



Piperine content variation in different *Piper longum* germplasms of North East India determined through RP-HPLC method

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Abstract: The present experiment was conducted at Assam Agricultural University, Jorhat with ten accessions including check variety of *Piper Longum* germplasm collected from different states of North Eastern region during 2013-14 and 2014-15. The piperine, a major alkaloid used in different therapeutic treatment, per cent content was determined from dried plant materials. Deionised water was used throughout the experiment and the chromatographic separation was carried out in an isocratic elution mode on RP-18 column with 5µm particle size, 4.6mm internal diameter and 250mm length. The mobile phase was a mixture of methanol and water (80: 20). The solvent flow rate was 1.0 ml/min with injection volume 20 µL. The photo diode array detector wavelength was set at 342 nm for the identification of piperine in all extracts. The per cent piperine content in extract was found to be 42.36 per cent in germplasm PLJ-30. The highest per cent piperine content in fruit was recorded in PLJ-11 (7.85%) followed by germplasm PLJ-9 (7.64) and significantly superior over check variety "Viswam" (5.15%). The descending order of piperine content in fruits among germplasm was PLJ-11 > PLJ-09 > PLJ-30 > PLJ-17 > PLJ-03 > PLJ-19 > check variety > PLJ-01 > PLJ-20 and PLJ-16. It can be revealed from the present experiment that piper germplasm with high piperine content has a great scope for commercial cultivation as alkaloid piperine has high demand in pharmaceutical use.

Keywords: Northeast India, Phytochemical, *Piper longum*, Piperine, RP-HPLC method

INTRODUCTION

Medicinal plants are used in different countries and are a source of many and varied chemical compounds leading to synthesized of many potent and powerful drugs. A total of 12,000 such compounds have been isolated so far (Tapsell *et al.*, 2006). *Piper longum* L. (Piperaceae) commonly known as long pepper or pippali is native of the Indo-Malaya region. It occurs in the hotter and humid parts of India, from Central Himalayas to Assam, Khasi and the Mikir hill, the lower hills of West Bengal, and the evergreen forests of the Western Ghats from Konkan to Travancore and has also been recorded in the Car Nicobar Islands (Manoj *et al.*, 2004). Long pepper is a slender, aromatic, perennial climber, with large woody roots and numerous creeping, jointed stems with thickened node bearing cordate leaves (Mishra, 2010). The male and female spikes are produced on different plants. The inflorescence is a cylindrical, pedunculate and flowers grow on solitary spike. The female spike is up to 2.5 cm long and 4-5 mm in diameter but the male spikes is larger, slender and narrow. The fruits are small, ovoid berries, shiny blackish green, embedded in fleshy spikes (Zaveri *et al.*, 2010).

Indian pippali plays an important role in the increased

thyroid hormone level and has a thermogenic response, i.e. the release of metabolic heat energy. Several biological activities like immunostimulatory, anti-ulcer (Sharma *et al.*, 2012), anti-amoebic, anti-oxidant, hepatoprotective, cardiovascular and anti-inflammatory activities (Chauhan *et al.*, 2011) were reported from the fruit of this plant. Active biomolecules from *P. longum* proved to be a source to develop MDR (multiple drugs resistant) reversal agents of natural origin to contain the development and spread of plasmid borne multiple antibiotic resistances (Kumar *et al.*, 2013). Effect of *Piper longum* includes analgesic and diuretic effects, relaxation of muscles tension and alleviation of anxiety (Sunila and Kuttan, 2004). The anti-inflammatory activity of two varieties of *Pippali* in acute and sub-acute experimental models of inflammation had been studied in albino rats. It was found that *Chhoti* variety of *Pippali* suppressed inflammation of both acute and sub acute phase, while *Badi* variety of *Pippali* only of acute phase (Kumari *et al.*, 2013). Piperine is extensively used for enhancing the bioavailability and bioefficacy of various drugs (Atal and Bedi, 2010). The piperine and its analogues are potential pesticides and possess a number of medicinal properties (Okwute and Egharevba, 2013). The people of North East have a rich source of traditional knowledge system and are

known to use herbal medicines for treatment of diseases such as cold, cough like respiratory problem, antidiabetic, headache, fever, pneumonia, skin diseases, stomach ache and many others (Choudhury *et al.*, 2012).

The medicinal properties of *P. longum* is mainly due to the presence of secondary metabolites in fruit that contains a large number of alkaloids and related compounds, the most abundant of which is piperine, together with methyl piperine, piperonaline, piperettine, asarinine, pellitorine, piperundecalidine, piperlongumine, piperlonguminine, refractomide A, pregumidiene, brachystamide, brachystamide-A, brachystine, pipericide, piperderidine, longamide and tetrahydropiperine, tetrahydropiperlongumine, dehydropiperonalinepiperidine, piperine, tetrahydropiperlongumine and trimethoxy cinnamoyl-piperidine and piperlongumine have been found in the root of *P. longum* (Zaveri *et al.*, 2010). Piperidine is used as a marker compound in *P. longum* (Saraf and Saraf, 2013). Different plant parts of *P. longum* revealed the presence of various important bioactive compounds viz. alkaloid, flavonoids, glycosides, tannin, phenol and sterol (Rami *et al.*, 2013). A large number of compounds now referred to as piperine-type alkaloids or alkamides or piperamides have been isolated commonly from species belonging to the genus piper (piperaceae). Family Piperaceae species specialize in the production of amides, phenylpropanoids, lignans and neolignans, benzoic acids and chromenes, alkaloids, polyketides, and a plethora of compounds of mixed biosynthetic origin (Kato and Furlan, 2007). Among the different extraction method of piperine that was confirmed by qualitative analysis and TLC in *Piper longum*. The purity of the compound can be ascertained by UV, FTIR studies and by differential scanning calorimetry (DSC). (Swapna *et al.*, 2012). A method was developed for the isolation of piperine from the fruit of *Piprenigum* (Kanakiet *et al.*, 2008). A simple, rapid, accurate and specific HPTLC method developed and validated and proposed to used for the routine analysis of *P. longum* fruit material and its formulations (Hamrapurkar *et al.*, 2011). The RP-HPLC method to standardize the various parts of *P. Guineense* used in isolation of piperine was characterized by carrying out NMR, UV and mass spectroscopy analyses (Adosraku *et al.*, 2013). Establishment of libraries by HPLC chromatograms, where such sample tracing can be compared with reference as fingerprints in the evaluation of authentication and standardization methods has also been developed (Menghaniet *et al.*, 2010).

North East India is one of the plant biodiversity hotspots (50% of total India biodiversity) that harbours many rare and endemic Medicinal plants. *P. longum* is rarely cultivated in North Eastern region. It is still extensively collected from the wild, threatening the very existence of the plant (Nair, 2000). Survey of the

literature indicates that not much attention has been paid to the spatio-temporal distribution of piperine in *P. longum*. In view of this, the present topic is of great concern to explore the piperine content in different *P. longum* germplasms collected from selected location of North East region in order to find a prominent germplasm for safe and more holistic alternatives to the piperine extraction leading to synthetic drugs so often used now-a-days with the objective to determine the piperine content variation in different *P. longum* germplasms collected from different locations of North East India through RP-HPLC method.

MATERIALS AND METHODS

General experimental procedures: The experimental field was ploughed 2-3 times and then laid in Randomized Block design with three replications in a net area of 120sqm. The rooted cuttings of already collected ten numbers of *P. longum* germplasms from some selected areas of North East region were planted in pits @ two numbers per pit at spacing of 60cm x 60cm (Plant to plant and row to row) having individual plot size of 2m x 2m and eighteen numbers of plants was incorporated per plot. The present germplasms were collected from different North East region on the basis of availability of that location for further study under research (Table 1). Gap filling was done as per the need till one month after planting. The second crop was maintained by following recommended cultural practices including fertiliser application from the first crop due to the perennial nature of the crop.

The Bio-chemical analysis for piperine content of the selected ten *Piper longum* germplasms was conducted in the Bio-chemical laboratory of Bio-chemistry and Agricultural Chemistry, Assam Agricultural University (AAU), Jorhat for two successive years 2013-14 and 2014-15.

The per cent piperine content in fruit of the selected *Piper longum* germplasm was determined in High Performance Liquid Chromatography (HPLC) method (Hamrapurkar *et al.*, 2011) by using the purity (%) formula (Purity (%))= (Amount of concentration found/ Amount of concentration taken)*100. HPLC grade solvent methanol was purchased from Merck, Mumbai, India. Deionized water used throughout the experiment obtained from a Millipore water purification system (Millipore, Mili-Q, France). Standard Piperine was procured from Sigma Aldrich, Bangalore, India.

Extraction and purification of piperine: Fruits of *P. longum* were dried in shade and ground into fine powder and was used for preparation of extract. Plant samples were extracted by refluxing at 100 °C with 4% water in methanol (sample:solvent ratio 1:20) on water bath for 3 hours (Adosraku *et al.*, 2013). Extracts thus obtained were concentrated under reduced pressure (768-7mmhg). Concentrated extracts obtained were

Table 1. List of collected germplasms for making rooted cutting.

SI No.	Germplasms	Collected location
1	PLJ-01	Khetri, Assam
2	PLJ-03	Namgui, Arunachal Pradesh
3	PLJ-09	Umrangso, Meghalaya
4	PLJ-11	Sarupathar, Assam
5	JPL-16	Dharmanagar, Tripura
6	PLJ-17	East kameng, Arunachal Pradesh
7	PLJ-19	East kameng, Arunachal Pradesh
8	PLJ-20	Jagirod, Assam
9	PLJ-30	Boxirhat, Assam
10	Check (Viswam)	Kerela

Table 2. Piperine content (%) in extract and fruit among different germplasm of *P. longum*.

Germplasm	Piperine in extract (%)			Piperine in fruit (%)		
	1 st year	2 nd year	Pooled mean	1 st year	2 nd year	Pooled mean
PLJ-01	22.38	22.54	22.46	4.34	4.5	4.42
PLJ-03	22.86	23.02	22.94	5.38	5.54	5.46
PLJ-09	31.11	31.27	31.19	7.56	7.72	7.64
PLJ-11	26.51	26.67	26.59	7.77	7.93	7.85
JPL-16	5.61	5.77	5.69	1.25	1.41	1.33
PLJ-17	22.89	23.05	22.97	6.25	6.41	6.33
PLJ-19	39.83	39.99	39.91	5.16	5.32	5.24
PLJ-20	6.90	7.06	6.98	1.59	1.75	1.67
PLJ-30	42.28	42.44	42.36	6.44	6.60	6.52
Check (Viswam)	21.43	21.59	21.51	5.07	5.23	5.15
S. Ed (±)			0.59			0.33
CD (5%)			1.21			0.68

further vacuum dried (70°C) to remove traces of residual solvent.

Preparation of standard: A 10mg amount of piperine standard was weighed accurately for preparation of standard stock solution and dissolved in 10 ml methanol in a 50 ml volumetric flask to get 1000µg/mL. From the standard solution, working solutions was prepared for required concentration of 40, 50, 60, 70, 80, 90, 100µg/mL on serial dilutions method.

Preparation of samples: Stock solutions of different extracts were prepared by dissolving extract in methanol (1000 µg/mL) and filtered through a 0.45-µm membrane filter. For stock solutions, 10 mg of piperine sample was accurately weighed and quantitatively transferred into a 100 ml volumetric flask. Approximately 50 ml methanol was added and the solution was sonicated for 30 min. The flask was filled upto 100 ml with methanol then mixed and filter.

Stock solutions of the standards and extract samples were stored at 4° C and were brought to room temperature before use.

Chromatographic conditions and method validation: HPLC system for chromatographic analysis consisted of a separation module (Waters 600E) equipped with Empower software (Waters) and comprising of quaternary pump, an in-line vacuum degasser and a photodiode array detector (Waters 2996). The chromatographic separation was carried out in an isocratic elution mode on RP-18 column (Lichro Cart, Merck, Mumbai, India) with 5 µm particle size, 4.6 mm internal diameter and 250 mm length. The mobile phase was a mixture of methanol and water (80: 20). The solvent flow rate was 1.0 mL/min and the injection volume was 20 µL. The photo diode array detector wavelength was set at 342 nm for the identification of piperine in all extracts. Chromatographic

peak was identified on the basis of 5.6 minute retention time. Concentration of piperine in extract samples was calculated by comparing the integrated peak area of standard piperine with that of a standard curve prepared from standard piperine.

Calibration curve of piperine: Seven different concentrations of stock solution (40, 50, 60, 70, 80, 90, 100 ppm) after dilution with methanol were injected in triplicates and regression equation and co-efficient of correlation (r^2) was derived. Calibration curve for piperine (5.0-100 μ g/mL) was prepared by injecting the different concentrations of standard samples, recording their peak areas and plotting peak areas obtained versus concentration.

Regression analysis was used to access the linearity of the developed HPLC method using the equation (1).

$$y = mx + c \quad \dots\dots\dots 1$$

where, x corresponds to the concentration of standard solution (μ g/ml), y to the peak area and m is slope of the line and c is the intercept of the straight line with y axis.

Calibration curve was linear in this concentration range with regression coefficient (r^2) 0.9946 and showed good linear regression equation (2).

$$y = 148532x - 754677, r^2 = 0.9946 \quad \dots\dots\dots 2$$

Statistical analysis: A Complete Randomized Block (CRD) design was considered for analysis of observed data. All data were expressed as the mean of three replications under laboratory condition. Values were analysed by following SPSS-16 computer based software at 5 % probability level and were considered to be significantly different.

RESULTS AND DISCUSSION

This present investigation was an attempt to isolate piperine content (%) in both extract and dried fruits of different *P. longum* germplasms available in North Eastern region. The pooled mean in the extract ranged in between 5.69 to 42.36 per cent and presented in table 2 and figure 1 to 7. The analysed data indicated that higher per cent piperine in extract was in

germplasm PLJ-30 (42.36) followed by germplasm PLJ-19 (39.91), PLJ-09(31.19), PLJ-17(22.97), which were significantly ($p=0.05$) superior over check variety (21.51). Determination of piperine in fruits revealed that piperine content varied from 1.33 to 7.85 per cent. The highest percent piperine content in fruits was observed in germplasm PLJ-11 (7.85) and differed significantly ($p=0.05$) from germplasms PLJ-01, PLJ-03, PLJ-16, PLJ-17, PLJ-19 and PLJ-20. The germplasm PLJ-09, PLJ-30, PLJ-17, PLJ-03 and PLJ-19 showed 7.64, 6.52, 6.33, 5.46 and 5.24 per cent piperine, respectively and was found to be better than check variety that recorded only 5.15 per cent of piperine content. Piperine content is 3-5% (on dry weight basis) in the fruits of *Piper longum* of commerce (Madhavi et al., 2009). The percentage yield of 2.07% w/w of isolated piperine from *Piper guineense* compares favorably with the 2.50% w/w isolated from *Piper longum* (Deepthiet et al., 2012). The difference in piperine content may be due to the methods of extraction and genetic character of the particular germplasm. The crude extract of *P. longum* contains 3-8% of piperine (James, 1999) that confirms the present findings. The highest per cent piperine content in JPL-11 may be for the particular genotype that has a potentiality of more piperine yield.

The highest percentage of piperine in extract than the piperine percentage in fruit may be due to presence of some other compounds like isopiperolein B, piperine, piperchabamide D, dehydropipermolanine, N-isobutyl amide of octadeca-trans-2-cis-4-dienoic acid and dehydropipermoline along with prepared extract. *P. longum* fruit extract confirmed the presence of chemical components including N-isobutyl amide of octadeca-trans-2-cis-4-dienoic acid (Zaveri et al., 2010), piperidine (Saraf and Saraf, 2013), isopiperolein B, piperine, piperchabamide D, dehydropipermolanine and dehydropipermoline (Siddique et al., 1997), alkaloid piperine (1-piperoyl piperidine) and piper longamine (Storhet et al., 2001) that confirms the present findings. The presence of various important

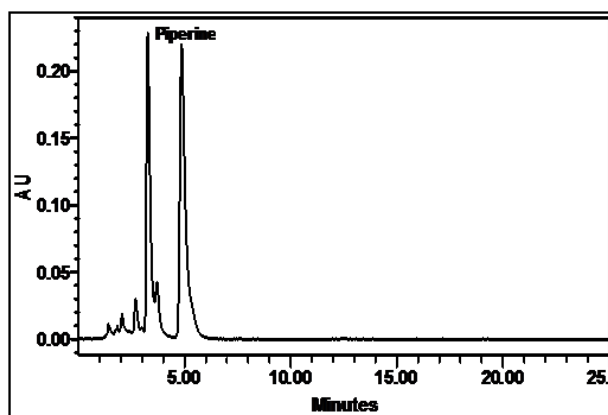


Fig. 1. HPLC chromatogram of PLJ-03.

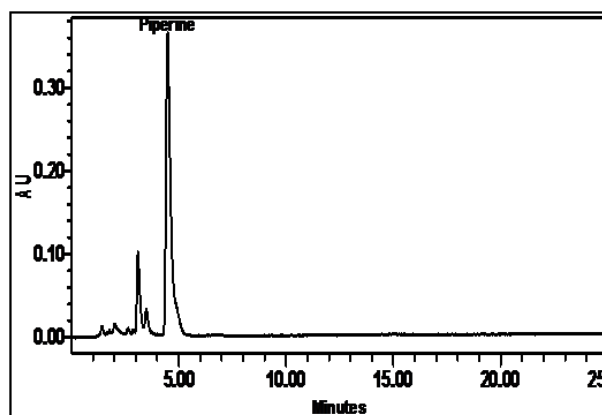


Fig. 2. HPLC chromatogram of PLJ-09.

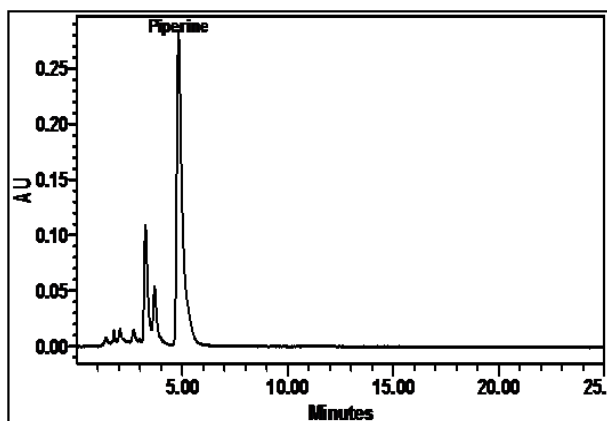


Fig. 3. HPLC chromatogram of PLJ-11

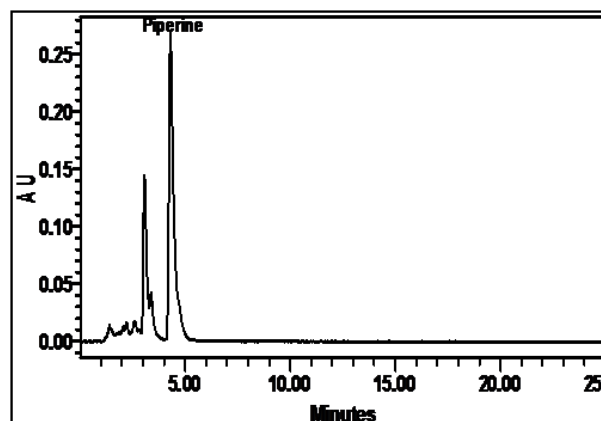


Fig. 4. HPLC chromatogram of PLJ-17.

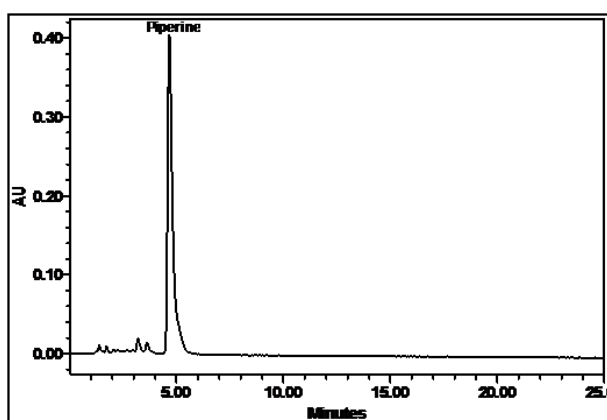


Fig. 5. HPLC chromatogram of PLJ-19.

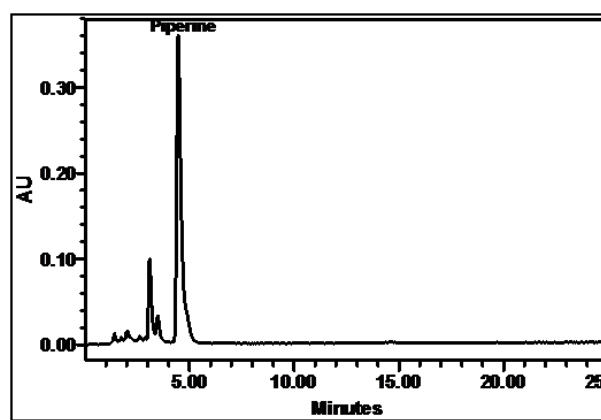


Fig. 6. HPLC chromatogram of PLJ-30.

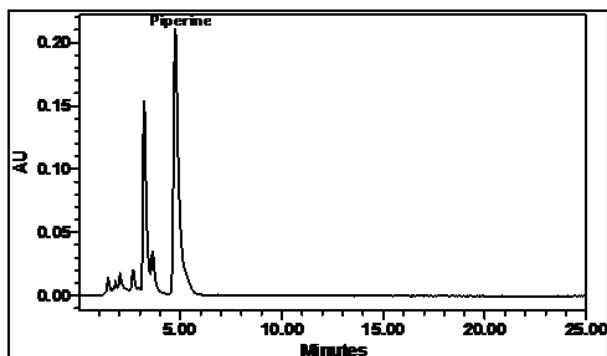


Fig. 7. HPLC chromatogram of check variety "Viswam".

AU= Absorbance Unit (milli absorption units mAU) (Fig. 1-7)

bioactive compounds viz. alkaloid, flavonoids, glycosides, tannin, phenol and sterol in plant parts of *P. longum* was also reported by Rami et al. (2013) in support of the present experimental result.

Conclusion

The present experimental findings revealed that the highest per cent piperine content in extract was found in germplasm PLJ-30. The highest per cent piperine content in fruit was recorded in PLJ-11 followed by

germplasm PLJ-9 and significantly superior over check variety "Viswam" at 5% probability level. It can be revealed from the present experiment that these two piper germplasmsthat yielded high piperine content has a great scope for commercial cultivation as alkaloid piperine has great demand in pharmaceutical useand may be the future source for preparation of modern drugs. Proper management and conservation of this promising and threatened *P. longum* germplasm along with large scale propagation for commercial cultivation at farmers field can be the cost effective future source of piperine.

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