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Development and Validation of an HPTLC Method as per ICH Guidelines Followed by GC-MS Characterization of Lupeol from Arisaema tortuosum (Wall.) Schott

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ABSTRACT

Arisaema tortuosum (Wall.) Schott holds significant ethnomedicinal value, yet its phytochemical profile has remained scientifically underexplored. This study provides a comprehensive analysis of its tuber extracts, revealing substantial solvent-dependent variations in composition. Extraction through maceration yielded 2.5% (chloroform), 2.9% (ethyl acetate), 6.3% (ethanol), and 11.2% (aqueous) w/w of crude extract. Phytochemical screening demonstrated the presence of alkaloids, flavonoids, phenols, and tannins across various extracts. Quantitative analysis established the ethanol extract as superior, containing the highest total phenolic (0.878 mg GAE/100 mg) and flavonoid (0.805 mg QE/100 mg) content.

A novel HPTLC method for lupeol quantification was developed and validated per ICH guidelines, showing excellent linearity (R^2 = 0.999) across 50-175 ng/spot. The method demonstrated high precision (intra-day %RSD 0.04-0.09, inter-day %RSD 1.60-1.75) and accuracy (recovery 99.92-100.05%), with LOD and LOQ of 1.22 and 3.70 ng/spot respectively. Lupeol was identified at Rf 0.67 and quantified highest in ethanol extract (0.067% w/w), followed by chloroform (0.055%) and ethyl acetate (0.052%) extracts. GCMS analysis confirmed lupeol identity through matching retention times (sample: 31.85 min, standard: 31.86 min) and characteristic molecular ion (m/z 426).

This study validates A. tortuosum as a rich source of bioactive compounds. The ethanol extract emerges as a particularly promising candidate for further drug development. These findings successfully bridge traditional knowledge with scientific validation, advocating for deeper exploration of its phytochemical mechanisms. The proposed HPTLC method provides a dependable tool for quality control and standardization of this vital medicinal plant.

Keywords*: Arisaema tortuosum, Lupeol, HPTLC, Method validation, GC-MS, Isolation, ICH guidelines.*

INTRODUCTION

The enduring value of plant-based natural products in drug discovery is well established, serving as an essential source for a diverse range of bioactive compounds that provide templates for new therapeutics.[1-6] This is particularly relevant for species within the Arisaema genus, which are gaining scientific recognition for their potential as sources of immunity-boosting compounds, moving from botanical curiosities to subjects of serious pharmacological inquiry.[7] This focus forms part of a broader renaissance in herbal medicine, where a renewed appreciation for traditional knowledge systems is driving researchers to apply sophisticated tools to validate ancient wisdom.[8-9] A prime candidate for this approach is Arisaema tortuosum, a plant whose leaf extracts demonstrate a rich antioxidant profile, providing a molecular basis for its historical use in traditional healing practices.(10) However, translating traditional promise into evidence-based therapy requires unwavering scientific rigor, beginning with the fundamental principle of standardization to ensure consistent and reproducible plant material. (11-13) This process involves creating a unique chemical fingerprint, for which techniques like High-Performance Thin-Layer Chromatography (HPTLC) are essential, confirming botanical identity and ensuring chemical consistency across batches. (14-16) Such foundational work enables significant discoveries, as demonstrated by

the recent isolation of anticancer molecules from Arisaema flavum, a close relative, signaling the genus's potential for yielding novel therapeutic entities. (17) To fully contextualize A. tortuosum, its deep-rooted ethnobotanical legacy must be considered; comprehensive reviews document its traditional use against conditions ranging from inflammation to tumors, providing a historical roadmap for modern research. (18). Building on this, systematic reviews confirm the genus chemical diversity and broad bioactivity, strengthening the scientific rationale for focused investigation on specific species like A. tortuosum. (19) Further supporting its pharmacological value, research reveals that the plant's utility is not confined to a single domain, with leaf extracts showing significant antiviral activity and thus revealing a broader defensive capability. (20) Additionally, the identification of nutritional metabolites in some Arisaema species suggests a dual role that complements targeted therapy with foundational health support. (21)

Empirical validation extends to its antibacterial properties, where laboratory studies using bacterial growth kinetics have quantitatively confirmed its efficacy, translating traditional knowledge into modern scientific terms. (22) Beyond establishing its biological activity, understanding how it acts is crucial; investigations have begun to unravel its mechanism, identifying specific lectins that bind to cell surface sugars, thereby inhibiting cancer cell proliferation. (23) Discoveries of this nature elevate plant extracts from simple remedies to valuable lead compounds, which are essential starting points for developing optimized pharmaceutical agents. (24) The validation pathway often integrates traditional insight with modern technology, as seen in studies on A. tortuosum that combined computational prediction with laboratory work to confirm its antioxidant and specific anti-breast cancer activities. (25) Furthermore, the plant's pharmacological repertoire appears extensive, with related species demonstrating anxiolytic effects, indicating potential applications in neurological and emotional well-being. (26) The activity is not limited to isolated compounds; studies on crude tuber extracts have confirmed a combination of anti-proliferative, antioxidant, and anti-inflammatory properties, illustrating the therapeutic potential of the plant's complete chemical profile.(27) The foundational evidence for this research trajectory was established by a pioneering study that successfully isolated a lectin from A. tortuosum and demonstrated its direct anticancer activity in vitro, providing the first mechanistic evidence for its traditional use against tumors. (23) Building upon this sequentially established foundation, the present study aims to integrate these diverse threads ethnobotanical clues, chemical standardization, compound discovery, and mechanistic insight into a unified investigation.

The objective of the present study was to investigate the phytochemical profile of Arisaema tortuosum tubers and to evaluate their potential as a source of bioactive compounds. The study aimed to develop and validate a simple, precise, and reproducible HPTLC method for the quantification of lupeol in different solvent extracts. It also sought to isolate lupeol from the selected extract and confirm its identity through GC-MS analysis. In addition, the work focused on comparing the extraction yields and phytochemical contents of the selected extracts, with an emphasis on supporting quality control applications in the pharmaceutical and herbal industries. Overall, the study was designed to support the standardization and quality evaluation of A. tortuosum tubers and to provide a scientific basis for further pharmacological exploration.

MATERIAL AND METHOD

Reagents and standards

All chemicals and solvents used were of analytical grade and obtained from Merck (Darmstadt, Germany). From this, solution was applied using Linomat applicator on TLC aluminum plates precoated with silica gel 60 F254 (10 · 10 cm, 0.2 mm thick) obtained from E. Merck Ltd. (Mumbai, India). (28-29)

Plant material

Fresh tubers of Arisaema tortuosum (Wall.) Schott were collected from the Raisen Region of Madhya Pradesh, India, in December 2022. The plant was authenticated by a botanist at the Minor Forest Produce Processing and Research Centre, Bhopal, Madhya Pradesh, as shown in

Fig. 1. (30)

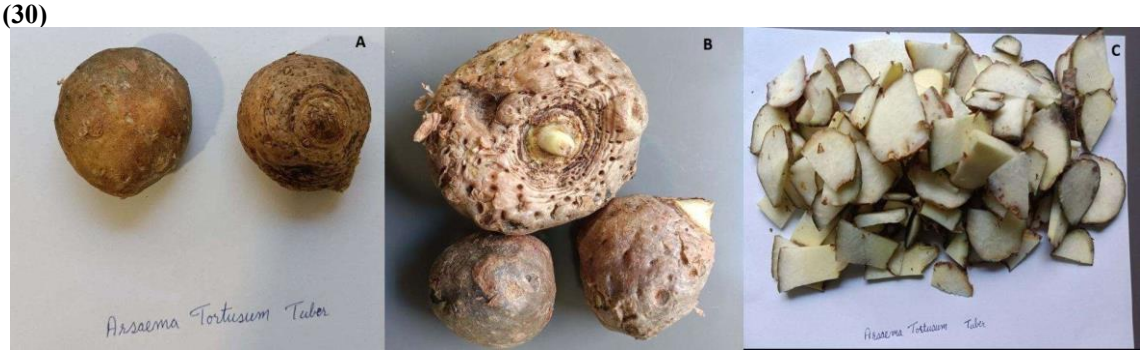

Figure 1. Sequential steps in the preparation of Arisaema Tortuosum tubers: (A) Collection of tubers, (B) Cleaning process, and (C) Cutting the tubers into slices for experimental use.

The tubers were thoroughly washed. The cleaning procedure was divided into the following stages: The decayed or deteriorated plant matter was removed first. The samples were then washed with tap and purified water. To extract excess water, the washed plant materials were covered with blotting paper. Plant material was stored for drying in the sun, but in the shade, shortly after washing. The primary goal of drying is to extract the water content of the plant for processing. The dried plant parts were finely powdered using an electric grinder, sieved, and stored in polyethylene bags. (30-31)

2.3 Extraction by Maceration Process

Dried powder (40 g) was extracted with different solvents (Chloroform, Ethyl acetate, Ethanol and Water) using the maceration method. The samples were left for 2 days in a sterile environment. The liquid extract was then filtered through Whatman filter paper (no. 40). The filtrate was kept in a water bath at 80-90°C till the extract was dried. (30-33)

2.4 Biochemical Assays

Preliminary screening of phytochemical tests was performed for testing various phytochemicals found in plants. The crude extracts were tested for the presence or absence of secondary metabolites, such as alkaloids, phenolic compounds, flavonoids, saponins, tannins,
and glycosides. (30-34)

2.5 Quantitative Studies of Phytoconstituents

2.5.1 Estimation of Total Phenol Content

The total phenol content of the extract was determined using the modified Folin–Ciocalteu method. Gallic acid (10 mg) was dissolved in 10 ml methanol, and various aliquots of 1050µg/ml were prepared in methanol. Dried extract (10 mg) was dissolved in 10 ml methanol and filtered. Two milliliters (1 mg/ml) of this extract was used for the estimation of phenol. The extract and each standard (2 ml) were mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 10min for colour development. The absorbance was measured at 765 nm using a spectrophotometer. (30-35)

2.5.2 Estimation of Total Flavonoids Content

The total flavonoid content was determined using the aluminum chloride method. Quercetin (10 mg) was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol. Ten milligrams of the dried extract was dissolved in 10 ml methanol and filtered. Three ml (1 mg/ml) of this extract was used for the estimation of flavonoids.1 ml of 2% AlCl3 solution was added to 3 ml of extract and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm. (30-35)

2.6 Preparation of plant extracts

To extract bioactive compounds from Arisaema tortuosum (AT), powdered plant material (300 mg) was separately dissolved in four different organic solvents: ethyl acetate, chloroform, ethanol, and methanol. Each mixture was subjected to sonication for 15 min to enhance solubility, followed by centrifugation at 3000 rpm for 10 min to separate the insoluble residue. The resulting supernatant was carefully collected and transferred to vials for subsequent analysis. (36)

2.7. Chromatography conditions

Chromatographic analysis was performed using high-performance thin-layer chromatography (HPTLC) on TLC aluminum plates pre-coated with silica gel 60 F_{254} (Merck). Samples and standards were applied as bands onto the plates using a CAMAG Linomat V automatic thinlayer chromatography (TLC) applicator. The plates were developed in a CAMAG twin-trough glass chamber previously saturated with the mobile phase vapor for 20 min at room temperature, with an ascending development distance of 70 mm. For the fingerprinting, identification, and quantification of lupeol, as well as for subsequent elution, a mobile phase of Ethyl Acetate: Formic Acid: Toluene (4:0.3:6, v/v/v) was employed. After development, the plates were dried and derivatized for visualization using anisaldehyde sulfuric acid reagent (ASR). The derivatized chromatograms were documented under white and UV light at 366 nm, and densitometric scanning was performed using a CAMAG TLC Scanner III controlled by win CATS software. Furthermore, the prominent bands corresponding to the identified compounds were eluted from the plate using a CAMAG TLC-MS Interface 2 for hyphenated analysis. (27,34,37)

2.7.1 Preparation of standard solution

An initial stock solution of Lupeol was prepared by dissolving 2 mg of the standard compound in 2 mL of methanol, yielding a concentration of 1 mg/mL (denoted as Sample A).

Sample A was then subjected to a 1:1 dilution using the appropriate diluent, resulting in a secondary solution with a concentration of 0.5 mg/mL (Sample B).

Sample B was further diluted at a 1:10 ratio with the diluent to obtain a working concentration of 0.05 mg/mL (Sample C). This final dilution (Sample C) was used for quantitative analysis. (37)

2.7.2 Detection and Quantitation

After sample application plates were developed in a Camag twin through a glass chamber presaturated with the mobile phase (20 ml Ethyl Acetate: Formic Acid: Toluene (4:0.3:6, v/v/v) for 20 min. The plate was developed in Camag horizontal developing chamber (20 x 10 cm) at the room temperature up to 7cm. Ascending mode was used for the development of Thin Layer chromatography. After development, plates were dried with a hair dryer and then derivatization of the chromatogram was performed by Camag dip tank using Anisaldehyde Sulphuric Acid reagent. The plate was observed under Camag UV cabinet (254, white light and 366 nm). Quantitative analysis of the compounds was done by scanning the plates at 545 nm using Camag TLC scanner III equipped with Vision-CATS software (Camag). The identification of Lupeol was confirmed by spiking study. A densitometry HPTLC analysis was also performed for the development of characteristic fingerprint profile, which may be used as a marker for quality evaluation and standardization of the drugs. (37)

2.7.3 Calibration of Lupeol

The lupeol content was determined using a calibration curve established over the range of 50175 ng/spot. A stock solution of standard lupeol (1 mg/mL) was prepared in methanol and further diluted to 0.01 mg/mL. Then, 5, 7.5, 10, 12.5, 15, and 17.5 µL of the working solution were applied on the HPTLC plate to obtain 50, 75, 100, 125, 150, and 175 ng/spot, respectively. The peak area for each concentration was plotted against the corresponding applied amount of lupeol. The linear regression of the standard curve was determined with $R^2 = 0.999$. The linear regression line is $y = 0.00003x - 0.0005$.

The regression data have shown a good linear relationship over the concentration range of 50–175 ng/spot. The linearity of the calibration graph and adherence of the system to Beer's law were validated by the high value of the correlation coefficient. The low %RSD values for peak areas at each concentration level
(ranging from 0.41% to 0.89%) indicate excellent repeatability. (37)

2.8 Validation of HPTLC Method

2.8.1. Precision

ICH guidelines were followed for the validation of the analytical method developed for precision, repeatability and accuracy. Instrumental precision, intraday precision and interday precision of the method were determined. Instrumental precision was measured by replicate (n = 6) applications of same Lupeol solution. Intraday assay precision was evaluated by analysis of replicate (n = 6) applications of freshly prepared standard solution of same concentration (50-175 ng/spot), on the same day. Intermediate precision was evaluated by analysis of replicate (n = 6) applications of standard solution of the same concentration (50-175 ng/spot) on six different days. The repeatability of sample application and measurement of peak area have been expressed in terms of % RSD. (38-42)

2.8.2. Limit of Detection and Limit of Quantification

To evaluate the limit of detection and limit of quantification, different concentrations of the standard solutions of lupeol were applied along with chloroform as a blank and determined based on the signal-to-noise ratio. The LOD was determined at an S/N ratio of 3:1, and the LOQ was determined at an S/N ratio of 10:1. (41-43) 2.8.3 Specificity

The specificity of the method was ascertained by analyzing standard Lupeol and extracts. The spot for Lupeol in the sample was confirmed by comparing the Rf and spectra of the spot with that of sample. The peak purity of Lupeol was assessed by comparing the spectra at three different levels, i.e., peak start, peak middle and peak end positions of the spot/ bands. (41,43) 2.8.4. Robustness

The estimation was performed by varying the selected parameters (mobile phase composition, mobile phase volume and duration of mobile phase saturation) within certain limits (±10%) and there has been no notable alteration found in method performance and in results obtained. The results were indicated by the %RSD between the data at each variable condition. (40-42) 2.8.5. Accuracy

The accuracy of the method was measured by performing recovery experiments at three different levels (80%, 100% and 120% addition of Lupeol) using the standard addition method.

The known amounts of Lupeol standard (80,100,120 ug/spot) were added by spiking. The values of % recovery and average value of % recovery for lupeol were calculated. (41-43)

2.9 Isolation of tuber extract from Arisaema tortuosum

For isolation of lupeol, 10 g of the crude ethanolic tuber extract of Arisaema tortuosum was adsorbed onto silica gel (60–120 mesh) by mixing and concentrating on a water bath until a dry mass was obtained. The dried, impregnated material was powdered and applied onto a glass column (60 cm × 3.5 cm) pre-packed with 100 g of silica gel (230–400 mesh) in petroleum ether. (44-45) The column was eluted under gravity with a petroleum ether–ethyl acetate gradient (100:0 to 60:40, v/v). Fractions of about 20 mL were collected and monitored by TLC on silica gel 60 F254 plates using Ethyl Acetate: Formic Acid: Toluene (4:0.3:6, v/v/v) as the mobile phase. After development, plates were derivatised with

anisaldehyde–sulphuric acid and heated at 105 °C for 5 min. Fractions showing a single band with an Rf identical to standard lupeol under the same conditions were pooled for further work. (46-47) 2.10 GC-MS Characterization

GC-MS analysis was conducted utilizing a Thermo Fisher TSQ9000 GC-MS/MS (Triple Quad S.No. TSQ92107008) equipment, which was outfitted with an HP 5 MS capillary column with a 5% phenyl polysiloxane as stationary phase (30.0 m × 0.25 mm, film thickness 0.25 µm). Different oven temperatures were evaluated to get optimal separation in the standard lupeol and sample. The instrument was initially set to 60°C for 2 minutes, after which the temperature was elevated at a rate of 10°C per minute till reaching 300°C. The helium carrier gas flow rate was maintained at 1.0 mL/min. Mass spectra were analyzed in scanning mode within the range of 50-1000 m/z. The interpretation of GC-MS spectra was acknowledged and validated using the NIST Mass Spectrometry Data Centre, 2019. The name, Molecular Weight (MW), Retention Time (RT), and structure of the active components were determined. (48-50)

RESULTS AND DISCUSSION

3.1 Solvent extraction yields

The percentage yield obtained from each solvent extract is summarized in Table 1 Among the four solvents tested, the aqueous extract produced the highest yield (11.2% w/w), indicating superior extraction of water-soluble constituents. This was followed by ethanol (6.3% w/w), which also showed good extraction efficiency. Comparatively lower yields were obtained with ethyl acetate (2.9% w/w) and chloroform (2.5% w/w), suggesting limited solubility of plant components in these less polar solvents.

Table 1: Percentage yield of extract of tubers of Arisaema Tortuosum

S. No	Extract	% Yield (W/W)
1.	Chloroform	2.5%
2.	Ethyl acetate	2.9%
3.	Ethanol	6.3%
4.	Aqueous	11.2%

Profiling of Secondary Metabolites in Plant Extracts

A series of qualitative biochemical tests were conducted to determine the presence of major groups of secondary metabolites in the different solvent extracts. The analysis included screening for alkaloids, glycosides, flavonoids, diterpenes, phenolic compounds, proteins, carbohydrates, saponins, and tannins using standard phytochemical procedures. The patterns of positive and negative reactions varied across the extracts, reflecting differences in the solubility and distribution of these metabolites. A complete summary of the outcomes for each test and extract is provided in Table 2.

Table 2: Result of phytochemical screening of extract of Arisaema Tortuosum.

S. No.	Constituents	Chloroform extract	Ethyl acetate extract	Ethanol extract	Aqueous Extract
1.	Alkaloids Wagner's Test: Hager's Test:	 -ve -ve	 +ve -ve	 +ve +ve	 -ve +ve
2.	Glycosides Legal's Test:	-ve	-ve	-ve	-ve
3.	Flavonoids Alkaline Reagent Test: Lead acetate Test:	 -ve +ve	 -ve +ve	 -ve +ve	 -ve +ve
4.	Diterpenes Copper acetate Test:	-ve	-ve	+ve	+ve
5.	Phenol Ferric Chloride Test: Folin Ciocalteu Test:	 -ve +ve	 -ve +ve	 +ve +ve	 +ve +ve
6.	Proteins Xanthoproteic Test:	+ve	-ve	+ve	+ve
7.	Carbohydrate Fehling's Test: Benedict's Test:	 -ve -ve	 -ve -ve	 +ve +ve	 -ve -ve
8.	Saponins Froth Test:	 -ve	 -ve	 +ve	 -ve

9.	Tannins Gelatin test:	+ve	+ve	+ve	+ve

3.3 Estimation of Total Flavonoids Content and Total Phenolic Content

Total flavonoid content and phenolic content expressed as quercetin equivalent and gallic acid equivalent, respectively, arrived at from the standard calibration line Eqns. y = 0.036x + 0.015; R^2 = 0.999 and y = 0.014x - 0.013; R^2 = 0.999, respectively (Figure 2).

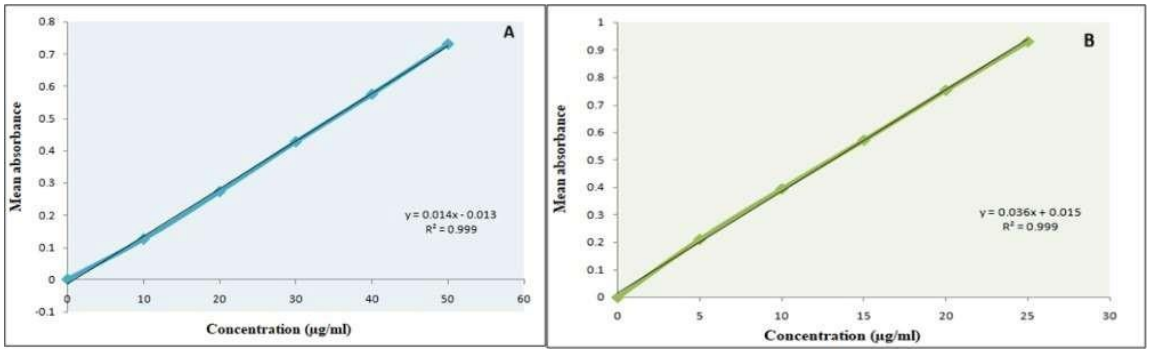

Figure 2. Calibration Curve of Gallic Acid and Quercetin.

The calibration data for both standards showed a regular increase in absorbance with rising concentration, confirming their suitability for quantifying total phenolics and flavonoids in Arisaema tortuosum extracts (Table 3). In the phenolic assay, gallic acid (standard for total phenolic content) in the range of 10–50 µg/mL gave mean absorbance values from 0.125 to 0.732. In the flavonoid assay, quercetin (standard for total flavonoid content) over 5–25 µg/mL produced mean absorbance values from 0.211 to 0.931. When plotted, these data yielded linear calibration curves with regression equations $y = 0.014x - 0.013$ for gallic acid and $y = 0.036x + 0.015$ for quercetin, each with a correlation coefficient of $R = 0.999$, indicating excellent proportionality between concentration and absorbance in the tested ranges and supporting accurate calculation of phenolic and flavonoid levels in the plant extracts.

Table 3. Mean absorbance values for gallic acid and quercetin standards used to construct calibration curves for total phenolic and total flavonoid content estimation.

S.NO	Total Flavonoid Content.		Total Phenolic Content.	
	Concentration (µg/ml)	Mean Absorbance	Concentration (µg/ml)	Mean Absorbance
1.	5	0.211	10	0.125
2.	10	0.395	20	0.273
3.	15	0.572	30	0.428
4.	20	0.754	40	0.576
5.	25	0.931	50	0.732

Ethanolic extract of Arisaema Tortuosum contained higher amount of total flavonoid content (0.805mg/100 mg) and total phenolic content (0.878mg/100 mg) as compare to other extract as shown in Table 4

Table 4: Total phenolic and total flavonoid content of different extract of Arisaema Tortuosum.

S. No	Extract	Total phenol content	Total flavonoids content
		mg/ 100 mg	mg/ 100 mg
1.	Chloroform	0.392	0.552
2.	Ethyl acetate	0.485	0.422
3.	Ethanol	0.878	0.805
4.	Aqueous	0.671	0.594

3.4 HPTLC Method Development

3.4.1 Chromatographic Condition

Chromatographic analysis was performed on pre-coated TLC aluminium plates of silica gel 60 F254 supplied by Merck. Sample and standard solutions were applied in the form of bands using a CAMAG Linomat applicator fitted with an automatic sampling system. The band length was maintained at 8 mm, with a distance of 4 mm between two bands, 1.5 cm from the side edge, and 2 cm from the bottom edge of the plate. The chromatographic chamber was saturated with mobile

phase vapour for 20 minutes at room temperature before development. Plate development was carried out by ascending technique up to a migration distance of 70 mm. After development, the plates were dried and derivatized with anisaldehyde sulphuric acid reagent to improve visualization of resolved bands. The derivatized plates were observed under white light, at 254 nm, and at 366 nm to record the fingerprint profile of the extracts. Densitometric scanning was carried out using CAMAG TLC Scanner III controlled through VisionCATS software, and quantitative evaluation of lupeol was performed at 545 nm.

3.4.2 Method Development and Optimization of Mobile Phase

The HPTLC procedure was developed to generate a clear, reproducible fingerprint of the aqueous, ethanolic, chloroform and ethyl acetate extracts of Arisaema tortuosum tubers and to select a suitable marker compound for standardization. In the course of marker screening, lupeol, quercetin and rutin were evaluated; however, only lupeol gave a consistent, well-defined response in the chromatographic system, so the method was ultimately focused on lupeol as the primary analytical marker. The method was optimized by systematically adjusting key chromatographic variables, including stationary phase, mobile phase composition, chamber saturation time, migration distance, sample application conditions and densitometric settings. Several binary and ternary solvent systems of different polarity were tested, and a mixture of Ethyl Acetate:Formic Acid:Toluene (4:0.3:6, v/v/v) on silica gel 60 F_{254} plates was finally selected, as it produced a sharp, compact lupeol band at Rf ≈ 0.67 with good resolution from neighbouring constituents and minimal tailing after derivatisation with anisaldehyde–sulphuric acid reagent. For routine analysis, samples and standard lupeol were applied as 8-mm bands on pre-coated silica gel 60 F_{254} aluminium plates using an automated applicator, with 4 mm spacing between bands, 1.5 cm from the side edge and 2 cm from the lower edge of the plate. Plates were developed by ascending chromatography to a distance of 70 mm in a chamber pre-saturated with the optimized mobile phase for 20 min at room temperature, then dried, derivatised with anisaldehyde sulphuric acid and examined under white light, 254 nm and 366 nm. Densitometric scanning was performed with a TLC scanner controlled by dedicated software, and lupeol was quantified at 545 nm under these conditions.

3.4.3 Fingerprint profile and identification

The optimized HPTLC conditions yielded a clear and reproducible fingerprint for the methanolic, ethanolic, chloroform and ethyl acetate extracts of Arisaema tortuosum tubers. Using Ethyl Acetate: Formic Acid: Toluene (4:0.3:6, v/v/v) as the mobile phase followed by derivatisation with anisaldehyde sulphuric acid, the plate viewed under white light showed multiple well-resolved coloured bands across all tracks, reflecting the underlying phytochemical complexity of the different solvent extracts (Figure 3A). Distinct bands were observed at several *R* positions, indicating qualitative differences in constituent profiles between the more polar (methanol, ethanol) and less polar (chloroform, ethyl acetate) extracts. When the same plate was examined at 366 nm prior to derivatisation, additional fluorescent bands became evident, particularly in the mid- to high-*R* region, providing an additional level of discrimination among the extracts and confirming the robustness of the fingerprint pattern (Figure 3B). Under these optimized conditions, comparison of extract tracks with reference standards showed that a prominent band at $R \approx 0.67$ co-migrated with lupeol after derivatisation, whereas quercetin and rutin did not produce corresponding bands in the sample lanes, thereby establishing lupeol as the most suitable chromatographic marker for A. tortuosum tuber.

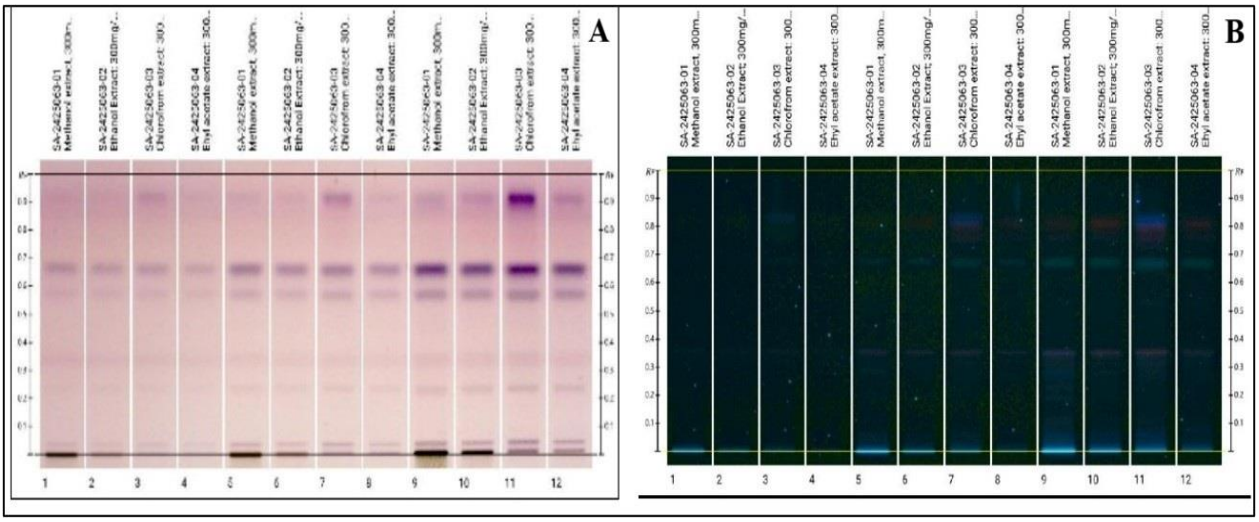

Figure 3. HPTLC fingerprint of Arisaema tortuosum tuber extracts under optimized conditions. (A) Chromatogram visualised under white light after development with Ethyl Acetate: Formic Acid: Toluene (4:0.3:6, v/v/v) and derivatisation with anisaldehyde–sulphuric acid, showing multiple-coloured bands for methanolic, ethanolic, chloroform and ethyl acetate extracts. (B) The same plate observed at 366 nm before derivatisation, highlighting characteristic fluorescent bands and the lupeol zone at $Rf \approx 0.67$.

3.4.4 Densitometric estimation of lupeol in multiple extracts

The validated HPTLC method was subsequently applied to quantify lupeol in the chloroform, ethyl acetate, ethanol and aqueous extracts of Arisaema tortuosum tubers and the corresponding densitograms are presented Figure 5 and Figure 5. A distinct peak corresponding to lupeol was observed in the standard as well as in all tested extracts, confirming the presence of lupeol in each fraction. Quantification was performed by interpolating the peak areas of the sample bands from the regression equation obtained from the multilevel calibration plot of the lupeol standard.

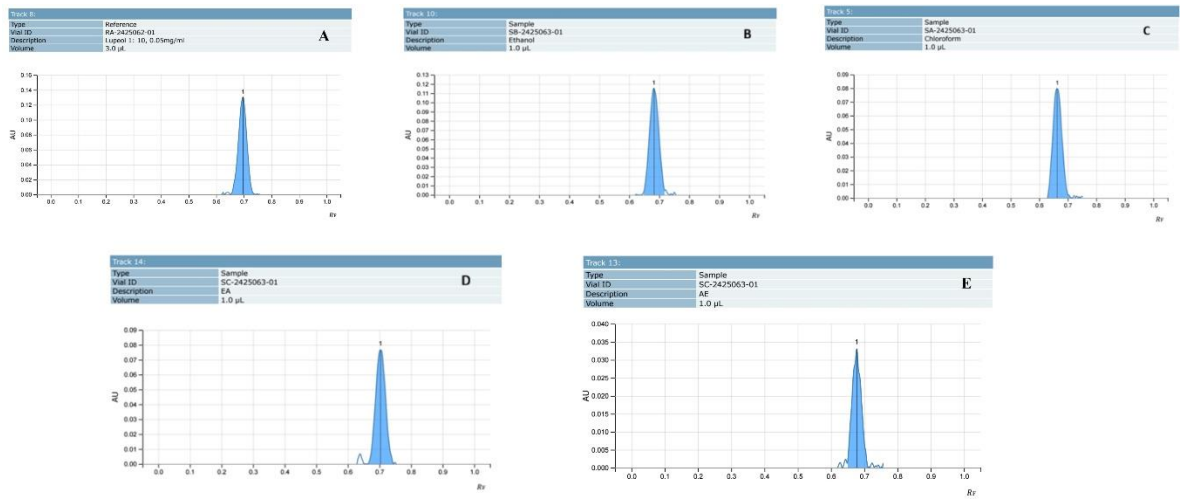

Figure 4. Densitometric chromatograms showing lupeol in different extracts of Arisaema tortuosum tubers: A, lupeol standard; B, ethanol extract; C, chloroform extract; D, ethyl acetate extract; and E, aqueous extract

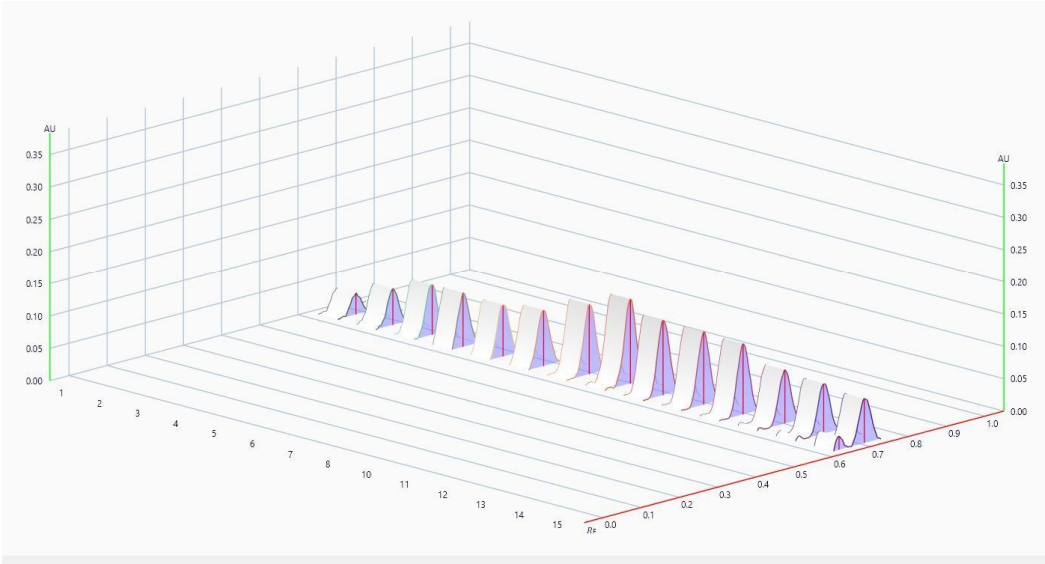

Figure 5. Three-dimensional densitometric overlay showing the chromatographic peak pattern of lupeol and its distribution in different extracts of Arisaema tortuosum tubers obtained by HPTLC densitometric scanning.

Among all extracts, the ethanolic extract showed the highest lupeol content, with 1.332 mg in 200.0 mg of dried extract, corresponding to 0.667% w/w. This was followed by the chloroform extract, which contained 1.050 mg in 200.0 mg of extract, equivalent to 0.525% w/w, and the ethyl acetate extract, which contained 982.5 µg in 200.0 mg of extract, equivalent to 0.491% w/w. In contrast, the aqueous extract showed only a comparatively low lupeol content of 149.12 µg in 200.0 mg of extract, corresponding to 0.074% w/w as shown in Table 5.

Table 5. Quantitative estimation of lupeol in different solvent extracts of Arisaema tortuosum tuber.

S. No	Sample Extract	Lupeol content in 200 mg extract	Percentage
1	Ethanol	1.332 mg in 200.0 mg	0.667%
2	Chloroform	1.050 mg in 200.0 mg	0.525%
3	Ethyl Acetate	982.5 µg in 200.0 mg	0.491%
4	Aqueous	149.12 µg in 200.0 mg	0.074%

This gradient is consistent with the physicochemical nature of lupeol as a lipophilic triterpenoid that is most efficiently extracted by ethanol which offers both non-polar and hydrogen-bonding interactions and to a somewhat lesser extent by chloroform and ethyl acetate, whereas its recovery into water remains low. The higher lupeol content in the ethanolic fraction, together with its previously demonstrated enrichment in phenolics and flavonoids, underscores ethanol as the most appropriate solvent for obtaining chemically rich, lupeol standardized extracts of A. tortuosum for further pharmacological evaluation.

3.5 Validation of HPTLC Method

3.5.1 Linearity

Linearity of the validated HPTLC method was established by analyzing standard lupeol at six concentration levels, namely 50, 75, 100, 125, 150, and 175 ng/spot, with triplicate application at each level (Table 6). A progressive increase in mean peak area was observed with increasing concentration, confirming a proportional densitometric response over the working range. The %RSD values for all levels were low, indicating acceptable repeatability of the method. The calibration curve exhibited excellent linear regression, with the equation $y = 0.0000291x - 0.000261$ and $R = 0.9998$, thereby confirming the linearity of the method for lupeol
quantification (Figure 6).

Table 6. Linearity data of lupeol by HPTLC

Conc. of standard lupeol (mg/ml)	On-plate amount (ng/spot)	AUC Rep-1	AUC Rep-2	AUC Rep-3	Mean AUC	S.D.	R.S.D. (%)
0.05	50	0.001215	0.001218	0.001198	0.00121	0.000010	0.8911
0.05	75	0.001918	0.001922	0.001894	0.00191	0.000015	0.7923
0.05	100	0.002612	0.002631	0.002645	0.00262	0.000016	0.6299
0.05	125	0.003453	0.003351	0.003363	0.00338	0.000055	1.6450
0.05	150	0.004136	0.004102	0.004123	0.00412	0.000017	0.4163
0.05	175	0.004844	0.004827	0.004795	0.00482	0.000024	0.5159

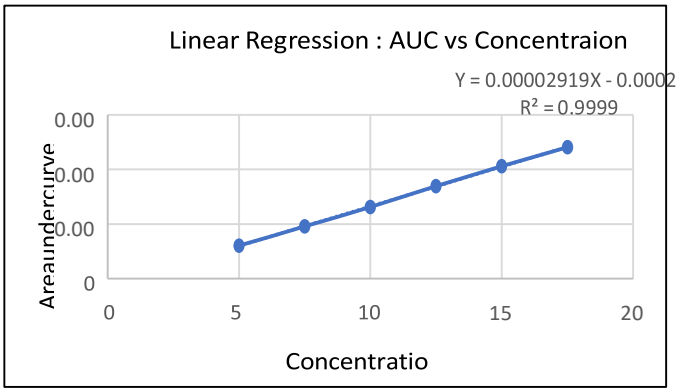

Figure 6. Calibration curve of lupeol showing the linear relationship between on-plate amount (50175 ng) and mean AUC by HPTLC.

3.5.2 Specificity

The developed HPTLC method was found to be specific for lupeol, as a well-resolved band was observed at the characteristic *R* value in both standard and sample tracks without interference from other matrix constituents.

3.5.3 LOD and LOQ

The sensitivity of the developed HPTLC method was determined in terms of limit of detection (LOD) and limit of quantification (LOQ). Using the slope of the calibration curve (0.0000291) and the standard deviation of the response (0.00001), the LOD and LOQ were calculated according to ICH guidelines. The LOD and LOQ values were found to be 1.22 ng/spot and 3.70 ng/spot, respectively, indicating that the method is sufficiently sensitive for the detection and quantification of lupeol at low concentrations.

3.5.4 Precision

The precision of the developed HPTLC method for lupeol was evaluated by assessing both repeatability (intra-day precision) and intermediate precision (inter-day precision) at 50, 75, and 100 ng/spot (Table 7). The intra-day study showed excellent consistency, with very low SD and %RSD values ranging from 0.04 to 0.09, indicating highly reproducible

densitometric responses within the same day. The inter-day study also demonstrated acceptable precision, with %RSD values between 1.60 and 1.75, confirming good day-to-day reproducibility. Although the inter-day variability was slightly higher than the intra-day variability, all values remained below 2%, which is well within acceptable limits. These results confirm that the developed HPTLC method is precise, reliable, and suitable for routine quantification of lupeol.

Table 7. Intraday precision and inter day precision of HPTLC method

Concentration of standards (µg/spot)	Intraday precision		Inter day precision	
	SD in area	%RSD	SD in area	%RSD
50	0.000001	0.09	0.000019	1.60
75	0.000001	0.05	0.000032	1.70
100	0.000001	0.04	0.000046	1.75

3.5.5 Accuracy

The accuracy of the developed HPTLC method for lupeol was evaluated by recovery studies at 80%, 100%, and 120% spiking levels presented in The mean recoveries at these levels were 99.92%, 100.05%, and 99.96%, respectively, showing excellent agreement between the added and recovered amounts of lupeol. The very low %RSD values obtained at each level confirm minimal variability among replicate measurements. These results demonstrate that the method is accurate, reliable, and not significantly affected by matrix interference, making it suitable for quantitative estimation of lupeol (Table 8). The mean recoveries at these levels were 99.92%, 100.05%, and 99.96%, respectively, showing excellent agreement between the added and recovered amounts of lupeol. The very low %RSD values obtained at each level confirm minimal variability among replicate measurements. These results demonstrate that the method is accurate, reliable, and not significantly affected by matrix interference, making it suitable for quantitative estimation of lupeol.

Table 8. Recovery studies of lupeol at 80%, 100%, and 120% addition by the proposed TLC densitometric method.

Concentration standards of (µg/spot)		Area spotted		Total area (sample + standard)	Total area obtained	% Recovery
Sample content	Spiked amount	Mean Sample area	Mean Spiked area			
100	80	0.002629	0.002103466	0.004732799	0.004729115	99.92%
100	100	0.002629	0.002629333	0.005258666	0.005261086	100.05%
100	120	0.002629	0.003155200	0.005784533	0.005782490	99.96%

3.5.6 Robustness

Robustness of the developed HPTLC method was evaluated by introducing small deliberate variations in the mobile-phase composition and chamber saturation time. Slight changes in the mobile-phase ratio of Ethyl Acetate: Formic Acid: Toluene around the optimized composition of 4:0.3:6 (v/v/v) produced only minimal variation in chromatographic performance, with *R* values ranging from 0.671 to 0.682, peak area values from 0.00276 ± 0.00002 to 0.00283 ± 0.00002, and %RSD between 0.36 and 0.72. Similarly, variation in chamber saturation time from 18 to 22 min resulted in *R* values between 0.673 and 0.680 and peak area values between 0.00277 ± 0.00002 and 0.00281 ± 0.00002, with low %RSD values. These results indicate that minor changes in analytical conditions did not significantly influence lupeol migration or densitometric response, confirming the robustness of the method for routine analysis.

Table 9. Robustness study by change in mobile phase ratio and chamber saturation time for lupeol standard

Change in mobile phase ratio, Ethyl Acetate: Formic Acid: Toluene (4:0.3:6, v/v/v)				
Lupeol Standard	Mobile phase ratio	Rf	Peak Area ± SD	%RSD
	3.9:0.3:6.1	0.671	0.00276 ± 0.00002	0.72
	4.0:0.3:6.0	0.677	0.00279 ± 0.00001	0.36
	4.1:0.3:5.9	0.682	0.00283 ± 0.00002	0.71
Change in the chamber saturation time				
Lupeol Standard	Time in minutes	Rf	Peak Area ± SD	%RSD
	18	0.673	0.00277 ± 0.00002	0.72
	20	0.677	0.00279 ± 0.00001	0.36
	22	0.680	0.00281 ± 0.00002	0.71

3.6 Isolation of Lupeol from Arisaema tortuosum

The ethanolic extract of Arisaema tortuosum tuber was selected for lupeol isolation because it showed the highest lupeol content among all tested extracts. After ethanol extraction and concentration, the crude extract was subjected to silica gel column chromatography using petroleum ether:ethyl acetate as the elution system. TLC/HPTLC monitoring identified fractions 56-85 as lupeol-rich, showing a single purple band at *R* 0.67 corresponding to the standard. Pooling of these fractions yielded 64 mg of a white amorphous compound, and its purity was confirmed by HPTLC along with a melting point of 213-215°C, consistent with lupeol.

Table 10. TLC profile of column fractions obtained from A. tortuosum ethanolic extract

Fraction No.	Petroleum ether: Ethyl acetate (v/v)	Volume (mL)	TLC profile (Rf)
1-15	100: 0	300	No detectable spots
16-25	98: 2	200	Single spot (Rf 0.91) non-polar compound
26-40	95: 5	300	Single spot (Rf 0.82) non-polar compound
41-55	90: 10	300	Two spots (Rf 0.67 and 0.45) - mixture containing lupeol
56-70	85: 15	300	Single spot (Rf 0.67) pure lupeol
71-85	80: 20	300	Single spot (Rf 0.67) -pure lupeol
86-100	75: 25	300	Multiple spots -polar compounds
101-120	70: 30	400	Multiple spots

3.6 Characterization by Mass Spectrometry

This isolated fraction was analysed by GC-MS to confirm the identity of the compound. Chromatographic analysis revealed a sharp, well-resolved peak in the plant-derived sample with a retention time (RT) of 31.85 minutes (Figure 7). This aligned almost identically with the RT of 31.86 minutes observed for the authentic lupeol standard (Figure 8), demonstrating excellent chromatographic concordance under the employed analytical conditions.

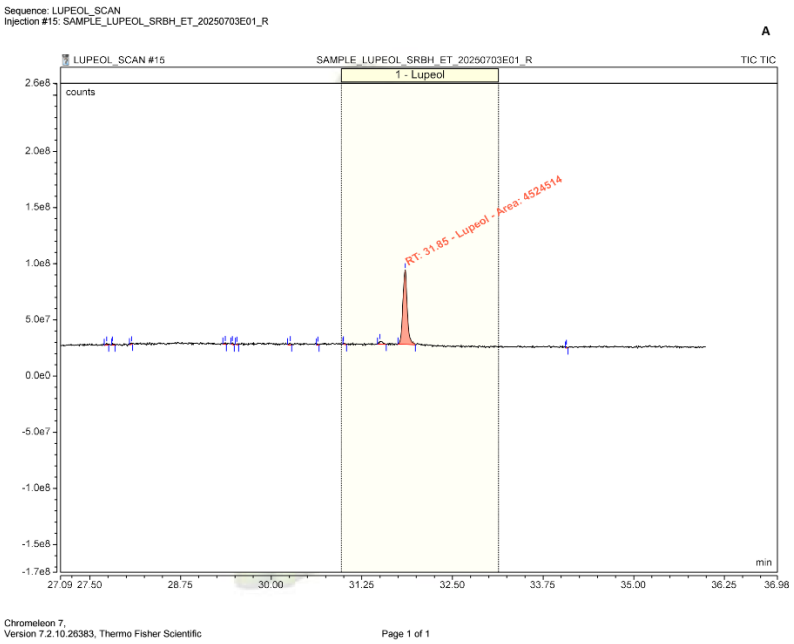

Figure 7. Total ion chromatogram (TIC) of the compound isolated from Arisaema tortuosum tuber extract showing a peak at retention time 31.85 min, identified as lupeol (peak area: 4,524,514).

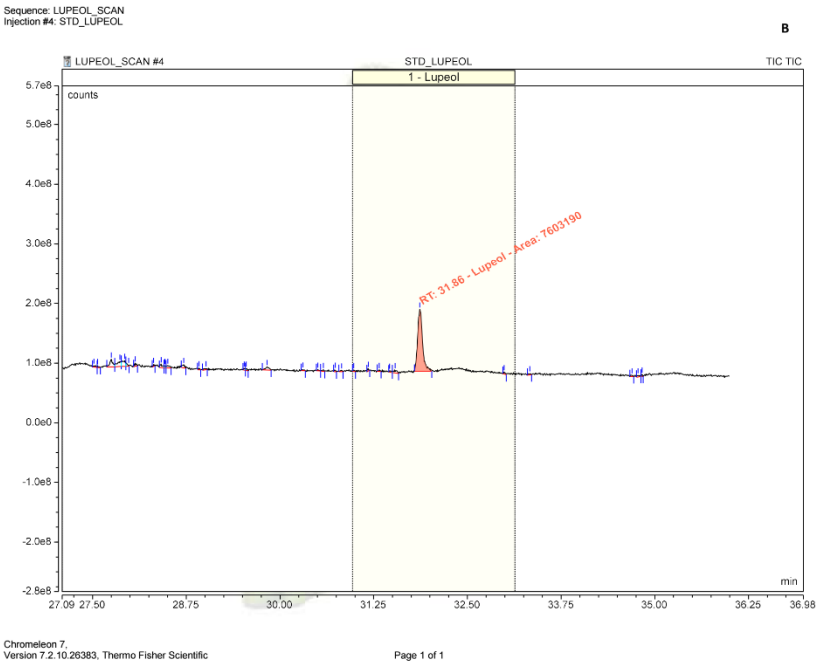

Figure 8. Total ion chromatogram (TIC) of the authentic lupeol standard showing a peak at retention time 31.86 min (peak area: 7,603,190) under identical GC-MS conditions.

Mass spectrometric examination provided definitive structural confirmation. The plant isolate exhibited a prominent molecular ion peak [M] at m/z 426 (Figure 9), corresponding to the molecular formula $C_{30}H_{50}O$. This mass spectral signature was identical from that of the reference lupeol (Figure 10). Furthermore, a diagnostic base peak at m/z 218 arising from retro-Diels-Alder cleavage of ring C, characteristic of the lupane skeleton. Additional fragments at m/z 207, 189, 161, 147, 135, 109, 95, 81, 69, 55, and 43 were consistent with the NIST library spectrum of lupeol (forward match factor 792, reverse match factor 876). These data collectively confirm the identity of the isolated compound as lupeol.

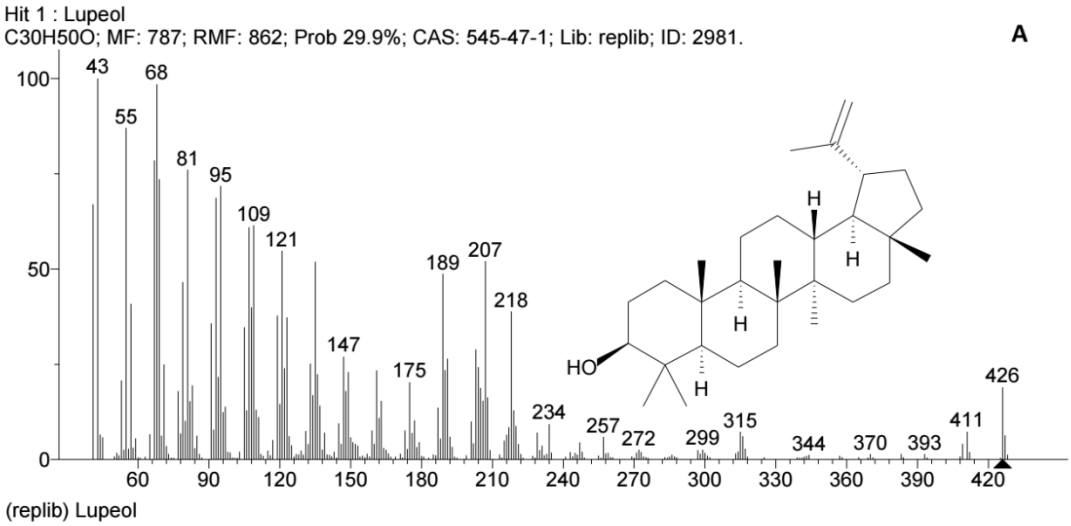

Figure 9. Electron Ionization Mass Spectra of the compound isolated from Arisaema tortuosum tuber extract

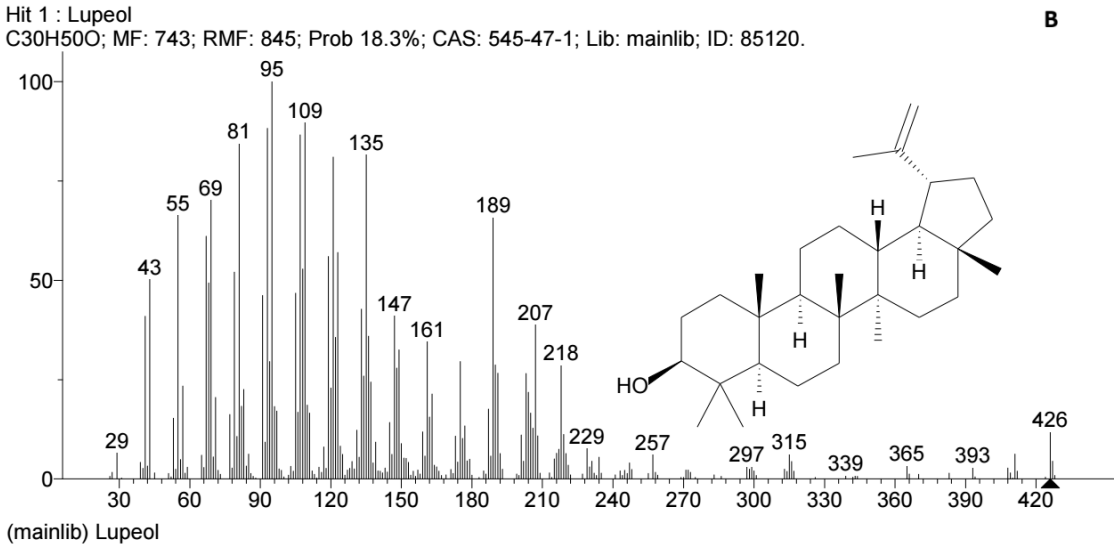

Figure 10. Electron Ionization Mass Spectra of the standard lupeol

CONCLUSION

The present study successfully established a validated HPTLC method for the quantification of lupeol in Arisaema tortuosum tuber extracts, followed by isolation and GC-MS/MS confirmation of the compound. Among the four solvents tested, the ethanolic extract gave the highest total phenolic (0.878 mg GAE/100 mg) and flavonoid content (0.805 mg QE/100 mg), and also contained the maximum lupeol – 1.332 mg in 200 mg of extract (0.667% w/w). The developed HPTLC method proved reliable, showing excellent linearity ($R^2 = 0.999$), high precision (%RSD < 2%), and good accuracy (recovery 99.92–100.05%), with LOD and LOQ of 1.22 and 3.70 ng/spot, respectively. Using silica gel column chromatography, lupeol was isolated as a white amorphous powder (64 mg from 10 g crude extract; 0.64% w/w), and its identity was confirmed by GC-MS through matching retention times (sample 31.85 min, standard 31.86 min) and the characteristic molecular ion at m/z 426 with a diagnostic base peak at m/z 218. Collectively, these findings validate A. tortuosum tubers as a promising natural source of lupeol and provide a robust analytical tool for its routine quality control. For future work, the isolated lupeol and the standardized ethanolic extract should be evaluated for their in vitro and in vivo pharmacological activities, particularly anticancer, anti-inflammatory, and antimicrobial effects, as suggested by the plant's traditional uses. Additionally, the HPTLC method can be extended to quantify lupeol in other plant species or herbal formulations. LC-MS/MS-based metabolomic profiling of the extract could uncover other bioactive minor constituents, and mechanistic studies (e.g., molecular docking, enzyme assays) would help elucidate the mode of action.

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Declaration of Competing Interest

No conflict of interest is declared by the authors.

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