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Isolation and characterization of flavonoids and ascorbic acid from aqueous extract of leaves of *Ocimum sanctum* L.

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ABSTRACT

Ocimum sanctum Linn., a holy plant in India, has given out a special status because of its various pharmacological activity. It has nearly more than 250 activities reported in different articles. Its leaf contains several chemical constituents of which flavonoids and ascorbic acid are the most important constituents. Due to antioxidant activity of these chemicals they are more popular in maintaining health and protection. But as the constituents can be changed as per the environment, the study was carried out on the leaves of *Ocimum sanctum* L. for the presence of flavonoids and ascorbic acid. From HPTLC fingerprint analysis it was found to contain four major flavonoids in a fixed ratio as well as it was found to be a good source of ascorbic acid.

KEYWORDS

Flavonoids;
Ascorbic acid;
Ocimum santum;
HPTLC;
Fingerprint analysis.

INTRODUCTION

Ocimum sanctum Linn. belongs to family Lamiaceae has habitat in India, it is an annual shrub obtained up to the height of 6000 feet in Himalayas and grows all over the country[1]. It consists of wide range of chemical constituents like aldehydes, alkaloids, ascorbic acid, beta-carotene, beta-sitosterol, glycosides, hexouronic acid, linoleic acid, linolenic acid, oleic acid, palmitic acid, pentoses, phenols/phenolic compounds, proteins, saponins, steric acid, tannins and xylose[2]. The plant also contains carvacrol, cineole, essential oil, eugenol, eugenol-methyl-ether, linalool, methyl chavicol[3]. Ascorbic acid present in the plant plays an important role as an antioxidants and used widely in several Ayurvedic medicines[4,5] as well as it also found to control the toxicity of the plant by controlling the oxidation of phenolic compounds[6].

EXPERIMENTAL

HPTLC and UV analysis were carried out on CAMAG HPTLC system at Anchrom Laboratories, Mulund, Mumbai. HPLC analysis was carried out on Jasco isocratic HPLC system while IR analysis was carried out on Jasco FTIR system at Appasaheb Birnale college of Pharmacy, Sangli. GCMS was recorded on GCMS – 2010 Shimatzu at Shivaji University, Kolhapur and NMR were recorded on Varian Mercury YH – 300 at AISSMS Pune. All chemicals and reagents used for different methods are of analytical, HPLC or IR grade.

Plant were collected from the Sangli region and authenticated at Department of Botany, Willingdon College, Sangli, the leaves were shade dried and finely powdered through sieve 80.

Extraction of Flavonoids: About 100 g of powdered

drug was subjected to cold maceration with chloroform water (10%) in 1000 ml conical flask for about 7 days at room temperature. The flask was securely plugged with absorbent cotton and was shaken periodically till complete maceration. After maceration, the mark was pressed in a muslin cloth and the filtrate was concentrated to residue at low temperature.

The dried aqueous extract of *Ocimum sanctum* L. was extracted with 50 ml methanol for 5 min on water bath at about 60°C and filtered, the filtrate was concentrated on water bath to 5 ml[7].

Two-dimensional paper chromatography (2-D PC)[8]: Extracts for analysis were redissolved in 1 ml 80% methanol and applied as 8-10 spots of 2 µl in the corner of quarter sheets of Watman No. 1 chromatography paper. The chromatogram was run in descending mode in BAW (n-Butanol, Acetic acid and Water = 4:1:5; v/v; upper layer) for the first dimension, and in 15% aqueous acetic acid for the second. After drying, the chromatogram was viewed under UV light at 360 nm, and again after fuming with ammonia vapour.

Preparative paper chromatography (PPC)[8]: Prior to HPLC analysis, crud extract was partially purified by PPC to remove rosmarinic acid and caffeic acid from the flavonoid fraction. Extract was applied as a narrow band at the top of a quarter sheet of Watman No.3 chromatography paper, and the paper was run in descending mode in BAW. After drying the chromatogram and viewing then in UV light, the bottom quarter of the paper (containing rosmarinic and caffeic acid, which have high Rf values in BAW and shows blue fluorescence under UV light) was removed. The remainder of the paper containing flavonoid glycosides was cut into 1 cm^2 pieces, which were eluted in 80% methanol. After 24 h the elute was filtered, evaporated and redissolved in 1 ml 80% methanol, for HPLC analysis. PPC was also used for the purification and isolation of flavonoid glycosides. The crude extract were applied onto whole sheet of Watman No. 3 paper and developed in BAW. The band containing flavonoids were cut out, eluted and subjected to a second stage of PPC using 15% acetic acid. Isolation and percentage of each flavonoid in the fractions was monitored by HPLC.

Extraction of Ascorbic acid: The powdered leaves were subjected to maceration with water for 48 h in 1000 ml beaker. After the maceration the marc was pressed in muslin cloth and again that filtrate was filtered through Watman filter paper no. 3, the impurities from the filtrate were removed by adding neutral lead acetate solution (400 ml of 10% lead acetate solution was prepared which was acidic in nature, neutralized by adding dilute sodium hydroxide solution). The precipitate that formed was removed by filtering through Watman filter paper no. 3, in the filtrate obtained, ammonia solution was added to bring the pH to 7.6 and at the same time again neutral lead acetate solution was added. The vitamin-C was precipitated as lead salt; the latter was treated with dilute H2SO4, whereby lead was removed as PbSO4 and then filtered. The solution so obtained was concentrated under vacuum to 1/3rd of its volume. It was further purified by fractional precipitation by taking the aqueous portion into the separating funnel and treating with ether. The aqueous portion was separated and decolorized with animal charcoal 5-6 times and filtered. The clear solution of ascorbic acid was obtained (adjusted the volume to 50 ml)[9]. Then it was subjected for the identification test.

Qualitative Identification: The extracts were identified qualitatively by chemical tests as follows…

Tests for flavonoids[10]: Ferric chloride test, shinoda test, zinc - hydrochloric acid-reduction test, alkaline reagent test, lead acetate solution test.

Tests for Ascorbic acid: Solution of Ascorbic acid decolorizes 2-6-dichlorophenol indophenol solution. It also reduces silver nitrate solution immediately in the cold, producing black precipitate and in 2 ml of solution few drops of nitric acid and few drops of silver nitrate produces dark grey precipitate[11].

Identification of Ascorbic acid (vitamin-C) by paper chromatography[12]: The ascorbic acid after preliminary chemical investigation subjected for paper chromatography by using 20 x 8 cm paper size and developed in a mixture of n-butanol saturated with water and oxalic acid. The spot was visualised by 2, 6-dichlorophenol indophenol solution at Rf 0.34

Characterization of flavonoids and ascorbic acid: Flavonoids were characterized by HPTLC, HPLC, and IR. Number of flavonoids and there relative percentages were determined on HPLC. Ascorbic acid was

characterized by HPTLC, UV, FTIR, GCMS and NMR.

HPTLC of flavonoids: Flavonoids were analyzed on HPTLC by using Silica gel GF 254 plates and ethyl acetate: formic acid: GAA: water (100:11:11:26) as a mobile phase. They are detected at 366 nm. TABLE 1 shows the fingerprint data of flavonoid fraction and chromatogram is shown in Figure 1.

TABLE 1 : Fingerprints of flavonoids and ascorbic acid containing fractions.

Sr. No.	Name of Phytochemicals	Wavelength in nm.	R_f values
1.	Flavonoids	366	0.01, 0.61, 0.67, 0.77, 0.79, 0.86, 0.99.
2.	Ascorbic acid	254	0.08, 0.18, **0.29**, 0.33, 0.43, 0.83, 0.91
3.	Std. Ascorbic acid	254	**0.29**

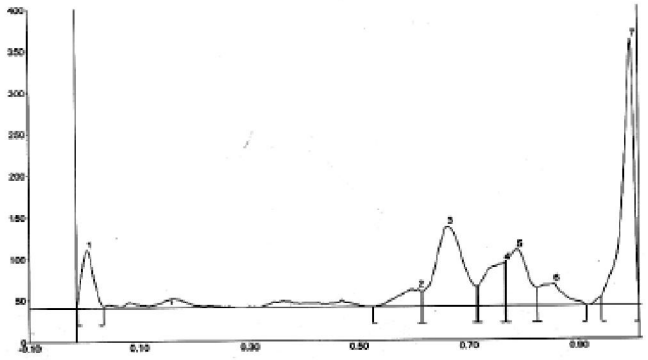

Figure 1 : HPTLC Fingerprint of flavonoids fraction.

HPLC of flavonoids[13]: Isolated flavonoids were analysed on isocratic HPLC system. The HPLC system consist of Jasco LC pump, UV-Vis detector and RP C18 column (5 µm) were used; 4.6 mm internal diameter x 250 mm length. Isocratic profile based on a mixture of solvents as methanol, glacial acetic acid and water were used. Mobile phase was prepared by mixing and degassing the mixture of methanol, GAA and water in a proportion of 18:1:1. The base line was observed before injecting the sample and the process was carried out at room temperature. The flow rate was adjusted to 1 ml/min and detection was carried out at 268 nm. After obtaining the base line, 20 µl of sample was injected and the chromatogram was recorded up to 20 min as shown in Figure 2. From the nature of peak, percent area and the Rf values, as shown in TABLE 2, the flavonoids are identified.

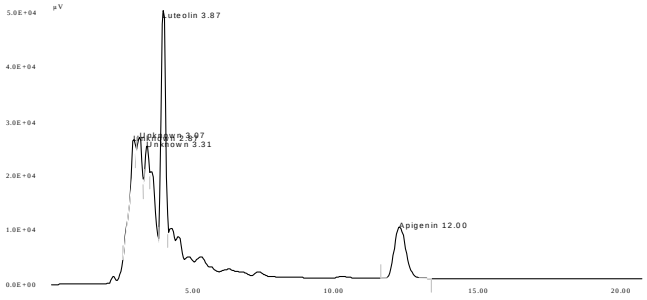

Figure 2 : HPLC analysis of flavonoids.

TABLE 2 : HPLC data for the flavonoids from aqueous extract of *O. sanctum* leaf.

Sr. No.	Retention time (R_t)	Area of peak	Height of peak	% area of peak	Conclusion
1.	2.87	40419.5	55.98	5.75	May be Vicenin-2
2.	3.07	35608.5	5452	5.06	May be Luteolin-5 -O-glucoside
3.	3.31	34951.5	5999	4.97	Unknown
4.	3.87	377728.5	41874	49.43	Luteolin-7-O-glucuronide
5.	12.00	244833.0	9384	34.80	Apigenin-7-O-glucuronide

IR of flavonoids: Flavonoid fraction of aqueous extract was spray dried, small quantity of dried amorphous free flowing powder of the extract was triturated with KBr and a pallet was scanned for IR spectrum. The spectrum is shown in Figure 3 and the data is given in TABLE 3.

TABLE 3 : IR data for the flavonoids from aqueous extract of *O. sanctum* leaf.

Wavelength	% T	Interpretation
3548.86	21.4816	O-H str (phenolic OH)
3477.03	12.5961	O-H str asym. (phenolic OH) broad
3415.80	9.8111	O-H str sym. (phenolic OH) broad
3241.75	39.9700	C-H str asym. (aromatic C-H)
3239.34	39.8210	C-H str sym. (aromatic C-H)
1637.75	31.2405	C=O str (Quinone type C=O)
1556.27	55.5761	C=C str (aromatic C=C)
1511.92	71.8974	C=C str (aromatic C=C)
1415.49	63.4528	C=C str (aromatic C=C)
1267.97	77.2329	C-O str (phenolic C-O)
1218.31	86.8743	O-H def (phenolic OH)
1127.67	87.5331	C-O str (cyclic ether not epoxide)

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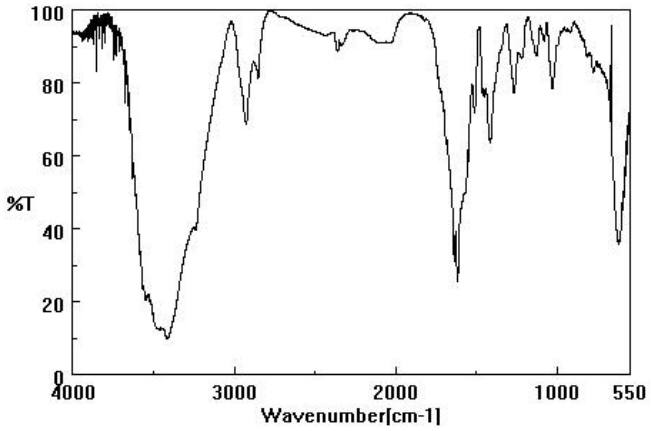

Figure 3 : IR spectra of flavonoid fraction.

HPTLC of ascorbic acid: Ascorbic acid was analyzed on HPTLC by using Silica gel GF 254 plates and n-butanol saturated with water and oxalic acid (80:20) as a mobile phase. Vitamin C was detected on 254 nm and was derivatised by spraying 2,6-dichlorophenol indophenol reagent and observed visibly. HPTLC scan at 254 nm was shown in Figure 4 and the data in TABLE 1.

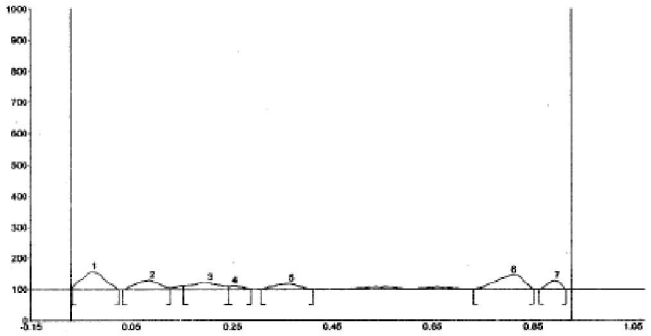

Figure 4 : HPTLC Fingerprint of ascorbic acid fraction.

IR of ascorbic acid: Ascorbic acid isolated from the leaf was confirmed by IR spectroscopy. The spectrum is shown in Figure 5 and the data is given in TABLE 4.

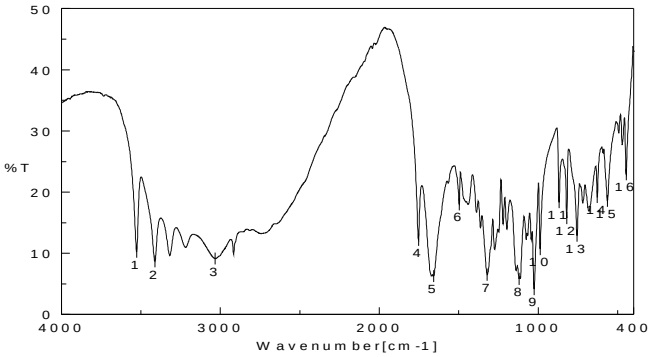

Figure 5 : IR spectrum of ascorbic acid:

TABLE 4 : IR data for ascorbic acid from aqueous extract of *O. sanctum* leaf.

Peak No.	Wave Number (cm^{-1})	% Transmission	Conclusion
1	3526.20	10.2729	O – H str (ring OH-CH=CH)
2	3412.42	8.7030	O – H str (OH-CH, OH-CH_2)
3	3033.48	9.1447	C – H str (enolic ring CH)
-	~ 2930	~ 10.0	C – H str (Aliphatic CH, CH_2)
4	1753.94	12.2199	C = O str (α-β unsaturated 5 membered ring ketone)
5	1658.48	6.2909	C = O str (ring enolic β diketone)
7	1321.00	6.5307	C – O str (alcoholic C-OH)
8	1120.44	5.8516	C – O – C str (Cyclic ether not epoxide)

GC-Mass spectrometry of ascorbic acid: A small quantity of sample was dissolved in distilled water and 1.0 µl of solution was injected as a sample. The gas chromatogram was obtained by using Helium as a carrier gas at a flow rate of 1.0 ml/min with programmed temperature started at 80°C followed by gradual increase by 7°C/min to reach 200°C hereafter the increment was increased to 10°C/min and finally the temperature was reached to 280°C where the chromatogram was stopped. Simultaneously the mass spectrum was obtained by using electronic ionization technique. The analysis were carried out on GCMS – 2010 Shimatzu with coloum RTX-SMS 60 m in length and 0.25 mm diameter, adsorbent coating were 0.25 µm in thickness. Quadrupole analyzer was used.

Figure 6 shows the mass spectrum of isolated ascorbic acid in comparison with the library standard

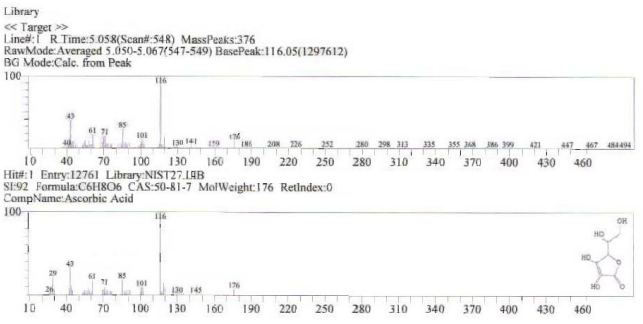

Figure 6 : Mass spectrum of ascorbic acid with library spectrum:

spectrum of ascorbic acid. The data of the spectrum is given in TABLE 5.

TABLE 5 : Mass data for isolated ascorbic acid:

Sr. No.	m/e	m/e for standard	Fragment
1	176	176	Molecular ion peak
2	116	116	Base peak after losing side chain.
3	101	101	Rearranged ion after losing side chain and an oxygen.
4	85	85	Loss of oxygen from the previous fragment.
5	61	61	A separated side chain.
7	43	43	A fraction (CH_2CHO) separated from the ring.

NMR spectrometry of ascorbic acid: NMR spectrum was obtained by dissolving a small quantity of isolated ascorbic acid in D2O. The spectrum was recorded on Varian Mercury YH – 300 with a pulse sequence of s2pu1. The resolved spectrum was shown in Figure 7 and data is given in TABLE 6.

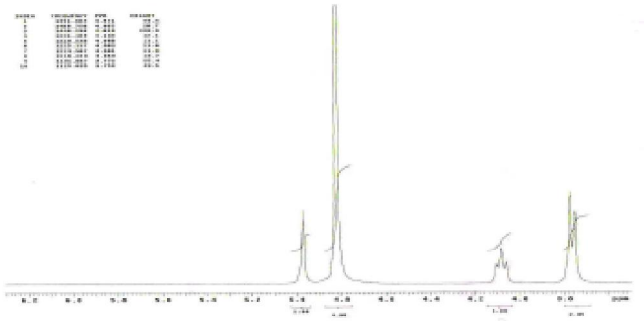

Figure 7 : H^1NMR spectrum of isolated ascorbic acid:

TABLE 6 : H^1NMR data for isolated ascorbic acid:

Sr. No.	Peaks	Indication
1	3.752-3.772	Doublet, 2H, deshilded due to OH and adjacent CHOH.
2	4.060-4.103	Quartet, 1H, deshilded due to OH, adjacent CH2OH and double bond present in ring.
3	4.820	Singlet, 4H, all hydroxyl groups (OH)
4	4.968-4.971	Doublet, 1H, highly deshilded due to adjacent CHOH, adjacent ring double bond and electro-negativity of ring Oxygen.

RESULT AND DISCUSSION

In HPTLC analysis of flavonoids the spots obtained at 0.61 and 0.67 showed the presence of Luteoline-7-O-glucoronoids and Apigenin-7-O-glucoronoids, while in analysis on HPLC the presence of Luteoline-7-O-glucoronoids and Apigenin-7-O-glucoronoids showed clearly at Rf 3.87 and 12.0 respectively. Percent area also confirms that these two flavonoids are the major constituents amongst the extract.

The IR spectrum showed presence of all functional groups necessary to detect the flavonoids. Spectrum shows O-H str, C-H str, Quinone type of C=O str, three different bands usually obtained for aromatic C=C str, O-H def, and most important two different bands for C-O str; out of which a band at 1268/cm is due to phenolic group while a band at 1127 is due to cyclic C-O but not an epoxide.

In the characterization of ascorbic acid, HPTLC showed its presence at Rf 0.23 which was compared with standard ascorbic acid. Isolated ascorbic acid and standard ascorbic acid were analysed on UV spectra the scans of both solutions showed an identical λmax at 254 nm indicating the presence of ascorbic acid in extract. Finally the IR spectrum of isolated ascorbic acid was compared with standard IR spectrum. IR spectrum was found to be concord exactly with the spectrum given in IP.

Gas chromatogram of isolated fraction of ascorbic acid shows absence of any detectable impurities in the fraction. Spectra shows the only retention time at 5.061 min. with 100% peak area. Mass spectrum of the same confirms the presence of ascorbic acid. Peak obtained at 176 as a mass peak and the highest peak at 116 as a base peak itself confirms the presence of ascorbic acid. All other fragment peaks are similar to those peaks reported in the library spectra.

Finally the presence of ascorbic acid in the isolated fraction was confirmed by NMR spectrum of the sample. A doublet at 3.76 belongs to C**H**$_2$OH in the side chain, Quartet at 4.082 belongs to proton in the side chain as C**H**OH, while a doublet with very small coupling constant at 4.965 – 4.971 belongs to a proton in the ring at the junction of side chain. All hydroxyl protons together recorded a large peak at 4.820 as they are well leaving protons they reported a wide singlet.

Hence from the spectral and chromatographic data, the presence of flavonoides i.e. Luteoline-7-O-glucoronoids and Apigenin-7-O-glucoronoids and ascorbic acid in the aqueous extract of the leaves of *Ocimum sanctum* L. were confirmed.

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