper longum* Linn.

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ABSTRACT

The l-phellandrene and caryophyllene found in *Piper longum* fruits are effective against a wide range of infections, including stomachic, bronchitis, spleen, cough, tumor, and asthma. This study reports preparing and characterizing alcohol-soluble extract of *P. longum* L. fruit powder. FTIR spectroscopy and TLC were used to characterize an alcoholic extract of *P. longum. P. longum* L. alcohol soluble extract was tested for its anti-tussive efficacy using the standard mouse cough model caused by ammonia liquor. Cough frequency was inhibited by 49.51% (Marketed formulation), 50.84% (Extract 100 mg/kg), 62.71% (Extract 200 mg/kg). Statistically significant ($p < 0.001$) difference was observed in %inhibition of cough frequency of both doses of extract when compared with the marketed formulation. This study reveals an anti-tussive activity of alcohol soluble extract of *P. longum* L.

Keywords: Alcohol soluble extract, Anti-tussive activity, *Piper longum* Linn, Total Phenolic content.

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INTRODUCTION

One type of coughing is triggered by mechanical stimulation, whereas chemicals, including sulphur dioxide, ammonia, citric acid, and capsaicin, trigger the other type. Some evidence suggests that cough suppressants work best when used to temporarily alleviate symptoms. The anti-tussive effect is just one of several conditions for which herbal medicines are increasingly being employed as a treatment.[1]

Cough suppressants are available in a variety of forms and are frequently used in combination. Before getting into the special type of medicine used, it's essential to look more carefully at nature of coughing, its function in disease, and the benefits of suppressing it. Since prehistoric times, people have used traditional medicine derived from plants to treat a broad variety of illnesses. Early humans realized their need for nature in both good health and unhealthy lifestyles. Throughout history, nature has served as a valuable reservoir of medicinal resources, with numerous contemporary pharmaceuticals being derived from traditional healing traditions. Numerous contemporary pharmaceuticals have been derived from natural origins, with plants being particularly noteworthy in this regard. The application of medicinal chemistry, combinatorial chemical techniques, and biosynthetic technology will conduct the optimisation of novel natural product leads. This approach aims to develop chemotherapeutic agents and other bioactive medications that demonstrate enhanced efficacy. The popularity of alternative treatments, particularly herbal medicine, has skyrocketed in recent decades.

Piper longum Linn

The fruits contain approximately 1% of volatile oil, resin, alkaloids such as piperine and piperlonguminine, a waxy alkaloid known as nisobutyldeca-trans-2-trans-4-dienamide, and a terpenoid compound. The predominant factor contributing to the fruit's tart flavor is mostly attributed to the presence of piperine, a piperidine alkaloid. In addition to their nutritional composition, the fruits are found to include calcium (1230 mg/100 g), phosphorus (190 mg/100 g), and iron

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(62.1 mg/100 g). Roots of the plant species under consideration are found to possess the chemical compounds piperine, piperlongumine, piplartine, and dihydrostigmasterol.

In traditional medicine, the fruits of the *P. longum* plant are used as a stimulant, carminative, tonic, and for postpartum mothers. Infections, including stomachic, bronchitis, spleen, cough, tumor, and asthma can all be treated with the l-phellandrene and caryophyllene found in the fruits of the *P. longum* plant. The active ingredient in long pepper (*P. longum*) is piperine. *P. longum* contains 3 to 5% piperine (on a dry weight basis). As a substitute for tonic, you might use dried, unripe fruits. A decoction made from unripe fruits and roots is recommended in cases of persistent bronchitis, cough, and cold. Piperine, a piperidine alkaloid, and its analogues, including isopiperine and isochavicine, are among the volatile oils and alkaloids found in *P. longum* fruit. In addition, it is rich in a wide variety of chemical compounds, including phenolics, flavonoids, amides and steroids, lignans, neolignans, terpenes, and chalcones. *P. longum* L. fruit powder is characterized for identity (color, odor, texture), purity, quality, solubility, micrometric properties, total ashes, loss on drying and extractive value.[2,3]

MATERIALS AND METHODS

Physical Examination and Proximate Analysis

Foreign organic matter (FOM), ash and extractive values, swelling index, and foaming index are just few of the characteristics measured in proximate analysis. According to I.P. standards, we determined the total ashes, loss on drying, and extractive value.[4]

Extraction of *P. longum* L.

Preparation of ethanolic extract of P. longum L.

This 125 g *P. longum* L. fruit powder was packed in a filter paper and placed in the thimble of soxhlet extractor. The powder was exhaustively extracted by continuous soxhlet extraction using ethanol (99.99%) for 3 days at 45 to 50°C. Then extract was separated and concentrated on a water bath. Then extract was subjected to phytochemical screening and other half is kept for evaporation at 40 to 45°C. After complete evaporation, the solvent percentage yield was calculated.[5] (Equation 1)

Calculation of percentage yield

Weight of empty orcelain dish=A gm
Weight of orcelain dish with extract= B gm
Weight of extract= C= (B-A) gm

$$\%\ yield = \frac{C \times 100}{volume\ of\ liquid\ extract} \quad \text{----------(1)}$$

Characterization of Ethanolic Extract of *P. longum* L. (PLE)

Phyto-constituents test

- *Alkaloids (Dragendorff Test)*

In a test tube containing 10 mL of methanol, 200 mg of PLE was transferred and filtered through paper. This was then mixed with 1% HCl and 6 drops of Dragendorff's reagent before being inspected for the presence of brownish-red ppt.

- *Flavonoids (Sodium hydroxide test*

In order to prepare 200 mg of PLE, 10 mL of distilled water were heated with it for 5 minutes before being added to the mixture. One mL of the filtrate was treated with a few drops of a NaOH (20% w/v) solution and then inspected for a change in color from yellow to colorless.

- *Tannins (Ferric chloride test)*

For 5 minutes in a boiling water bath, 200 mg of PLE was combined with 20 mL of DW before being filtered through a fine mesh sieve. The solution was noted for its brownish-green or blue-black color when around 1-mL of the cool filtrate was combined with 5 mL of distilled water and a few drops of ferric chloride.

- *Saponins (Froth test)*

For 10 minutes, 200 mg of PLE was heated in 10 mL of distilled water in the test tube. After cooling to room temperature (about 8–120 °C), the mixture was filtered while still hot (using filtered paper). After adding 10 mL of distilled water to 2.5 mL of the filtrate, sealing the tube, and shaking vigorously for 30 seconds, the filtrate was found to be clear. The solution was checked for steady froth after being left to stand vertically for 5 to 10 minutes (Tables 1 and 2).

- *FTIR spectrum study*

In 0.25 to 0.5 g of PLE was used and was scanned over the 450 to 4,000 cm^{-1} and the spectrum was recorded and an attempt was made to find the presence of major functional groups of some phytoconstituents (Figure 1 and Table 3).

Thin Layer Chromatography

Preparation of sample

In 50 mg PLE was dissolved in about 2 mL ethanol contained in a sodium fusion tube. The extract was applied as a closely spaced series of individual spots in a horizontal line to form a continuous band (1 to 2 mm in length) using thin glass capillary with fine nozzle at a distance of 1-cm from the bottom of the chromatographic plate (glass coated with silica gel G 254 (20–30% W/V). Simultaneously, 100 mL of the mobile phase toluene: EA (7:3) was prepared and transferred into a clean and dry chromatographic chamber.

Table 1: Proximate characteristics of *P. longum L.* fruit powder and its compliance with the standards

Sr. No.	*Parameters*	*Reported values*	*Experimental values*
1.	Foreign organic matter (FOM)	NMT 2	0.6
2.	Total ash value	NMT 8	6.50
3.	Acid insoluble ash value (%)	NMT 1	0.9
4.	Water soluble ash value (%)	NMT 12	12
5.	Alcohol soluble extractive (%)	NLT 2	11
6.	Water-soluble extractive value (%)	NMT 12	11
7.	Swelling index (%)	3.3	3.7
8.	Foaming index (U)	333.3	355.2

Extraction of alcohol soluble extract of *P. longum* L.

Table 2 : Major phyto-constituents detected in the ethanolic extract of *P. longum L.*

Sr. No.	*Type of the phyto-constituent*	*Reported*	*Experimental finding*	*Inference*
1	Alkaloids (Hager's test)	+	White precipitate	Alkaloids may be present
2	Flavonoids (test with NaOH)	+	Change in colour i.e. yellow to colourless	Flavonoids may be present
3	Tannins (Test with FeCl3)	+	Formation of brownish green colour	Tannins may be present
4	Saponins (Foam test)	-	Does not form of foam above the liquid surface.	Saponins may be absent
5	Phenolic compounds (Test with FeCl3)	+	Bluish green colour	Phenolic compounds may be present

FTIR Spectrum of ethanolic extract of *P. longum L.*

Table 3: Interpretation of IR spectrum of EtOH PLE

Peak No.	Observed frequency (cm^{-1})	Probable functional group and type of molecular vibration
1	3328.75	O-H Stretching of alcohol
2	2925	C-H stretching of Alkanes
3	1581.78	N-H stretching of amides
4	1398	-C-O stretching of Carboxylic acid
5	1138	-C-O stretching aromatic cyclic ethers
6	1039	C = N stretching vibration of aliphatic amines.

Thin layer chromatography

Saturation of chromatography chamber with mobile phases

Sufficient volume (60 mL) of mobile phase was kept in well-closed to attain saturation in the well closed chromatography chamber for about 30 to 45 minutes so as to attain saturation.

Preparation of iodine chamber

A total of 10 to 15 g of iodine crystals were transferred carefully in a 100 mL glass beaker. The crystals were allowed to sublime at room temperature to form dense fumes and the chamber was tightly closed using a glass lid.

Visualization of separated spots/bands

The residual volume of mobile phase retained on the glass slide was allowed to evaporate at room temperature (30–35°C) for about (5–10 minutes). Subsequently, the dried separated spots of PLE were visualized by placing the plate into the iodine chamber.

Calculation of Rf values

The TLCV plates were removed from the iodine chamber and the separated components of PLE were located and marked

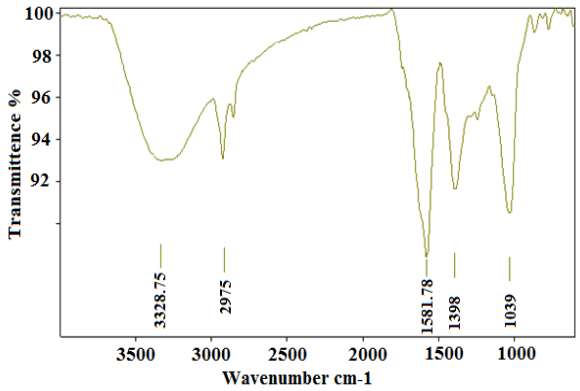

Figure 1: FTIR spectrum of ethanolic extract of *P. longum L.*

with the marker and the path length traveled (cm) by each clear separated component from the point of application was measured. Similarly, the distance traveled by (cm) the solvent was measured and the *Rf* values of individual separated components were measured by using (Equation 2). An attempt was made to find the presence of major functional groups of some known phytoconstituents.[6]

$$Rf = \frac{x}{y} \quad \text{--------(2)}$$

X= Distance traveled by sample
Y= Distance traveled by solvent

- *Total phenolic content (Gallic acid test)*

With the folin-ciocalteu reagent's help, we could calculate how many total phenols were present in the extract. By dissolving 10 mg of gallic acid into 100 mL of 50% methanol, a stock solution of gallic acid (100 µg/mL) was created. It was further diluted to give concentration 3.25, 6.25, 12.5, 25, 50, 70 µg/mL solutions from these concentrations 1-mL aliquot of each dilution was mixed with 5 mL of folin ciocalteu reagent (diluted 10 folds) and 4 mL of sodium carbonate solution (75 µg/litre). All dilutions were incubated at 40°C for 30 minutes. The total phenolic content of the extract was determined by diluting 0.1 gm of extract with ethanol to a final volume of 10 mL and measuring the absorbance at 765 nm on a UV-visible spectrometer (stock solution). The same reagent was added to 1-mL of stock solution, and the resulting mixture was maintained at 40°C for 30 minutes. With an absorbance reading of 765 nm. From the regression equation of the gallic acid calibration curve, the gallic acid equivalent (GAE) was calculated.[7]

Anti-tussive activity of extract for dose selection

The anti-tussive activity of *P. longum* L. was determined by the following method: Human equivalent dose was calculated based on body surface area. To convert animal dose in mg/kg to human equivalent dose (HED) in mg/kg; animal dose was divided by 12.3

Procedure

Thirty male albinos In the experiment, mice weighing 30 to 50 g were used. The animals were housed in a controlled setting with a 12 hour light/dark cycle, a room temperature of 22 ± 2°C, and a humidity level of 50 ± 5%. For the whole period of

the trial, which lasted 10 days, the animals (three rats or six mice per cage) were kept in colony cages with free access to food (commercial pellets containing a balanced diet) and water. Five days before the trial, all of the animals were acclimated to the conditions in the lab. The animals will be provided free access to water throughout the trial, despite having fasted for the previous night. The IAEC formed under CCSEA approved all of the experiments before they were conducted.[8-10]

Induction of Cough

Ammonium hydroxide induced cough: 6 Swiss albino mice in 5 groups.

Group I- Normal control
Group II- Control – Treatment with distilled water orally.
Group III- Positive control- Treatment with marketed preparation
Group IV- Test drug- Treatment with extract at doses of 100 mg/kg
Group V- Test drug- Treatment with extract at doses of 200 mg/kg

A standard mouse cough model using ammonia liquor was used to examine anti-tussive activity. The test drugs and vehicle to control group were administered 1-hour before the ammonia exposure. Each mouse was given 40 µL of 25% NH_4OH in a customised glass vessel holding 300 mL. During the 2-minute test period, the number of coughs that occurred was recorded. The coughing pattern and coughing delay were documented. The formula determines the cough reflex's frequency as a percentage. (Equation 3).

Percent inhibition frequency of cough $= \frac{1-Ta}{Ca} \times 100$ ----------(3)

Ta-Frequency of cough bout in tested herbal formulation
Ca-Frequency of cough bout in control group treated animals.

End point

From experimental study data, the dose of drug for the formulation was calculated.

RESULT AND DISCUSSION

Characterization of *P. longum* L. Fruit Powder

The plant material used in the study is official in Ayurvedic Pharmacopoeia. It is greenish black, aromatic and pungent fine cohesive free flowing powder. The powder is insoluble in distilled water, chloroform and pH 6.8 phosphate buffer, whereas soluble in ethanol, methanol and acetone.[11]

Yield% w/w of ethanolic extract of fruits of P. longum L.

Calculation of percentage yield
Weight of empty porcelain dish= A= 68.80 g weight of porcelain dish with extract= B= 5.44g
Weight of extract= C= (B-A) = 9 (85.44-68.80) = 16.64 g

Percentage yield= $\frac{C \times 100}{\text{Volume of PLE}} = 20.8\ \%$

Characterization of Alcoholic Extract of *P. longum L.*

Phyto-constituents test

- *Rf values of PLE neat sample and its ethanolic solution in selected mobile phases*

The experimental *Rf* values of neat samples as well as that of EtOH solution of PLE in the selected mobile phase compositions Toluene: Ethyl acetate (7:3) were almost identical with those reported in standard chromatograms for same mobile phase compositions The *Rf* 0.68 are reported and 0.61 are the experimental value in the corresponding solvent system (Table 4).

- *Total phenol content*

The standard curve of gallic acid was found to be linear over the range of 3.25 to 60 µg/mL (Figure 2). Hence, the calibration curve of gallic acid followed Beers- Lambert law over this range.

There were 1.2 mg of phenols per gramme of *P. longum* fruit extract. Piperine, a piperidine alkaloid, and its analogs, including isopiperine and isochavicine, are among the volatile oils and alkaloids found in *P. longum* fruit. In addition, it is rich in a wide variety of chemical compounds, including phenolics, flavonoids, amides and steroids, lignans, neolignans, terpenes, and chalcones.

Anti-tussive Activity of Extract for Dose Selection

Dose calculations

- *In-vivo animal study to select dose of a P. longum fruit powder extract*

There are numerous examples No matter the reason for administering a medicine, proper dose is essential. *P. longum* fruit powder is used in various formulations such as Vasavaleha, Vasu cough syrup, sitopaladi churna. All these formulations are polyherbal, which contain various other ingredients. Hence, dose of *P. longum* is not reported for the treatment of cough. Hence, animal study was performed to select a dose of ethanolic extract of *P. longum* fruit powder.

All results were compared by one way ANOVA followed by Tukey-Krammer test (Table 5). The ammonium liquor-induced cough model is widely used for evaluating anti-tussive activity of a compound. The cough incubation period and the frequency with which a cough occurs are common indices for usage in this framework. Coughing was reduced in the treatment groups given either the ethanolic extract (100 or 200 mg/kg) or the commercially available formulation. The anti-tussive effect of these extracts may be related to their active compound piperine, including flavonoids and phenolic compounds. The anti-tussive effects of the ethanolic extracts

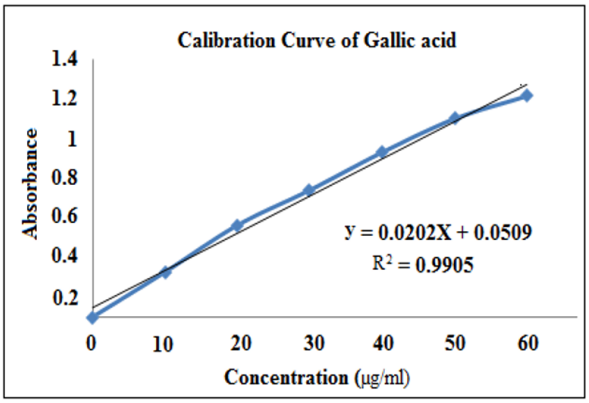

Figure 2: Calibration curve of gallic acid

Table 4: R*f* values of neat sample and solution of PLE in selected mobile phase compositions

Mobile phase	Rf value		Observation
	Reported	Experimental	
Toluene: Ethyl acetate (7:3)	0.68	0.61	Faint Brown colored spot

Table 5: The effect exhibited by the entire treated group on the ammonium hydroxide group induced cough in experimental animal

Sr. No.	*Treatment*	*Dose (mg/kg) (mL)*	*Cough bouts*	*Inhibition%*
1	Control group	0.4	60	-
2	Dextromethorphan	0.4	30	49.15
3	Ethanolic extract *Piper longum* L.100 mg/kg	0.4	30	50.84***
4	Ethanolic extract of *Piper longum* L.200 mg/kg	0.4	21	62.71***

from *P. longum* fruit powder on mice were shown in Table 4. Cough frequency was inhibited by 49.51% (Marketed formulation), 50.84% (Extract 100 mg/kg), 62.71% (Extract 200 mg/kg). Statistically significant ($p < 0.001$) difference was observed in %inhibition of cough frequency of both doses of extract when compared with marketed formulation. Though there was a significant difference in %inhibition of cough frequency of both doses of extract, a dose of 100 mg/kg was selected. Low dosage (as a spice) is safe for long-term use.

- *Dose calculation for pediatrics*

The safety of clinical trials can be improved by taking into account pharmacokinetics and physiological time when extrapolating doses from animals to humans. It is possible to determine an appropriate starting dose using one of four methods: the factor, similar drug, pharmacokinetically guided, or comparative approach. The NOAEL of medications from preclinical toxicological studies are used in the dosage by factor method, which is an empirical way to estimating HED. The human equivalent dosage is determined by employing a calculation method that takes into account the individual's body surface area, which was found to be 162 mg/kg.[12]

CONCLUSION

The ethanolic extract of *P. longum* L. belonging to family Piperaceae has demonstrated excellent anti-tussive activity. There is a need to develop suitable orally administered formulations using extract of *P. longum* L. and *in-vitro in-vivo* characterization of formulations to understand the safety and efficacy of developed formulations.

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