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COMPARISON AND ESTIMATION OF URSOLIC ACID IN DIFFERENT OCIMUM SPECIES BY RP-HPLC METHOD

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ABSTRACT

Ursolic acid is a potential anti-inflammatory, trypanocidal, antirheumatic, antiviral, antioxidant and antitumoral agent. The present study was designed to evaluate Ursolic acid among different varieties(CIMAP) of three Ocimum species using RP-HPLC method. Mean data from two different seasons[Winter(December-2017) and Summer (April-2018)]and as well as different parts (Aerial and Inflorescence) were evaluated in the study. The percentage of major chemical constituents in *O. Gratissimum, O.basilicum*(CIM-Saradha) and *O. africanum*(CIM-Jyothi)were also observed more in summer season. The average values of major chemical constituents from aerial part consists more in *O.Gratissimum, O. africanum*(CIM-Jyothi), *O. basilicum*(Vikarsudha) and except *O.basilicum*(CIM-Saradha).

KEYWORDS: *Ocimum*; Aerial part; Inflorescence; Winter season; Summer season; Ursolic acid.

1. INTRODUCTION

Ocimum sanctum often known as Holy Basil, tulsi. It is an aromatic perennial plant belongs to family Lamiaceae. It is considered as a religious plant in Hindu religion. Tulasi is used as medicinal plant due to its innumerable bio pharmacological activities. Because of its medicinal properties tulasi is known as queen of herbs. The *Ocimum* is grown all around the world for savoury and medicinal purposes. It holds numerous activities like Antimicrobial, Anticancer, Immunomodulatory, Antifungal, Hepatoprotective, Antibacterial, Antiarthritic, Antidiabetic, Anti-fertility, cardioprotective. Antipyretic, Anticataract, Adaptogenic, Antistress, Anti cholinesterase activity, Anti hyperlipidemic activity due to occurance of active phyto chemicals from different parts of the plant.

The different parts of *Ocimum*species contain varying amounts of different constituents. The phytochemical constituents of tulasi are Oleanolic acid, Ursolic acid, rosmarinic acid, carvacrol, linalool, beta caryophyllene. Tulasiessenetial oils consist mostly of eugenol, beta elemene, beta caryophyllene and germacrene.

According to literature survey Shanmuga Sundaram R.et.al., has reported a simple, rapid, accurate and reliable HPLC method for the determination of rosmarinic acid (RA) and ursolic acid (UA) in the leaves of ethanol extract of *Ocimum sanctum.* Vipul S. Patel. et.al., reported the developed and validated simple and rapid RP-HPLC Assay method for the estimation of Ursolic acid in *Ocimumsanctum* herb powders and extract in human plasma.

Hence, literature survey reveals that there no single method has been reported for the determination of Ursolic acid from different *Ocimum* species in different regions.

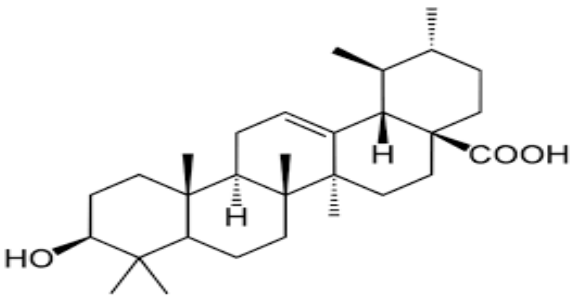

Figure 1: Structure of Ursolic acid.

2. MATERIALS AND METHODS

2.1: Reagents and Chemicals

The standards UrsolicAcid were procured from M/S Natural Remedies Pvt. Ltd., Banglore, India used as a reference standard for method development and analysis

for*ocimum* species. Methanol and Acetonitrile were HPLC-grade (Merck, Bombay, India) and were filtered through a Millipore filter (0.5µm) as required. Water used was HPLC grade (Rankem, New Delhi, India).

2.2: Apparatus

The HPLC analysis was performed using a Water modular system consisting of two model 1524 pumps, an automatic gradient controller, a model 717 plus injector, a model 2996 Photodiode array detector (PDA) and empower software. The injector, gradient controller and chromatography manager were integrated together to give reproducible results and Phenomenex Sphereclone ODS (2) column (4.6 X 250 mm, 5 µm particle size, made in USA) were used for the analysis.

2.3: Collection of Plant materials

Different *Ocimum* species (Aerial part and inflorescence) were collected from CSIR- CIMAP -Research Centre, Hyderabad. The collected plant materials were shade dried. The dried materials was powered by pulverizer.

2.4 Extraction and purification

The air-dried leaves of *O. gratissimum*(4.1 kg) and other collected species were extracted with ethanol andthe solventwas removed under reduced pressure given a solid which was submitted to a chromatographic silica gel column, sequentially eluted with hexane, dichloromethane, ethyl acetate and methanol. The ethyl acetate fraction was chromatographed on a silica gel column to yield ursolic acid (35.2 mg). The isolated Ursolic acid was extracted with methanol (125 mL) using a Soxhlet apparatus for three hours. Methanol extracts were concentrated and the extracts were diluted with methanol to 10 ml. The afforded solution was filtered through a 0.45 µm syringe filter prior to HPLC use.

2.5: Preparation of Standard Solution

5mgof different *Ocimum sanctum* ((Leaves, Stems and inflorescence) and other collected species powdered plant material accurately weighed and transferred into a conical flask and 30ml of methanol was added. This was extracted by sonication at 45^0 C for 30 min. Then the extraction was filtered through filter paper in to a round bottom flask and the residue was returned to the conical flask. The above extraction procedure was repeated for 3 times. Then the combined methanol extractions were concentrated under reduced pressure by Rotary evaporator. 25 mg of extract was accurately weighed and transferred in to a 25 mL volumetric flask and made up to the volume with methanol. Each sample solution was filtered through a 0.45 µm membrane filter.

2.6 Method Development

Preliminary studies involved trying in Phenomenex Sphereclone ODS (1) Phenomenex Sphereclone ODS (2) column and testing several mobile phase compositions were conducted for the separation Ursolic acid in different *Ocimum* species with a good chromatographic parameters (ex: minimized peak tailing, good symmetry, and good resolution between ursolic acid and adjacent peaks). A Phenomenex Sphereclone ODS (2) column (4.6 X 250 mm, 5 µm particle size) as a stationary phase with a mobile phase of acetonitrile and methanol at (80:20 v/v) at a flow rate of 0.4ml/min and detection wavelength of 210nm affords the best separation of ursolic acid from different species of *Ocimum.*

2.7 RP-HPLCAnalysis

HPLC was performed using a reversed-phase column (Phenomenex Sphereclone -ODS (2) 4.6 mm x 15 cm – particle size 5 □m, Made in USA) eluted at a rate of 0.5 mL/min with an A:B solvent system (A-acetonitrile; B-Methonal ; A:B = 80:20 (v/v), with a detection wavelength set at 210 nm. To prepare UA standard solution, this compound (7.8 mg) was dissolved in methanol (10 mL) for analysis. Standard solutions were injected (2, 4, 6, 8, 10, and 20 µL respectively) and run for calibration curves. To test plant leaves (5,000 g), appropriate amounts of UA were added to approximately double the contents of this compound in treated materials. The follow-up extractions and HPLC analysis were accomplished in the same manner. The recovery was determined as follows: recovery (%) = (A - B) / C x 100% where, A is the amount of detections, B is the amount of sample without added standard, C is the added amount of the standard. The relative standard deviations (RSD) of recoveries of the UA was 2.1 (n= 5; mean =98.0).

2.8 Method Validation

a. Linearity

A minimum of seven concentration levels along with minimum specified ranges are required. Sample injected for linearity study.

b. Accuracy

Accuracy was assessed by standard addition method, standard was added at three different levels (50%,100%.150%)to the sample solution.

c. Precision

Precision is the measure of the degree of repeatability of an analyte method under normaloperation and is normally expressed as relative standard deviation for a statistically significant number of samples.

1. Repeatability

It is determined from a minimum 6 determinations covering the specified range of the procedure. RSD for replicate injections should not be greater than 2%.

2. Intermediate precision(Ruggedness)

The intermediate precision of the current method was evaluated by calculating the percentage recovery of ursolic acid at 3 concentration levels (80%, 100%, 120%) by another analyst in different days.

d. Robustness

The robustness was assessed by altering the optimized chromatographic conditions such as by changing the flow rate, the mobile phase composition and wavelength.

e. LOD &LOQ

Limit of detection (LOD) and limit of quantification (LOQ) were determined by using the formula based on the standard deviation. LOD and LOQ were calculated by using the formula LOD=3.3x σ/S and LOQ= 10xσ/S where σ is standard deviation and S is he slope of corresponding calibration curve.

3 RESULTS AND DISCUSSION

3.1 Method Development

Where the retention time (Rt) of UA was 7.92 min. Calibration graphs for UA were constructed in the 3.60 – 72.00 µg/mL range. The regression equation of this curve and its coefficients of determination (R^2) were calculated as follows: Y=1.3050E+06X-1.5050E+04 (R^2=0.9999); limit of quantification 0.1 µg/mL; limit of detection 0.04 µg/mL; relative standard deviations (RSD) less than 2.0 %. The eight different sample solutions were analyzed in the same manner, the peaks were identified by comparison of the retention time corresponding to authentic UA purified from *O. gratissimum*.and other species. Regarding the extraction efficiency, repetition of the work-up three times was deemed sufficient, since it allowed over 98,00 % extraction of the UA(Ursolic Acid)

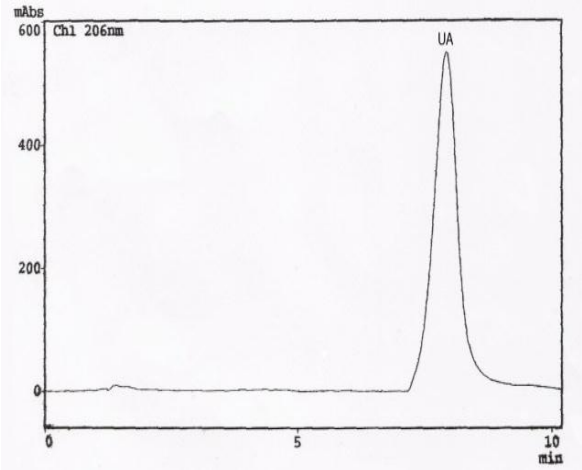

Figure 2: Chromatogram of Ursolic acid.(UA).

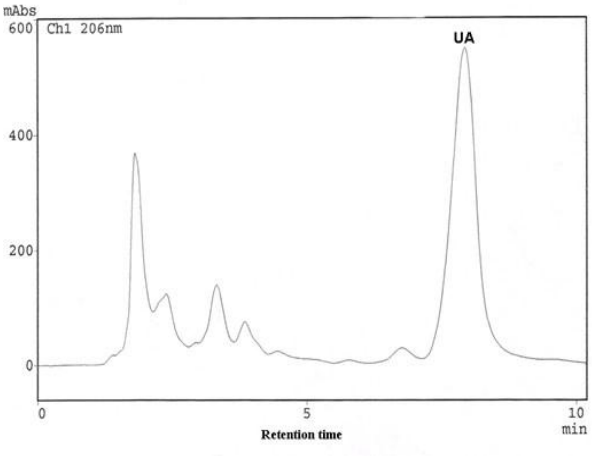

Figure 3: Chromatogram of a methanol extract of O. gratissimum.

3.2 Method validation

a. Linearity

The calibration curve was constructed by plotting the mean peak area versus the concentration of analyze the calibration graph for Ocimum species was within in the concentration range of 10, 20, 30, 40, 50 and 60 µ/ml for ursolic Acid and correlation coefficient for ursolic acid is 0.991.

Table 1: Linearity data of Ursolic acid.

S. No	Parameter	Ursolic acid
1	slope	20595.25
2	intercept	16675
3	R^2	0.995

b. Accuracy (% Recovery)

The average recovery and the RSD for each level have been calculated. results has shown that the current method has good recovery (from 96 to 105) from ursolic acid at the three concentration levels studies(32, 40, 48µ/ml) and within the RSD lower than 1%.

Table 2: % Recovery of Ursolic acid.

Drug	Level(µ/ml)	Amount added(µ)	Amount Recovered(µ)	%Recovered	Main Recovery
Ursolic acid	32	1449.98	1396.81	0.8	96
	40	1805.49	1859.02	0.5	103
	48	2225.89	2334.90	0.9	105

c. Precision

1. Repeatability

Current method for determination of ursolic acid was evaluated the RSD of the peak areas of 2 replicate injections of three standard solutions with three concentrations (80%, 100%, 120%) was found to be less than 2%.

Table 3: Repeatability data of Ursolic acid.

Concentration	Injection	Ursolic acid
80%	1	0.7032
	2	0.7015
100%	3	0.7236
	4	0.6988
120%	5	0.7008
	6	0.6963
	Average	**0.7040**
	SD	**0.00987**
	%RSD	**1.4**

d. Robustness

The method was found to be robust with change of ±2 % in wavelength, flow rate and mobilephase ratio.

Table 4: Robustness of Ursolic acid.

Parameters	nm/Rt	Mean area	%RSD of area	% ursolic acid(w/w)
wavelength	209	38195	1.4	0.355
	210	37567	1.3	0.363
	211	38407	0.9	0.357
Flow rate	0.36	36003	0.8	0.818
	0.4	37397	1	0.849
	0.44	39216	1.4	0.891

e. LOD & LOQ

The LOD and LOQ of ursolic acid using this method where determine by preparing dilute solutions of ursolic acid (10, 20, 30, 40, 50 & 60 µ/ml) and injecting these into the liquid chromatography and recording the S/N ratio. The LOD and LOQ values (ursolic acid) were found to be 0.6µg/ml respectively.

4. CONCLUSION

Three different *Ocimum*species harvested in winter and summer seasons. Twelve common chemical constituents were identified in all the species by RP-HPLC analysis..Each species has variations in the chemical compositions. Some have major chemical constituents greater than 80 % (*O. basillicum*-CIM-Saradha**).** The more percentage of the major chemical constituents are noticed in summer season especially in inflorescence part. The day temperature exhibited a significant positive influence on the yield and significant variations of chemical compositions.

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