

Research Article



Pharmacognostic and Phytochemical Evaluation of *Chrysophyllum cainito* Linn. Leaves

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ABSTRACT

Chrysophyllum cainito L. (Sapotaceae); commonly known as Star Apple is a tropical tree native to lowland of Central America and West Indies and exotic to the warmer parts of India. Leaves of *C. cainito* are reported to possess antidiabetic and antioxidant properties as well as have been used against articular rheumatism. A decoction of leaves is taken as pectoral. In the present study, macroscopic, physicochemical, phytochemical and safety profile of *C. cainito* leaves has been evaluated and discussed. Findings of this study can be useful as reference information in order to evaluate quality of *C. cainito* leaves.

Keywords: *Chrysophyllum cainito* Linn., Gallic acid, Physicochemical evaluation, Safety evaluation, Triterpenoids.

INTRODUCTION

Chrysophyllum Linn. (Sapotaceae), is a genus of evergreen trees, distributed mainly in the tropics especially America with a few species in West Africa and Australia. *C. cainito* is one of the species of this genus which occur in warmer parts of India¹ and is commonly known as Star Apple. The plant is highly desired throughout the tropics as an ornamental tree and for the production of its large edible fruits.² The ripe fruit, because of its mucilaginous character, is eaten to sooth inflammation in laryngitis, pneumonia and given as a treatment for diabetes mellitus. The bark is considered as a tonic, stimulant and its decoction is used as an antitussive.¹ Infusion of the leaves has been used against diabetes, articular rheumatism and its decoction is taken as pectoral. *C. cainito* leaves have been chemically screened and reported to possess alkaloids, flavonoids, phenols, sterols and triterpenes.³

Pharmacognosy, in recent years has gained immense importance as it is an efficient tool for the authentication and identification of plant raw materials and therefore evaluation of pharmacognostic parameters is an indispensable step when dealing with herbal drugs.⁴ A thorough literature survey revealed that, though *C. cainito* plant is of high therapeutic value, scientific data on pharmacognostic parameters of *C. cainito* leaves remains an unexplored issue. Hence, establishment of a pharmacognostic profile of *C. cainito* leaves will assist in its standardization in terms of quality, purity and sample identification.⁵ Therefore, the aim of this study was to standardize *C. cainito* leaves by carrying out macroscopic, physicochemical, phytochemical, chromatographic and safety evaluation.

Based on the reported therapeutic activities of ursolic acid (anticancer, anti-inflammatory, anti-oxidative and hepatoprotective, etc.⁶), β -sitosterol (anticancer, estrogenic, hypolipidemic, etc.⁷), lupeol (anti-

inflammatory, anticancer, antimicrobial, hepatoprotective, cardioprotective activity, etc.⁸) and gallic acid (antioxidant, anti-allergic, anti-inflammatory, anti-mutagenic, anti carcinogenic, apoptotic and wound healing, etc.⁹); chromatographic characterization of *C. cainito* leaves was carried out in terms of these triterpenoids (ursolic acid, β -sitosterol and lupeol) and phenolic compound (gallic acid) content. Further, in order to evaluate safety of *C. cainito* leaves, acute oral toxicity study (as per OECD guideline no. 420) was conducted in Albino Wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

Ursolic acid, β -sitosterol, lupeol and gallic acid (98 % purity each) were procured from Sigma Aldrich Chemical Company, (Steinheim, Germany). Chemicals of analytical grade were purchased from Merck Specialties Private Limited, Mumbai.

Plant sample

Fresh leaves of *C. cainito* were collected from two different locations of Mumbai (Sion and Ruia College campus, Matunga). The taxonomic identification of a representative sample was confirmed by Agharkar Research Institute, Pune (Authentication no. Auth 13-020). The plant sample was shade dried for a week followed by drying in an oven preset at 45°C for 4 days. The samples were powdered in a mixer grinder, sieved through 85 mesh (BSS) and stored in an air tight containers.

Macroscopic evaluation

Macroscopic characters of fresh leaves such as type of the leaf base, presence or absence of petiole, characters of lamina, venation, margin, apex, base, surface and texture were studied.



Physicochemical evaluation

The physicochemical parameters of the leaves such as foreign organic matter, ash content (total, acid insoluble and water soluble) and extractive values were determined using standard Pharmacopoeial methods.¹⁰⁻¹¹

Phytochemical evaluation

Phytochemical screening of some major secondary metabolites (flavonoids, essential oils, tannins, glycosides, alkaloids and resins) in *C. cainito* leaves was carried out by performing preliminary phytochemical tests as per the methods reported¹² and further the powder of dried *C. cainito* leaves was subjected to phytochemical evaluation by successively soxhlet extraction with various organic solvents in order to analyze the percent extract of major class of compounds present in the plant raw material as per the method reported.¹³

Chromatographic characterization

Extraction of phytochemical constituents from *C. cainito* leaves

The powdered sample (0.2 g) was extracted with methanol (5.0 mL), vortex mixed for 1 min and sonicated for 20 min followed by filtration through Whatman filter paper No. 1. The filtrate was subjected to HPTLC analysis for the development of a phytochemical fingerprint.

In order to extract triterpenoids namely ursolic acid (Figure 1A), β -sitosterol (Figure 1B) and lupeol (Figure 1C) from the complex matrix of *C. cainito* leaves, the powdered sample (0.5 g) was extracted with petroleum ether (10.0 mL¹⁴), vortex mixed for 1 min and then sonicated for 20 min followed by filtration through Whatman filter paper No. 1. Filtrate was evaporated under vacuum using rotary evaporator to dryness at 40^o C, reconstituted in equal volume of methanol and subjected to HPTLC analysis for optimized separation of these triterpenoids.

In order to extract a phenolic compound gallic acid (Figure 1D), the powdered sample (1.0 g) was extracted with methanol (10.0 mL), vortex mixed for 1 min and sonicated for 20 min followed by filtration through Whatman filter paper No. 1. The filtrate was subjected to HPTLC analysis for optimized separation of gallic acid.

Preparation of standard stock solutions

Standard stock solution of ursolic acid, β -sitosterol, lupeol and gallic acid (1000 μ g/mL each) was prepared in methanol. Serial dilution of the stock solution in methanol was carried out in order to prepare calibrant/ quality control samples.

Optimized chromatographic conditions for phytochemical fingerprint and quantitation of markers

The HPTLC system used consisted of CAMAG TLC Scanner 4 supported by winCATS software version 1.4.7 equipped with CAMAG Linomat 5 sample spotter and CAMAG Reprostar 3 system for photo-documentation. A Denver

analytical balance (Goettingen, Germany) was used to weigh the standard. Chromatographic separation of the phytochemical constituents was achieved on TLC plates (E. Merck) pre-coated with silica gel 60 F₂₅₄ (0.2 mm thickness) on aluminium sheet support.

To develop an HPTLC fingerprint of *C. cainito* leaves, the sample (10.0 μ L) was applied to the plate as a band of 8.0 mm wide and at a distance of 15.0 mm from the edges. Plate was developed up to a distance of 85.0 mm in CAMAG twin trough glass chamber pre-saturated with the mobile phase chloroform: toluene (8: 2, v/v) for 15 min. After development, the plate was dried in a current of air at room temperature. The plate was derivatised using 10% methanolic sulphuric acid and dried in oven preset at 110^o C for 10 min. For densitometric scanning, the source of radiation was a mercury lamp (366 nm). All measurements were performed at 22 \pm 1^o C. Plate was photo-documented at 366 nm.

For simultaneous separation of ursolic acid, β -sitosterol and lupeol from *C. cainito* leaves, the sample was diluted using methanol (1:1, v/v) and 10.0 μ L of it along with the standards- ursolic acid (10.0 μ g/mL), β -sitosterol (10.0 μ g/mL) and lupeol (20.0 μ g/mL) of 10.0 μ L each were spotted on TLC plate as bands of 8.0 mm wide and at a distance of 15.0 mm from the edges under similar instrumental conditions. Plate was developed up to a distance of 85.0 mm in CAMAG twin trough glass chamber pre-saturated with mobile phase toluene: methanol (8:1, v/v) for 15 min and derivatised using 10% methanolic sulphuric acid. Post derivatization procedure was kept similar for the development of fingerprint and estimation of these markers.

To separate gallic acid from *C. cainito* leaves, sample (10.0 μ L) and gallic acid standard (100.0 μ g/mL, 10.0 μ L) were spotted on TLC plate as bands of 8.0 mm wide and at a distance of 15.0 mm from the edges under similar instrumental conditions. Plate was developed up to a distance of 85.0 mm in CAMAG twin trough glass chamber pre-saturated with mobile phase toluene: ethyl acetate: formic acid (2:7:1, v/v/v) for 15 minute. The plate was scanned and photo documented at 254 nm.

Safety evaluation (acute oral toxicity study)

Acute oral toxicity¹⁵ of *C. cainito* leaves (aqueous slurry and ethanolic extract) was conducted and the results were compared with the animals of control group treated with distilled water [guidelines: OECD guideline no. 420, ethical committee approval no.: CPCSEA-315/DG-130624-02, animals: Albino Wistar rats, female (200-225 g), n=6/group, parameters observed: cage side observations, change in body weight along with food and water intake and mortality].

Statistical analysis

Microsoft Excel-2007 was used to determine mean, standard deviation (SD), relative standard deviation (RSD) and mean difference during the analysis.



RESULTS AND DISCUSSION

Macroscopic characters

The leaves of *C. cainito* are evergreen, alternate, simple, oval, entire (Figure 2A). These are elliptic or oblong-elliptic, 5–15 cm long, rich green and glossy on the upper surface, coated with silky, golden-brown pubescence beneath when mature (Figure 2B). Leaves appear dull green (upper surface) and dull golden (lower surface) when dried and light green when powdered (Figure 2C). Leaves also showed different types of apex when collected from the same plant (Figure 2D).

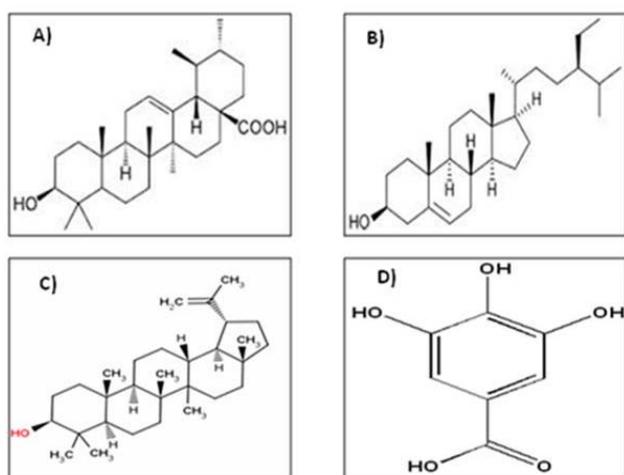


Figure 1: Structures of A) Ursolic acid B) β -sitosterol C) Lupeol and D) Gallic acid

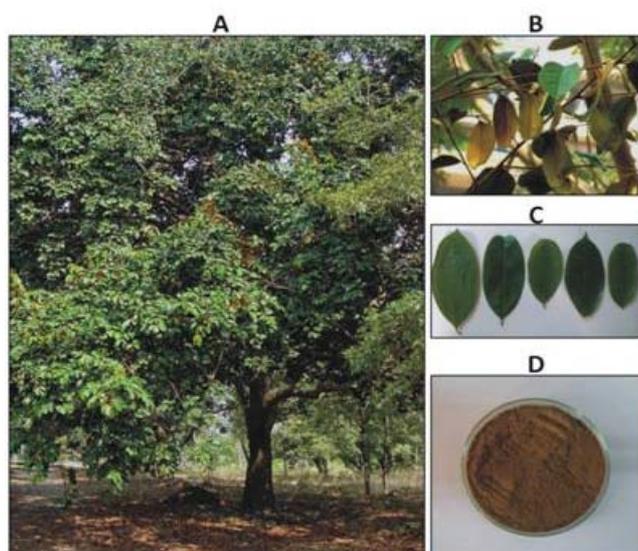


Figure 2: (A) Habit of *Chrysophyllum cainito* (B) Twig showing leaves (C) Leaves of *C. cainito* with different apex (D) Powdered *C. cainito* leaves

Track details: a- Ursolic acid (10.0 $\mu\text{g/mL}$); b- β -sitosterol (10.0 $\mu\text{g/mL}$); c- Lupeol (20.0 $\mu\text{g/mL}$); d- Mixture of ursolic acid, β -sitosterol and lupeol (10.0 $\mu\text{g/mL}$, 10.0 $\mu\text{g/mL}$ and 20.0 $\mu\text{g/mL}$ respectively); e- *C. cainito* leaves collected from Sion; f- *C. cainito* leaves collected from Ruia College campus, Matunga.

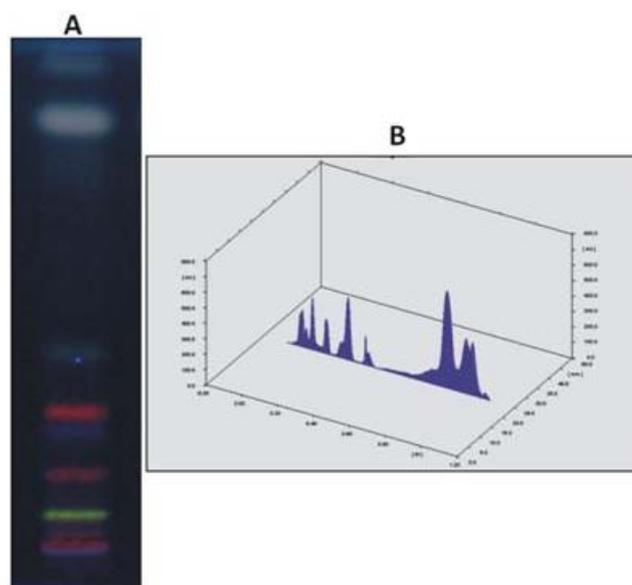


Figure 3: (A) HPTLC plate photo and (B) overlay of the phytochemical fingerprint pattern of *C. cainito* leaves at 366 nm.

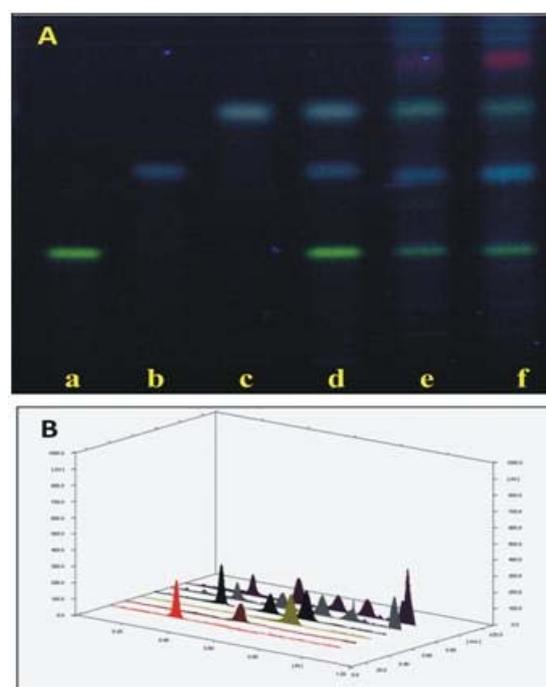


Figure 4: (A) HPTLC plate photo and (B) overlay showing presence of ursolic acid, β -sitosterol and lupeol at 366 nm in leaves of *C. cainito* collected from two different regions of Mumbai with standards

Physicochemical and phytochemical analysis

Proximate parameters such as foreign organic matter, ash values (total, acid insoluble and water soluble) and extractive values (ethanol soluble, water soluble and ether soluble) of *C. cainito* leaves were determined and results are shown in Table 1A. The ether soluble extractive values were found maximum when compared with ethanol soluble and water soluble extractives. This suggests the presence of more non polar components in the plant (approx. 20%). In preliminary phytochemical

evaluation, tannins and resins were found to be present in the aqueous extract of *C. cainito* leaves, whereas in methanolic extract of *C. cainito* leaves, flavonoids, alkaloids, essential oils and glycosides were found absent. Amongst all the phytochemicals fractions extracted, leaves of *C. cainito* were found to be rich in quaternary alkaloids and N-oxides fraction, whereas the fractions of fats and waxes as well as alkaloids were least. The leaves were also found source of terpenoids and phenolics, hence were further subjected to chromatographic characterization using validated HPTLC technique (Table 1B).

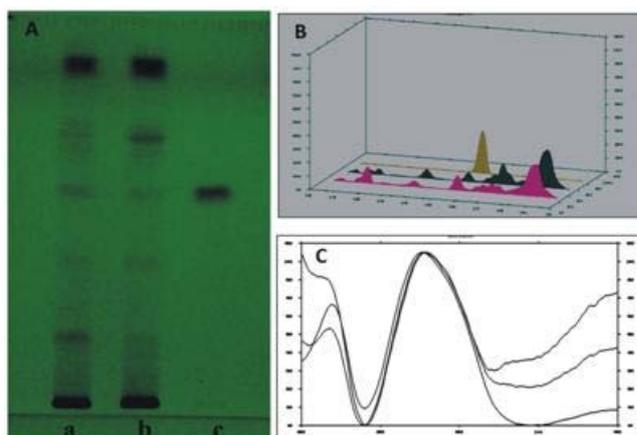


Figure 5: (A) HPTLC plate photo (B) overlay and (C) spectral confirmation showing presence of Gallic acid at 254 nm in leaves of *C. cainito* collected from two different regions of Mumbai with standard gallic acid

Track details: a- *C. cainito* leaves collected from Sion; b- *C. cainito* leaves collected from Ruia College campus, Matunga; c- Gallic acid (100.0 µg/mL)

Table 1A: Results of physicochemical parameters of *C. cainito* leaves

Parameters	Results
Foreign organic matter	0.214 ± 0.018
Total ash	9.376 ± 0.629
Acid insoluble ash	2.022 ± 0.035
Water soluble ash	7.325 ± 0.143
Ethanol soluble extractive value	8.786 ± 0.012
Water soluble extractive value	9.455 ± 0.135
Ether soluble extractive value	19.268 ± 0.240

Values are (% Mean ± S.D., n=3)

Table 1 B: Content of phytochemical fractions obtained from *C. cainito* leaves

Phytochemical fractions	% Content
Fats and waxes	0.934 ± 0.045
Terpenoids and phenolics	4.004 ± 0.122
Alkaloids	0.166 ± 0.068
Quaternary alkaloids and n-oxides	10.678 ± 0.035
Fibres	71.122 ± 0.136

Values are (% Mean ± S.D., n=3)

Chromatographic evaluation

Leaves of *C. cainito* have been reported to possess compounds like alkaloids, sterols and triterpenes³, but no report is available on individual phytochemical constituents belonging to these compounds. Based on the fact that these phytochemicals are reported to have therapeutic activities, in this research work, chromatographic characterization was carried out by developing a phytochemical fingerprint and two HPTLC methods for characterization of *C. cainito* leaves in terms of their triterpenoids (ursolic acid, β-sitosterol, and lupeol) and phenolic compound (gallic acid).

HPTLC fingerprint was developed from methanolic extract of *C. cainito* leaves using chloroform: toluene (8:2, v/v) as a mobile phase and it showed 12 peaks at R_f values of 0.04, 0.07, 0.15, 0.23, 0.27, 0.37, 0.38, 0.50, 0.74, 0.83, 0.93 and 0.97 with blue, yellow, red, purple, light pink coloured bands, which will be helpful in identification of plant raw material (Figure 3A and 3B). Mobile phase composition of toluene: methanol (8:1, v/v) gave good resolution of ursolic acid, β-sitosterol and lupeol from other phytoconstituents in *C. cainito* leaves extract. Ursolic acid was detected at R_f = 0.29, β-sitosterol at R_f = 0.50 and lupeol at R_f = 0.66. Identity of these three phytoconstituents in the leaves extract was confirmed by overlay and colour of band with that of the standard (Figure 4A and 4B). The method was validated as per ICH guidelines and was found to be rapid, specific, precise, sensitive and rugged (Table 2). Content of ursolic acid and β-sitosterol was found to be more in *C. cainito* leaves collected from Ruia College campus, Matunga whereas content of lupeol was found to be more in sample collected from Sion (Table 3). This clearly indicates the impact of regional variation over marker content of plants and results were in compliance with the other published reports.¹⁶

For separation of gallic acid from the leaves of *C. cainito*, toluene: ethyl acetate: formic acid (2:7:1, v/v/v) was used as mobile phase which gave well resolved band of gallic acid at R_f = 0.53. Presence of gallic acid in sample was confirmed by overlay, colour of band with that of the standard as well as spectral scan (Figure 5A, 5B and 5C). The method was validated as per ICH guidelines and was found to be rapid, specific, precise, sensitive and rugged (Table 2). Content of gallic acid was found to be more in *C. cainito* leaves collected from Sion than leaves collected from Ruia College campus, Matunga (Table 3). Here too, the impact of regional variation over marker content was evident.

Safety evaluation

In acute toxicity study, oral administration of *C. cainito* leaves (aqueous slurry and ethanolic extract) did not cause any mortality as well as no significant change in the body weight, food and water intake was observed when compared with the animals of control group. The cage side observations also appeared normal.

Table 2: Results of method validation experiment for simultaneous estimation of triterpenoids (ursolic acid, β -sitosterol and lupeol) and phenolic compound (gallic acid)

Parameters	Ursolic acid	β - sitosterol	Lupeol	Gallic acid
Mobile phase	Toluene: methanol (8:1, v/v)			Toluene: ethyl acetate: formic acid (2:7:1, v/v/v)
R _f	0.29	0.50	0.66	0.53
LOD (μ g/mL)	2.5	1.0	2.0	10
LOQ (μ g/mL)	5.0	5.0	5.0	20
Linearity (μ g/mL)	5.0 – 100.0	5.0 – 60.0	5.0 – 75.0	20.0-150.0
Regression equation	y = 30.80x + 132.5	y = 39.76x + 194.0	y = 24.81x + 35.10	y= 56.67x-56.74
Coefficient of determination (r ²)	0.999	0.993	0.995	0.992
Instrumental precision (% RSD), n=7	1.82	1.63	1.57	1.12
Repeatability (% RSD), n=5	1.09	0.96	1.28	1.12
Intraday Precision (% RSD)	1.73	0.50	0.89	1.37
Interday Precision (% RSD)	1.99	0.93	1.15	1.94
Recovery (%)	97.23	96.54	98.16	98.12
Specificity	Specific			
Ruggedness	Rugged			

Table 3: Content of triterpenoids (ursolic acid, β -sitosterol and lupeol) and phenolic compound (gallic acid) in *C. cainito* leaves collected from two different regions of Mumbai

Region	Content of markers (mg/g) in <i>C. cainito</i> leaves			
	Ursolic acid	β -sitosterol	Lupeol	Gallic acid
Sion	0.165 \pm 0.003	0.261 \pm 0.005	0.453 \pm 0.008	0.500 \pm 0.007
Matunga	0.220 \pm 0.004	0.507 \pm 0.009	0.382 \pm 0.007	0.280 \pm 0.006

Values are (Mean \pm S.D., n=3)

CONCLUSION

The evaluation of pharmacognostical parameters may ensure the identity and authenticity of *C. cainito* leaves. The plant was found to be a good source of ursolic acid, β -sitosterol, lupeol and gallic acid by HPTLC analysis. The validated HPTLC methods can be applied to various herbal drugs for the quantitation of pharmacologically active markers- ursolic acid, β -sitosterol, lupeol and gallic acid. Data generated from the acute oral toxicity study may ensure an adequate safety margin of *C. cainito* leaves for their intended use. Thus, findings of this study could be useful for the compilation of a monograph in a suitable pharmacopoeia for its proper identification and quality control, which can be used globally.

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