Research Article

QUANTIFICATION OF ASCORBIC ACID IN LEAVES OF *ANNONA SQUAMOSA*

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ABSTRACT

Annona squamosa Linn is a multipurpose tree with edible fruits & a source of the medicinal & industrial products. *Annona squamosa Linn* is used as an antioxidant, antidiabetics, hepatoprotective, cytotoxicactivity, genetoxicity, antitumor activity, antilice agent. The leaves of *Annona squamosa* contains valuable quantity of ascorbic acid and tannins. Vitamin-C, chemically known as ascorbic acid, is an important component of a healthy diet. The history of vitamin-C revolves around the history of the human disease scurvy, probably the first human illness to be recognized as a deficiency disease. Its symptoms include exhaustion, massive hemorrhaging of flesh and gums, general weakness and diarrhea. Vitamin C (ascorbic acid, AA) is a water-soluble organic compound involved in many biological processes. A number of methods are available and are being developed for the extraction and characterization of Ascorbic acid from plants. This article deals with extraction and to develop a simple, efficient, reliable and cost-effective high performance liquid chromatographic (HPLC) and UV spectrophotometric methods for the quantization of vitamin C in leaves of *A.squamosa*. The method is simple, rapid and has high specificity to ascorbic acid. Each analysis require less than 5 min. Qualitative estimation was carried out by thin layer chromatographic (TLC) method. HPLC separation was performed on a Cyber Lab C-18 column (250 x 4.0 mm, 5μ) using Water(A) and 0.34% o-phosphoric acid (B) using an isocratic elution as follow: 0–30 min, 40%A–80% A, 60%B–20% B. The flow rate was 1.5 mL/min, column temperature 25°C, the injection volume was 25μl and UV detection was effected at 266 nm.

Keywords: Ascorbic acid, *Annona squamosa*, HPLC method, UV spectrophotometric.

INTRODUCTION

The therapeutic efficacy of many indigenous plants, for various diseases has been described by traditional herbal medicinal practitioners[1]. Natural products are the source of synthetic and traditional herbal medicine. They are still the primary health care system in some parts of the world[2]. In India, local impirical knowledge about medicinal properties of plants is the basis for their uses as a home remedies. It is generally accepted by many Indians and elsewhere in the world that beneficial medicinal effects can be obtained by ingesting plant products. Plants have bear the basis of many traditional medicines throughout the world for thousands of years and continue to provide new remedies to mankind[3]. *Annona squamosa (A. squamosa)* L. (Family: Annonaceae), commonly known as custard apple. *Annona squamosa syn.* Arabic (gishta); Bengali (ata);German (Rahm Annone, Rahmapfel, Zimtapfel, Süßsack); Hindi (sitaphal, ata, sharifa); Lao (Sino-Tibetan) (khièb); Malay (nona sri kaya,sri kaya, buah nona); Mandarin (fan-li-chi); Portuguese (atta, fructa do conde); Sanskrit (sitaphal); Spanish (candongo, chirimoya, fructo do conde, anón, anona blanca, pinha, saramuya, anona).It is cultivated throughout India, mainly for its edible fruits. It is a semi-evergreen shrub or small tree reaching 6–8 meters (20–26 ft) tall. The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, hemorrhage, antibacterial infection, dysuria, fever, and ulcer. It also has antifertility, antitumor and abortifacient properties[4-9]. Studies of medicinal plants based on ancient literature and its investigation in modern light is under process. The medicinal importance of a plant is due to the presence of some special substances like alkaloids, glycosides, resins, volatile oils, gums and Tannins and ascorbic acid etc. These active principles usually remain concentrated in the storage organs of the plants viz., roots, seeds, bark, leaves etc.

Vitamin C (chemical names: ascorbic acid and ascorbate) is a six-carbon Lactone and a valuable food component because of its antioxidant and therapeutic properties. It helps the body in forming connective tissues, bones, teeth, blood vessels and plays a major role as an antioxidant that forms part of the body defense system against reactive oxygen species and free radicals, thereby preventing tissue damage. It is widely used in the treatment of certain diseases such as scurvy, common cold, anemia, hemorrhagic disorders, wound healing as well as infertility [10]. Vitamin C (ascorbic acid), which must

be obtained from the diet, is an essential micronutrient required for normal metabolic functioning of the body. Therefore, a deficiency of this vitamin results in the symptoms of scurvy and death. This potentially fatal disease can be prevented with as little as 10 mg of vitamin C per day, an amount easily obtained through consumption of fresh fruits and vegetables. However, the current recommended dietary allowance (RDA) for vitamin C is set at 60 mg per day to provide an adequate margin of safety, as 60 mg/day would prevent the development of scurvy for about one month in a diet lacking vitamin C[11].

Fig. 1: *Annona squamosa*

Vitamin C is a cofactor for several enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters. A deficiency in vitamin C results in a weakening of collagenous structures, causing tooth loss, joint pains, bone and connective tissue disorder and poor wound healing, all of which are characteristic of scurvy. Vitamin C is an important water-soluble antioxidant in biological fluids. It readily scavenges reactive oxygen and nitrogen species such as superoxide and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals and hydrochlorous acid, thereby effectively protecting other biomolecules from oxidative damage[12].
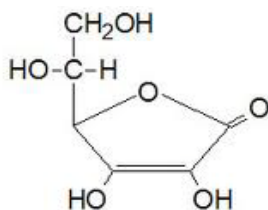
Fig. 2: Ascorbic acid

The main objective of the present study is to extract and develop a simple, efficient, reliable and cost-effective high performance liquid chromatographic (HPLC) method for the quantization of vitamin C in leaves of the *Annona squamosa*.

MATERIALS AND METHODS

Plant material:- Leaves were collected from Medicinal garden of L.N.C.P. Bhopal(M.P.)

Reagents and chemicals

Ethanol, 1.0% w/w or w/v acetic acid, n-butanol, phenol, ammoniacal silver nitrate, O-phosphoric acid and water (HPLC grade). All chemicals were obtained from Shyam Brothers, 27-Sindhi market, Bhopal (M.P.).

Extraction of vitamin C

For vitamin C determination, leaves were sliced(5mm^2), frozen into liquid nitrogen and stored at -80ºC until the analyses were carried out. Frozen pulverized leaves samples were weighed (2g) and mixed with 2.5 ml of the extractant solution (3% O-Phosphoric acid (OPA) and 8% acetic acid for OPA-acetic acid extraction. The mixture was homogenized in a Politron PT 6000 high-speed blender at 18000 g (in ice and darkness) for 1 min and then centrifuged at 9000 rpm (refrigerated at 4ºC) for 20 min. This procedure was repeated twice and the two resulting supernatants were mixed together. All extractions were carried out in quintuplicate.

Qualitative Profile (TLC & Paper Chromatography)

For Thin layer chromatographic studies of Ascorbic acid, precoated Silca gel F$_{254}$ aluminum plates (20 X 20cm) were used. The Ascorbic acid was separated using ethanol: 1.0% acetic acid (9:1) as a mobile phase. The detection was carried out by UV 254nm.

For Paper Chromatography Whatmann filter paper was used as stationary phase. The Ascorbic acid was separated using two different solvents systems. Firstly n-butanol: acetic acid: H$_2$O (4:1:5) taken as mobile phase. Second mobile phase Phenol: 1% acetic acid was taken. Detection was carried out by spraying with ammoniacal silver nitrate solution.

Preparation of Calibration curve

The UV analysis was performed using UV spectrophotometer Shimadzu 1700. A Calibration equation for AA was constructed by plotting the UV absorbance against the AA concentration at five concentration levels (analyzed in triplicate). UV absorbance (*y*) of AA over a concentration (*x*) range of 05-30 ppm was linear y = 0.0253 x with a regression coefficient (r^2) of 0.9905 (Fig: 3).

$$\% \text{ Vitamin C} = A_{Test} * Vol.\text{made up} * Dilution\ factor\ /\ A^{1\%}{}_{1cm} * Weight\ taken$$

Quantitative estimation of Ascorbic acid by HPLC

The HPLC analysis was performed using a LC-100, Cyberlab™, Salo Torrace, Millburry, MAO 1527, USA with LC-UV-100 UV detector. A CAPCELL (C-18) HPLC-packed column (4.6 mm I.D.X 250 mm), type

MG 5 µm, number AKAD/05245 was used for the chromatographic separations. The mobile phase consisted of Water (A) and o-phosphoric acid(B) using a isocratic elution as follow: 0–10 min, 90% A–99% A, & 0.1 %B–0.2% B. The flow rate was 1.5 mL/min, and a column temperature of 25°C. The injection volume was 25µl, and UV detection was effected at 266 nm. After extraction, the ascorbic acid (10µg/ml) was subjected to HPLC column and the obtained records were superimposed on the retention time values of the standard ascorbic acid.

$$\text{Vitamin C content \%} = \frac{A_1\ X\ W_2\ X\ P}{A_2\ X\ W_1}$$

Where, A$_1$ = Peak area of sample solution

A$_2$ = Peak area of standard solution

W$_1$ = Weight in g of sample

W$_2$ = weight in g of standard

P = Purity of standard Ascorbic acid

RESULT AND DISCUSSION

Vitamin C is the most important vitamin for human nutrition that is supplied by fruits and vegetables. L-Ascorbic acid (AA) is the main biologically active form of vitamin C. AA is reversibly oxidized to form L-dehydroascorbic acid (DHA), which also exhibits biological activity. The extracted sample was subjected for qualitative estimation by TLC analysis (Table 1) and Paper chromatography (Table 2).UV scanning of leaves samples was carried out at 200-400nm(Fig:2). Calibration equation for AA was constructed by plotting the UV absorbance against the AA concentration at five concentration levels (analyzed in triplicate). UV absorbance (*y*) of AA over a concentration (*x*) range of 05-30 ppm was linear y = 0.0253 x with a regression coefficient (*r^2*) of 0.9905 (Fig: 3 & Table:3).The concentration of the ascorbic acid found was to be 59.05 µg/ml and % vitamin C in sample was found be 3.5.The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines. HPLC separations of isolated samples with reference to standard were performed on a Cyber Lab C-18 column (250 x 4.0 mm, 5µ). Thus, chromatographic fingerprint should be considered to evaluate the quality of herbal medicines globally considering multiple constituents present in the herbal medicines[13]. Good results were obtained using a mixture of water and orthophosphoric acid as the mobile phase. The concentration of orthophosphoric acid was studied over the range 0-0.5% and a concentration of 0.2% was found to be optimal. The flow-rate significantly influenced AA retention time; the best flow rate was 1.0 ml/min (optimized between 0.4 and 1.6 ml/min) due to the better retention time (4 ± 0.06 min) and resolution for AA and other compounds. The retention time of leaves component of *A.squamosa* was tabulated in table 4 and figure 4. The HPLC analysis revealed that % vitamin C content was found to be 3.4.

CONCLUSION

The 3% O-Phosphoric acid (OPA) and 8% acetic acid method affords enough sensitivity and selectivity in ascorbic acid determination in leaves of *A.squamosa*. The quantification of Ascorbic acid was carried out by HPLC and UV spectrometry methods. The % Vitamin C was found in both the methods was almost same. The present work revealed that this developed method was simple, efficient, reliable and cost-effective for the quantization of vitamin C in leaves of the *A.squamosa*. The method is simple, rapid and has high specificity to Ascorbic acid.

Table 1: TLC Profile of Ascorbic acid

S. No.	Sample	Solvent System	Detection	R$_f$ of Sample	Inference
1.	Std ascorbic acid	ethanol: 1.0% acetic acid (9:1)	UV 254nm	0.51 (Blue spot)	Ascorbic acid
2.	Vit.C(sample)	ethanol: 1.0% acetic acid (9:1)	UV 254nm	0.50	Ascorbic acid

Table 2: Paper chromatographic Profile of Ascorbic acid

S. No.	Sample	Solvent System		Detection	R_f value of Sample		Inference
		Mobile Phase A	Mobile Phase B		A	B	
1.	Std ascorbic acid	n butanol:Acetic acid: H_2O(4:1:5)	Phenol-1% acetic acid	Sprayed with Ammoniacal $AgNo_3$	0.37	0.36	Ascorbic acid
2.	Vit.C(sample)	……"……..	……"……..	……"……..	0.38	0.33	Ascorbic acid
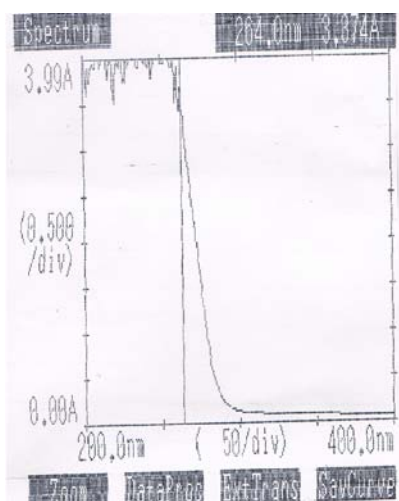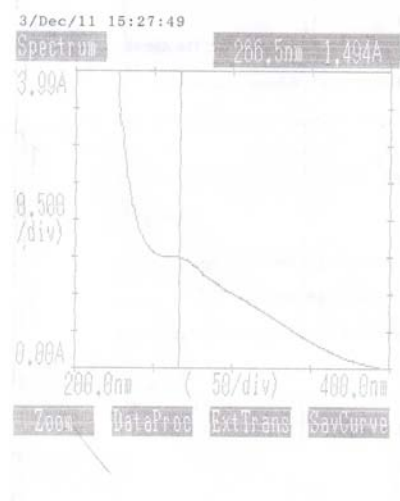
(Standard)   (Sample)

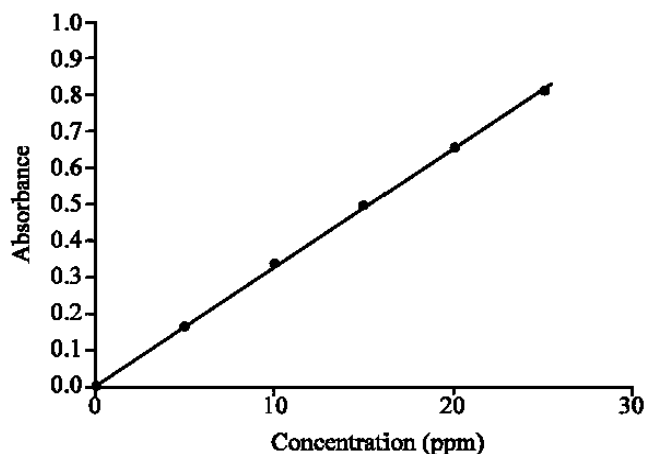**Fig. 2: UV scanning of Standard and Samples**

Fig. 3: Standard Curve of Ascorbic acid

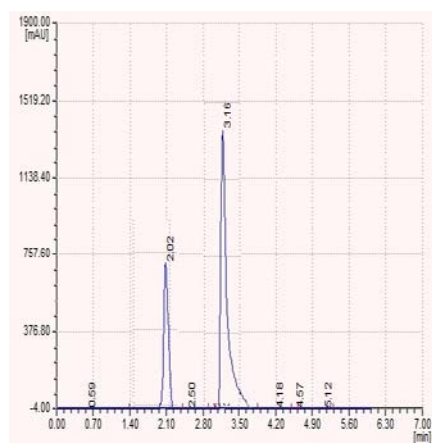**Table 3: Quantitative estimation of Ascorbic acid from UV spectrometry**

S. No.	Sample	Absorbance at 266nm	Statistical Analysis	Concentration (µg/ml)
1.	Leaves of *A.squamosa*	1.494	Correlation coefficient $R^2= 0.9905$ Straight Line equation $y= 0.0253x$	59.05
2.	% Vitamin C content			3.5

Table 4: HPLC Analysis of Ascorbic acid

S. No.	Sample	Height	Area	Conc.	RT	Inference
1.	Standard Ascorbic acid	51422	582540.3	97.7197	4.14	Ascorbic acid
2.	Ascorbic acid(sample)	827	10078.6	43.2844	3.16	Ascorbic acid
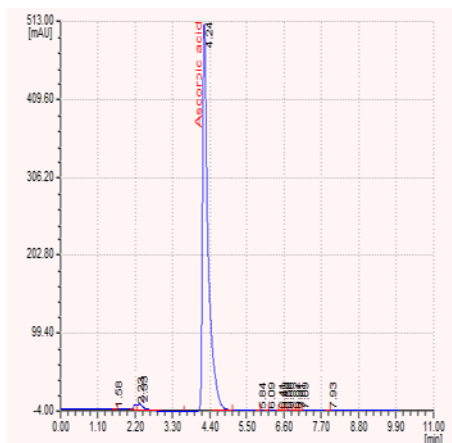
| HPLC Chromatogram of Ascorbic acid (standard) | HPLC Chromatogram of Ascorbic acid (sample) |

Fig. 4: HPLC Chromatogram of Ascorbic Acid

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