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COMPARATIVE APPRAISAL OF PIPERINE CONTENT IN VARIOUS FEMALE SPIKES OF *PIPER LONGUM* LINN., A RET MEDICINAL PLANT OF ODISHA

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ABSTRACT

Piperine [$C_{17}H_{19}O_3N$], a medicinally active bio-molecular principle compound, elicits diverse pharmacological activities like bioavailability enhancer, immune-stimulant, hepatoprotective, antioxidant and many more. Piperine is generally extracted or traditionally obtained from female spikes i.e. fruits (Pippali) of wild *Piper longum*, a RET medicinal plant of the family Piperaceae. Present study elucidates the Piperine content in fruit spikes of *Piper longum* grow in three different agro-climatic zones of Odisha. Quantitative estimation of piperine was done through Spectrophotometric and High Performance Liquid Chromatography (HPLC) methods. Piperine content was found within a range of 0.99-2.46% dry weight when extracted with Soxhlet apparatus (extraction-1). In case of magnetic stirrer extraction using Methanol: Water & Ethyl Acetate: Water (extraction-2) Piperine content was ranged from 0.839-2.7% dry weight. Through HPLC method, Piperine content was found within a range of 1.93-5.29% dry weight in extraction-1 and 4.24-5.72% dry weight in extraction-2. Furthermore, samples collected from Keonjhar region (NCP: K_2) showed highest yield of piperine followed by G. Udayagiri (NEG: G) and finally by Khurda (ESCP: K_1). Amongst all the selected solvent systems, Methanol prevailed to be the most efficient solvent for extracting good amount of piperine. Irrespective of methods of extraction & quantification, piperine content was found higher in Keonjhar region followed by G. Udayagiri and Khurda.

KEY WORDS: RET, *Piper longum*, Female Spikes, Piperine, Spectrophotometric, HPLC.

INTRODUCTION

Piper longum Linn is an aromatic medicinal herb in the family Piperaceae & popularly known as pippali. It is cultivated for its fruits i.e. female spikes and roots, which are usually used as a spice and in preparation of indigenous system of medicine in India. It is native in the hotter parts of the country and found in wild as well as cultivated extensively in all parts of India.[1] It is also found in various agro-climatic zones of Odisha but in a very sporadic manner. Mostly this plant is available in Keonjhar, Khurda, Phulbani, Mayurbhanj areas.[2] Usually the plant bears unisexual flowers in a specialized spike form, where the male spikes are larger and slender & female spikes are comparatively shorter.[2] The main active constituent of *P longum* is an alkaloid called *Piperine*.[3-5] The bioavailability enhancing property of *Piperine* indicates its potential to be used as an adjuvant in therapeutic drugs preparations.[6] The pharmacological studies on Piperine have revealed that this compound elicited diverse pharmacological activities; analgesic, anti-pyretic, anti-inflammatory & CNS-depressant activities [7-8]. It acts as bioavailability enhancer.[9-10] antioxidant.[11-12] and anti-inflammatory properties. Due to multidimensional effect on various systems of body, it has been described as antipyretic, diuretic, aphrodisiac, immune-stimulant, hepatoprotective, digestive, counter irritant, antiseptic, antispasmodic.[13-14] There are several methods reported for quantitative estimation of Piperine.[14-21] But taking into consideration of various extraction & analysis methods and source of experimental samples, present study was under taken to yield a comprehensive account of piperine content in fruits of *P longum*, a RET & high valued medicinal plant of Odisha.

MATERIALS AND METHODS

Materials

Female Spikes of *P longum* plants were collected from various agro-climatic zones of Odisha viz. North Central plateau zone-Keonjhar (NCP: K_2), East and South eastern coastal plain zone-Khurda (ESCP: K_1) and North East Ghats zone-Phulbani (G. Udayagiri) (NEG: G). The samples were compared with the herbarium specimens (9613) present in the herbarium of Regional Plant Resource Centre store and also identified through the reference book The Flora of Odisha.[2] The characteristic features of selected agro-climatic zones are described below.

North Central Plateau (NCP) covers the district Mayurbhanj, major parts of Keonjhar (except Anandapur & Ghasipura block). Basically the climate condition in this agro-climatic

zone is hot & moist with sub-humid condition. Broad soil groups found in this climatic zone are lateritic, red & yellow and mixed red & black. Mean annual rainfall in this zone is 1534 mm. Temperature range varies in between 36.6-11.1°c.

East & South-Eastern Coastal Plain (ESCP) covers the districts Kendrapara, Khurda, Jagatsinghpur, part of Cuttack, Puri, Nayagarh & part of Ganjam. Basically the climate condition in this agro-climatic zone is hot & humid. Broad soil groups found in this climatic zone are alluvial, red & mixed red and black. Mean annual rainfall in this zone is 1577 mm. Temperature range varies in between 39.0-11.5°c.

North Eastern Ghats (NEG) covers the districts Phulbani, Rayagada, Gajapati, part of Ganjam & small patches of Koraput. Basically the climate condition in this agro-climatic zone is hot & moist with sub-humid condition. Broad soil groups found in this climatic zone are brown forest, lateritic alluvial, red, mixed red & black. Mean annual rainfall in this zone is 1597 mm. Temperature range varies in between 37.0-10.4°c.

Standard Preparation

Standard stock solution (1 mg/ml) was prepared with Piperine (SIGMA, Aldrich) in Methanol (HPLC Grade) and kept in amber vials. These standard solutions were stored at 4°C protected from light and brought to room temperature before use.

SAMPLE EXTRACTION

Preparation of Extract-1

Powdered spike samples were extracted using Soxhlet apparatus for a minimum of 10-12 hrs with Methanol and Ethanol. The total extract was condensed and kept as Piperine sample stock solution.[15-16]

Preparation of Extract-2

Powdered sample was extracted with Ethyl acetate: Water [EA: W], Methanol: Water [M: W] in equal ratio sequentially by means of mechanical stirring for a minimum of 12-16 hrs and preserved in the same manner as that of extract-1.[17]

Estimation of Piperine through Spectrophotometric Method

For quantitative estimation of piperine, to the samples, 0.1 ml of Gallic acid was added followed by 5 ml of Conc. H_2SO_4. The mixture was incubated in water bath at 47 °c for 3-4 mins. The absorbance of this mixture was measured through spectrophotometer (Analytic Jena, Model No- SPEKOL-2000) by measuring the OD values at 656 nm wavelength.[18]

Estimation of Piperine through HPLC Method

Identification & Isolation through Thin Layer Chromatography Procedure

The crude Piperine samples were chromtographed on pre-coated TLC silica gel 60 F_{254} sheets and TLC plates using the mobile phase containing a mixture of Toluene: Ethyl Acetate (90: 10). Bands were visible at 365 nm wavelength. After run, the plates were viewed with chromatogenic solution containing vanillin in methanolic sulfuric acid.[22] The Rf value of drug was determined to check the presence of Piperine in the extracted sample while run against the standard.

Quantitative Estimation of Piperine through High Performance Liquid Chromatography Procedure

HPLC was performed in a Waters make HPLC system equipped with a binary pump (Model-1525) and porous Silica with 5 µm diameter C_{18} (4.6 × 150 mm column). The mobile phase consisted of a mixture of Acetonitrile: water, at a flow rate of 1 ml/min. The peaks eluted were detected at 344 nm wavelengths and identified with authentic standard Piperine sample that was obtained from Sigma Aldrich, Germany.[18] By this method retention time was evaluated for standard and each sample. The reproducibility of quantitative analysis was verified by carrying out five replicate injections of standard and three replicate injections of each extract**.** The HPLC method was validated by defining the linearity, peak purity, and correlation coefficient, limit of quantification and limit of detection, relative standard deviation, accuracy and specificity (Table 1). For the qualitative purposes, the method was evaluated by taking into account the precision in the retention time, peak purity and selectivity of Piperine elutes.

Table 1: Statistical Data for Validation of HPLC

Parameters	**Values**
Absorption maxima	344 nm
Correlation coefficient (r^2)	0.9997
Regression equation (Y=bx+c)	Y=34346x+0
Intercept (c)	0
Slope (b)	34346
LOD mg/ml	0.057
LOQ mg/ml	0.067
Retention Time	1.4-1.5
Precision (% RSD)	0.01
Accuracy (%)	98.64%
Relative Standard Deviation (RSD)	0.010%

Statistical analysis

In present investigation, the Piperine content, estimated in both the ways (Spectrophotometric and HPLC), was analyzed through TWO WAY ANOVA (Repetitive Measures) along with Tukey's multiple comparisons test using GRAPHPAD PRISM software version 6.0. All the results are expressed as Mean ± SD. The variations in both Spectrophotometric and HPLC results were observed at 99% significant level.

RESULTS AND DISCUSSION

Piperine content as estimated through spectrophotometric analysis

In extraction-1, Piperine content was found to be within the range of 0.99-2.46% dry wt. in female spikes. Methanol extracted samples of NCP: K_2 yielded the highest amount of Piperine content (2.46% dry wt.) Whereas sample of ESCP: K_1 gave the minimum amount of Piperine (2.12% dry wt.). Ethanol extracted samples collected from NCP: K_2 gave maximum amount of Piperine content (1.86% dry wt.). Whereas sample of ESCP: K_1 possessed the minimum amount (0.99% dry wt.). Similarly in case of extraction-2 samples, Piperine content was found to be within the range of 0.839-2.7% dry wt. in female spikes. Samples extracted with EA: W of NCP: K_2 gave the highest amount of Piperine (2.7% dry wt.) Whereas sample of ESCP: K_1 gave the minimum amount of Piperine (1.01% dry wt.). Moreover samples extracted with M: W of NCP: K_2 gave the highest amount of Piperine content (1.77% dry wt.) and sample of ESCP: K_1 yielded the minimum amount of Piperine (0.839% dry wt.) (Table 2, Fig 1). All the data were analyzed through TWO WAY ANOVA (Repetitive Measures) along with Tukey's multiple comparisons test and the results depicted significant variation at P-Value = 0.0132,

Table 2: Piperine Content (% Dry Weight) from various Agro-Climatic Zones of Odisha Estimated through Spectrophotometric Method

Collection Source	Piperine Content (% Dry Weight)			
	Extract-1		Extract-2	
	Methanol	Ethanol	EA: W	M: W
ESCP:K1	2.12±0.6605	0.99 ±0.392	1.01±0.4411	0.839±0.3056
NCP:K2	2.46±0.3290	1.86 ±0.023	2.7±0.2645	1.77±0.1509
NEG:G	2.24±0.006	1.466±0.388	1.61±0.1946	1.235±0.2057

NB-Results expressed as Mean ± SD. The statistical differences were tested by Two-way RM ANOVA at 0.05% significant level

Abbreviations NEG: G-G. Udayagiri, ESCP: K_1-Khurda, NCP: K_2-Keonjhar, EA: W-Ethyl Acetate: Water, M: W-Methanol: Water

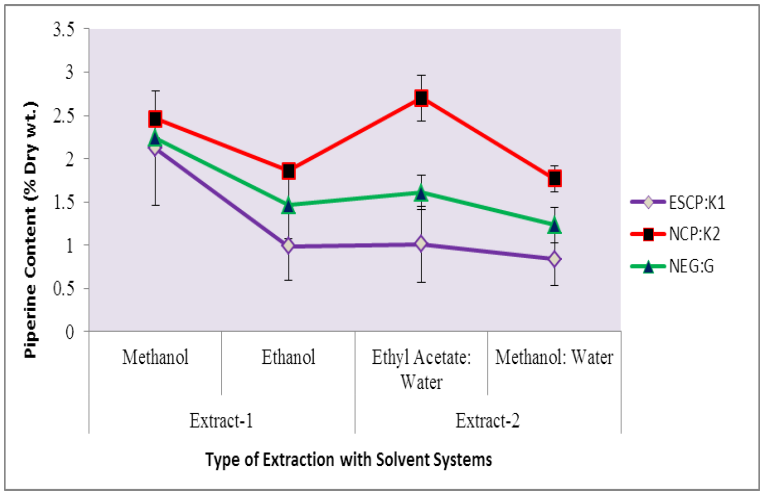

Fig 1: Piperine Content (% Dry weight) from various Agro-climatic Zones of Odisha through Spectrophotometric method

Piperine content as estimated by HPLC analysis

During the course of isolation of pure Piperine for HPLC, the Rf values of the standard and the extracted samples were found to be 0.23 through TLC (Fig: 3). In extraction-1 Piperine content was found to be within the range of 1.93-5.29% dry wt. in female spikes. Methanol extracted samples of NCP: K_2 gave the highest amount of Piperine content (5.29% dry wt.) Whereas sample of ESCP: K_1 yielded the minimum amount (3.62% dry wt.). Ethanol extracted samples collected from NCP: K_2 gave maximum amount of Piperine content (3.36% dry wt.) Sample of ESCP: K_1 possessed the minimum amount (1.93% dry wt.). In case of extraction-2 samples, Piperine content was found to be within the range of 4.24-5.72% dry wt. in female spikes. Samples extracted with EA: W of NCP: K_2 yielded the highest amount of Piperine (5.72% dry wt.) Whereas sample of ESCP: K_1 gave the minimum amount of Piperine (4.28% dry wt.). Samples extracted with M: W of NCP: K_2 gave the highest amount of Piperine (5.11% dry wt.) and sample of ESCP: K_1 gave minimum amount of Piperine (4.24% dry wt.) (Table: 3, Fig: 2). All the data were analyzed through TWO WAY ANOVA (Repetitive Measures) along with Tukey's multiple comparisons test and the results depicted significant variation at P-Value < 0.0001.

Table 3: Piperine Content (% in Dry weight from various Agro-climatic zones of Odisha Estimated through HPLC method

Piperine Content (% Dry Weight)				
Collection Source	Extract-1		Extract-2	
	Methanol	Ethanol	EA: W	M: W
ESCP:K1	3.62±0.08	1.93±0.153	4.28±0.021	4.24±0.11
NCP:K2	5.29±0.01	3.36±0.131	5.72±0.203	5.11±0.105
NEG:G	4.89±0.515	2.7±0.4	5.18±0.030	4.63±0.096

NB-Results expressed as Mean ± SD. The statistical differences were tested by Two-way RM ANOVA at 0.05% significant level

Abbreviations NEG: G-G. Udayagiri, ESCP: K_1-Khurda, NCP: K_2-Keonjhar, EA: W-Ethyl Acetate: Water, M: W-Methanol: Water

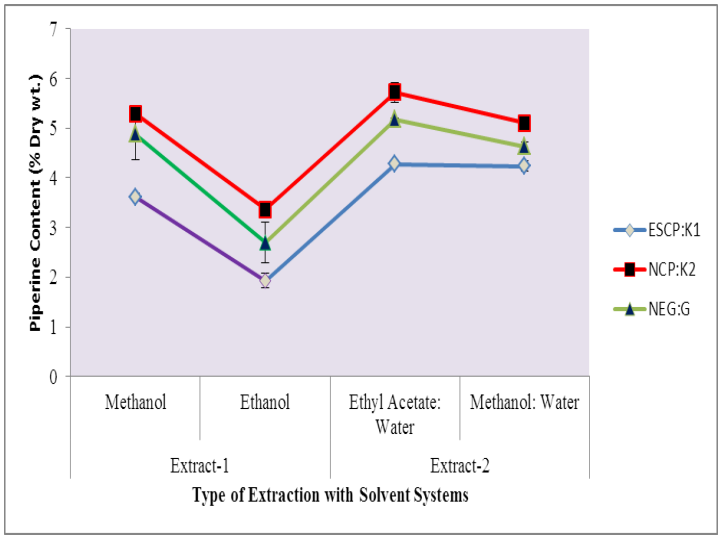

Fig 2: Piperine Content (% Dry weight) from various Agro-climatic Zones of Odisha through HPLC method

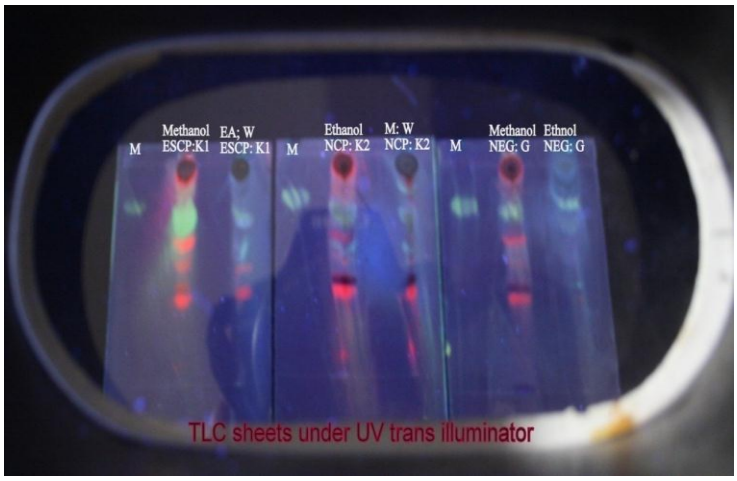

Fig 3: TLC sheets showing Piperine standard & extracted samples.

Accuracy/Recovery Test of Piperine

To check the accuracy of the developed method and to study the interference of samples, recovery experiment was carried out by standard addition method (Table: 4). A known amount of sample was taken. To each tube known amount of Piperine was added. Each sample was analyzed by the developed HPLC method and the amount of Piperine recovered for each level, was calculated.[16, 19]

Table 4: Recovery/Accuracy Test of Piperine through HPLC Analysis

Sl. No.	In Sample [mg/ml]	Added [mg/ml]	Estimated [mg/ml]	% RSD	% of Recovery
1	50	100	147.25±0.005	0.0034	98.2
2	100	150	245.3±0.052	0.021	98.12
3	150	200	348.5±0.021	0.006	99.6

NB-Results expressed as Mean ± SD [n=3]

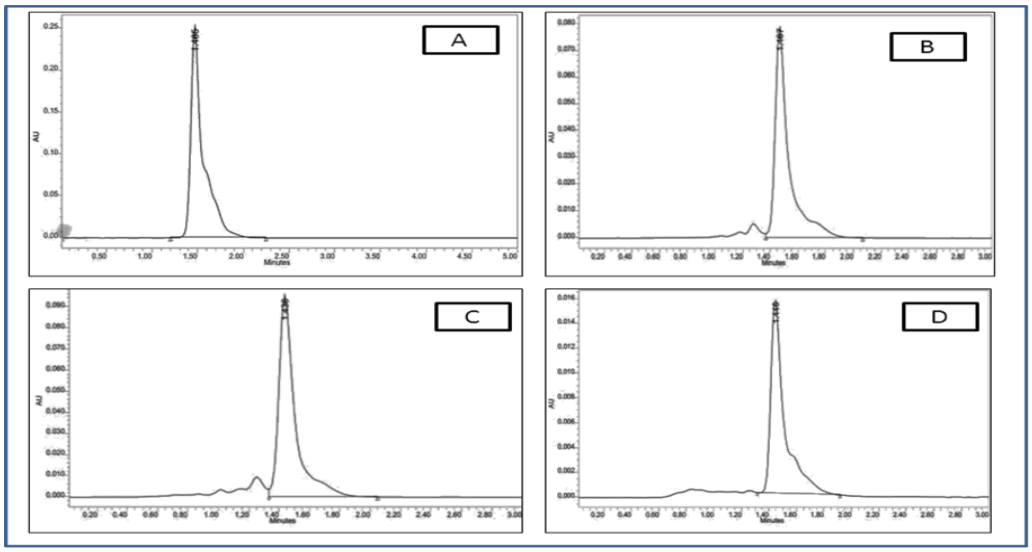

Fig 4: Chromatograms of Piperine. A. Standard Piperine, B. Ethanolic spike extracts from NEG: G, C. M: W spikes extracts from NCP: K_2 & D. EA: W spikes extracts from ESCP: K_1

It has been reported that the Piperine content in the fruits of P. longum, through Soxhlet extraction procedure was 4.32% dry wt. which corroborates our findings [15]. In fruit samples of P. longum Piperine content evaluated was 0.879% w/w [20]. In another finding, quantitative estimation of Piperine content in the powdered samples of Piper longum was validated to be within range 0.68-4.90% w/w.[23]

In this experiment, Piperine content in spikes of P. longum varied from one agro-climatic zone to another. The zonal variation for change in alkaloid concentration may be due to the climatic and environmental change in different agro-climatic regions. This fact was corroborated by other findings. Similar type of zonal difference was also reported on different medicinal plant.[24-25] In a recent report, the amount of embelin content in fruits of *Embelia tsjeriam*-cottam was shown varied significantly amongst the selected agro-climatic zones of Odisha.[24] In another study the proximate, anti-nutrient and elemental analysis of *Ganoderma lucidum*, from various agro-climatic zones of Haryana also depicted the zonal variation clearly.[25]

The time required for extraction of Piperine by Soxhlet method was 10-12 hrs whereas for Magnetic Stirring method was 12-16 hrs. This showed Soxhlet extraction has more extraction efficiency and reduced extraction time than Magnetic Stirring method. Precision in extraction procedure of Piperine content was also validated by others; where the superficial extraction procedure was found to be superior to that of Soxhlet extraction procedure.[15] From the above experiment it was found that the methanolic and EA: W extracts showed higher Piperine content than that of the ethanolic and M: W extracts respectively.

CONCLUSIONS

Piperine content was assessed from the female spikes of P. longum, collected from different agro-climatic zones of Odisha, through Spectrophotometric and HPLC methods. From this experiment comparative analysis of two types of extraction methods was done. Highest amount of Piperine content was found in the agro-climatic zone NCP: K2, as the rich source of Piperine obtained in both methods. The P. longum plants of this geographic zone may be mass multiplied and domesticated properly to meet market demand of *P longum* fruits & Piperine as a vital ingredient of drug formulations. The Soxhlet extraction process and Methanol solvent system prevailed to be most appropriate for higher yield of Piperine. The HPLC method, for quantification of Piperine reported in this work from P. longum collected from different agro-climatic zones, is sensitive, simple and reliable for routine use. The amount of Piperine content was found to be more in the HPLC technique than in the Spectrophotometric technique.

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