Pharmacognostic and Phytochemical Evaluation of Chrysophyllum cainito Linn. Leaves

Sunita Shailajan*, Deepti Gurjar
Herbal Research Lab, Ramnarain Ruia College, Matunga (E), Mumbai, India.
*Corresponding author’s E-mail: sunitashailajan@gmail.com

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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 India 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. The ripe fruit, because of its mucilaginous character, is eaten to soothe 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. 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.

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. 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. 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.), β-sitosterol (anticancer, estrogenic, hypolipidemic, etc.), lupeol (anti-inflammatory, anticanccer, antimicrobial, hepatoprotective, cardioprotective activity, etc.) and gallic acid (antioxidant, anti-allergic, anti-inflammatory, anti-mutagenic, anti-carcinogenic, apoptotic and wound healing, etc.); 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°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 µg/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 µL) 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°C for 10 min. For densitometric scanning, the source of radiation was a mercury lamp (366 nm). All measurements were performed at 22 ± 1°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 µL of it along with the standards- ursolic acid (10.0 µg/mL), β-sitosterol (10.0 µg/mL) and lupeol (20.0 µg/mL) of 10.0 µL 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 µL) and gallic acid standard (100.0 µg/mL, 10.0 µL) 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 µg/mL); b- β-sitosterol (10.0 µg/mL); c- Lupeol (20.0 µg/mL); d- Mixture of ursolic acid, β-sitosterol and lupeol (10.0 µg/mL, 10.0 µg/mL and 20.0 µg/mL respectively); e- *C. cainito* leaves collected from Sion; f- *C. cainito* leaves collected from Ruia College campus, Matunga.

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

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 phytochemical 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).

**Chromatographic evaluation**

Leaves of C. cainito have been reported to possess compounds like alkaloids, sterols and triterpenes, 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 Rf 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 Rf = 0.29, β-sitosterol at Rf = 0.50 and lupeol at Rf = 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.

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 Rf = 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.
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 β-sitosterol content in 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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REFERENCES


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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ursolic acid</th>
<th>β-sitosterol</th>
<th>Lupeol</th>
<th>Gallic acid</th>
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<tr>
<td>Rf</td>
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<td>0.50</td>
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<td>LOD (µg/mL)</td>
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<td>5.0</td>
<td>5.0</td>
<td>20</td>
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<tr>
<td>Linearity (µg/mL)</td>
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<td>5.0 – 75.0</td>
<td>20.0-150.0</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y = 30.80x + 132.5</td>
<td>y = 39.76x + 194.0</td>
<td>y = 24.81x + 35.10</td>
<td>y = 56.67x-56.74</td>
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<tr>
<td>Coefficient of determination (r²)</td>
<td>0.999</td>
<td>0.993</td>
<td>0.995</td>
<td>0.992</td>
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<tr>
<td>Instrumental precision (% RSD), n=7</td>
<td>1.82</td>
<td>1.63</td>
<td>1.57</td>
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<tr>
<td>Repeatability (% RSD), n=5</td>
<td>1.09</td>
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<td>1.28</td>
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<td>Intraday Precision (% RSD)</td>
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<td>Interday Precision (% RSD)</td>
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<td>96.54</td>
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<tr>
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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

<table>
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<th>Region</th>
<th>Content of markers (mg/g) in C. cainito leaves</th>
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<tr>
<td></td>
<td>Ursolic acid</td>
</tr>
<tr>
<td>Sion</td>
<td>0.165 ± 0.003</td>
</tr>
<tr>
<td>Matunga</td>
<td>0.220 ± 0.004</td>
</tr>
</tbody>
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Values are (Mean ± S.D., n=3)


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