



**EVALUATION OF PIPERINE CONTENT FROM UN-EXPLORED LEAF PARTS OF
PIPER LONGUM LINN**

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ABSTRACT

The Ayurvedic formulation “Pippali” we intake during Cold, Cough and Asthma is generally prepared from the fruit and root of the plant *Piper longum*. Its main active constituent is Piperine, the well known alkaloid, frequently used for preparation of several drug formulations in medicament industries. But in this experimentation the active ingredient Piperine is being searched from its leaf parts of *Piper longum* plant of Piperaceae family through spectrophotometric and high performance liquid chromatography methods. Leaf samples were extracted through cold-stirring method using different solvent systems. Piperine content was estimated to be within the range of 0.032-0.078% dry weight through spectrophotometric analysis and 0.018-0.054% dry weight through HPLC analysis. From this experiment it could be opined that the leaf parts of *P. longum* also contains piperine. This validated new result would be helpful in finding suitable alternate non-destructive source of piperine, which will lessen the threat status of the concerned species.

KEYWORDS: HPLC, Leaf, *Piper longum*, piperine, Spectrophotometer.

INTRODUCTION

Piper longum is one of the mostly used RET medicinal plants, used for preparation of several herbal medicines in Ayurvedic system and drug development industries. It is an herbal vine enlisted in endangered group of RET category.^[1] Piperine is the principal bio-active compound found in this plant. Piperine (1-piperoylpiperidine) is the major piper-amide occurring basically in the fruits and roots of *Piper* species that shows diverse pharmacological activities.^[2-15] There are several methods reported for assessment of piperine from the fruits and roots of *P. longum*^[16-23] But no valid evidences are available till now regarding the quantification of piperine in leaf parts of *P. longum*. The present study is a validated method that describes the quantitative assessment of piperine from leaves of *P. longum* through consumption of less detrimental plant materials, i.e. the un-explored leaves.

MATERIALS AND METHODS

Materials

Leaf samples of *P. longum* were collected from three different locations of Odisha via Keonjhar (21°21'32.1356"N 85°23'52.3336"E), Khurda (20°18'207"N 85°48'251"E) and G. Udayagiri (20°07'48.154"N 84°22'47.354"E). The samples were compared with institutional herbarium specimen (9613)

and identified through reference book ‘The Flora of Odisha’.^[24]

HPLC Instrumentation

The HPLC analysis was performed in a HPLC system, Model no. 1525 (Waters Co., USA) with UV detector. The instrument was loaded with BREEZE software for data collection and acquisition. The separation was achieved by using porous Silica with 5 µm diameter (4.6×150 mm) C₁₈ column (Merck, Germany) maintained at room temperature and equipped with a binary pump. Processing was done using HPLC Syringe (Model-Hamilton 1702 RNR) of 25 µl capacity from SIGMA Aldrich, Germany.

Preparation of Standard solution

Standard Piperine solution was prepared taking standard Piperine (SIGMA Aldrich, Germany) in HPLC Grade Methanol (1 mg mL⁻¹) and kept at 4°C temperature for further use.

Sample Extraction & Pre-treatment

Powdered leaf samples were extracted with Ethyl acetate: Water (EA: W), Methanol: Water (M: W) in equal ratio sequentially by means of stirrer for a period of 18-20 h.^[21, 25]

Estimation of piperine (Spectrophotometrically)

Piperine content was assessed from the extracted crude samples through UV-Vis spectrophotometer (Analytik Jena, Model No- SPEKOL-2000) at 656 nm wavelength.^[16]

Mobile Phase Optimization

The optimisation of mobile phase was done based on the level of resolution of the analytes that to be separated, peak properties (retention time), ease of preparation, and applicability of the method involved. To achieve this, the effect of different solvent systems in different ratios was investigated.

Identification & Isolation

For identification and isolation of pure piperine, the crude extracts were chromatographed using TLC method. The mobile phase used was a mixture of Toluene and Ethyl Acetate in 90: 10 ratios.^[26] After run the chromatogenic reagent used was vanillin sulfuric acid solution and the R_f value was viewed at 365 nm wavelength to validate the presence of piperine.

Estimation of piperine (HPLC)

Chromatographic separation was performed on a Waters make HPLC system equipped with a binary pump (Model-1525) and porous Silica with 5 µm diameter C₁₈ (4.6×150 mm) column. The selected mobile phase was a mixture of Acetonitrile and water. Isocratic conditions were employed at a flow rate of 1 mL min⁻¹. The peaks eluted were monitored at 344 nm wavelength and identified with authentic standard Piperine. An injection volume of 10 µL was set for the optimized method. By this method retention time was evaluated for standard taking ten replications and each sample taking five replications.^[16]

Method Validation

The purposed HPLC method was validated by defining the linearity, peak purity, co-relation coefficient, limit of quantification, limit of detection, relative standard deviation, accuracy, specificity, peak purity, specificity, recovery, sensitivity, selectivity and precision in the retention time (Table 1).

The reproducibility of piperine and potentiality of chromatographic interference analysis was verified by carrying out ten replicate injections of standard and three replicate injections of each extracted samples.^[20] The equation of the calibration curve was developed by least-square regression for the peak area and concentration of analyte. For determining the intra-day accuracy and precision, five replicates of each sample were analyzed twice on the same day. The inter-day accuracy and precision were assessed by analysis of five replicates of samples on three different days. Limit of quantification and limit of detection were determined from the standard deviation of response and the slope of calibration curve.

Table 1: Statistical Data for Validation of HPLC Method.

Parameters	Values
Absorption maxima	344 nm
Correlation coefficient (r ²)	0.997
Regression equation	Y=34346x+0
Intercept (c)	0
Slope (b)	34346
LOD mg mL ⁻¹	0.534
LOQ mg mL ⁻¹	1.62
Retention Time	1.4
Precision (% RSD)	0.01
Accuracy (%)	98.64%

Linearity

A linear calibration curve was developed for the concentration range of 50 µg mL⁻¹ to 500 µg mL⁻¹. The relative standard deviation (% RSD) values did not exceed 0.01 for any of the concentrations. After linear regression analysis, the slope (±SD of the mean) for the calibration curve of piperine were found to be 34346 (±0.67) with a regression coefficient (r²) value of 0.997. The homogeneity of the highest variance with the variance of the remaining calibration curve points was checked with ANOVA test (Table 1).

Accuracy and Extraction Recovery

All three quality control samples (LOQ=1.62 µg mL⁻¹, LOD=0.534 µg mL⁻¹) showed 98.64% accuracy of the method for the determination of piperine. Percent recoveries of piperine from the leaf parts of *P. longum* were between 98-99%. Low percentage RSD values established the extraction efficiency for the selected solvents used in combination for precipitation and also affirmed the robustness of the method.

Statistical Interpretation

In present study, the Piperine content, estimated through both Spectrophotometric and HPLC methods, were analyzed through TWO WAY ANOVA (Repetitive Measures) along with Tukey's Multiple Comparisons Test using GRAPHPAD PRISM software version 6.0. All the data are expressed as Mean ± SD. The variations in both Spectrophotometric and HPLC results were observed at 99.9% significant level.

RESULTS

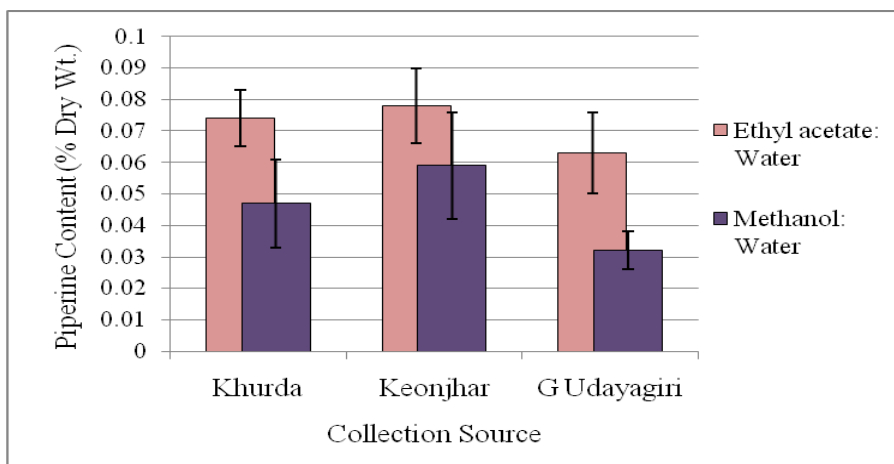
Estimation of Piperine (Spectrophotometrically)

Piperine content in the leaves of *Piper longum* was evaluated to be within the range of 0.032-0.078% dry wt., when extracted through spectrophotometric analysis. Leaf samples were extracted through cold stirring method. Ethyl acetate: Water extracted leaf samples collected from Keonjhar region gave highest amount of Piperine (0.078% dry wt.), whereas Methanol: Water extracted leaf samples collected from Khurda region yielded minimum amount of Piperine (0.032% dry wt.). All data were analyzed statistically at 99.9% interval and the P value was found to be significant at P<0.0001 (Table 2; Fig. 1).

Table 2: Piperine content in leaves of *Piper longum* estimated spectrophotometrically.

Collection Source	Piperine Content (% Dry Weight)	
	Ethyl acetate: Water	Methanol: Water
Khurda	0.074±0.009	0.047± 0.014*
Keonjhar	0.078± 0.012****	0.059±0.017
G Udayagiri	0.063±0.013	0.032±0.006

NB-Results expressed as Mean ± SD. The statistical differences were tested by Two-way RM ANOVA followed by Tukey's Multiple Comparisons Test, where ****P< 0.0001, *P=0.1553

**Fig. 1: Piperine content in leaves of *Piper longum* (Spectrophotometrically).****Estimation of Piperine (HPLC)**

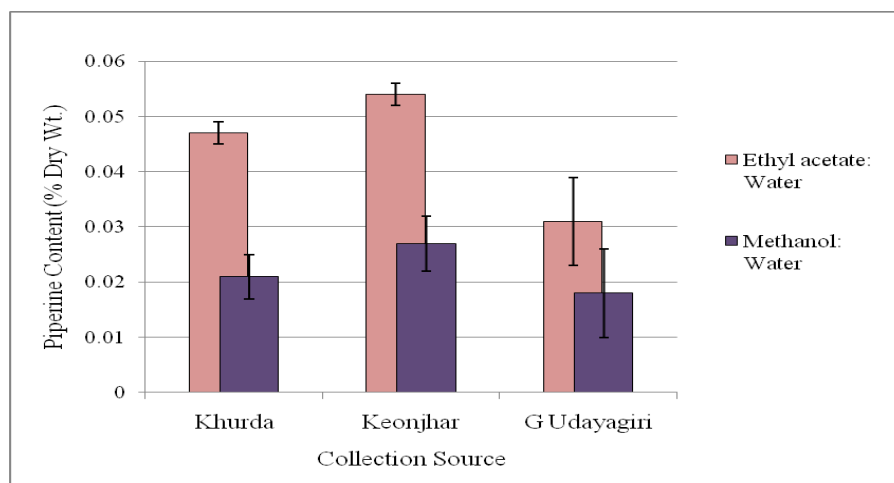
Piperine content in the leaves of *Piper longum* was evaluated to be within the range of 0.018-0.054% dry wt., when extracted through HPLC analysis. Leaf samples were extracted through cold stirring method. Ethyl acetate: Water extracted leaf samples collected from Keonjhar region gave highest amount of Piperine

(0.054% dry wt.), whereas Methanol: Water extracted leaf samples collected from Khurda region yielded minimum amount of Piperine (0.018% dry wt.). All data were analyzed statistically at 99.9% interval and the P value was found to be significant at P<0.0001 (Table 3; Fig. 2).

Table 3: Piperine content in leaves of *Piper longum* estimated through HPLC.

Collection Source	Piperine Content (% Dry Weight)	
	Ethyl acetate: Water	Methanol: Water
Khurda	0.047±0.002	0.021± 0.004*
Keonjhar	0.054± 0.002****	0.027±0.005
G Udayagiri	0.031±0.008	0.018±0.008

NB- Results expressed as Mean ± SD. The statistical differences were tested by Two-way RM ANOVA followed by Tukey's Multiple Comparisons Test, where ****P< 0.0001, *P=0.1073

**Fig. 2: Piperine content in leaves of *Piper longum* (HPLC Method).**

During the course of isolation of pure Piperine for HPLC, the R_f values of the standard and the extracted samples were found to be 0.23 through TLC (Fig. 3).

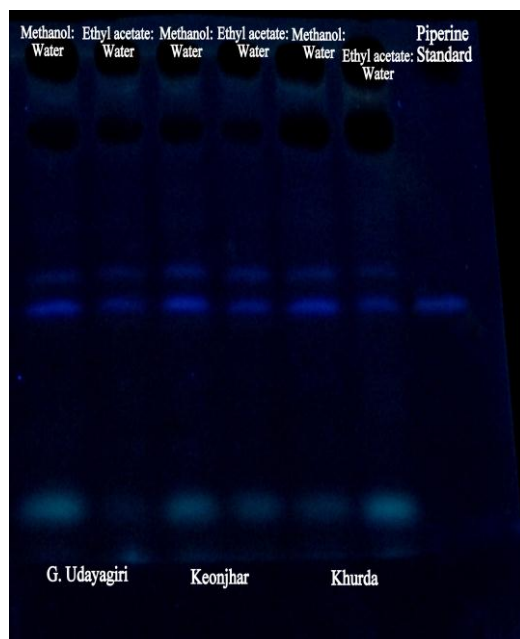


Fig. 3: TLC Sheet showing presence of Piperine in extracted samples against standard.

The HPLC chromatograms of analyzed leaf samples of *P. longum* from various geographical locations in different extraction methods and different solvent systems are illustrated in Fig. 4,

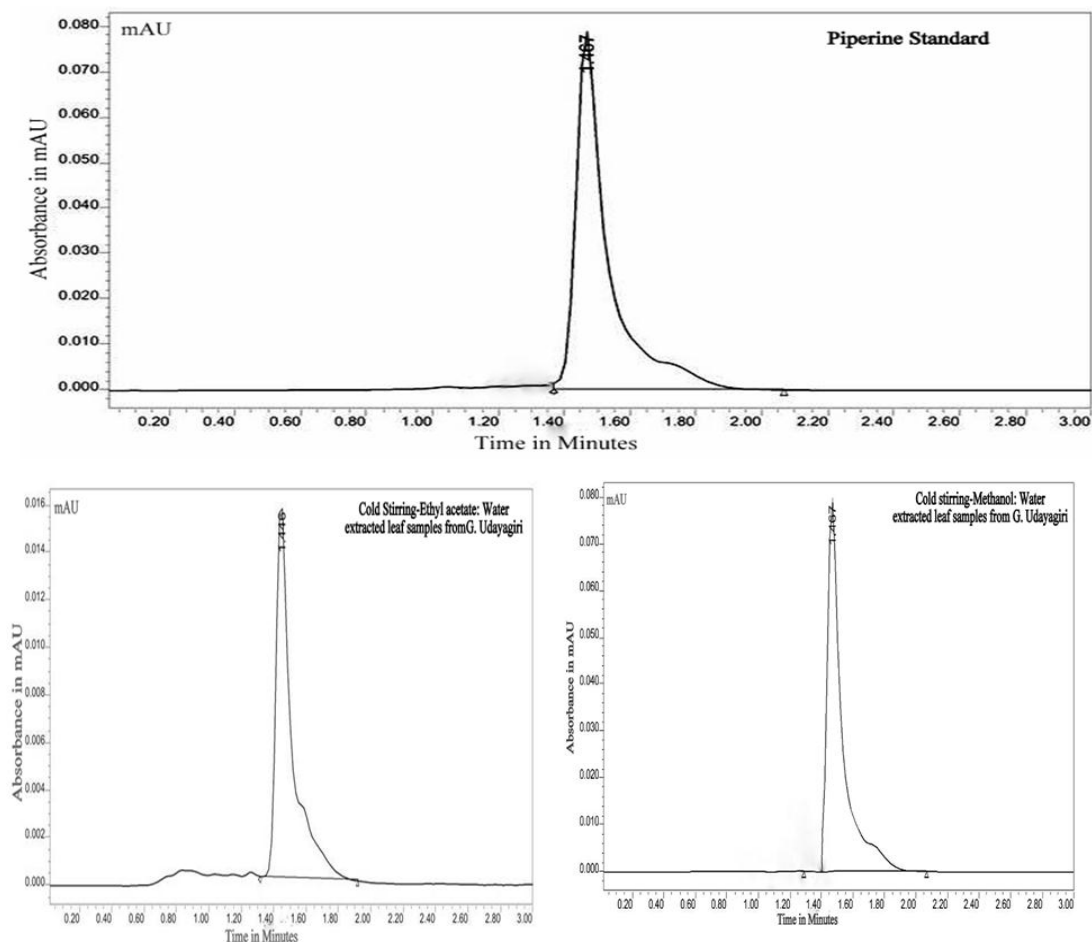


Fig. 4: HPLC Chromatograms of leaf sample of *P. longum* Linn.

DISCUSSION

Besides the extensive study on the root and fruit part of *P. longum* for piperine content, [4, 9, 12, 16, 27] rarely used and less popular leaf part is considered in this experiment for detailed investigation. As the variation in selected extraction process does have varying efficiency rate during the process, hence the vitality to choose a particular process during extracting bio-active compounds have a significant role. Previously various research reports are available regarding the quantification of Piperine from *Piper longum* plants (both roots & fruits) using various solvent systems. [3, 8, 12, 13, 15-23, 27-30] Estimation of Piperine content in the roots and fruits, particularly the short spikes were also previously reported. [31-33]

However some reports regarding the quantification of Piperine from leaf samples of *Piper longum* have been published. [8, 34, 35] But till now detailed method regarding quantification of Piperine from the leaf parts of various agro-climatic zones of Odisha, using several extraction methods have not been elucidated yet. In this experiment, however, the Piperine content in the leaf parts of *Piper longum*, collected from three geographical location of Odisha, was evaluated both through spectrophotometric and HPLC analysis. From the results Piperine content in the leaf samples were found to be in a range of 0.032-0.078% in case of spectrophotometric method and 0.018-0.054% in case of HPLC analysis. However the findings of our result corroborated with other researchers as they have shown the presence of Piperine in the leaf parts, [8,34,35]

CONCLUSION

Piperine content was assessed from the leaf parts of *P. longum*, collected from different agro-climatic zones of Odisha, through Spectrophotometric and HPLC methods. From the above experiment it could be opined that the leaf samples of *P. longum* does contain piperine, though in a lesser quantity. Furthermore, quantitative assessment of piperine would definitely be helpful as an alternate substitute source for use in drug formulation process in both Ayurvedic and modern medicament industries.

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