5 Chapter 5: Collection, authentication & extraction of *Piper longum* fruits, and marker-based standardization by validated HPLC method

5.1 Background

Taxonomical and DNA-based molecular authentication and identification were carried out. Extraction was carried out by cold maceration method using absolute ethanol. The residual solvent content of the extract was analyzed by headspace gas chromatography (GC-HS) to avoid solvent-related toxicities. Chemical marker-based standardization with respect to PIP and PLGN was carried out by a validated HPLC method in order to provide batch-to-batch consistency, dosage uniformity, and reproducible therapeutic response. The validation of the HPLC method was carried out as per ICH Topic Q 2 (R1) to achieve reproducible and accurate quantification of the intended analytes.

5.2 Objectives

- Collection and authentication (taxonomical and molecular) of *Piper longum* fruits
- Extraction of *Piper longum* fruits and residual solvent analysis by GC-HS analysis
- Marker-based standardization of PLFEE by validated HPLC as per ICH Topic Q 2 (R1)

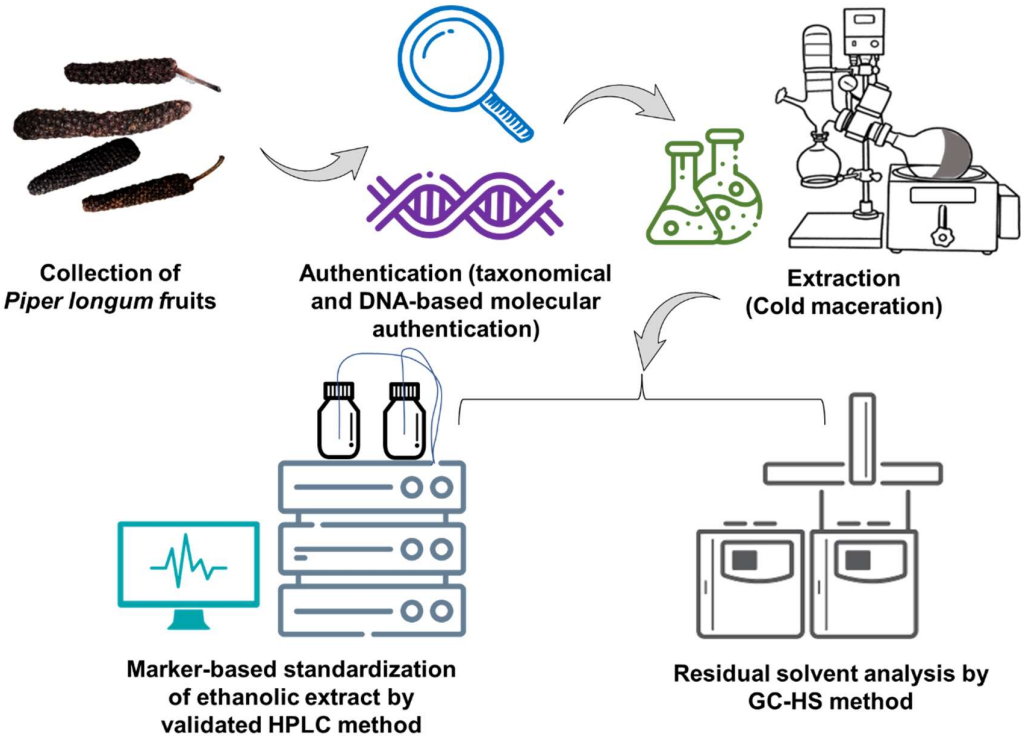

Figure 5. 1 Schematic representation of workflow of chapter 5

5.3 Methodology

5.3.1 Collection and authentication (Taxonomical and Molecular) of *Piper longum* fruits

Fruits of *Piper longum* were collected from a cultivated source from Jayachandrapur village, Odisha, India.

5.3.1.1 Taxonomical authentication

The collected fruits of *Piper longum* were authenticated by Prof. N.K Dubey, Department of Botany, Institute of Science, Banaras Hindu University, and the voucher specimen (Pipera.2021/6) was deposited in the Departmental Herbarium.

5.3.1.2 Molecular authentication of fruits of *Piper longum*

Further, the authenticity of the fruit was verified by DNA-based molecular characterization. The online Basic Local Alignment Search Tool (BLAST) study (NIH, National Library of Medicine, USA) was executed for the investigation of

nucleotide sequence homology with authenticated sequences of ribulose-bisphosphate carboxylase gene (rbcL). The DNA-based molecular authentication was carried out by (i) DNA isolation, (ii) DNA quality check, (iii) Polymerase Chain Reaction (PCR), (iv) agarose gel electrophoresis of PCR products, (v) removal of unwanted primers and deoxynucleoside triphosphate (dNTPs) from a PCR product, (vi) sequencing by the terminator, (vii) post-sequencing PCR clean up, and (viii) sequence analysis. DNA of dried fruits was isolated utilizing NucleoSpin® Plant II Kit (Macherey-Nagel, Germany) as per the protocol of the manufacturer. The isolated DNA quality was examined using agarose gel electrophoresis with 0.5X Tris-borate-Ethylenediaminetetraacetic acid (TBE) as electrophoresis buffer at 75 V till the front of bromophenol dye migrated to the bottom of the gel. The PCR reaction of the isolated DNA of the rbcL gene was carried out using primers, such as RBCL-AF (direction: forward, sequence (5' to 3'): ATGTCACCACAAACAGAGACTAAAGC) and RBCL-724R (direction: reverse, sequence (5' to 3'): TCGCATGTACCTGCAGTAGC). The PCR analysis was executed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, USA) using 5 µL of 2X Phire Master Mix (Thermo Fisher, USA), 4 µL of distilled water (D/W), 0.25 µL of RBCL-AF, 0.25 µL of RBCL-724R, and 1µL of purified isolated DNA. The amplification profile in PCR includes (a) denaturation at 98℃ for 30 sec, (b) denaturation at 98° C for 5 sec (40 cycles), (c) annealing at 58° C for 10 sec (40 cycles), (d) extension at 72° C for 15 sec (40 cycles), (e) final extension for 60 sec at 72° C, and (f) final hold at 4° C. Then the gel electrophoresis was carried out to check the quality of PCR product in agarose gels (1.2%) prepared in 0.5 X TBE buffer comprising 0.5 µg/mL ethidium bromide using 2-log DNA ladder (New England BioLabs Inc., USA) as molecular standard. The bands were envisaged in a UV

transilluminator (Genei, Bangalore, India), and the image was acquired under UV light using a gel documentation system (Bio-Rad, USA). Then the removal of unwanted primers and dNTPs from the PCR product was carried out by treating with ExoSAP-IT (GE Healthcare, Illinois, USA) as per manufacture protocol. Further, sequencing was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, USA) utilizing BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following the manufacture's protocol. Then, the post-sequencing PCR cleanup was carried out using D/W (5 µL), 3M sodium acetate (1 µL), EDTA (0.1 µL), and 100% ethanol (44 µL). The quality of the sequence was verified using "Sequence Scanner software" v1 (Applied Biosystems, Life Technologies, USA). The sequence alignment and necessary editing of the obtained sequences were performed using Geneious Pro software (v 5.1, Auckland, New Zealand). The analyses of sequence homology were carried out using the nucleotide BLAST search (NIH, National Library of Medicine, USA).

5.3.2 Extraction of *Piper longum* fruits and residual solvent analysis by Headspace Gas Chromatography (GC-HS) analysis

5.3.2.1 Extraction

The collected fruits of *Piper longum* were dried at room temperature. The dried fruits were coarsely powdered using a mixture grinder (HL7756/00, Phillips, India) and extracted via microwave-assisted exposure of crude powdered drugs at 450 W for 5 min (MC35J8085PT/TL, domestic microwave oven, HOTBLAST^{TM}, Samsung, South Korea) followed by triple maceration by soaking the partially grounded PL with absolute ethanol at a proportion of powder to solvent 1:4 w/v. The extraction was facilitated by placing the crude drug-solvent dispersion on an orbital shaker (REMI, RS 12 plus) for 72 h. Then it was filtered through a vacuum membrane filter (pore

size: 0.45 µM, diameter: 47mm), filtrates of all maceration events were mixed thoroughly and concentrated in a rotary vacuum evaporator (IKA®, RV 10 digital, vacuum pump: IKA® MVP 10 basic, water bath: IKA® HB10 digital, and chiller system: IKA® RC 2 Basic) at 40°C with 60 rpm and stored in a refrigerator until usage. The extraction yield of *Piper longum* fruits ethanolic extract (PLFEE) was calculated by Equation 5.1.

$$\%\ Extraction\ yield = \frac{Weight\ of\ extract\ obtained}{Weight\ of\ powdered\ PL\ taken} \times 100 \quad (5.1)$$

5.3.2.2 GC-HS analysis

The residual absolute ethanol (99.99%) in the dried ethanolic extract (PLFEE) was analyzed by GC-HS instrument (7890, Agilent, USA) equipped with a headspace injector, CombiPAL automatic headspace sampler, capillary column (HP-5, 30 m length, 0.28 mm id, and 0.25 µm film thickness), temperature- controlled oven, capillary flow technology (CFT), and a flame ionization detector (FID). The ethanol was diluted with xylene to produce a series of concentrations (10-500 ppm). One milliliter of each diluted sample was placed individually in GC headspace vials, tightly closed, and subjected to an oven at 60°C for 15 min for partitioning to the headspace until attaining equilibrium. For analysis of ethanol content in the PLFEE, it was kept in the headspace vial and subjected to an oven for partitioning the residual ethanol to the headspace in a similar way as stated above. Then 250 µL of each specimen from the head space was injected into the GC column via autosampler using Helium (He) as mobile phase with a flow rate of 1 mL/min. A run time of 20 was given, and the analysis was carried out using EZChrom software (Agilent, USA). The analysis was performed in triplicate, and the mean was reported.

5.3.3 Marker-based standardization of PLFEE by validated HPLC as per ICH Topic Q 2 (R1)

5.3.3.1 HPLC methodology

The analysis was performed by an Agilent HPLC system (Agilent 1260 Infinity II, Agilent, USA) equipped with a quaternary pump (1260 Quat Pump VL)), diode array detector (1260 DAD WR), autosampler (1260 Vial Sampler), column (Quasar^{TM} C18 LC column, PerkinElmer, 250 × 4.6 mm with 5 µm particle size), a standard flow cell (13 µL, 10 mm, and 120 bar), and Open LAB CDS EZChrom Workstation VL software. The isocratic elution was executed at a flow rate of 1.00 mL/min using HPLC grade methanol and water (Finar Limited, India) as mobile phase at a ratio of 80:20 v/v with a run time of 10 min. Quantification of two markers, i.e., PIP and PLGN, were carried out by the developed HPLC method at their respective absorption maxima (λmax of PLGN: 340 nm and PIP: 342 nm). The individual primary stock solutions of PIP and PLGN, each at a concentration of 1 mg/mL, and their working standard solutions (2-30 µg/mL) were prepared by diluting the pure PIP (>97% Purity, Sigma Aldrich) or PLGN (≥98% purity, Cayman Chemical Company) with HPLC grade methanol. The standard solutions were vortexed, bath sonicated for 5 min, filtered through a syringe filter (0.22 µm), stored in the amber-colored volumetric flask, and preserved at 4°C before the HPLC analysis. Twenty microliters of working standards were injected into the HPLC system using an autosampler.

5.3.3.2 Validation of the HPLC method as per ICH Topic Q 2 (R1)

The analysis was performed in triplicate, and the calibration curve was made by plotting the average area (mAU) of three independent analyses v/s the corresponding concentration (µg/mL). The developed HPLC method was validated for linearity, range, accuracy (% recovery), precision (repeatability and intermediate precision: intra-day and inter-day), limit of detection (LOD), limit of quantification (LOQ),

system suitability, and robustness test as per International Conference on Harmonization Harmonised Tripartite Guideline (ICH Topic Q 2 (R1), Step 4 version, Validation Of Analytical Procedures: Text And Methodology) [74].

5.3.3.2.1 Linearity and range

The linearity of the developed HPLC method was identified by observing the correlation coefficients obtained from the calibration curves of PIP and PLGN. The range of linearity was determined by fitting the data of average area (mAU) of three independent analyses with the corresponding concentration (µg/mL) to the linear equation and determining the concentrations range that follows the linearity.

5.3.3.2.2 Accuracy

The accuracy of the HPLC method was investigated from the percentage recovery by the standard addition method at three levels (50, 100, and 150%). Accurately 4, 8, and 12 µg/mL of working standard solution were added to pre-analyzed samples (8 µg/mL) to produce 6, 8, and 10 µg/mL, respectively. The samples were analyzed by the developed HPLC method for quantifying the PIP or PLGN. The percent recovery was calculated by Equation 5.2.

$$\%\, recovery = \frac{Experimental\ or\ Recovered\ concentration}{Theoretical\ concentration\ after\ addition} \times 100 \qquad (5.2)$$

5.3.3.2.3 Precision

The repeatability and intermediate precision studies were performed to determine the precision of the developed HPLC method. Six replicates of the working standard solution (12 µg/mL) of PIP and PLGN were chromatographed by the HPLC method, and the repeatability of the method was assessed by determining the percent relative standard deviation (% RSD) of the obtained area. The intermediate precisions (intra-day and inter-day) were assessed by analyzing the chromatographic area of three

concentrations of working standard solution (6, 12, and 18 µg/mL) and calculating the % RSD.

5.3.3.2.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of the HPLC method were estimated by the standard deviation of the response and the slope method using Equation 5.3 and Equation 5.4, respectively.

$$LOD = \frac{3.3\ SD}{S} \qquad (5.3)$$

$$LOQ = \frac{10\ SD}{S} \qquad (5.4)$$

Where the SD is the average standard deviation of responses (y-intercept), and S is the average slope of calibration curves

5.3.3.2.5 Robustness

The robustness of the developed HPLC method was studied by intentional changes in different chromatographic settings, such as wavelength (340, 342, and 344 nm for PIP; 338, 340, and 342 nm for PLGN), run times (8, 10, and 12 min), flow rate (0.8, 1, and 1.2 mL/min), mobile phase composition (78:22, 80:20, and 82:18 v/v of methanol: water), and analyzing the changes in the peak area and retention time (Rt) PIP and PLGN each of 12 µg/mL.

5.3.3.2.6 System Suitability

The consistency in the performance of the HPLC method was studied by system suitability constraints. Six replicates of working standard solution (12 µg/mL) of PIP or PLGN were injected, and parameters like % RSD of peak area, % RSD retention time (Rt), the number of theoretical plates, peak purity, capacity factor, and tailing factor were considered for system suitability analysis.

5.3.3.3 Marker-based standardization

Marker-based standardization was performed to quantify the amount of PIP and PLGN in the PLFEE. Accurately 20 mg of PLFEE was weighed and dissolved with 10 mL of HPLC grade methanol, vortexed (SPINIX^{TM} Vertex shaker, Tarsons Products Pvt. Ltd., Kolkata, India) for 5 min, and sonicated in an ultrasonic bath (GT Sonic Ultrasonic Cleaner, Guang Dong GT Ultrasonic Co., Ltd., China) for 10 min to achieve complete solubilization of phytoconstituents. Then, it was filtered through a syringe filter (0.2 µm, PVDF), diluted suitably with HPLC grade methanol to obtain 1 mg/mL and 50 µg/mL for estimation of PLGN and PIP, respectively. Accurately, 20 µL of the sample was sampled by autosampler and injected into the HPLC system for the estimation of two markers using their respective calibration curves obtained from the validated HPLC method.

5.4 Results and Discussion

5.4.1 Taxonomical and Molecular authentication of collected *Piper longum* fruits

5.4.1.1 Taxonomical authentication

The taxonomical authentication of fruits ensured the identity of the received sample as fruits of *Piper longum* Linn. (Family: Piperaceae).

5.4.1.2 DNA-based molecular characterization

The results of gel electrophoresis under UV illumination via a gel documentation system are represented in Figure 5. 2a. The left lane showed the 2-log DNA ladder, and the right lane displayed the PCR amplification fragment of the rbcL gene. The single band in the gel documentation data represents the proper amplification of PCR product with good quality, purity, and a molecular size of 800 bp. The ".ab1" file of the FASTA sequence of the rbcL gene was obtained and then processed through

Codon Code Aligner software (Version 9.0.2, CodonCode Corporation, USA) for obtaining the DNA sequencing chromatograms of PCR product. The resultant DNA sequencing chromatogram is shown in Figure 5. 2b. The chromatogram is a visual representation of nucleotide sequences. Each of the colors in the chromatogram represents a nucleotide. The unclean peaks or multiple broad peaks at the same region are typically found at the beginning and the end of the sequence. In the complete chromatogram, the initial portions up to the 71st nucleotide showed an unclean signal of nucleotide with superimposed broad peaks, after which the sharp distinguished peaks were found representing the clean region with very low backgrounds (noise) up to the 301st number nucleotide. The BLAST analysis was carried out using the nucleotide sequences from the clean region. The BLAST analysis results of the rbcL sequence showed a percentage identity of 100% to *Piper longum* (Accession: ON720789.1), confirming the authenticity of the collected fruits. The sequence match result of the sample sequence with the reported sequence (ID: ON720789.1) was given in Table 5. 1. All the base pairs of query subjects matched with the reference database sequence. The matching score (of 231 nucleotides) showed a value of 100% and a 0% gap with the reference data. The detailed outcome of BLAST analysis of the rbcL gene of dried Piper longum fruit is represented in Table 5. 2.

Table 5. 1 Sequence matches the result of the sample sequence with the reported sequence (ID: ON720789.1)

Score 427 bits (231)	**Expect 3e-115**	**Identities 231/231 (100%)**	**Gaps 0/231 (0%)**	**Standard Plus/Plus**
Query 1	GATATCTTGGCAGCATTCCGAGTAACTCCGCAACCCGGAGTTCCGCCCGAAGAAGCAGGG			60
Sbjct 89	GATATCTTGGCAGCATTCCGAGTAACTCCGCAACCCGGAGTTCCGCCCGAAGAAGCAGGG			148
Query 61	GCTGCAGTAGCTGCCGAATCCTCTACTGGTACATGGACAACTGTATGGACCGACGGACTT			120
Sbjct 149	GCTGCAGTAGCTGCCGAATCCTCTACTGGTACATGGACAACTGTATGGACCGACGGACTT			208
Query 121	ACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCCCGTTGCTGGGGAGGAA			180
Sbjct 209	ACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCCCGTTGCTGGGGAGGAA			268
Query 181	AATCAATATATTTGCTATGTAGCTTATCCTTTAGACCTTTTTGAAGAAGGT 231			231
Sbjct 269	AATCAATATATTTGCTATGTAGCTTATCCTTTAGACCTTTTTGAAGAAGGT 319			319

A: adenine, G: guanine, C: cytosine, and T: thymine

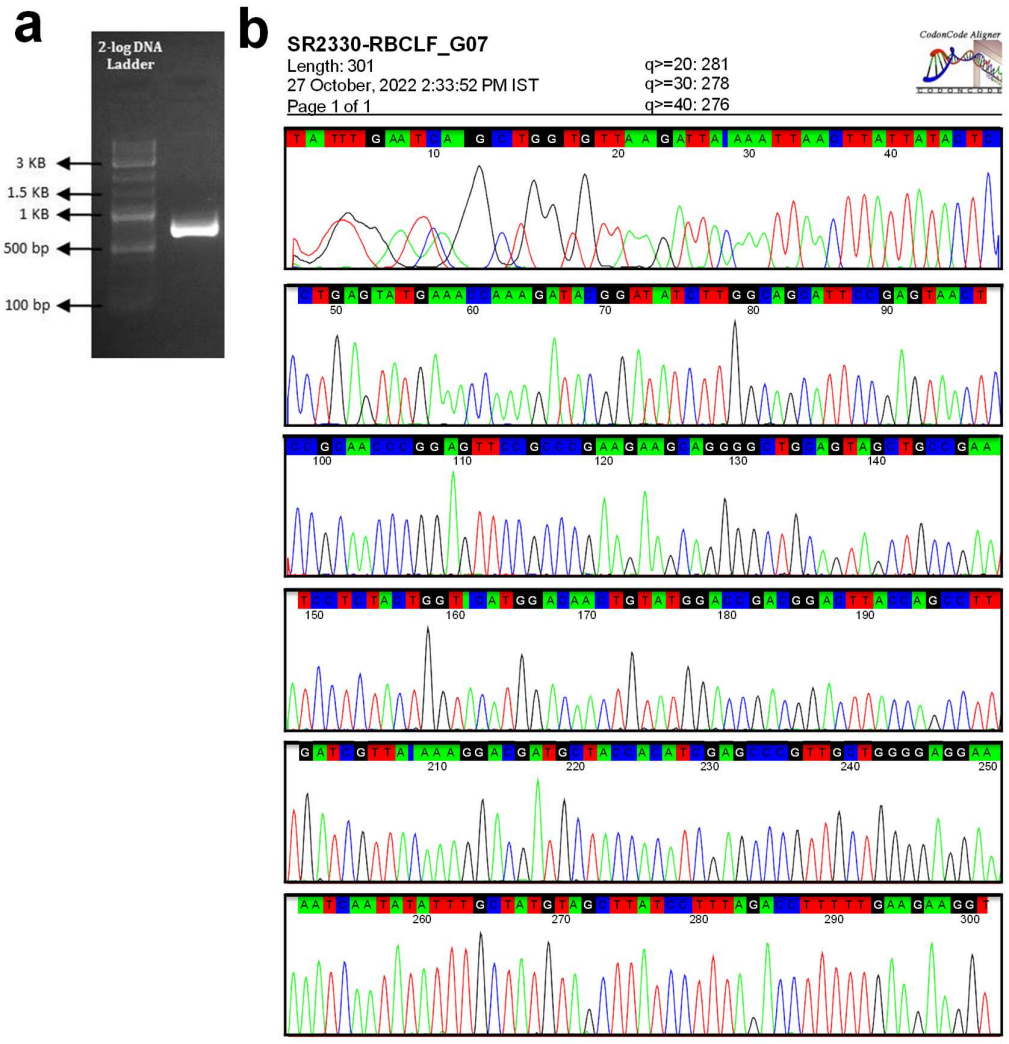

Figure 5. 2 Gel electrophoresis and DNA sequencing chromatogram (a) gel electrophoresis results under UV light using a gel documentation system: Left Lane: 2-log DNA ladder and right lane: the PCR amplification fragment of rbcL, (b) DNA sequencing chromatogram of rbcL gene.

Table 5. 2 Outcomes of BLAST analysis

Parameters of BLAST analysis	Outcomes	Description
Accession	ON720789.1	
Max score	427	It is the uppermost alignment score among the database sequence and query sequence. It signifies sequence similarity and is linearly related to the raw alignment score. The higher the score, the higher the significance of the match.
E- value (Expect)	3e-115	E- value represents the probability that a given sequence match is purely by chance. The lower the E- value, the more significant the match.
Percentage identity	100%	The higher the percentage, the more the match is related

5.4.2 Extraction of *Piper longum* fruits and residual solvent analysis

5.4.2.1 Extraction

Various steps involved in the extraction of *Piper longum* fruits via cold maceration using absolute ethanol have been schematically represented in Figure 5. 3. Microwave irradiation was utilized to enhance the yield of extraction. To avoid the toxicity issues of extracting solvents, it is of utmost importance to use non-toxic or less-toxic solvents and to remove the solvent up to the extent possible [75, 76]. The yield of the obtained dark brown colored ethanolic extract (PLFEE) was found to be 16.532%.

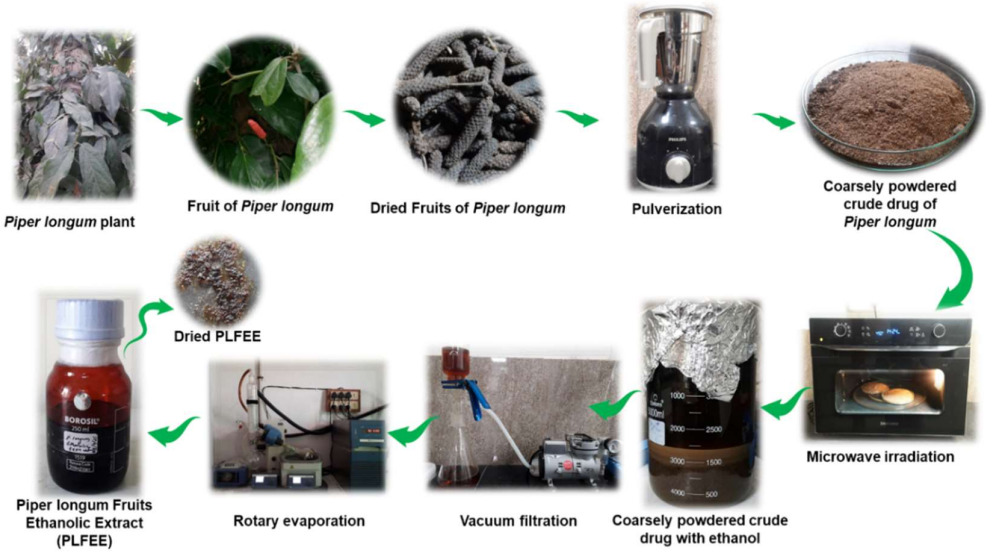

Figure 5. 3 Schematic representation of extraction of *Piper longum* fruits.

5.4.2.2 Residual solvent analysis by GC-HS

The aforementioned GC conditions were selected based on the preliminary works on GC-HS analysis of ethanol with the best resolution. The GC chromatogram (Figure 5. 4a) showed a broad, easily recognizable intense peak (starting from 1.46 min up to 2.93 min) of absolute ethanol at 2.04 ± 0.005 min. The obtained calibration curve was found to be linear within the selected concentration range (10-500 ppm) with a linear regression equation y = 77066.494 x – 707353.883 with a correlation coefficient of 0.999 (inset Figure 5. 4a). The limit of detection (LOD) and limit of quantification (LOQ) was found to be 3.253 ppm and 9.859 ppm, respectively. The residual ethanol in PLFEE (Figure 5. 4b) showed no obvious GC-chromatogram at the retention time (R_t) of standard ethanol, reflecting very low (below the quantification limit) or almost absence of ethanol in the extract (PLFEE).

As per the ICH Q3C(R8) guideline (guideline for residual solvents), the residual solvents should not exceed recommended levels except in exceptional circumstances. Class 3 solvents (e.g., ethanol, ethyl acetate, acetic acid, acetone, etc.) are regarded as less toxic and of lower risk to human health than Class 1 (e.g., carbon tetrachloride,

1,2-Dichloroethane) and Class 2 (e.g., acetonitrile, chlorobenzene, chloroform, cyclohexane, etc.) residual solvents. The amounts of Class 3 residual solvents of 50 mg per day or less (corresponding to 5000 ppm) would be acceptable without justification [77].

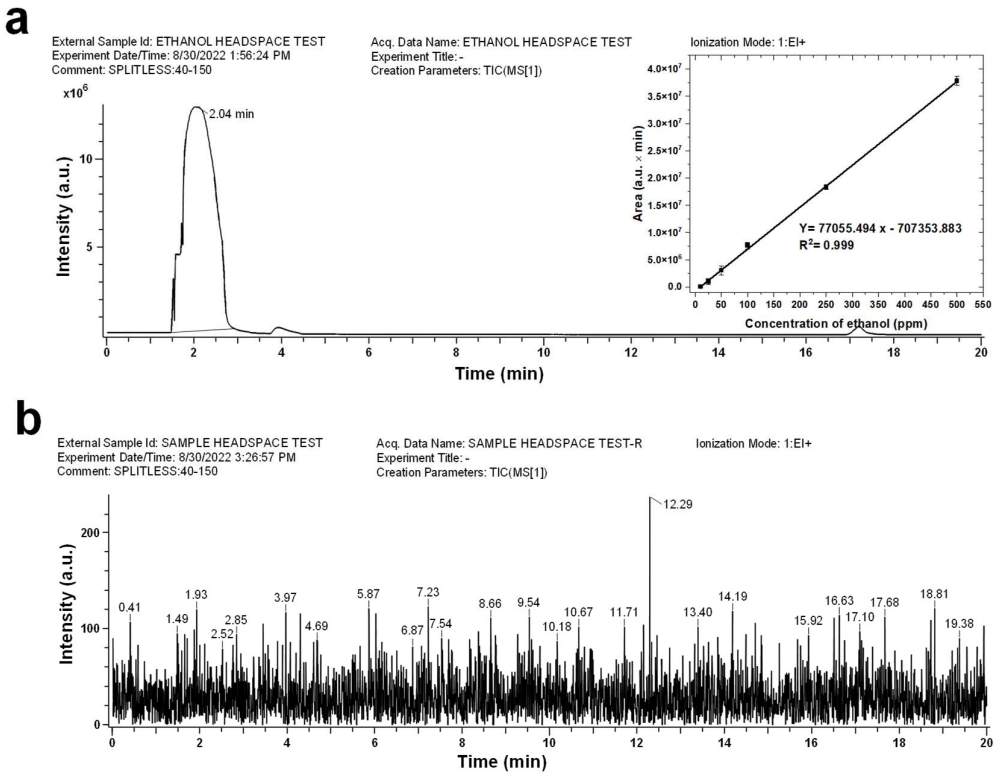

Figure 5. 4 GC-HS analysis for residual ethanol content (a) GC-HS-chromatogram of absolute ethanol with the retention time of 2.04 min and the inset image showing the calibration plot, (b) chromatogram of ethanol in the dried extract (PLFEE)

5.4.3 Marker-based standardization of PLFEE by validated HPLC as per ICH Topic Q 2 (R1)

5.4.3.1 HPLC analysis

Methanol and water at various volume ratios (65:35, 68: 32, 70:30, and 80: 20 v/v) were preliminarily tried as mobile phases in the HPLC method. At a higher proportion of methanol in the mobile phase (80:20 v/v), the retention time of the compounds was found to be within 10 min. Decreasing the proportion of methanol in the mobile phase (65:35 and 70:30 v/v), the Rt was found to exceed 10 min. We have tried to obtain the

best-resolved peaks with minimum Rt times. Based upon the observation of the retention time of peaks and the extent of separation, methanol and water at a volume ratio of 80:20 was selected. Very sharp and well-distinct chromatograms of PIP (Figure 5. 5a) and PLGN (Figure 5. 5b) were observed with the selected mobile phase at Rt of 7.087 ± 0.053 min and 6.413 ± 0.014 min, respectively. The absorption maxima (λmax) obtained from DAD spectra of resolved compounds were found to be 342 nm for PIP (Figure 5. 5c) and 340 nm for PLGN peak (Figure 5. 5d), confirming the identity of PIP and PLGN [78, 79].

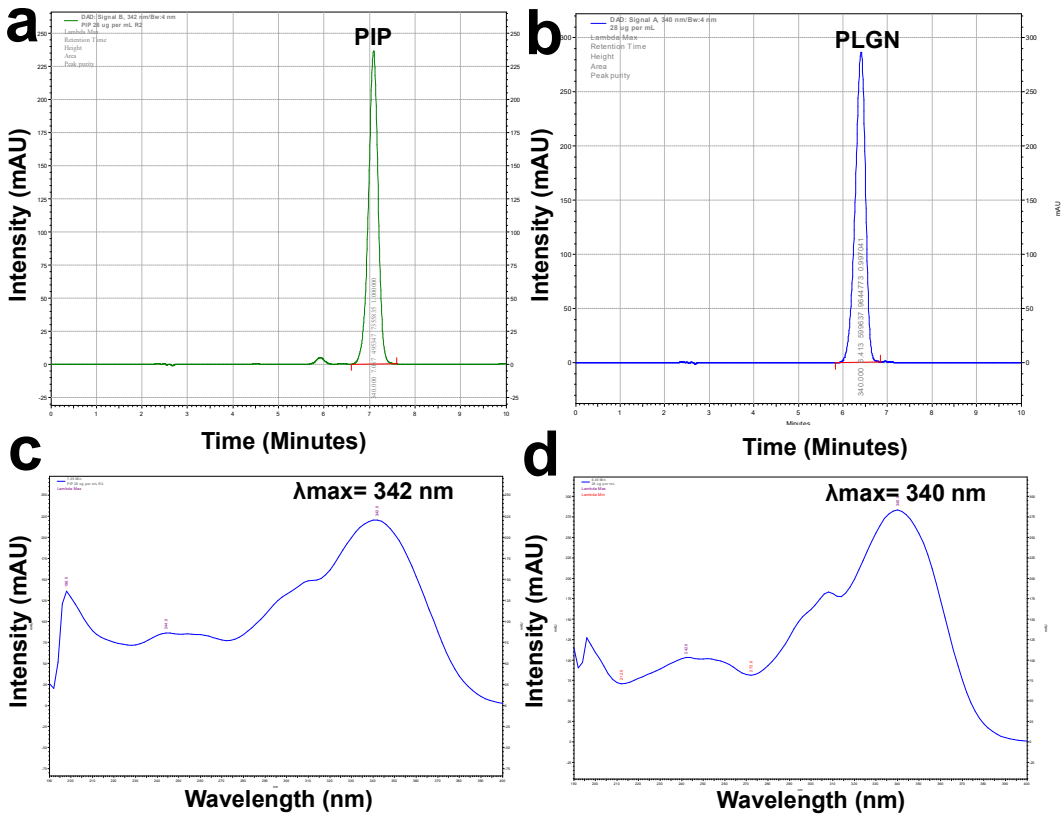

Figure 5. 5 HPLC chromatograms and absorption spectra of PIP and PLGN each of 28 µg/mL in mobile phase methanol and water (80:20 v/v) (a) chromatogram of PIP (Rt = 7.087 ± 0.053 min) at wavelength 342 nm, (b) chromatogram of PLGN (Rt = 6.413 ± 0.014 min) at wavelength 340 nm, (c) spectrum of PIP showing the maximum absorbance at 342 nm, and (d) spectrum of PLGN showing the absorption maxima at 340 nm

The two-dimensional (2-D) and three-dimensional (3-D) chromatograms of PIP (Figure 5. 6a and Figure 5. 6b) and that of PLGN (Figure 5. 6c and Figure 5. 6d) confirmed the absence of any peaks around the elution time of PIP or PLGN. However, very min spots around Rt of 2 min and 4 min were due to the mobile phase, whose intensity is very low and absent in the chromatograms (Figure 5. 5a and Figure 5. 5b) due to the higher signal-to-noise ratio of markers. The red region in the contour and 3-D plots represents the highest intensity of the marker compounds with their corresponding retention times and wavelengths.

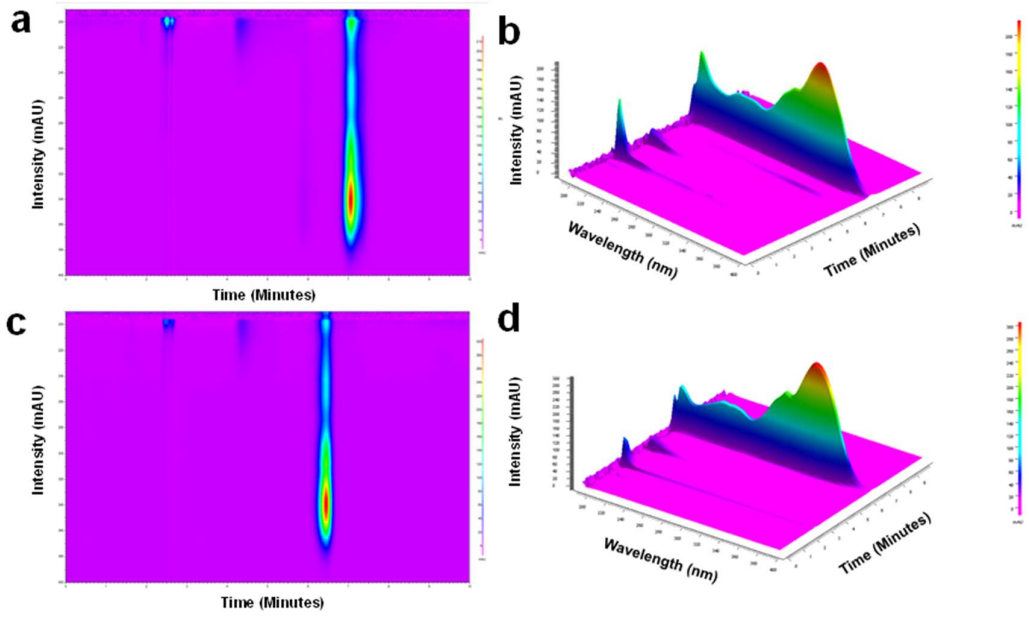

Figure 5. 6 Two-dimensional (2-D) and three-dimensional (3-D) chromatograms of PIP and PLGN (a) 2-D contour chromatogram of PIP (b) 3-D chromatogram of PIP, (c) 2-D contour chromatogram of PLGN, and (d) 3-D chromatogram of PLGN

5.4.3.2 Validation

5.4.3.2.1 Linearity and range

The average area of three repetitive measurements for PIP and PLGN plotted against their respective concentrations is presented in Figure 5. 7a and Figure 5. 7b. A linear relationship was found between the selected concentrations of PIP (2 µg/mL to 30 µg/mL) and the chromatographic area (at 342 nm) with correlation coefficients (r^2 =

0.997) and regression equation y = 276947.424 x - 200378.812 (n = 3). Similarly, a linear relationship was found between the used concentrations of PLGN (2 µg/mL to 30 µg/mL) and the corresponding chromatographic area (at 340 nm) with correlation coefficients (r^2 = 0.996) and regression equation y = 349329.568 x - 272061.184 (n = 3). The high correlation coefficient values (r^2 = 0.99) in both cases of the fitted model indicate a strong relationship among the variables.

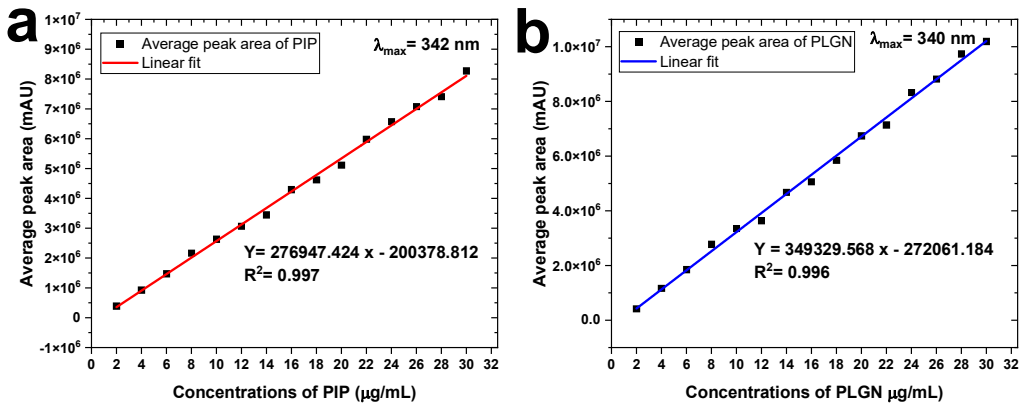

Figure 5. 7 HPLC-based calibration curves (a) calibration curve of PIP measured at wavelength 342 nm and (b) calibration curve of PLGN measured at wavelength 340 nm

5.4.3.2.2 Accuracy

The closeness of theoretical and experimental values indicates the accuracy of the analytical method. The results of the accuracy of PIP and PLGN in terms of % recovery and percentage relative standard deviation (% RSD) were represented in Table 5. 3. The high recovery values and low percent RSD (<2%) reflect the excellent accuracy of the developed HPLC method [78].

Table 5. 3 Accuracy studies of the developed HPLC method (n = 3)

Concentration of PIP (µg/mL)	**Level (%)**	**PIP spiked concentration (µg/mL)**	**Theoretical concentration (µg/mL)**	**Experimental concentration (µg/mL)**	**% Recovery**	**% RSD**
8	50	4	6	5.991 ± 0.045	99.85	0.757
8	100	8	8	8.082 ± 0.136	101.031	1.694
8	150	12	10	9.934 ± 0.123	99.345	1.242
Concentration of PLGN (µg/mL)	**Level (%)**	**PLGN spiked concentration (µg/mL)**	**Theoretical concentration (µg/mL)**	**Experimental concentration (µg/mL)**	**% Recovery**	**% RSD**
8	50	4	6	6.359 ± 0.027	105.986	0.425
8	100	8	8	7.998 ± 0.016	99.976	0.201
8	150	12	10	10.026 ± 0.124	100.268	1.241

5.4.3.2.3 Precision

Precision indicates the degree of scattering (closeness of agreement) among a series of readings obtained from multiple specimens of the same homogeneous sample under specified analytical conditions. In the present study, the repeatability and intermediate precision (intra-day and inter-day) studies were performed to examine the precision of the HPLC method for various concentrations (6, 12, and 18 µg/mL) of working standard solution. The % RSD values obtained from the repeatability study were found to be 0.16% for PIP and 0.119% for PLGN (Table 5. 4).

The results of intermediate precision studies are shown in Table 5. 5. From intra-day precision studies, the % RSD values of the PIP peak area ranged from 0.146 to 0.299%, and for inter-day precision, the % RSD values ranged from 0.258 to 0.369%. Similarly, the % RSD values of the PLGN peak area were found to be between 0.064 and 0.88% (intra-day precision) and between 0.721 and 1.155% (inter-day precision). In all cases, the % RSD values were lesser than 2%, which fulfilled the acceptance criteria and showed a high degree of precision of the developed HPLC method [78].

Table 5. 4 Repeatability study of developed HPLC method (n = 6)

Concentration of PIP (µg/mL)	**Mean Area (mAU)**	**SD**	**RSD (%)**
12	3230831.167	5177.835	0.16
Concentration of PLGN (µg/mL)	**Mean Area (mAU)**	**SD**	**RSD (%)**
12	3643443.167	4354.112	0.119

Table 5. 5 Intermediate precision (Intra-day and inter-day precision) studies of the developed HPLC method

Concentration of PIP (µg/mL)	**Intra-day (at an interval of 6 h)**		**Inter-day (Day1- Day3)**	
	Obtained Area ± SD (mAU)	**RSD (%)**	**Obtained Area ± SD (mAU)**	**RSD (%)**
6	1618408 ± 3798.53	0.234	1622421 ± 5989.94	0.369
12	3241725.333 ± 4740.057	0.146	3256179.333 ± 10828.978	0.332
18	4714037.333 ± 14102.094	0.299	4707917 ± 12164.467	0.258
Concentration of PLGN	**Intra-day (at an interval of 6 h)**		**Inter-day (Day1- Day3)**	
	Obtained Area ± SD (mAU)	**RSD (%)**	**Obtained Area ± SD (mAU)**	**RSD (%)**
6	1880619 ± 1216.598	0.064	1850762.667 ± 13350.252	0.721
12	3687662.667 ± 32458.115	0.88	3639347.333 ± 37043.657	1.017
18	5872113.333 ± 22782.816	0.387	5845683.333 ± 9105.994	1.155

5.4.3.2.4 LOD and LOQ

The LOD and LOQ values for the determination of PIP were found to be 0.259 and 0.786 µg/mL, respectively. Similarly, the LOD and LOQ values for PLGN were found to be 0.27 and 0.819 µg/mL, respectively. The lower values of LOD and LOQ of the developed HPLC method indicated the sensitivity of the optimized method for analysis of PIP and PLGN.

5.4.3.2.5 Robustness and Ruggedness

It is a measure of the ability of the HPLC method to remain unaffected by slight but deliberate changes in the method variables that offer its reliability during normal usage. Robustness and ruggedness of the developed HPLC method investigated by deliberate changes in different chromatographic conditions showed the lower % RSD values (<2%) of the area and retention time (Table 5. 6 and Table 5. 7), which confirm the robustness of the developed HPLC method.

Table 5. 6 Robustness and ruggedness study of developed HPLC method for PIP (12 µg/mL)

Parameters	**Variations made**	**Area ± SD (**mAU)	**RSD (%)**	**Retention time (Rt) ± SD (min)**	**RSD (%)**
Wavelength (nm)	340	3390391.333 ± 20376.823	0.601	7.133 ± 0.021	0.303
	342	3377712 ± 19944.805	0.59	7.133 ± 0.021	0.303
	344	3340953.333 ± 19354.762	0.579	7.133 ± 0.021	0.303
Run Time (min)	8	3382801.333 ± 11055.37	0.326	7.135 ± 0.027	0.387
	10	3227710 ± 501.352	0.015	7.092 ± 0.021	0.305
	12	3375691.667 ± 16612.698	0.492	7.173 ± 0.03	0.426
Flow rate (mL/min)	0.8	4190108.667 ± 8244.626	0.196	8.888 ± 0.041	0.472
	1	3223921.667 ± 4744.925	0.147	7.095 ± 0.026	0.366
	1.2	2833650.333 ± 12476.467	0.44	6.028 ± 0.019	1.818
Mobile phase composition (Methanol: Water) v/v	78:22	3450097 ± 13660.3214	0.395	8.125 ± 0.07	0.984
	80:20	3257255 ± 42512.258	1.305	7.099 ± 0.022	0.317
	82:18	3459427.66 ± 25292.359	0.731	6.52 ± 0.015	0.234

Table 5. 7 Robustness and ruggedness study of developed HPLC method for PLGN (12 µg/mL)

Parameters	**Variations made**	**Area ± SD (mAU)**	**RSD (%)**	**Retention time (Rt) ± SD (min)**	**RSD (%)**
Wavelength (nm)	338	3711673.667 ± 43144.199	1.162	6.411 ± 0.007	0.121
	340	3688361.33 ± 66729.35	1.809	6.405 ± 0.015	0.24
	342	3856646 ± 17489.522	0.453	6.409 ± 0.009	0.146
Run Time (min)	8	3746709.667 ± 55753.844	1.488	6.402 ± 0.003	0.058
	10	3644321.667 ± 5695.461	0.156	6.412 ± 0.012	0.198
	12	3716112 ± 54328.062	1.461	6.401 ± 0.009	0.147
Flow rate (mL/min)	0.8	4657913 ± 41988.913	0.901	8.072 ± 0.062	0.778
	1	3655028 ± 19590.404	0.535	6.419 ± 0.004	0.07
	1.2	3157162.333 ± 11512.149	0.364	5.308 ± 0.018	0.357
Mobile phase composition (Methanol: Water) v/v	78:22	3775809 ± 13947.62	0.369	6.828 ± 0.034	0.508
	80:20	3642568 ± 32103.463	0.881	6.415 ± 0.009	0.154
	82:18	3785191.667±22562.0006	0.596	5.729±0.089	0.455

5.4.3.2.6 System Suitability

The system suitability checks the specificity and validity of the developed analytical method. The % RSD of various validation parameters, like retention time (Rt), peak area, the number of theoretical plates, peak purity, tailing factor, and capacity factor, was estimated for system suitability analysis (Table 5. 8 and Table 5. 9). The % RSD of all examined parameters was within the permissible limits (<2%) [78]. The number of theoretical plates (N>2000) and tailing factor (<2) were within the acceptable limit [78]. The co-elution of degradants or impurities was explored by examining the peak purity. The peak purity curve for PIP at 7.087 min (Figure 5. 8a) and PLGN at 6.413 min (Figure 5. 8b) signifies nothing was co-eluting along with PIP or PLGN and no interference at their retention times. Such purity reports were also reported elsewhere [80]. The peak purity of PIP and PLGN was found to be 1, representing a high degree of purity [80]. The purity factor was greater than the purity threshold, representing that no additional compounds were co-eluted with the markers [81]. The capacity factor (K^1) represents the degree of interaction of the analyte with the stationary phase in the column. A capacity factor of zero indicates no interaction, whereas a capacity factor >1 indicates a certain degree of interaction with the stationary phase. Ideally, a capacity factor of an analyte varies from 1-5. A higher value of capacity factor (>20) indicates a greater degree of interaction of analytes with the stationary phase, which leads to prolongation of elution time. In both cases (i.e., PIP and PLGN), the K^1 values were found to be within optimal range, thus require less run time (10 min) for analysis. The system suitability results disclose the suitability of the developed HPLC system for quantitative analysis of PIP and PLGN.

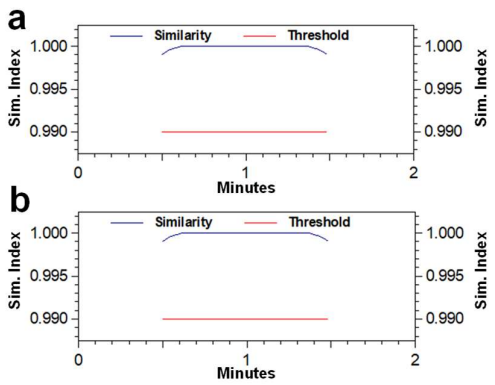

Figure 5. 8 Purity curve of PIP and PLGN (a) purity curve of PIP and (b) purity curve of PLGN

Table 5. 8 System suitability study of developed HPLC method for analysis of PIP (12 µg/mL), n = 6

Parameters	**Average Value ± SD**	**RSD (%)**
Area (mAU)	3230831.167 ± 5177.835	0.16
Retention time (Rt)	7.047 ± 0.023	0.425
Number of theoretical plates	3592.834 ± 43.26	1.204
Peak purity	0.998 ± 0.002	0.2
Tailing factor (Asymmetry)	0.9235 ± 0.0017	0.194
Capacity factor (k^1)	1.448 ± 0.009	0.646

Table 5. 9 System suitability study of developed HPLC method for analysis of PLGN (12 µg/mL), n = 6

Parameters	**Average Value ± SD**	**RSD (%)**
Area (mAU)	3643443.167 ± 4354.112	0.119
Retention time (Rt)	6.406 ± 0.041	0.646
Number of theoretical plates	3541.5 ± 31.17	0.88
Peak purity	0.99 ± 0.007	0.775
Tailing factor	0.868 ± 0.007	0.838
Capacity factor (k^1)	1.135 ± 0.002	0.193

5.4.3.3 Marker-based standardization of PLFEE

As herbal drugs are complex mixtures of phytoconstituents, sufficient efforts are needed to guarantee a constant and adequate quality [82]. So, it is very important to standardize the plant extract with reference to chemical markers. Chemical markers

(either active markers or analytical markers) refer to phytoconstituents, including primary and secondary metabolites and other macromolecules [83]. Standardization ensures that each dosage unit of the herbal product will deliver the same amount of phytoconstituents, which is a prerequisite for reproducible therapeutic effects. The peak of PIP and PLGN in the PLFEE was verified from their respective retention time (Rt) and corresponding absorption maxima (λmax). The Rt of PIP and PLGN in the PLFEE was obtained at 7.087 ± 0.023 min and 6.340 ± 0.046 min, respectively (Figure 5. 9a and Figure 5. 9b), which matches with the Rt of pure PIP at Rt = 7.087 ± 0.053 min and pure PLGN at 6.413 ± 0.014 min (Figure 5. 9a and Figure 5. 9b). The peak for PIP was found to be very intense compared to the peak of PLGN, reflecting the comparatively higher amount of PIP in the PLFEE than PLGN. The corresponding λmax obtained from the DAD detector for PIP and PLGN in the PLFEE was found to be at 342 nm and 340 nm, respectively (Figure 5. 9c and Figure 5. 9d). The obtained λmax corresponding to PIP and PLGN in PLFEE is in accordance with the obtained λmax of PIP (Figure 5. 5c) and PLGN (Figure 5. 5d), confirming the existence of two selected markers in the PLFEE. However, the spectral intensity of PLGN (Figure 5. 9c) is very low due to its low quantity in PLFEE. Further, the contour plots (Figure 5. 9e and Figure 5. 9f) and 3-dimensional chromatograms (Figure 5. 9g and Figure 5. 9h) matched with the chromatograms of pure PIP and PLGN (Figure 5. 6). The area of the respective peak was considered for quantitative estimation of PIP and PLGN by analyzing the samples in triplicate. The chromatogram (Figure 5. 9a) for PLFEE (1 mg/mL) was selected for the quantification of PLGN at 340 nm, and the chromatogram (Figure 5. 9b) for PLFEE (50 µg/mL) was selected for the quantification of PIP at 342 nm. The quantity of PIP was found to be 377.0687 ± 1.453 mg, and PLGN was found to be 6.72 ± 0.108 mg per gram of dried PLFEE.

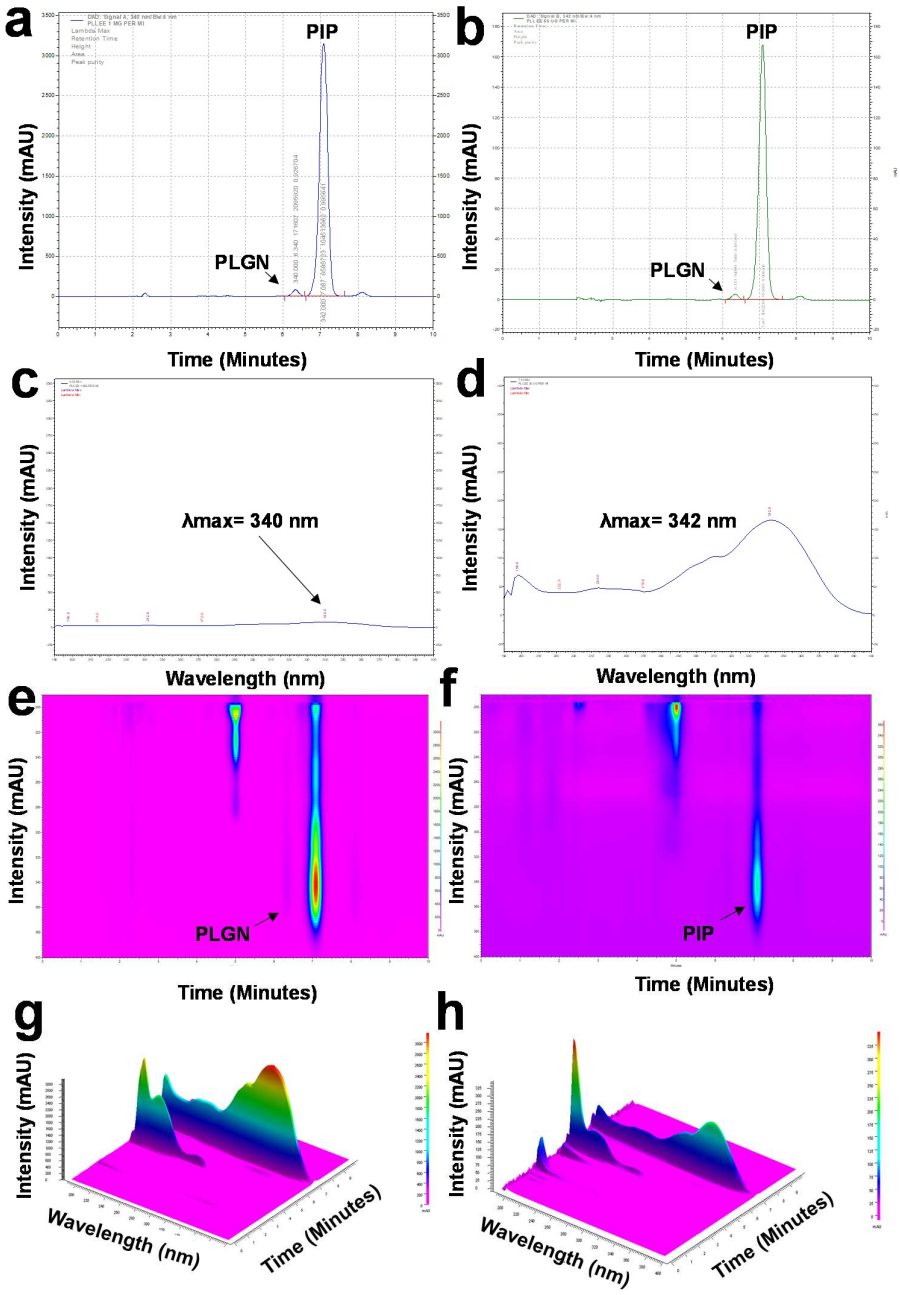

Figure 5. 9 HPLC results of PLFEE 1mg/mL and 50µg/mL in mobile phase methanol and water (80:20 v/v) (a) Chromatograms of PIP (Rt = 7.087 ± 0.023 min) and PPLGN (Rt = 6.340 ± 0.046 min) in 1 mg/mL of PLFEE at wavelength 340 nm (b) Chromatogram of PIP (Rt = 7.087 ± 0.023 min) at wavelength 342 nm and chromatogram of PLGN (Rt = 6.333 ± 0.027 min) at wavelength 342 nm, (c) spectrum of PLGN in 1 mg/mL of PLFEE showing the maximum absorbance at 340 nm, (d) spectrum of PIP in 50 µg/mL of PLFEE showing the absorption maxima at 342 nm, (e) 2-D contour chromatogram of PIP and PLGN in 1 mg/mL of PLFEE (f) 2-D contour chromatogram of PIP and PLGN in 50 µg/mL of PLFEE, (g) 3-D chromatogram of PIP and PLGN in 1 mg/mL of PLFEE, and (h) 3-D chromatogram of PIP and PLGN in 50 µg/mL of PLFEE

5.5 Conclusions

The taxonomical and DNA-based molecular authentication of fruits ensured the identity of the received sample as fruits of *Piper longum* Linn. (Family: Piperaceae). The extraction was carried out successfully using absolute ethanol via microwave irradiation, followed by the cold maceration method. The GCHS analysis of PLFEE revealed the absence of ethanol in the extract, hence deprived of ethanol-related toxicities. Also, as per the ICH Q3C(R8) guideline (guideline for residual solvents), the amounts (5000 ppm per day) of Class 3 residual solvents would be acceptable without justification. The marker-based standardization was successfully carried out using a validated HPLC method. The overall results of validation reflected the suitability of the developed HPLC method for the quantification of PIP and PLGN accurately. The amount of a chemical marker is a signifier of the quality of an herbal product. The standardization of extract will ensure that each dosage unit of the herbal product will deliver the same amount of phytoconstituents (dosage uniformity), which is a prerequisite for reproducible therapeutic effects.

5.6 Summary points:

- Authentication of collected fruits by taxonomical and DNA-based molecular authentication ensured the identity of the received sample as fruits of *Piper longum* Linn. (Family: Piperaceae).
- The extraction yield of the PLFEE via microwave-assisted cold maceration method was found to be satisfactory.
- The GC-HS analysis of PLFEE revealed the residual ethanol below the permissible limit.
- The overall outcomes of validation reflected the suitability of the developed HPLC method for the quantification of PIP and PLGN accurately.
- The marker-based standardization of extract will ensure batch-to-batch consistency, dosage uniformity, and reproducible therapeutic effects.