ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info/jasr

ISSN
0976-9595
Research Article

EVALUATION OF MORPHOLOGICAL AND GEOGRAPHICAL VARIATION IN THE CONTENT OF URSOLIC ACID FROM *CARISSA CARANDAS* LINN. USING HPTLC

Sunita Shailajan*, Dipti Singh, Bhavesh Tiwari

Herbal Research Lab, Ramnarain Ruia College, Matunga, Mumbai 400 019, India

**Corresponding author: sunitashailajan@gmail.com*

ABSTRACT

Ursolic acid (UA), a hydroxyl pentacyclic triterpenoic acid found in *Carissa carandas* Linn., a folk medicine used for the treatment of various diseases and also an important component of plant based medicine. In the present work, a precise, accurate and reproducible densitometric HPTLC method is developed and validated in terms of sensitivity, linearity, recovery, specificity and ruggedness for the estimation of ursolic acid from various samples of *Carissa carandas* Linn. Chromatographic separation was achieved on silica gel 60 F_{254} plates with toluene: methanol (8: 1, v/v) as a mobile phase. Detection of ursolic acid was achieved by derivatizing the plate with 10% methanolic sulphuric acid followed by heating at 110°C for 10 min. Camag TLC scanner 4 equipped with winCATS software was used for densitometric scanning at 366 nm. The method was found applicable to evaluate the impact of morphological and geographical variations in the ursolic acid content from *Carissa carandas* ripe fruits and different morphological parts of *Carissa carandas*. Ursolic acid content in different morphological parts of *Carissa carandas* was found in the following order: Fruits > leaves > stems > flowers > seeds. Samples collected from Rajapur showed the maximum content while sample from Lonavla had minimum. The developed method was found useful for quantitation of bioactive marker ursolic acid from different samples of *Carissa carandas* and can be used as a routine quality control tool for the assessment of botanicals.

Keywords: *Carissa carandas*, Geographical variation, HPTLC, Morphological variation ripe fruit, Ursolic acid.

1. INTRODUCTION

Carissa carandas Linn. (Apocynaceae) commonly known as 'Karanda', is a large dichotomously branched evergreen shrub with short stem and strong thorns in pairs. The plant is native and common throughout India, Sri Lanka, Java, Malaysia, Myanmar and Pakistan. It is a popular medicinal plant having long history of use in various indigenous systems of medicine like Ayurveda, Unani and Homoeopathy [1].

Fruits of *Carissa carandas* are extensively used in various Ayurvedic, Unani and herbal formulations. Fruits are reported to possess various phytoconstituents like luteolin, n-hentriacontane, nonacosane, p-hydroxybenzoic acid, 5-oxyisophthalic acid, carisol, epimer of α-amyrin, linalool, β-caryophyllene, carissone, carissic acid, carindone, ursolic acid, carinol, ascorbic acid, lupeol and β-sitosterol [2].

Ursolic acid, a pentacyclic triterpenoid is the bioactive phytoconstituent found abundantly in the aerial parts of *Carissa carandas*. Ursolic acid is reported to exhibit various biological activities such as anti-inflammatory, antimicrobial, antibacterial and antifungal activity [3], hepatoprotective [4, 5], hypoglycemic [6], anti-hyperlipidemic, anti-tumor [7] and anti-cancer activities [8].

Estimation of ursolic acid from various plant samples has been reported using various analytical techniques [9-12] such as HPLC, HPTLC, GC etc. The literature revealed that, till date, chromatographic characterization of *Carissa carandas* and the impact of regional variation in terms of ursolic acid content has not been carried out using validated HPTLC technique. Therefore, the present investigation deals with the development of HPTLC method for the estimation of ursolic acid content from different samples of *Carissa carandas* spread across an altitudinal gradient, in the different geographical regions of India.

2. MATERIALS AND METHODS

2.1. Plant material

Carissa carandas ripe fruits were collected from Kalyan, Maharashtra authenticated by Agharkar Research Institute, Pune (Auth 11-1234) and a voucher specimen was deposited for further reference. Ripe fruits were also collected from different regions of Maharashtra like Dapoli, Igatpuri, Kalyan, Karjat, Lonavla, Malshej, Rajapur, Ratnagiri in order to study the impact of regional variation on marker (ursolic acid) content. Also, different morphological parts of the *Carissa carandas* were collected from Karjat. Samples were shade dried for 15 days, then dried at 37 ± 2°C for a week, powdered, sieved through BSS sieve (85 mesh) and stored in air-tight containers.

2.2. Chemicals

Analytical grade solvents like toluene and methanol were procured from Merck Specialities Pvt. Ltd., Mumbai. Ursolic acid (purity ≥ 90%, HPLC grade, Figure 1) was procured from Sigma-Aldrich Private Limited, India.

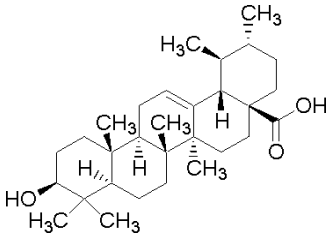

Fig. 1: Structure of Ursolic acid

2.3. HPTLC conditions

Chromatographic separation was achieved on TLC plates pre-coated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminium sheet support. Samples were spotted using CAMAG Linomat 5 sample spotter (Camag Muttenz, Switzerland) equipped with syringe (Hamilton, 100 µL). Plates were developed in a glass twin trough chamber (CAMAG) pre-saturated with mobile phase toluene: methanol in the ratio of 8: 1, v/v. Scanning device used was CAMAG HPTLC Scanner 4 equipped with winCATS software. The experimental condition was maintained at 25 ±2°C. CAMAG reprostar 3 was used for photodocumentation.

2.4. Preparation of standard stock solutions and extraction conditions for plant samples

A stock solution of ursolic acid (1000.0 µg/mL) was prepared in methanol. Seven calibrant samples ranging from 5.0 µg/mL-100.0 µg/mL and three quality control samples of ursolic acid namely low, mid, and high (6.5, 25.0, 80.0 µg/mL respectively) were prepared in methanol using the stock solution. Accurately weighed powdered sample (0.5 g) of leaves, stems, flowers, fruits and seeds were extracted with methanol (5.0 mL). The samples were vortexed for 1-2 mins, kept standing overnight at room temperature and filtered through Whatmann filter paper No. 1 (E. Merck, India). The filtrate was then subjected to HPTLC analysis. The same extraction technique was used to extract ursolic acid from different morphological parts of *C. carandas*.

2.5. Method validation

The developed HPTLC method for estimation of ursolic acid was validated as per ICH guidelines [13] for the parameters like sensitivity, linearity, precision, recovery, specificity and ruggedness [14].

2.6. Assay of ursolic acid from the different samples of *Carissa carandas*

Relative response for the characteristic band of ursolic acid in various samples of *Carissa carandas* ripe fruits collected from different geographical regions was obtained using validated HPTLC technique. In order to evaluate the morphological and geographical variation in the content of ursolic acid, the data obtained (response) was analyzed using the regression equation and the content of ursolic acid was determined.

2.7. Statistical analysis

Microsoft Excel-2007 was used for the statistical evaluation of results.

3. RESULTS AND DISCUSSION

Carissa carandas is commonly used in traditional systems of medicine. Its therapeutic benefits are largely based on folkloric rather than scientific evidences. Ursolic acid is found in the aerial parts of *Carissa carandas*. It also has various therapeutic potential. A wide number of medicinal plants have been characterized using various analytical tools for their ursolic acid content. In this research work, some of the existing analytical methods were tried to separate the ursolic acid from the plant matrix, but use of these methods were found to be unsatisfactory. The problems faced during this work were, the separation between two adjacent bands, peak shape, improper resolution, compactness of the bands. We have recently published a validated HPTLC method for the estimation of ursolic acid from *Chrysophyllum cainito* [15]. This method was found applicable for the separation of ursolic acid from the complex matrix of *Carissa carandas.*

Table 1: Results of method validation experiments conducted using HPTLC for the estimation of ursolic acid

Parameters	Results
LOD and LOQ (µg/mL)	2.5 and 5.0
Linear range (µg/mL)	5.0 – 100.0
Regression equation	y= 30.80x+132.5
Mean coefficient of determination (r^2)	0.999
System suitability (% CV , n = 6)	
R_f	0.36
Area	1.20
Precision (% CV , n = 3)	
Intra day	0.31-0.93
Inter day	0.09-1.35
Recovery using QC samples (% mean ± SD, n = 3)	
Low (6.5 µg/mL)	97.83 ± 0.0062
Mid (25.0 µg/mL)	98.20 ± 0.0156
High (80.0 µg/mL)	97.42 ± 0.0395
Stability	
Standard Stock Solution short term stability (For 12 h)	Stable at (4±1°C)
Standard Stock Solution long term stability (For 15 days)	Stable at (4±1°C)
Bench top stability (For 6.00 h)	Stable at (22±2°C)
Ruggedness	Rugged

Briefly, of the various solvent systems tried, mixture containing toluene: methanol (8: 1, v/v) gave the best resolution and separation of ursolic acid (R_f = 0.36) from the other components in the methanolic extract of *Carissa carandas.* The identity of band of ursolic acid in the plant matrix was confirmed by comparing the R_f and the colour of the band with that of the standard ursolic acid. The method was validated as per ICH guidelines and the findings are

summarized in Table 1. The method was found linear over the range of 5.0-100.0 µg/mL, precise during intra-day and inter-day precision studies, sensitive with limit of detection and limit of quantification values 2.5 µg/mL and 5.0 µg/mL, respectively. The average recovery for quality control samples of ursolic acid was found to be 97.82 %. The method was also found rugged for the parameters like change in analysts, change in mobile phase composition and change in spotting volume.

As an application of this method, various morphological parts of *Carissa carandas* have been characterized for their ursolic acid content. Content of ursolic acid in different morphological parts of *C. carandas* is shown in table 2.

Table 2: Ursolic acid content in the different plant parts of *Carissa carandas*

Sample	Content (Mean ± S. D., n=3) (mg/g)
Leaves	1.08 ± 0.0188
Fruits	1.24 ± 0.0440
Flowers	0.64 ± 0.0216
Seeds	0.26 ± 0.0333
Stems	0.73 ± 0.0204

The HPTLC plate photo and the 3-D overlay of the chromatograms of *Carissa carandas* ripe fruits and different morphological parts with ursolic acid are shown in fig. 2A and 2B. Content of ursolic acid was found to be present in all the five parts analyzed. The highest content of ursolic acid was observed in the fruits followed by leaves, stems, flowers and seeds, respectively. The advantage of the observation is that instead of using the other aerial parts of the plant in formulation, ripe fruits can be used.

Also, study on phytochemicals of wild populations at different altitudes was performed from the complex matrix of *Carissa carandas*. The sample collected from Rajapur (low altitude region) showed the maximum content of ursolic acid whereas sample collected from Lonavla had the least. The impact of geographical variation was clearly evident on the content of ursolic acid. The results are given in table 3.

Table 3: Ursolic acid content in the *Carissa carandas* ripe fruits collected from different geographical regions

Sample	Content (Mean±S.D, n=3) (mg/g)
Ratnagiri	1.24 ± 0.0170
Rajapur	1.45 ± 0.0163
Dapoli	1.13 ± 0.0145
Lonavla	0.39 ± 0.0078
Karjat	1.24 ± 0.0220
Malshej	0.53 ± 0.0086
Kalyan	0.73 ± 0.0216
Igatpuri	1.05 ± 0.0175

HPTLC plate photo and the 3-D overlay of the chromatograms of *Carissa carandas* ripe fruits collected from different geographical regions of India is shown in Fig. 3A and 3B. *Carissa carandas* grows in different geographical regions of India and environmental factors may fluctuate at various altitudes. Since, *Carissa carandas* is distributed in different regions of India, contents of its bioactive markers vary greatly depending on their geographical locations, climatic conditions and other factors. In our previous studies, we have reported the morphological and geographical variation in β-sitosterol, stigmasterol and lupeol content from various samples of *Carissa carandas* [16]. In this study, we observed the morphological and geographical variation in a new bioactive marker (Ursolic acid) found abundantly in *Carissa carandas*.

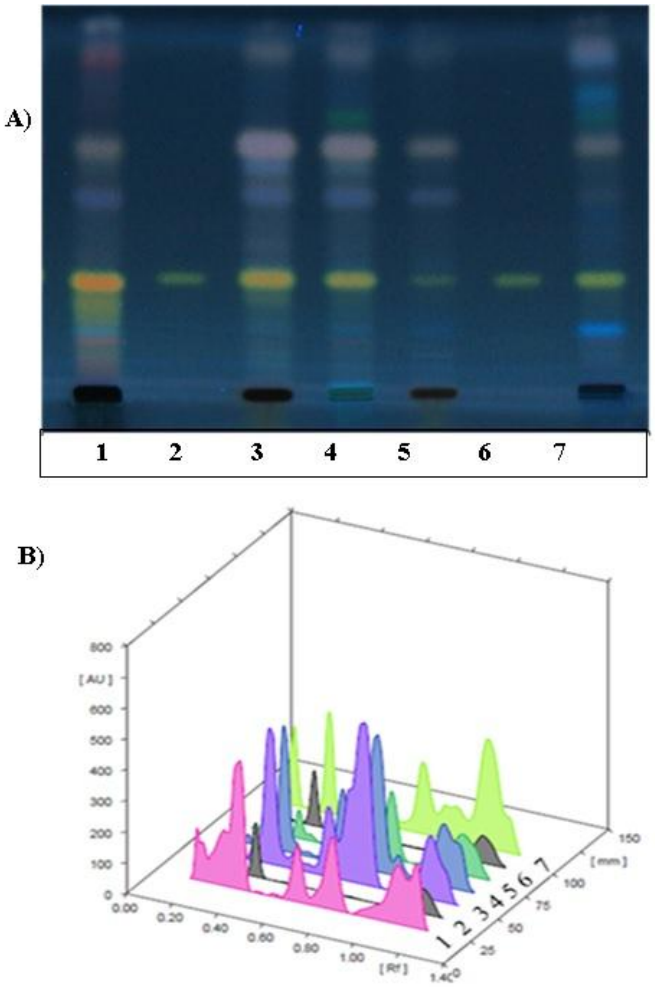

Fig. 2: HPTLC plate photo (A) 3D overlay of the chromatograms and (B) *Carissa carandas* ripe fruits and different morphological parts with ursolic acid at 366 nm.
Track details: 1: Leaves, 2: Ursolic acid (10 µg/mL), 3: Fruits, 4: Flowers, 5: Seeds, 6: Ursolic acid (10 µg/mL), 7: Stems

At this juncture, it is not conclusive whether the observed variation are a response of individual plants to environmental factors related to altitude or the adaption of the plants growing at different altitudes to their specific environment [17-19]. Further studies may throw light on it.

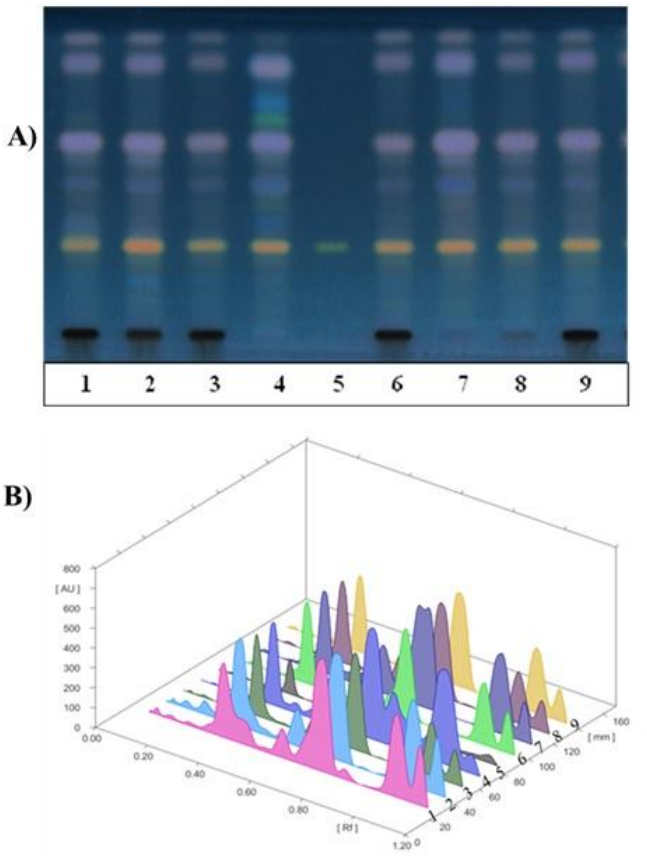

Fig. 3: HPTLC plate photo (A) and 3D overlay of the chromatograms (B) of *Carissa carandas* ripe fruits collected from different geographical regions with ursolic acid at 366 nm.

Track details: 1: Ratnagiri, 2: Rajapur, 3: Dapoli, 4: Lonavla, 5: Ursolic acid (10 µg/mL), 6: Karjat, 7: Malshej, 8: Kalyan, 9: Igatpuri

4. CONCLUSION

The developed HPTLC method for the estimation of ursolic acid from various sources of *Carissa carandas* is rapid, reliable, accurate, reproducible, selective and economic and can be used for routine assessment of quality of *Carissa carandas*. The simplicity of this method allows for the application in laboratories that lack sophisticated analytical instruments such as HPLC, LC-MS and GC-MS. From the study, it is concluded that the geographical variation may have direct influence in the content of chemical constituents of the plants. Using such validated methods, *Carissa carandas* with precise quality can be encouraged in herbal and food industries. The reported method is expected to stimulate interest and open the possibility of clinically effective drugs or food products from this plant because of the presence of potentially active phytoconstituent, ursolic acid. Although ursolic acid is not a plant specific marker, it was chosen for its proven therapeutic efficacy against various ailments and for the quality evaluation of *Carissa carandas* fruits.

5. ACKNOWLEDGEMENTS

The authors are highly thankful to the team of Herbal Research Lab, Ramnarain Ruia College for helping us in the collection of the fruits from various regions. A special mention to Dr. Neelam Naik and Deepti Gurjar for providing us with the sample from Malshej and Rajapur.

6. REFERENCES

1. Itankar PR, Lokhande SJ, Verma PR, Arora SK, et al. *J Ethnopharmacol*, 2011; **135(2):**430-433.
2. Bhaskar VH, Balakrishnan N. *DARU Journal of Pharmaceutical Sciences*, 2009; **17:**168-174.
3. Liu J. *J Ethnopharmacol*, 1995; **49(2):**57-68.
4. Kataki MS, Murugamani V, Rajkumari A, Mehra PS, et al. *Pharm Crop*, 2012; **3:**38-46.
5. Hegde K, Thakker SP , Joshi AB, Shastry CS, et al. *Trop J Pharm Res*, 2009; **8(2):**117-125.
6. Bnouham M, Merhfour FZ, Ziyyat A, Mekhfi H. *Fitoterapia*, 2003; **74(7-8):**677-681.
7. Shetty P, Mangaonkar K, Sane RT. *J. Planar. Chromatogr. - Mod. TLC,* 2007; 20(1):65-68.
8. Yuanyuan X, Guangli W, Duanyun S, Changxiao L. *J Chromatogr B*, 2011; **879(2):**219-224.
9. Anandjiwala S, Kalola J, Rajani M. *J AOAC Int*, 2006; **89:**1467-1474.
10. Simone CB, Eloir PS, Valquiria LB. *J. Braz. Chem. Soc*. 2005; **16(4):**723-726
11. Liao Q, Yang W, Jia Y, Chen X, et al. *Yakugaku Zasshi*, 2005; **125(6):**509-515.
12. Zou S, Chen W, Li K. *Acta Agri Univer Jiangxiensis*, 2004; **26:**808-810.
13. Shailajan S, Yeragi M, Tiwari B. *Int J Green Pharm*, 2013; **7(1):**62-65.
14. International Conference on Harmonisation, Q2B. Validation of analytical procedures. Geneva US FDA Federal Register, 1997; **62:**27463-467.
15. Shailajan S, Gurjar D. *Int J Pharm Sci Rev Res*, 2014; **26(1):**106.
16. Shailajan S, Menon S, Sayed N, Tiwari B. *Int J Green Pharm*, 2012; **6(3):**241-47.
17. Ruhland, Day. *Plants and Climate Change*; 2000.
18. Zidorn C, Schubert B, Stuppner H. *Biochem. Syst. Ecol,* 2005b; **33:**855-872.
19. Zidorn C, Stuppner H. *Taxon 50*, 2001a; 115-133.