ORIGINAL ARTICLE

Simultaneous estimation of three triterpenoids-ursolic acid, β-sitosterol and lupeol from flowers, leaves and formulations of *Rhododendron arboreum* Smith. using validated HPTLC method

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Background: This paper enfolds a rapid and sensitive high-performance thin-layer chromatographic (HPTLC) method for the simultaneous estimation of three triterpenoids namely ursolic acid, β-sitosterol and lupeol from the leaves, flowers and herbal formulations of *Rhododendron arboreum* Smith., an ethnomedicinal Himalayan tree. All the three phytoconstituents have high therapeutic value. **Aims and Objectives:** The main aim is to separate, resolve and simultaneously quantitate the three markers-ursolic acid, β-sitosterol and lupeol from *R. arboreum* using normal phase HPTLC. **Materials and Methods:** Separation was performed on TLC aluminium plates precoated with silica 60 F_{254} followed by detection of ursolic acid, β-sitosterol and lupeol carried out by derivatizing the plate with 10% methanolic sulphuric acid reagent followed by heating at 110°C for 7 min. Camag TLC scanner 4 equipped with winCATS software was used for densitrometric scanning at 366 nm. The proposed method was further validated in terms of linearity, precision, accuracy and sensitivity as per the International Conference on Harmonisation (ICH) guidelines. **Results:** A good linear relationship was obtained for the calibration plots with r^2 = 0.999, 0.993 and 0.995 for ursolic acid, β-sitosterol and lupeol, respectively. Accuracy of the method was checked by recovery study conducted at three different levels with the average recovery between 95% and 98% for all the three markers. **Conclusion:** The developed method can be used for the assessment of the quality of botanicals in terms of bioactive content.

Key words: Ethnomedicinal, *Rhododendron arboreum*, simultaeneous estimation, triterpenoids

INTRODUCTION

Various herbs and herbal extracts contain a large number of biologically active compounds like vitamins, amino acids, carotenoids, terpenoids, alkaloids, tannins and phenols which are responsible for their therapeutic effects.[1] Among these various classes of phytoconstituents, triterpenoids are widely distributed in plants. Triterpenoids namely ursolic acid, β-sitosterol and lupeol [Figure 1] are compounds with a carbon skeleton based on six isoprene units which are derived biosynthetically from the acyclic C_{30} hydrocarbon.[2] β-sitosterol has anticancer,[3] estrogenic[4] activities and lupeol is reported to show antimalarial[5] and hepatoprotectant[6] activities while ursolic acid exhibits anti-inflammatory[7] and nephroprotective activity.[8] Thus, the quantitative analysis of triterpenoids from plant matrix and herbal formulations becomes an unavoidable necessity.

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Rhododendron arboreum Smith. (Ericaceae); a small evergreen tree endemic to the Southern Western ghats of penninsular India and Himalayas, occurs in the high altitudes from 4,500 ft to 10,500 ft.[9] The tree is bestowed with beautiful conspicuous red flowers having a sweet sour taste[10] which are widely used to treat dysentery and fever[1] and act as anti-inflammatory, anti-diarrhoeal[9] and anti-diabetic agent,[11] whereas the leaves are reported to be useful in the management of headache, gout and rheumatism[9] and myocardial infarction.[12] Herbal formulations, '*Rhododendron* Super' and 'Cardorium Plus' containing *R. arboreum* flowers are also available in market.

The leaves of *R. arboreum* are reported to contain glucoside, ericolin, ursolic acid, α-amyrin, epifriedelinol, campanulin, quercetin and hyperoside,[13] whereas quercetin-3-rhamnoside, coumaric acid, ursolic acid and resins[14] are the pharmacologically active markers

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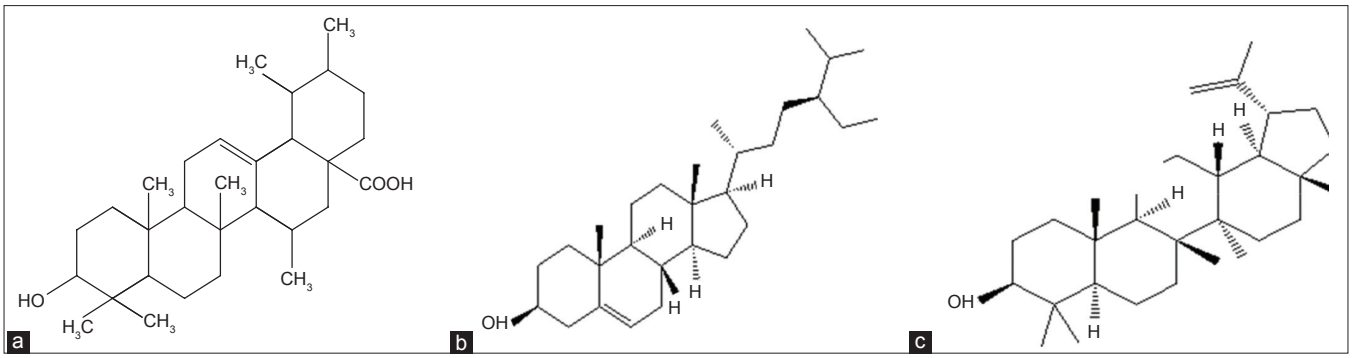

Figure 1: Structure of (a) Ursolic acid; (b) β-sitosterol and (c) Lupeol

present in flowers. β-sitosterol, ursolic acid, quercetin and friedlin have been isolated from the leaves of *R.arboreum*.[15,16] There are reversed-phase high-performance thin-layer chromatographic (RP-HPTLC) methods reported for simultaneous quantification of quercetin, rutin and coumaric acid[1] and hyperin from the ethyl acetate fraction[16] of *R. arboreum flowers* and epicatechin, syringic acid, quercetin-3-O-galactoside and quercetrin from leaves of the tree.[10]

But, so far there are no methods reported for the simultaneous estimation and quantification of three triterpenoids-ursolic acid, β-sitosterol and lupeol from the leaves and flowers of *R. arboreum*. Keeping this in view, the present study was done with an objective of developing a rapid and simple validated method for the simultaneous estimation of these three pharmacologically active compounds by using normal phase HPTLC using a single mobile phase from the leaves and flowers of *R. arboreum*. The method was also applied to the available liquid formulations as a part of quality control.

MATERIALS AND METHODS

Plant Materials

Fresh leaves of *R. arboreum* were collected from Assam and Garhwal, whereas the flowers were collected from Assam, Garhwal and Shillong in the month of April. The taxonomic identification of the plant was confirmed by Botanical Survey of India (BSI), New Delhi. The collected material was cleaned, shade dried for a week and kept in the oven at 45°C for one more week. It was then powdered in a mixer grinder, sieved through 85 mesh (BSS) sieve and preserved in an air tight container at room temperature.

Two herbal formulations containing flowers of *R. arboreum* namely '*Rhododendron* Super' (Sanjivani Herbs and Nature Care, Batch no. 101N) and 'Cardorium Plus' (Alaknanda Herbals Pvt Ltd, Batch no. 012) were purchased from the market.

Chemicals

Chemicals of analytical grade were purchased from Merck Specialities Private Limited, Mumbai. Reference standards-ursolic acid (98.5% purity), β-sitosterol (98% purity) and lupeol (97% purity) were procured from Sigma Aldrich Chemical Company, (Steinheim, Germany). Derivatising reagent i.e., 10% methanolic sulphuric acid reagent was prepared as per the procedure described by Reich and Schibli.[17]

Preparation of Standard and Sample Solutions

Stock solutions of pure compounds (1000 µg mL^{-1}) were prepared by dissolving 10 mg of accurately weighed standards in small amount of methanol and making the volume up to 10 mL in a standard volumetric flask. The stock solutions were further diluted for the preparation of working solutions.

Accurately weighed 50 mg and 100 mg of dried powder of leaves and flowers, respectively, of *R. arboreum* was separately extracted in 10 mL of methanol, vortexed for 1 min and kept standing overnight. Next day, the extracts were filtered using Whatman filter paper no. 1 in dry stoppered test tubes and the filtrate (10 µL) was used for HPTLC analysis.

In the case of formulations, 4 mL of both the formulations was extracted in 4 mL of chloroform, vortexed for 1 min and kept standing overnight. Next day, the bottom layer of chloroform was separated from the upper aqueous layer, taken in two separate glass beakers and evaporated to dryness in a water bath at 54°C. The residue was then reconstituted in 0.5 mL (for *Rhododendron* Super) and 1 mL (for Cardorium Plus) of methanol, respectively, and the solutions were then filtered through Whatman filter paper no. 1 and the filtrate (10 µL) was used for HPTLC analysis.

High-Performance Thin-Layer Chromatographic Analysis

Instrumentation and operating conditions

A CAMAG HPTLC system equipped with Linomat V Automatic Sample Spotter (Camag Muttenz, Switzerland) and CAMAG TLC Scanner IV with winCATS planar

chromatography manager software version 1.4.7 was used for the analysis. Samples were applied on TLC plates precoated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminium sheet support in 7 mm bands at 10 mm from the bottom, 15 mm from the sides and 7 mm space between two bands. Plates were developed in a twin trough chamber presaturated with mobile phase of toluene: Methanol (8:1, v/v) for 30 min to a height of 8.5 cm from the base. After development, the plate was dried and derivatised with 10% methanolic sulphuric acid reagent. Quantitative evaluation of the plate was carried out in the reflectance mode at 366 nm with following conditions: Slit width 6 mm × 0.45 mm, scanning speed 20 mm/s and data resolution 100 μm/step.

Method Validation

International Conference on Harmonisation (ICH) guidelines were followed for the validation of developed analytical method.[18] The parameters evaluated were linearity, precision, specificity, accuracy, sensitivity and ruggedness.

Specificity

Specificity of the method was confirmed by comparing the bands of the sample solutions with that of the respective reference standards in terms of R_f and colour in fluorescence mode.

Sensitivity

Sensitivity of the method was determined with respect to limit of detection (LOD) and limit of quantification (LOQ). Different dilutions of the standard solutions of ursolic acid, β-sitosterol and lupeol were applied on TLC plates along with methanol as blank and analysed on the basis of signal-to-noise (S/N) ratio. LOD was determined at an S/N of 3:1 and LOQ at an S/N of 10:1.

Calibration and quality control samples

For designing the calibration curve, appropriate dilutions were prepared from the stock solutions to get desired concentrations in the quantification range. The working standards in the range of 5-100 μg mL^{-1}, 5-60 μg mL^{-1}, 5-75 μg mL^{-1} for ursolic acid, β-sitosterol and lupeol, respectively, were applied on TLC plate for obtaining a seven point linear calibration curve. Further, quality control samples low quality control (LQC), medium quality control (MQC) and high quality control (HQC) for ursolic acid, β-sitosterol and lupeol were prepared for precision, accuracy and ruggedness studies.

Precision

Instrumental precision

Instrumental precision was checked by repeated scanning ($n = 7$) of 5 μg mL^{-1} of ursolic acid, β-sitosterol and lupeol and further expressed as relative standard deviation (% RSD).

Repeatability

The repeatability of the method was affirmed by analysing 5 μg mL^{-1} of all the three markers on a HPTLC plate ($n = 5$) and expressed as % RSD.

Inter- and intra-day precision

Variability of the method was studied by spotting the quality control samples of ursolic acid, β-sitosterol and lupeol on the same day (intra-day precision-spotting each concentration thrice within 24 hour) and on different days (inter-day precision-spotting each concentration three times at 3 days interval) and the result was expressed as % RSD.

Accuracy

The accuracy of the method was assessed by spiking the quality control samples of ursolic acid, β-sitosterol and lupeol in plant matrix. The spiked samples were extracted in triplicate and percent recovery for each was calculated.

Ruggedness

Ruggedness of the method was assessed by deliberately incorporating small variations in the optimized chromatographic conditions. Effect of change in analyst, change in mobile phase composition (Toluene:Methanol (7.9:1.1 v/v) and Toluene:Methanol (8.1:0.9 v/v)) and change in spotting volume (9 μL and 11 μL), on the response and R_f of quality control samples was observed. Results were expressed in terms of percent mean difference.

Assay

The content of all the three markers from the flowers, leaves and formulations of *R. arboreum* was determined by applying the samples (10 μL) in triplicate along with pure standards.

RESULTS AND DISCUSSION

In order to obtain a good separation amongst three triterpenoids viz. ursolic acid, β-sitosterol and lupeol, various solvent systems consisting of toluene, ethyl acetate, methanol, formic acid, glacial acetic acid were tried on normal phase HPTLC, out of which toluene: Methanol (8:1, v/v) successfully resolved the three markers ursolic acid ($R_f = 0.30$), β-sitosterol ($R_f = 0.47$) and lupeol ($R_f = 0.58$) and enabled their simultaneous estimation from complex sample matrix. The plate derivatised in 10% methanolic sulphuric acid was visualised in 366 nm [Figure 2]. The method developed was found to be selective with good baseline resolution of each compound [Figure 3].

The developed method was validated in terms of specificity, precision, sensitivity, ruggedness and accuracy. Specificity of the method was confirmed by matching the colour and

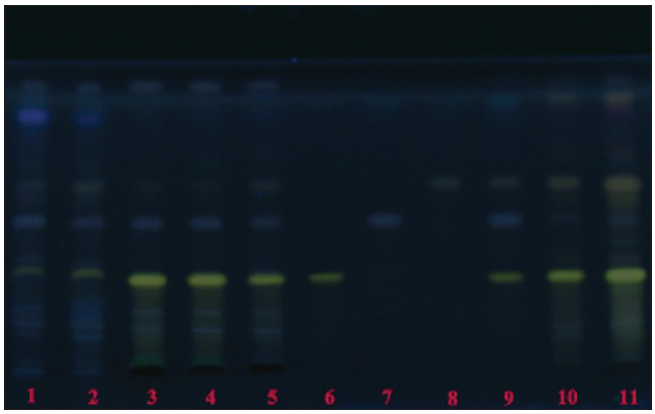

Figure 2: Detection and quantitation of ursolic acid, β-sitosterol and lupeol from flowers, leaves and formulations of *R. arboreum* at 366 nm. Track details: 1: *Rhododendron* Super, 2: Cardorium Plus, 3: *R. arboreum* flowers (Assam), 4: *R. arboreum* flowers (Garhwal), 5: *R. arboreum* flowers (Shillong), 6: Ursolic acid (10 ppm), 7: β-sitosterol (10 ppm), 8: Lupeol (20 ppm), 9: UBL Mixture, 10: *R. arboreum* leaves (Assam), 11: *R. arboreum* leaves (Garhwal)

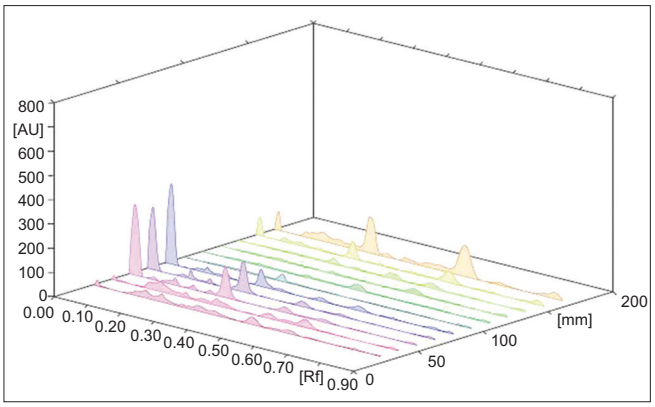

Figure 3: HPTLC densitometric profile as a 3D overlay of *R. arboreum* flowers, leaves and formulation with ursolic acid, β-sitosterol and lupeol at 366 nm. Track details: 1: *Rhododendron* Super, 2: Cardorium Plus, 3: *R. arboreum* flowers (Assam), 4: *R. arboreum* flowers (Garhwal), 5: *R. arboreum* flowers (Shillong), 6: Ursolic acid (10 ppm), 7: β-sitosterol (10 ppm), 8: Lupeol (20 ppm), 9: UBL Mixture, 10: *R. arboreum* leaves (Assam), 11: *R. arboreum* leaves (Garhwal)

R_f of the bands from samples with that of the reference standards. Absence of any interfering bands indicated that the method was specific. The seven point calibration curves for three reference compounds were found to be linear in the range of 5-100, 5-60 and 5-75 µg mL^{-1} for ursolic acid, β-sitosterol and lupeol, respectively. Regression equation and coefficient of determination for the reference compound were: y = 30.80x + 132.5, r^2 = 0.999 for ursolic acid, y = 39.76x + 194.0, r^2 = 0.993 for β-sitosterol and y = 24.81x + 35.10, r^2 = 0.995 for lupeol [Table 1]. The LOD and LOQ values obtained were 2.5 µg mL^{-1} and 5 µg mL^{-1} for ursolic acid, 1 µg mL^{-1} and 5 µg mL^{-1} for β-sitosterol and 2 µg mL^{-1} and 5 µg mL^{-1} for lupeol indicating that the developed method was more sensitive for β-sitosterol as compared to ursolic acid and lupeol [Table 2]. The % RSD was found to be less than 2% when minute deliberate changes were made in the mobile phase composition and spotting volume of the samples stating that the method was rugged.

The values for repeatability, instrumental, intra-day and inter-day precision were found to be in the range of 0.5-2% demonstrating good precision and repeatability of the proposed method using the quality control samples of reference compounds. Good recoveries were obtained by the enrichment of plant matrices at three different concentration levels after sample processing and applying. It is evident from the results that the average percent recoveries were in the range of 95-98% [Table 2].

Ursolic acid, β-sitosterol and lupeol were simultaneously quantitated from the complex matrix of *R. arboreum* flowers, leaves and formulations. Leaves of *R. arboreum* showed maximum content of all the three markers as compared to flowers. Leaves collected from Garhwal region were rich in ursolic acid (18.86 ± 1.47) while leaves collected from Assam were rich in lupeol (11.064 ± 1.79) as compared to other markers. Flowers collected from all the three regions i.e., Assam, Garhwal and Shillong showed the highest content of ursolic acid as compared to β-sitosterol and lupeol. The method when applied to aqueous herbal formulations containing flowers of *R. arboreum* showed a good separation, resolution and quantitation of three triterpenoids [Table 3].

Table 1: Calibration parameters for examined phytoconstituents

Phytoconstituents	Linear working range (µg mL^{-1})	Regression equation	Coefficient of determination (r^2)
Ursolic acid	5–100	y=30.80x+132.5	0.999
β-sitosterol	5–60	y=39.76x+194.0	0.993
Lupeol	5–75	y=24.81x+35.10	0.995

Table 2: Method validation parameters for ursolic acid, β-sitosterol and lupeol

Parameters	Ursolic acid	β-sitosterol	Lupeol
R_f	0.30	0.47	0.58
LOD (µg mL^{-1})	2.5	1.0	2.0
LOQ (µg mL^{-1})	5.0	5.0	5.0
Instrumental precision (% RSD), *n*=7	1.82	1.63	1.57
Repeatability (% RSD), *n*=5	1.09	0.96	1.28
Intraday precision (% RSD)	1.73	0.50	0.89
Interday precision (% RSD)	1.99	0.93	1.15
Recovery (%), *n*=3		For leaves	
	97.23	96.54	98.16
		For flowers	
	95.33	98.21	96.83
Specificity		Specific	
Ruggedness		Rugged	

LOD – Limit of detection ; LOQ – Limit of quantification; % RSD – Relative standard deviation

Table 3: Content of ursolic acid, β-sitosterol and lupeol in *R. arboreum* flowers, leaves and formulation

Samples	Content of ursolic acid (mg g^{-1})	Content of β-sitosterol (mg g^{-1})	Content of lupeol (mg g^{-1})
		Mean±SD, *n*=3	
Rhododendron super	0.056±1.09	0.057±0.44	0.048±1.63
Cardorium plus	0.08±0.49	0.034±1.39	0.136±1.27
R. arboreum flowers (Assam)	7.241±0.33	0.912±1.13	0.881±0.17
R. arboreum flowers (Garhwal)	6.656±1.29	0.918±0.80	0.578±1.22
R. arboreum flowers (Shillong)	3.718±0.21	0.531±0.98	1.83±0.65
R. arboreum leaves (Assam)	7.020±0.17	1.260±1.85	11.064±1.19
R. arboreum leaves (Garhwal)	18.861±1.47	1.020±0.79	14.260±1.82

CONCLUSION

The developed method is an initiative for the estimation of ursolic acid, β-sitosterol and lupeol simultaneously using normal phase HPTLC from the flowers, leaves and formulations of *R. arboreum*. This method may be applied for the quantification of these three triterpenoids from any other plant material or its compound matrix and to assess the quality of the raw materials used in the formulations in terms of bioactive markers as a part of routine analysis and quality assurance.

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REFERENCES

1. Swaroop A, Gupta AP, Sinha AK. Simultaneous determination of quercetin, rutin and coumaric acid in flowers of *Rhododendron arboreum* by HPTLC. Chromatographia 2005;62:649-52.
2. Shailajan S, Joshi H. Optimized separation and quantification of pharmacologically active markers quercetin, kaempferol, β-sitosterol and lupeol from *Cuscuta reflexa* Roxb. J Pharm Res 2011;4:1851-3.
3. Imanaka H, Koide H, Shimizu K, Asai T, Kinouchi SN, Ishikado A, *et al*. Chemoprevention of tumor metastasis by Liposomal β-Sitosterol intake. Biol Pharm Bull 2008;31:400-4.
4. Malini T, Vanithakumari G. Comparative progesterone study of the effects of beta sitosterol, estradiol and on selected biochemical parameters of the uterus of ovariectomized rats. J Ethnopharmacol 1992;36:51-5.
5. Fotie J, Bohle DS, Leimanis ML, Georges E, Rukunga G, Nkengfack AE. Lupeol long-chain fatty acid esters with antimalarial activity from holarrhena floribunda. J Nat Prod 2006;69:62-7.
6. Prasad S, Kalra N, Shukla Y. Protective effects of lupeol and mango extract against androgen induced oxidative stress in Swiss albino mice. Asian J Androl 2008;10:313-8.
7. Checker R, Sandur SK, Sharma D, Patwardhan RS, Jayakumar S, Kohli V, *et al*. Potent anti-inflammatory activity of ursolic acid, a triterpenoid antioxidant, is mediated through suppression of NF-kappaB, AP–1 and NF-AT. PLoS One 2012;7:e3131.
8. Pai PG, Nawarathna SC, Kulkarni A, Habeeba U, Reddy SC, Teerthanath S. Nephroprotective effect of ursolic acid in a murine model of gentamicin-induced renal damage. ISRN Pharmacol 2012;2012:410902.
9. Srivastva P. *Rhododendron arboreum*: An overview. J Appl Pharm Sci 2013;02:158-62.
10. Sharma N, Sharma UK, Gupta AP, Sinha AK. Simultaneous determination of epicatechin, syringic acid, quercetin-3-O-galactoside and quercetrin in the leaves of *Rhododendron* species by using a validated HPTLC method. J Food Comp Anal 2012;214-9.
11. Verma N, Amresh G, Sahu PK, Rao CV, Singh AP. Antihyperglycemic and antuhyperlipidemic activity of ethyl acetate fraction of *Rhododendron arboreum* Smith. flowers in streptozotocin induced diabetic rats and its role in regulating carbohydrate metabolism. Asian Pac J Trop Biomed 2012;696-701.
12. Mudagal, MP, Karia S, Goli D. Preventive effect of *Rhododendron arboreum* on cardiac markers, lipid peroxides and antioxidants in normal and isoproterenol-induced myocardial necrosis in rats. Afr J Pharm Pharmacol 2011;5:755-63.
13. Popescu R, Kopp B. The genus Rhododendron: An ethnopharmacological and toxicological review. J Ethnopharmacol 2013;147:42-62.
14. The Wealth of India, 1972. Raw materials, Publications and Information Directorate, CSIR, Hill side road, New Delhi, 521.
15. Sonar PK, Singh R, Khan S, Saraf SK. Isolation, characterization and activity of the flowers of *Rhododendron arboreum* (Ericaceae). E J Chem 2012;9:631-6.
16. Verma N, Singh AP, Gupta A, Sahu PK, Rao CV. Antidiarrheal potential of standardized extract of *Rhododendron arboreum* Smith flowers in experimental animals. Indian J Pharmacol 2011;43:689-93.
17. Reich E, Schibli A. High-performance thin-layer chromatography for the analysis of medicinal plants. New York: Thieme Medical publishers; 2006.
18. Shailajan S, Menon S, Sayed N, Tiwari B. Simultaneous quantitation of three bioactive triterpenoids Lupeol, Stigmasterol and β-sitosterol from *Carissa carandas* using RP-HPLC method and its validation as per ICH guidelines. Int J Green Pharm 2012;6:241-7.

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