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Research article

Cold Extraction of *Carissa congesta* Wight monitored by a comparative revision of HPLC and HPTLC

Gaurav M. Doshi,[1,2*] Pratip K. Chaskar[1], Sandeep Zine[1] and Hemant D. Une[3]

[1]Assistant Professors, Department of Pharmacology and Pharmaceutical Chemistry, Vivekanand Education Society's College of Pharmacy, Mumbai, India
[2]Pacific Academy of Higher Education and Research University, Udaipur, Rajasthan, India
[3]Associate Professor, Vice Principal, Department of Pharmacology, Y. B. Chavan College of Pharmacy, Rouzabagh, Aurangabad, Maharashtra, India

ABSTRACT: **Background:** *Carissa congesta* is an important medicinal plant in India. Lupeol, an active constituent of this species, has been reported to possess good amount of pharmacological as well as therapeutic potential. **Objective:** In the current studies enlighten we focussed on determining the percentage of the lupeol present in the plant root extract by sophisticated analytical techniques. **Materials and Methods:** Shade-dried roots of *Carissa congesta* were subjected to a cold petroleum ether extraction followed by monitoring the lupeol contents by HPTLC and HPLC methods after carrying out preliminary phytochemical screening for the active constuients. **Results:** The extraction yield was found to be 1.8% (w/w). Phytochemical screening of the extract showed the presence of steroids, flavonoids, saponins, glycosides, tannins, phenolic compounds, fixed oils and fats.. The amount of lupeol present was found to be **25.80 % (w/w)** and **11.18 % (w/v)** by HPTLC quantification and HPLC analysis respectively. From HPLC analysis, the petroleum ether extract was found to a peak which eluted coincident with lupeol, indicating that the extract contained lupeol **Conclusion:** The extract contains significant amount of lupeol.

KEYWORDS: *Carissa congesta* ,Cold Extraction, , HPLC, HPTLC, lupeol

INTRODUCTION

Herbal medicines have served as a alternative source for pharmaceutical medicines and various healthcare products.[1] Medicinal plants are essential for drug discovery and development since isolated plant constituents play a key role either as starting material in drug synthesis or as pharmacologically active component in research intervention.[2] A new era of herbal renaissance has produced a profound effect on Western medicine, which is now trying to acknowledge methods of healing that have existed for millennia in the traditional system of medicine throughout the world, especially in Asia.[3,4] We have selected *Carissa* species for our study. These are evergreen shrubs with 2-10 m in height containing thorny twigs and latex and are cultivated as ornamental plants in America ranging from Florida to California. The shrubs of this genus are native to tropical and subtropical regions of Asia, Africa and Australia. . The roots are bitter and stomachic with good pharmacological properties. The paste of the roots is used as fly-repellent. Decoctions and extracts of the roots provide effective remedies in the management and control of convulsions as well as epilepsy.[5] The roots have yielded a number of volatile principles including 2-acetylphenol, lignans such as carinol, and a mixture of sesquiterpenes including carissone and carindone (a novel type of C-31 terpenoid).[6] In addition, Des-*n*-methylnoracronycine (an acridone alkaloid) has also been isolated along with other constituents such as lupeol.[7,8] Thus, taking a brief insight

*Correspondence
Gaurav M. Doshi
Assistant Professor
Department of Pharmacology
Vivekanand Education Society's College of Pharmacy
Mumbai, India
Mobile No: +91-9819771515
Email id: gaurav.pharmacology@gmail.com
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of the existing literature, our article attempts to find out how much percentage of the lupeol is present in the plant root extract. Since lupeol, a pentacyclic triterpene is known to be reported for number of important bioactivities such as antiarthritic, antiprotozal, anti-inflammatory, anticancer properties, hepatoprotective and chemopreventive agent mainly found in species of the Asteraceae family.[9,10] Although the literature reports several methods for the quantification of lupeol, no studies on bioactivity guided fractionation for the isolation and quantification of lupeol from *C. congesta* Wight has been reported.

MATERIALS AND METHODS

Part A: Collection, authentication and extraction

Fresh roots of *Carissa congesta* were collected from Jalgaon district, Maharashtra, in the month of April-May and shade-dried. They were authenticated by Agharkar Research Institute, Pune. A voucher specimen (No.3/187/2013/Adm.1692/081) was deposited in the botany department of Agharkar Research Institute, Pune. The roots were subjected to a cold extraction procedure consisting of two parts[11] as follows:

Part 1: Sample: 1.5 kg shade dried root powder

To 1.5 kg of coarsely powdered root 4.5 L ethyl alcohol was added. With occasional mixing/shaking, the sample was extracted in cold for 4 days. The ethyl alcohol was decanted after 4 days. Fresh solvent was added and the process was repeated for 4 times. The solvent from the extract was filtered. The concentrate was evaporated to dryness under reduced pressure and low temperature on a rotary evaporator. The ethanolic extract was collected and stored at 4-20°C to perform part 2.

Part 2: Solvent: Petroleum Ether Extract

The ethanol extract (90g) was suspended in distilled water and extracted with petroleum ether. All the fractions were washed with distilled water. The fractions were dried over anhydrous sodium sulphate to get petroleum ether soluble fraction. The fraction was then saponified by refluxing for 6 h in 500ml 5% methanolic KOH. Methanolic KOH fraction was allowed to stand for 20 h at room temperature. The unsaponifiable portion was extracted with diethyl ether. The ethereal fractions were washed with distilled water. The solvent was evaporated and dried over anhydrous sodium sulphate and stored in an airtight ambered colored glass container.

This petroleum ether extract of *Carissa congesta* (CC) roots was subjected to analytical studies by comparison with standard biomarkers after carrying out preliminary qualitative phytochemical screening as previously described.[12–14] All the standard biomarkers used for identification purpose in analytical studies were obtained from Sigma-Aldrich Private Limited, India and solvents from Merck India including HPLC grades. The general selection criterion for HPTLC and HPLC methods was to optimize the separation and identification of the bioactive compounds from the extract and to check the comparison of accuracy of the results obtained by HPTLC and HPLC. The basic advantage in selecting these powerful visualization techniques was its accuracy, preciseness, specificity, sensitivity and reproducibility.[15]

Part B: Analytical studies

Analytical studies comprises of Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and High Pressure Liquid Chromatography (HPLC) for determining the percentage of the active constituent.

(a) Thin Layer Chromatography (TLC)

Two Mobile Phases *n*-hexane and ethyl acetate (9:1) and Toluene, ethyl acetate and formic acid (9:1:0.5) were used for the study. The standard used was lupeol dissolved in petroleum ether. The sample was dissolved in ethyl alcohol and filtered before spotting the sample (10mg/ml). The Chamber was saturated for 30 mins

(b) High Performance Thin Layer Chromatography (HPTLC)

The HPTLC was performed at the Radiant Research Laboratories Private Limited, Bangalore. The analysis was carried out by application of the sample and standard dissolved in methanol on HPTLC plate's Silica gel 60 F254 (20 x 10 cm). Scanning of the developed plates was carried out at 333nm and 550 nm. The standard and sample were prepared by dissolving 5.16 mg and 47.5 mg in 5 ml of solvent each. Spots of extract (6 and 9 µg/l) and standard (3, 6 and 9 and 12 µg/l) were applied on the plates.

Instrument used was CAMAG Linomat 5 with spray gas as an inert gas, sample solvent as methanol, dosage speed of 150 nl/s and predosage volume: 0.2 µl. The HPTLC details applied were syringe size with 100 µl, number of tracks used were 10 with application position of 12.0 mm and band length of 8.0 mm. Calibration mode used was of single level, statistics mode was CV and evaluation mode was with peak height.

Formula: Percentage of lupeol = sample area x standard dilution x purity x 100/ standard area x sample dilution x 100

(c) High Pressure Liquid Chromatography (HPLC) analysis

HPLC instrument used was Shimadzu LC-10 ATVP with software as Chromtech N 2000 data with a detector of 280 nm and a flowrate of 1.5 ml/min. The injection volume was 20 µl and column dimensions were RP C-18, 250 x 4.6mm, 5 µ. Mobile phase used was acetonitrile and water (95:5). 100 µg of both, the standard and sample was dissolved in 1 ml of the solvent. From this stock solution, 20 µl was injected.

Formula: Percentage of lupeol = sample area x standard dilution x purity x 100/ standard area x sample dilution x 100

RESULTS

(a) Extraction yield

The extraction yield of the first step was found to be 10.74% w/w (ethanolic extract). The final extraction yield was 1.8% w/w (petroleum ether extract).

(b) Preliminary analysis of the plant extracts

The CC petroleum ether extract was been found to be positive for pentacyclic triterpenoids. Phytochemical screening of the extract showed the presence of steroids, flavonoids, saponins, glycosides, tannins, phenolic compounds, fixed oils and fats (Table I).

(c) Chromatographic analysis of extracts

(i) TLC reports

Mobile phase 1: Rf value for standard lupeol and the extract was found to be 0.21

Mobile phase 2: Rf value for standard lupeol and the extract was found to be 0.5

The Rf value was found to be consistent with lupeol in mobile phase 1 and 2 (Figure I).

(ii) HPTLC reports

Petroleum ether extract showed well resolved spots on the HPTLC plate at Tracks 7 and 8 in comparison to the standard at Tracks 1,2,3,4,9,10,11 and 12. The images were obtained at 333 nm before derivatisation and 550 nm after derivatization. The Rf value [Start Rf (0.49 and 0.48), maximum (0.54) and end(0.59)]. was found to be consistent with lupeol. (Table II and Figure II).

According to the formula mentioned in methodology and regression analysis, the amount of lupeol present in the extract was **25.80% w/w** (12.25 mg of lupeol present in 47.5 mg of extract).

(iii) HPLC reports

The CC extract showed a characterristic retention peak (15 min) at 280 nm identical to lupeol confirming the

Table I: Preliminary qualitative phytochemical analysis of the plant extract.

Sr.	Test for	Reagent	Observation
1	Carbohydrates	Molish's reagent	Absent
2	Reducing sugars	Fehling's reagent Benedict's reagent	Absent Absent
3	Saponin glycosides	Formation of Foam	Present
4	Flavonoids	Shinoda reagent	Present
5	Alkaloids	Dragendorff's reagent Hager's reagent Wagner's reagent Mayer's reagent	Absent Absent Absent Absent
6	Tannins and Phenolic compounds	5% $FeCl_3$ solution Bromine water Dilute iodine solution	Present Present Present
7	Mucilage with powdered drug material	Ruthenium red Swelling property	Absent Absent
8	Steroids	Salkowski reagent Liebermann-Burchard reagent Lieberman reagent	Present Present Present
9	Fats and Oils	Sudan Red III reagent Filter paper Saponification	Present Present Present

Table II: HPTLC analysis of Carissa congesta and standard lupeol.

Track No	Details	Height	Area
1	lupeol (3µg/l)	146.3	4123.5
2	lupeol (6 µg/l)	265.6	7144.5
3	lupeol (9µg/l)	332.6	9307.5
4	lupeol (12µg/l)	384.1	11146.7
9	lupeol (12µg/l)	370.9	10527.7
10	lupeol (9µg/l)	313.2	8656.6
7	*Carissa congesta* petroleum ether extract (6 µg/l)	559.9	16577.4
8	*Carissa congesta* petroleum ether extract (9 µg/l)	627.7	21718.3
11	lupeol (6 µg/l)	239.2	6272.9
12	lupeol (3µg/l)	130.6	3500.4

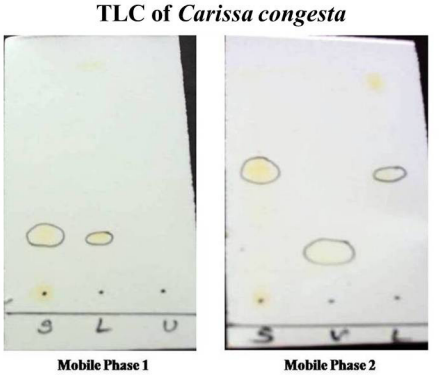

Figure I. TLC of *Carissa congesta*.

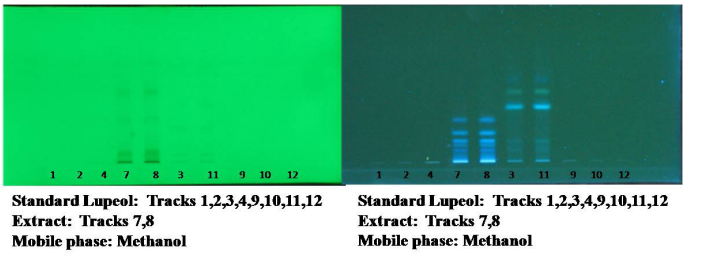

Figure II. HPTLC Image before and after Derivatization.

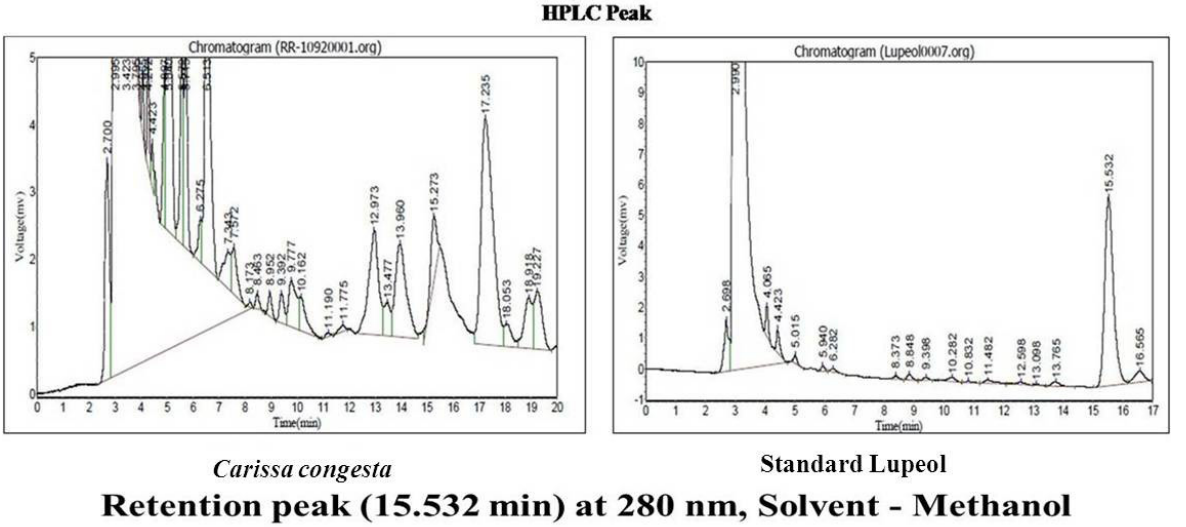

Figure III. HPLC Peaks for *Carissa congesta*.

Table III: HPLC analysis of *Carissa congesta* and standard lupeol

Sample Name	Value
(Retention) Sample area of lupeol	(15.237) 15503.427
(Retention) Standard area of lupeol	(15.532) 124739.67
Dilution of lupeol (Sample and Standard)	1:1
% of lupeol	**11.18 % w/v**

presence of the constituents at flow rate of 1 ml/min using methanol when HPLC studies were monitored (Table III, Figure III).

According to the formula mentioned in methodology and regression analysis, the amount of lupeol present in the extract was **11.18 % w/v**.

DISCUSSION AND CONCLUSION

Lupeol has been found to play a significant role from different plant extracts. Our studies draw the readers towards the constituent isolated in the petroleum ether extract of *Carissa congesta* roots which was found to be lupeol as confirmed by TLC, HPLC and HPTLC. The future prospect urges the burgeoning researchers that this plant may exert a strong pharmacological potential which could be explored by undertaking studies on enormous pharmacological models of different therapeutic categories.

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