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RESEARCH ARTICLE

Quantification of Bioactive Principles in Indian Traditional Herb *Ocimum Sanctum Linn*. (Holy Basil) Leaves by High Performance Liquid Chromatography

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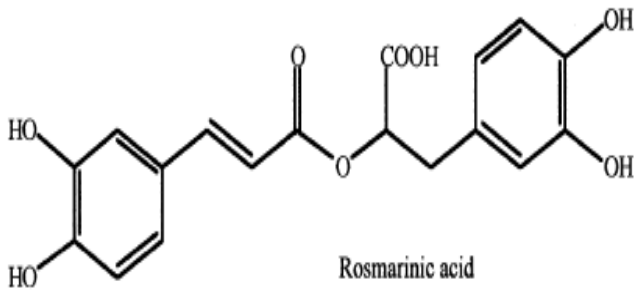
ABSTRACT

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* (EEOS) has been developed for the first time and validated. Several biological activities of EEOS have been attributed to the presence of phenolic compounds and terpenes in the crude extract. The separation system consisted of a Phenomenex C_{18} reversed-phase column, using phosphate buffer / acetonitrile (80:20) and acetonitrile / water / methanol (90:5:5) as mobile phase for the determination of RA and UA respectively. The flow rate was 1.0 ml min^{-1} and detection wavelength was set at 326 and 261 nm for RA and UA respectively. The recovery of the method was in the range of 93.85 to 95.77 % for RA and 92.76 to 97.40 % for UA, and all the compounds showed good linearity in a relatively wide concentration range. Using the optimized conditions, the quantity of RA and UA in EEOS was found to be 0.26 ± 0.01% and 0.40 ± 0.01% w/w, respectively. The method is simple, sensitive, reproducible and ideally suited for rapid routine analysis.

KEYWORDS: Rosmarinic acid, Ursolic acid, *Ocimum sanctum*, Tulsi, Holy basil, HPLC.

INTRODUCTION

Natural products have served as an important source of drugs since ancient times and a significant part of today's drugs are somehow derived from natural sources. In recent years, a renewed interest in obtaining biologically active compounds from natural sources has been observed. Holy basil (Tulsi, *O.sanctum* Linn. Family: *Labiatae*), one of the most popular culinary herb, is native to India and found in tropical and subtropical regions in the world and is used in several systems of medicine like *Ayurveda*, *Siddha*, *Unani*, *Greek* and *Roman* for vast therapeutic applications[1]. Indian Materia Medica describes the use of various extracts of *O.sanctum* leaves in a variety of disorders, like bronchitis, rheumatism and pyrexia[2-4]. It is classified as '*Rasayana'*, an herb that nourishes a person's health and promotes long-life. Medicinal, religious and culinary uses of *O.sanctum* have been documented for centuries in Asia, China, the Middle East, North Africa and Australia. After the herb was introduced in Europe from the Orient, it became known to Christians as sacred or Holy basil[5-7]. Several recent investigations of *O.sanctum* crude extracts indicating neuroprotective, antidepressive, antianxiety, antistress, antiulcer, adaptogenic, analgesic, antipyretic, anti-inflammatory, immunomodulatory, cardioprotective, hyolipedemic, hypoglycemic, hepatoprotective, diuretic, radioprotective, anticarcinogenic and antioxidant properties have been reviewed[4,8-10]. The diverse biological activities of the herb appears to be related to their of phenolic content, which in *O.sanctum* extracts, belong to three groups: phenolic di- and tri-terpenes; flavonoids and phenolic acids; and sterols[11], amongst which rosmarinic acid (RA, Fig 1) is an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid (hydroxylated phenolic acid)[12-18] and ursolic acid (UA, Fig 2), an important isomer of oleanolic acid, a triterpenoid compound[19-29].



Rosmarinic acid

Fig 1: Structure of rosmarinic acid.

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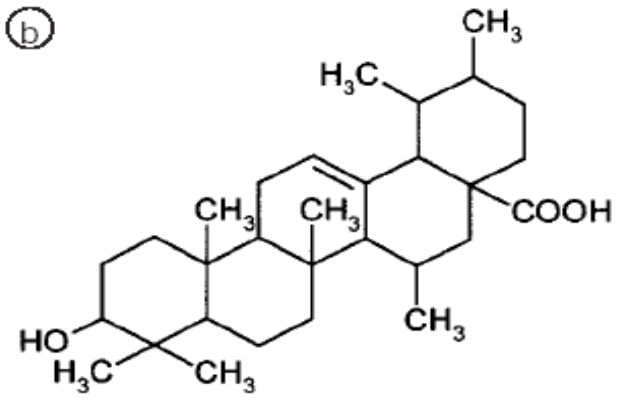

Fig 2: Structure of ursolic acid.

Despite many reports on the medicinal or functional properties of these aromatic leaves and their preparations, substantial papers have not been published on the determination of the phenolic and triterpenoid constituents of these materials by HPTLC, HPLC, GC or LCMS techniques in *O.sanctum* extracts. In order to obtain quantitative information on the amounts of these two molecules in the plant, we tested several methods described in the literature[30,31]. To date, some analytical methods have been reported on the analysis of various bioactive compounds in the leaves of *O.sanctum*. Although no literature is available for the quantification of RA from *O.sanctum* leaves, its estimation in a number of other herbs have been reported by various analytical techniques[32-34]. On the other hand, UA is reported to have been estimated from leaves of *O.sanctum* and rat plasma by HPTLC[35] and LC-MS[36] methods, respectively. Currently, the HPLC determination of natural compounds is time consuming, requires a large sample and entails liquid extraction with an organic solvent. Therefore, a reliable quality control method is needed for the qualitative and quantitative determination of triterpenoids and phenolic acid in the leaves of ethanol extract of *O.sanctum* (EEOS). This paper presents a simple analytical method to determine the bioactive compounds in the ethanol (90%) extract of *O.sanctum* dried leaves, using HPLC technique.

EXPERIMENTAL WORK

MATERIALS AND REAGENTS

Chromatographic separation was performed on a Shimadzu® liquid chromatographic system equipped with a LC-10AT-vp solvent delivery system, SPD M-10 AVP photo diode array detector and Rheodyne 7725i injector with 5µl loop (Koyoto, Japan). The analysis was performed on a reverse phase Phenomenex C_{18} column (25 cm × 4.6 mm i.d, 5µm) as stationary phase. Methanol (HPLC grade) acetonitrile (HPLC grade), were purchased from S.D. Fine chemicals Ltd (Biosar, India), potassium dihydrogen ortho phosphate (Analytical grade), rosmarinic acid (RA) and ursolic acid (UA) were purchased from Sigma Chemical Company (New Delhi, India). The water used in HPLC and for sample preparation was purchased from Ranbaxy Laboratories Ltd, (Mumbai, India).

COLLECTION AND EXTRACTION OF LEAVES OF *OCIMUM SANCTUM*

The aerial parts of the herb *O.sanctum* Linn. were collected as fresh plants from Bhavani, Erode district, Tamilnadu, India. The whole plant was washed and leaves were separated from other aerial parts of the plant, freed from earthy material and shade dried with occasional sifting at room temperature, powdered and was subjected to extraction by cold maceration with 90% ethanol (17.38% yield) at room temperature with continuous stirring (300 rpm) for 7 days, after defatting with pet ether (60-80°C). The solvents were evaporated using rotary vacuum and stored in desiccator. The chemical constituents of the extract were identified by qualitative analysis followed by their confirmation by thin layer chromatography.

PREPARATION OF STANDARD SOLUTION

Stock standard solutions were prepared by accurately weighing 10 mg of RA and UA reference standards into separate 100 ml volumetric flasks and dissolving in phosphate buffer / acetonitrile (80:20) and acetonitrile/ water/ methanol (90:5:5), respectively, with the aid of sonication to get a concentration of 1 mg/ml of stock solution. These solutions were serially diluted to get 100 µg/ml and filtered through Whatman filter paper.

PREPARATION OF SAMPLE SOLUTION

EEOS (100 mg) sample was accurately weighed into a 100 ml volumetric flasks, and extracted with 50 ml phosphate buffer / acetonitrile (80:20) and acetonitrile / water / methanol (90:5:5) for RA and UA respectively with the aid of sonication for 10 min. The resulting mixture was centrifuged at 4500 rpm for 5 min, and the supernatant transferred to a 50 ml volumetric flask. The residual solid was further extracted with 20 ml of the same phosphate buffer / acetonitrile (80:20) and acetonitrile /water/methanol (90:5:5) mixture for RA and UA respectively, with sonication for 5 min and centrifuged as above. The supernatants were combined, and made to 100 ml with water. All samples were centrifuged at 13,000 rpm for 10 min prior to injection for HPLC analysis.

ANALYTICAL CONDITIONS FOR HPLC

The analysis was performed with Phenomenex C_{18}, (25 cm × 4.6 mm i.d, 5µ) as reverse phase column and controlled at 20 °C. The flow rate of the mobile phase was 1.0 ml min^{-1}. The composition of mobile phase was Phosphate buffer / acetonitrile (80:20, v/v) and acetonitrile / water / methanol (90:5:5 v/v) for RA and UA respectively. The sample injection volume was 10 µl. Under these conditions almost all the components could be separated very well. The optimum detection wavelength was 326 and 261 nm for rosmarinic and ursolic acid respectively. The extract was injected three times. The chromatographic peaks of RA and UA were confirmed by comparing their retention times and UV spectra with that of their reference standards. Working standard solutions were injected into the HPLC and peak area responses obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph. The developed method was validated and studied with parameters such as accuracy, precision, specificity, LOD and LOQ. The standard solutions and the sample solutions were subjected to long term (3 days) stability studies. The changes in separation, retention and, asymmetry of the peaks were studied and compared with the pattern of the chromatogram of freshly prepared solutions. Calibration curve for rosmarinic and ursolic acid were constructed by plotting concentration versus area. Based on the peak area of standard and sample solutions, the amount (%) of RA and UA in the extract was calculated by using the following formula: % RA / UA = {Peak area of sample solution / Peak area of standard solution × dilution factor × 100} Quantification was carried out from integrated peak areas of the samples using the corresponding calibration curve. EEOS was standardized by using standard samples of RA and UA, as analytical marker compounds. The proposed HPLC method was validated as per ICH guidelines[37].

RESULTS AND DISCUSSION

The amount of RA and UA in EEOS were estimated by adopting optimized and validated chromatographic procedures. Several tests were performed for optimizing the components of mobile phase in order to achieve good chromatographic peak shape and resolution. A good separation condition should satisfy the need that the analyzed peaks have baseline separation with adjacent peaks within a short analysis time as far as possible. To obtain the chromatograms with the good separation, fixed phase, mobile phase, column temperature, detection wavelength and flow rate were, respectively, investigated. Using the optimized conditions, the amount of RA and UA in EEOS was found to be 0.26 ± 0.01 % and 0.40 ± 0.01 % w/w, respectively.

METHOD DEVELOPMENT AND CHROMATOGRAPHIC CONDITIONS

In order to obtain quantitative extraction of solid samples, variables involved in the procedure, such as solvent, extraction method and time were optimized. Various mixtures of phosphate buffer and acetonitrile for RA and acetonitrile/ water/ methanol for UA were used as mobile phase but separation was not satisfactory. It was also suggested that separation was better when column temperature was kept at 20 °C than 15, 30 and 40 °C. The most suitable flow rate was found to be at 1.0 ml min^{-1}. The mobile phases resolved RA and UA efficiently from other components present in EEOS. Peaks in the chromatograms were identified by comparing the retention times and on-line UV spectra with those of the standards. Retention time for RA and UA were found to be 25.568 and 6.542 min, respectively. The chromatograms of RA, UA and EEOS are shown in figures 3 to 6.

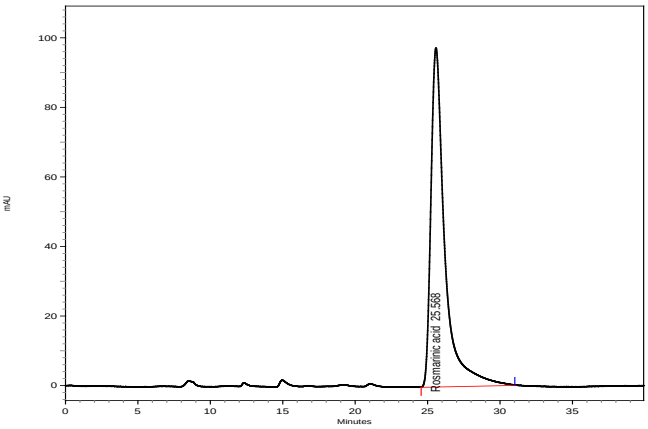

Fig 3: HPLC chromatogram of standard rosmarinic acid.

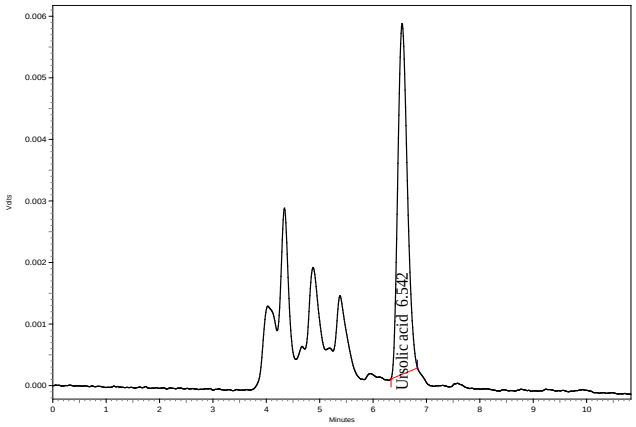

Fig 4: HPLC chromatogram of standard ursolic acid.

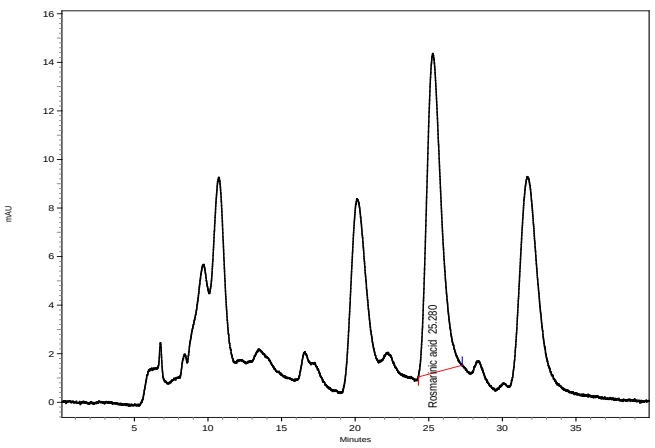

Fig 5: HPLC chromatogram of rosmarinic acid in EEOS.

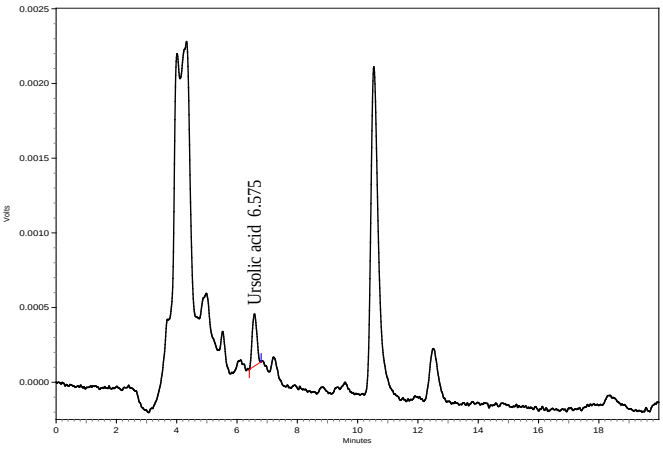

Fig 6: Chromatogram of ursolic acid in EEOS.

METHOD VALIDATION

CALIBRATION CURVES

The external standard method was used to get the regression equations. In the regression equation ($y = ax + b$), x is referred to the concentration of the standard compounds (μg ml^{-1}), y to the peak area, a is the intercept of the straight line with y-axis and b is the slope of the line. All the standard compounds showed good linearity in a relatively wide concentration range.

Calibration plot of peak area against concentration was linear in the range of 20 – 120 μg/ml of RA and UA. The slope and intercept value for calibration curve for RA and UA respectively, were, $y = 1065.5x - 187.47$ and $y = 923.12x + 390.59$, where x is peak area and y is the concentration. The correlation coefficient (r^2) for RA (0.9992) and UA (0.9996) indicates excellent correlation between the peak area and concentration. The data demonstrates that the methods have adequate sensitivity to the concentration of the analyte. Hence this method shows linearity over the range of ± 50%. The column efficiency and peak asymmetry were calculated for the standard solutions. The values obtained demonstrated the suitability of the system for the analysis of RA and UA in EEOS (Table 1). System suitability parameters may fall within ± 3 % standard deviation range during routine performance of the methods.

Parameters	Rosmarinic acid	Ursolic acid
Theoretical plates/meter	21536	18267
Asymmetry (10%)	1.02	1.05
LOD (ng/ml)	24	21
LOQ (ng/ml)	35	29

Table 1: System suitability studies of rosmarinic acid and ursolic acid by HPLC.

LIMIT OF DETECTION AND QUANTIFICATION

The limit of detection (LOD, the smallest concentration of the analyte that gives a measurable response, signal to noise ratio of 10) and limit of quantification (LOQ, the smallest concentration of the analyte, which gives response that can be accurately quantified, signal to noise ratio of 10), of the developed method were determined by injecting progressively low concentrations of the standard solutions. The LOD and LOQ for RA and UA were found to be 24 and 35 ng/ml, and 21 and 29 ng/ml respectively (Table 1). It can be seen from results of LOD and LOQ that the limits are low enough to determine RA and UA in EEOS. The recovery test was carried out by the addition of three different quantities (low: 80 ng/ml, medium: 100 ng/ml and high: 120 ng/ml) of standards into the samples. The resultant samples were then extracted and analyzed as described below. The quantity of each analyte was subsequently obtained from the corresponding calibration curve. The recovery of the standards ranged from 93.85 to 95.77 % for RA and 92.76 to 97.40 % for UA, showing the reliability and reproducibility of the method.

PRECISION AND SUITABILITY STUDIES

The precision test was carried out by the intra-day and inter-day variability. Three different concentration solutions (low, medium and high) of authentic standards were prepared. The quantity of each analyte was obtained from corresponding calibration curve. The relative

standard deviation (RSD) was taken as a measure of precision. The intra-day variability was examined within 1 day in five times and the result showed that RSD of intra-day variability was in the range of 0.00 – 0.29 % and 0.00-0.26 % respectively for RA and UA. The inter-day precision was calculated from nine determinations over 3 days for each concentration and the results were in the range of 0.16 – 0.49 % and 0.15 – 0.42 % respectively for RA and UA. The recovery test was carried as followings: three different quantities (low, medium and high) of authentic standards were added into samples. The resultant samples were extracted and analyzed as described and the quantity of each analyte was subsequently obtained from the corresponding calibration curve. The recovery of the six standards ranged from 95.1 to 104.8 %. From the results of precision test and recovery test, it was known that the method manifested good precision and accuracy.

For stability test, the same sample solution was analyzed every 12 h in 3 days at the room temperature, and the analyte were found to be rather stable within 72 h (R.S.D. < 5.4 %). The measured percent coefficient of variation (CV) for spectroscopic procedures is < 1.28 % for RA and < 1.07 % for UA, both being lower than the values determined for HPLC methodology. A volume of 20 µl of the filtered solution of each sample was injected into the instrument. Each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing the retention times and on-line UV spectra with those of the standards. Retention time for RA and UA were found to be 25.568 and 6.542 min, respectively (Figures 3 and 4). The content of each analyte was calculated from the corresponding calibration curve.

LINEARITY

Calibration graphs for rosmarinic and ursolic acid were constructed using seven levels of concentration which covered the concentration ranges expected in the various samples. The linearity range for RA and UA was determined to be 20–120 µg/ml. The of the square of correlation coefficient (r^2) was 0.9992 and 0.9996, for RA and UA respectively, and on-line linearity (LOL) was 99.72 % and 99.92 %, respectively, according to the following equation[38,39], LOL (%) = 100– RSD (*b*) Where, RSD (*b*) is the relative standard deviation of the slope (expressed in percentage). According to an ALAMIN program[39], analytical sensitivity (AS) is determined by the ratio of S_s/b, in which S_s is the residual standard deviation and *b* is the slope of the calibration curve. The limit of detection (LOD_{approx}) is determined by the following equation:

$$LOD_{approx} = 3(S_s/b) \times \left[\frac{(n-2)}{(n-1)}\right]^{1/2},$$

Where, *n* is the number of total measurements for the calibration set.

The results for the AS, LOD and LOQ were listed in table 1. It can be seen from these results that the limits are low enough to determine RA and UA in *O. sanctum*. Recovery was determined by spiking a sample with three different additions of standard solutions of RA and UA. The average recovery was found to be 93.85 to 95.77 % for RA and 92.76 to 97.40 % for UA, showing the reliability and reproducibility of the method.

To evaluate the precision of the system, a sample solution kept at ambient temperature, was analyzed three times in a single day for seven days. As a result, the intra-day precision was found to be 0.00-0.29 % and 0.00 – 0.26 %, and inter-day precision, 0.16 -0.49 % and 0.15 – 0.42 % for RA and UA, respectively.

CONCLUSION

In conclusion, the newly established HPLC method is validated for the quantification of the main bioactive triterpene (UA) and phenolic acid (RA), and the quality control of the plant materials such as *O.sanctum*, where the triterpenes and the phenolic acid is the dominant phytochemicals. This was the first report on the determination of RA and UA by HPLC from the dried leaves of *O.sanctum*. This method is simple and sensitive and the limits of detection and quantification (LOD & LOQ) were low enough to analyze RA and UA present in *O.sanctum*. This method is rapid, precise, reproducible, sample-saving, and maybe helpful for the quantitative analysis of phytochemical analogous to the triterpenes and the phenolic acids in aromatic herbs.

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