Pharmacogn. Res., 2025; 17(4):1308-1314.
https://www.phcogres.com

Original Article

Comparative HPTLC Analysis of Piperine Content in Roots of *Piper longum* L. Cultivated with Bio Formulations

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ABSTRACT

Background: Phytochemicals from medicinal plants significantly impact healthcare. Piperine, a bioactive compound in *Piper longum*, exhibits diverse pharmacological effects, including analgesic, antipyretic, antioxidant, immune-stimulant, hepatoprotective, and bioavailability-enhancing properties. Traditionally, Piperine is extracted from both fruits (*Pippali*) and roots (*Pippalimula*) of *Piper longum*. **Objectives:** To quantify the Piperine content in *Piper longum* roots cultivated using bio formulations-*Kunapa Jala* and Vermiwash-on Piperine yield. **Materials and Methods:** *Piper longum* was cultivated with Random Block Design with two treatment groups (*Kunapa Jala* and Vermiwash), each with 12 replications. Roots were harvested, cleaned, dried, and powdered. High-Performance Thin-Layer Chromatography (HPTLC) was used to detect and quantify of Piperine. **Results:** HPTLC analysis revealed the presence of Piperine in both groups. Quantitative comparison showed a higher Piperine concentration in the *Kunapa Jala* treated group than in the Vermiwash group. **Conclusion:** *Kunapa Jala* enhances Piperine content in *Piper longum* roots in comparison to Vermiwash. This supports the potential of *Vrukshayurveda* and traditional formulations in improving the phytochemical yield of *Pippali*.

Keywords: *Piper longum*, Piperine, HPTLC analysis, *Kunapa Jala*, Vermiwash, *Vrukshaayurveda*.

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Received: 24-04-2025;
Revised: 09-06-2025;
Accepted: 12-08-2025.

INTRODUCTION

Medicinal plants are utilized across various nations and serve as a source of numerous diverse chemical compounds, which have contributed to the synthesis of many effective and powerful pharmaceuticals.[1] *Piper longum* Linn., widely recognized as Long Pepper, is a threatened medicinal species within the *Piperaceae* family.[2] *Piper longum* is a slender, aromatic, perennial vine characterized by large woody roots and numerous creeping, jointed stems that bear thickened nodes with heart-shaped leaves. This South Asian herb grows wild and cultivated across warm regions of India, Malaysia, Singapore, Bhutan, and Myanmar.[3] The principle bioactive compound found in *Piper longum* is an alkaloid, 5-9% of piperine.[4-6] Researches have demonstrated that, this compound exhibits a range of pharmacological effects, including analgesic,[7] bio enhancer,[8] antipyretic, anti-inflammatory, antioxidant, hepatoprotective, anti-thyroid, anti-hypertensive, anti-tumor, anti-asthamatic, and CNS depressant activities.[9,10]

Bio formulations, may be defined as a mixture of physiologically active part of microbial biomass and its metabolites combined with the carrier material. It is employed in environmentally supportable ways for nutrient absorption, morpho physiological growth promotion, enhancing yield, bio-control etc. (Aamir, M., Rai, K. K., Zehra, A., Dubey, M. K. (2020). These formulations, which consist of beneficial microorganisms, organic compounds and plant extracts, are emerging as a promising alternative to conventional chemical-based approaches.

Traditional agricultural approaches have advocated many such bio formulations. One of traditional agriculture practice *Vrukshayurveda* mentioned by *Surapala* dates back to 1000A.D has mentioning of such preparations. *Vrukshayurveda*, a science of plant life, has dealt overall aspects of plant cultivation including land preparation, plant cultivation, productivity, disease and pest management etc. *Vrukshayurveda* has mentioned *Kunapa Jala* [KJ] to promote the plant growth, to enhance soil fertility and plant productivity. KJ is a fermented concoction prepared by using of animal and plant products. Recent research has not only proved its efficacy in enhancing the plant growth, yield but also higher concentration of phyto constituents/bio active molecules.[11,12] Vermiwash [VW] is a liquid prepared from vermi compost. Vermi compost is prepared by mixture of soil, cow dung, and foliage etc., later digested by earth worms. Vermi compost comprises of large population of decomposer bacteria,

DOI: 10.5530/pres.20252328

mucus, and macro and micro nutrients. It is commonly used bio formulation for cultivation; it has been shown to support plant growth and secondary metabolite production.[13,14]

Increased metabolite levels due to traditional bio-inputs may translate enhanced therapeutic efficacy, as supported by previous reports that herbs grown under organic regimens exhibit 20-30% stronger pharmacological responses.[15] So a study is planned to compare the piperine content of the roots of *piper longum* cultivated by two different bio formulations KJ and VW.

MATERIALS AND METHODS

Cultivation

Planting material were collected from local market of Shahapur, Belagavi, Karnataka which is located 752 M above sea level and has humidity is approximately 74%.[16]

Bio formulations preparation

Kunapajala is prepared by boiling the flesh of goat, sheep, fish, and pork in water until thoroughly cooked. Sesame seeds powder and black gram powder combined with milk are added to this concoction. After cooling of this concoction Ghee and honey is added. Later the mixture is moved to an earthen pot and left to ferment for around 40 days in a warm atmosphere, stirring occasionally to guarantee even breakdown. Following the fermentation process, the liquid is filtered and used as an organic bio fertilizer.[17]

Vermiwash is a liquid extract obtained from vermicompost units. To prepare vermiwash, a container is set up with a tap at the bottom for collection. The base is layered with gravel, followed by sand and then soil. Earthworms are introduced into this setup along with pre-decomposed organic waste. Water is sprinkled regularly to maintain moisture. As the water percolates through the vermicompost, it collects nutrients and microbial secretions, exiting through the tap as vermiwash. This liquid is collected periodically and used as a foliar spray or soil drench to promote plant growth.[18]

Figure 1: Harvested Plants of *Piper longum*.

Methodology

Pippali (*Piper longum*) was cultivated in two groups namely Group A Vermiwash, Group B *Kunapa Jala*. Standard package of practice followed for *Piper longum* cultivation. Both bio formulations, 10% drenched every fortnightly. Roots harvested after 9 months, washed thoroughly using running water to get rid of soil and other contaminants (Figure 1). The roots were dehydrated in a hot air oven under controlled temperatures until all moisture was removed and then powdered in a sterile environment to prevent contamination (Figure 2).[19]

Extract preparation

For the sample preparation, the root powder from each sample [1 g] was refluxed with 20 mL of methanol for 30 min. The mixture was filtered three times with Whatman filter paper no. 41 in order to guarantee complete extraction, and all the filtrates were combined for concentration. The combined filtrates were concentrated, the final volume done volumetric flask 10 mL was adjusted and methanol was added. The concentrates were methanol diluted to a 50% strength prior to HPTLC analysis. Piperine standard solution was simultaneously prepared by dissolving 10 mg of piperine [Sigma, USA] in10 mL of methanol to get a 1 mg/mL stock solution. Calibration and quantification were done with a working standard solution of 10 µg/mL from this stock solution.[20]

HPTLC Profile

HPTLC Instrumentation

The HPTLC analysis was done on a CAMAG HPTLC system equipped with a Linomat V sample applicator, TLC scanner 3, and WinCATS for data analysis. The analysis was performed on Silica gel 60 F254 plates of 20 by 20 cm. The mobile phase was prepared by mixing toluene with ethyl acetate and formic acid in a ratio of 3:1. Each extract sample was applied as a band of 6 mm, which was capped at 10 µL sample volume, and 8 cm in twin trough chamber. Post development, the plates were dried and visualized under UV light at 254 nm, and densitometric scanning was performed at the same wavelength R_f values were noted (Table 1). Piperine quantification was carried out using peak area comparison against samples with a standard piperine calibration curve. This process was repeated thrice to guarantee precision and reproducibility.

Validation and Data Analysis

Following ICH criteria, the analytical procedure for the HPTLC analysis of *Piper longum* roots was verified to guarantee accuracy, precision, and repeatability.[21,22] Six scans of the same piperine [40 ng] site were used to evaluate repeatability; the results were reported as the coefficient of variation [%CV]. Piperine aliquots ranging from 20 to 50 ng were analyzed on the same day and on different days in order to assess accuracy and variability. To ascertain consistency, precision was also represented as a percentage of CV. Recovery studies at three concentration levels-50%, 100%, and 150%-were used to verify accuracy. The mean recovery was calculated to assess technique accuracy, and the percentage recovery at each level was calculated. Where appropriate, descriptive statistical analyses were used, and all data were displayed as Mean±Standard deviation.

RESULTS

High-Performance Thin-Layer Chromatography (HPTLC) analysis facilitated a wide-ranging comparative appraisal of the phytochemical contents of *Piper longum* (Pippali) root samples that were Cultivated with two different organics: Vermiwash (Sample A) and *Kunapajala* (Sample B). Under densitometric scanning at 254 nm, several well-separated peaks were seen, indicating the occurrence of varied phytoconstituents. Both the samples exhibited an intense peak at R_f ~0.38, which coincided with the piperine marker (Table 2), where Sample B recorded higher intensity. Most importantly, *Kunapajala*-treated roots (Sample B) showed a more intricate and chemically rich profile, as attested by a wider spectral spread and higher band intensity across the chromatographic plate. This was confirmed by identification of other characteristic peaks at R_f values 0.59, 0.65, and 0.76, which were not present in the Vermiwash-treated group (Sample A), reflecting the existence of treatment-specific bio actives. Visual examination using UV light (366 nm and 245 nm) revealed finer, denser, and brighter fluorescent bands in Sample B than Sample A, indicating greater concentration and a broader diversity of secondary metabolites (Figures 3 and 4). A total of 12 peaks (R_f: 0.04-0.98) were detected in Sample A and 14 peaks (R_f: 0.02-0.99) in Sample B, with the highest peaks at R_f ~0.94-0.95 in both samples indicating the presence of major phytoconstituents at these positions. Bar graph comparisons of peak heights at different R_f values (Figure 5) reaffirmed the superiority of *Kunapajala* in sustaining a more diverse and more abundant phytochemical spectrum.

Quantitative determination of piperine content from a multilevel calibration curve (1 µg, 5 µg, and 10 µg standards) validated the qualitative findings. Sample B (*Kunapajala*-treated) had a piperine content of 0.41 mg/g of dry root powder and Sample A (Vermiwash-treated) had 0.35 mg/g, showing an increased accumulation of the bioactive marker in the *Kunapajala* group (Figure 2). The piperine peak was repeatedly seen at R_f 0.55±0.5 mm in both groups, which was authenticated by the standard tracks at 254 nm and 366 nm. The piperine peak in Sample A contributed to 38.6% of the chromatographic area, with the maximum height being 554.5 AU and the area being 24,858.2 AU. In Sample B, even though the peak height of piperine was slightly greater (631.1 AU), it accounted for 35.0% of the area in the chromatogram, amounting to 30,919.3 AU in area. Fluorescence detection at 366 nm yielded further confirmation: Sample A's peak of piperine was 494.7 AU in height and 21,416.1 AU in area (81.9% of total area), while Sample B's peak registered 574.8 AU in height and 29,628.1 AU in area (79.1% of total area), (Table 3). These results altogether imply that treatment of *Kunapajala* Improvised biosynthesis and amassing of piperine as well as conceivably other pharmacologically valuable constituents in roots of *Piper longum*.

DISCUSSION

This study evaluated the influence of two traditional organic bio formulations- *Kunapajala* and Vermiwash-on the phytochemical characteristics of *Piper longum* roots, with a focus on piperine content. HPTLC analysis showed that Kunapajala-treated roots had a higher piperine content [0.41 mg/g] compared to Vermiwash-treated roots [0.35 mg/g], suggesting a superior effect of *Kunapajala* in enhancing alkaloid accumulation. This observation is in agreement with previous reports where fermented bio-inputs significantly enhanced the secondary metabolite content in medicinal plants.[23,24] The increased phytochemical complexity in *Kunapajala*-treated roots is likely due to its rich

Table 1: HPTLC Analysis Parameters.

Parameter	Details
Instrumentation	CAMAG HPTLC System
Sample Applicator	Linomat V
Scanner	CAMAG TLC Scanner 3
Software	WinCATS
Stationary Phase	Silica gel 60 F254 TLC plates [20 × 20 cm]
Mobile Phase	Toluene: Ethyl acetate: Formic acid [6:3:1]
Sample Volume	10 µL per band
Band Length	6 mm
Development Distance	8 cm
Detection Wavelength	UV 254 nm
Standard Used	Piperine [Sigma, USA]
R_f Value of Standard	0.39
Quantification Method	Calibration curve of standard piperine
Replication	All experiments conducted in triplicate

Figure 2: Dry Roots of *Piper longum*.

composition of fermented animal and plant derivatives, which contribute essential nutrients, amino acids, microbial enzymes, and phytohormones. These compounds act as biochemical elicitors, stimulating pathways like the phenylpropanoid and shikimate pathways responsible for piperine biosynthesis.[25,26] The role of nutrient-rich organic inputs in modulating such metabolic routes has been previously documented in crops like Curcuma longa and *Andrographis paniculata*.[27,28] Furthermore, *Kunapajala* is known to improve microbial diversity and soil enzyme activity, factors directly linked to improved root metabolism and bioactive compound production.[29] These findings support earlier literature indicating that multi- component organic inputs outperform simpler ones in boosting medicinal phytoconstituents.[30] The higher piperine concentration in *Kunapajala*-treated plants aligns with HPTLC-based standardization approaches where such bioformulations have been shown to upregulate target metabolites.[31] These findings validate the use of Kunapajala not only as a sustainable soil amendment but also as a quality-enhancing agent for medicinal plant cultivation. The results also provide modern pharmacognostic evidence supporting classical *Vrikshayurveda* principles, demonstrating how indigenous agricultural wisdom can be scientifically harnessed for modern drug standardization.

This difference indicates that *Kunapajala* might have created a more conducive rhizospheric condition for the biosynthesis

Table 2: R_f Values of Methanolic Extracts at 254 nm.

Sample	Track ID	R_f Values
Sample A	Track 1	0.06, 0.07, 0.25, 0.31, 0.34, 0.44, 0.55, 0.69, 0.75, 0.83, 0.94, 0.96
Sample B	Track 2	0.04, 0.06, 0.08, 0.11, 0.25, 0.30, 0.34, 0.43, 0.54, 0.68, 0.75, 0.83, 0.94, 0.96
Marker [Std]	Track 3-5	0.54

and accumulation of therapeutic alkaloids in *P. longum* roots. The organic compounds of *Kunapajala*, which are generally rich in fermented pulses, and animal waste, are also responsible for adding micronutrients, enzymes, and phytohormones that synergistically stimulate root metabolism and secondary metabolite pathways, especially alkaloid biosynthesis. Confirming this, an Industrial Crops and Products [2024] paper noted the function of nutrient-rich organic formulations to upregulate the phenylpropanoid and polyketide pathways associated with piperine biosynthesis. The significance of these findings is multifarious. First, they affirm the utility of adopting indigenous organic farming practices, like the use of *Kunapajala*, in medicinal plant production protocols to increase pharmacological activity. Second, the enhanced piperine content and wider phytochemical

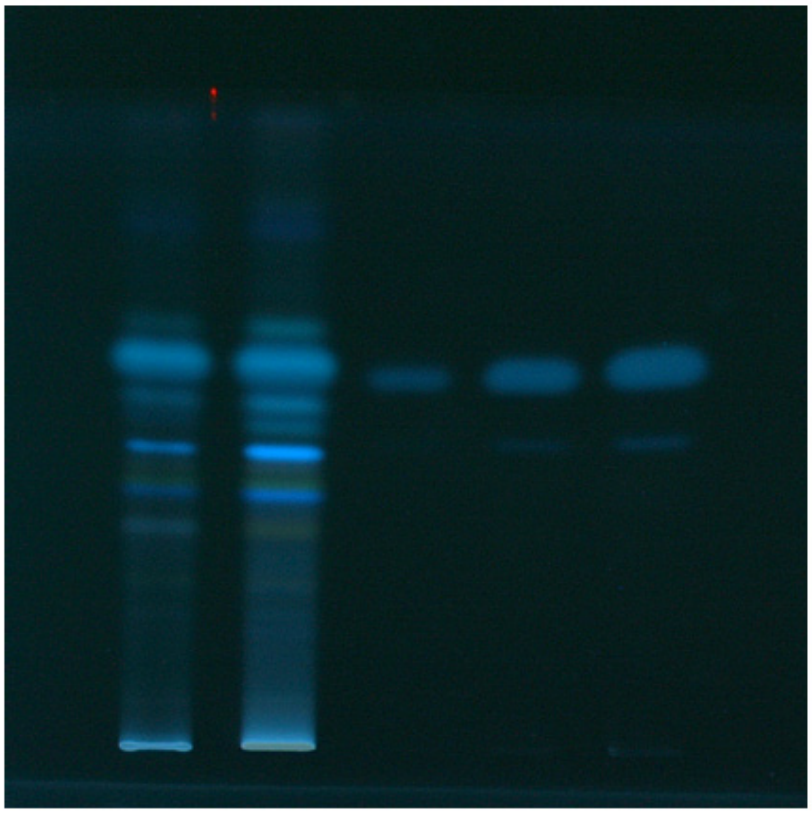

Figure 3: HPTLC chromatograms visualized at 254 nm.

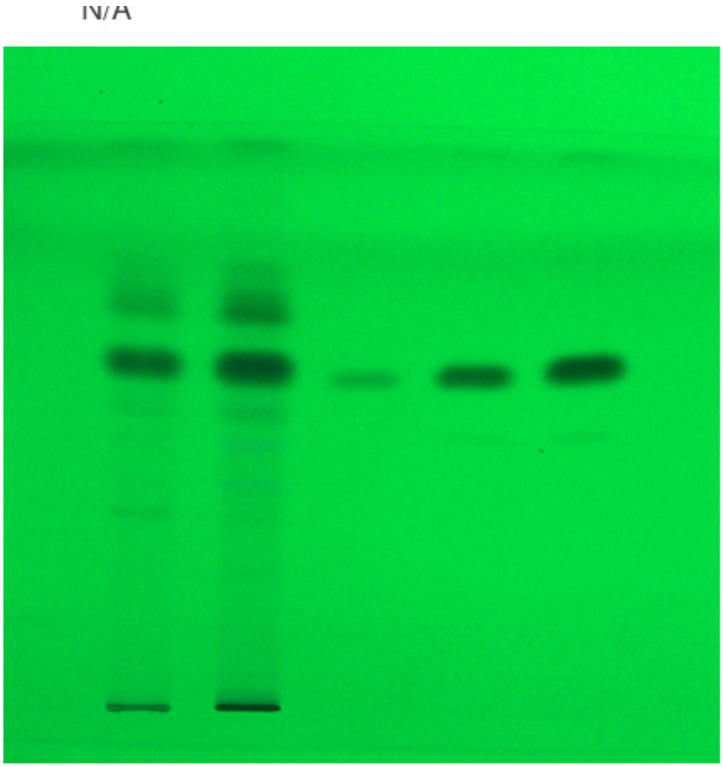

Figure 4: HPTLC chromatograms visualized at 366 nm.

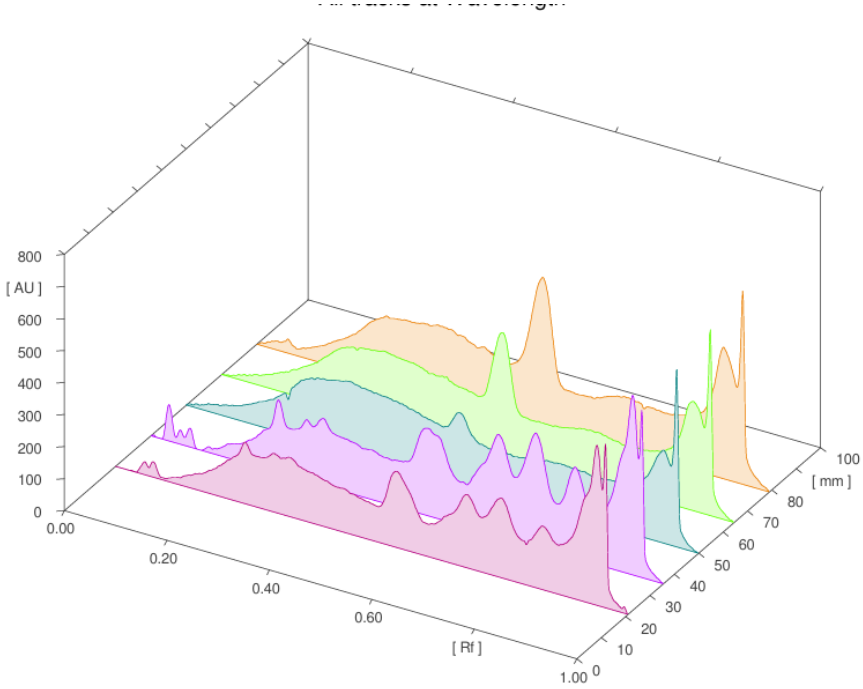

Figure 5: All tracks at Wavelength.

Table 3: R_f Values of Methanolic Extracts at 366 nm.

Sample	Track ID	R_f Values
Sample A	Track 1	0.06, 0.07, 0.25, 0.31, 0.34, 0.44, 0.55, 0.69, 0.75, 0.83, 0.94, 0.96
Sample B	Track 2	0.04, 0.06, 0.08, 0.11, 0.25, 0.30, 0.34, 0.43, 0.54, 0.68, 0.75, 0.83, 0.94, 0.96
Piperine [Std]	Track 3-5	0.54

profile may considerably influence the therapeutic value of *P. longum* derived drugs, rendering them more effective and polyfunctional. Additionally, *Kunapajala*, owing to its cost-effective and a self-preparative formula, is both sustainably agrarian-friendly as well as a pharmacognostically high-profile offering. In this regard, the current study provides a paradigm for how cultivation parameters can be scientifically confirmed and optimized to produce desired phytochemical profiles. In addition, these findings provide a foundation for subsequent studies of the mechanistic underpinnings of these results, possibly including metabolomic analysis, gene expression profiling of biosynthetic enzymes of interest [e.g., phenylalanine ammonia-lyase, piperine synthase] under various organic treatments.

CONCLUSION

HPTLC analysis of *Piper longum* roots cultivated with *Kunapajala* and Vermiwash revealed higher piperine content and greater phytochemical diversity in *Kunapajala*-treated samples in comparison with vermiwash. These findings highlight *Kunapajala's* effectiveness in enhancing secondary metabolite production, supporting the role of traditional organic practices in improving medicinal quality.

ABBREVIATIONS

KJ: *Kunapa Jala*; **PG:** *Panchagavya*; **CV:** Coefficient of variation; **HPTLC:** High Performance thin layer chromatography.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

The KLE Academy of Higher Education and Research, Belagavi, Karnataka.

SUMMARY

Using High-Performance Thin-Layer Chromatography (HPTLC), this novel study examines the impact of two traditional bio-formulations, *Kunapa Jala* and *Vermiwash*, on the piperine content of *Piper longum* roots. When grown under controlled settings, roots treated with *Kunapa Jala* showed more phytochemical diversity and higher piperine levels (0.41 mg/g) than roots treated with Vermiwash (0.35 mg/g). *Kunapa Jala's* superiority in boosting secondary metabolites was validated by HPTLC profiling. The study supports the incorporation of *Vrikshayurveda*-based methods into contemporary pharmacognostic procedures for herbal standardization and therapeutic optimization, and it confirms their effectiveness in enhancing the quality of medicinal plants. The results support

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the sustainable production of herbal medications with high potencies.

SCOPE AND LIMITATION OF STUDY

This study aimed to profile the phytochemical composition of *Piper longum* roots treated with Vermiwash and *Kunapajala*, illustrating the potential of traditional organic methods to enhance piperine content and bioactive diversity. Limitations include lack of control group.

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Cite this article: Holeyache A, Chaudhary V. Comparative HPTLC Analysis of Piperine Content in Roots of *Piper longum* L. Cultivated with Bio Formulations. Pharmacog Res. 2025;17(4):1308-14.