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Pavan C Akkiraju
Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni, Ahmednagar (Dt.), Maharashtra, India

Dipeeka D Suryawanshi
Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni, Ahmednagar (Dt.), Maharashtra, India

Aparna J Jawakekar
Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni, Ahmednagar (Dt.), Maharashtra, India

Harshad S Tambe
Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni, Ahmednagar (Dt.), Maharashtra, India

Srilakshmi Mamillapalli
Department of Pharmacy, Sarada College of Pharmaceutical Sciences, Kondakavuru, Narasarao pet, Guntur, Andhra Pradesh, India.

Correspondence
Pavan C Akkiraju
Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni, Ahmednagar (Dt.), Maharashtra, India

Phytochemical analysis and HPLC study of vitamin-C from *Punica granatum* L. Aarakta variety of India

Pavan C Akkiraju, Dipeeka D Suryawanshi, Aparna J Jawakekar, Harshad S Tambe and Srilakshmi Mamillapalli

Abstract
Punica granatum L. commonly known as Pomegranate is a plant with medicinal properties known from ancient times. The Indian variety Aarakta was investigated for its phytochemicals along with HPLC study of Vitamin-C. Methanol extracts of exocarp, mesocarp and seeds were evaluated for alkaloids, flavonoids, tannins, anthraquinones, glycosides, phycobilins, vitamin-C, amino acids, proteins, carbohydrates and saponins. All three parts of the fruit contained saponins, carbohydrates, alkaloids, phycobilins and vitamin-C. The same were found absent with glycosides, proteins, amino acids and anthraquinones. The flavonoids were not found in the seeds and gallotannins were present only in the seeds. HPLC analysis of Vitamin-C had TP contents ranged from 53.7 to 5917.3 µg/mg, 4.0 to 3957.3µg/mg and 497.4 to 2797.7µg/mg for exocarp, mesocarp and seed extracts, respectively. The contents of TF ranged from 0.65 to 1.6 µg/mg, 0.5 to 1.9 µg/mg and 0.60 to 1.61 µg/mg for exocarp, mesocarp and seed extracts, respectively.

Keywords: Pomegranate, phytochemistry, HPLC, ascorbic acid, alkaloids, flavonoids

1. Introduction
The role of fruits in alternate medicine is of great significance from ancient times worldwide. The common dietary fruits like banana, grapes, guava, pomegranate etc. have proven their phytochemical constituents. The pomegranate is one of the highly consumed fruit worldwide because of the seed's taste. Native to Persia, pomegranate is cultivated in India from ancient times and is mainly consumed in Mediterranean countries along with USA. Pomegranates have a wide spectrum of antimicrobial and antihelminthic properties. They also possess anticancer properties in the cases of prostrate, breast, colon and skin tumors [1]. The other medicinal applications of pomegranate are in treating cardiovascular disease, dental conditions, erectile dysfunction, ultraviolet radiation-induced skin damage, diabetes, male infertility, infant brain ischemia, arthritis, Alzheimer's disease, and obesity [2].
The medicinally important plants are being the therapeutic agents also contribute to certain essential chemical constituents, which can be later designed into drugs. This may enhance the possibility of designing novel drugs to life-threatening diseases [3]. The phenolic compounds can be extracted from different parts of pomegranate plant. The major parts associated with these extractions are exocarp, mesocarp, seeds, leaves, roots and tree bark. The major phytochemicals present in pomegranate with industrial application include ellagic acid, punicic acid, ellagitannins, flavonoids, anthocyanins, anthocyanidins, flavones and estrogenic flavones [4]. Vitamin-C is an essential nutrient for both human and animals. The antioxidant properties and acting as cofactor for at least eight enzymes made it as a highly influencing element in the biological systems [5]. As Vitamin-C deficiency leads to scurvy in humans, it is necessary to introduce it as a supplement in the diet to overcome the disease.
The preliminary phytochemical analysis of pomegranate peel extraction samples was performed in previous studies [6, 7]. These studies found that most of the biologically active Phytochemical were present in the ethanolic, aqueous and chloroform extracts of *Punica granatum* peel, whole fruit and seeds [6]. It is also evident that the total phenolic distribution of juice, peel, and seed extracts of four pomegranate cultivars can vary in their composition [8]. The physical and chemical characteristics of pomegranates were studied extensively [9].

Aarakta is a pomegranate variety of high demand internationally and mainly exported to Bahrain, Oman, Saudi Arabia, Kuwait, U.A.E., and Netherlands etc. [10]. It is a commercial variety cultivated in various regions of Maharashtra. It is a dark red skin bearing big fruit with high yield, sweet and soft seeds along with bold red arils. It is also an early variety with a harvesting time with 120 to 135 days and yields 30-35 kg per tree under better cultivation conditions [11].

The current study has focused on qualitative identification of different phytochemical constituents of Aarakta along with HPLC study of Vitamin-C.

2. Materials and Methods

2.1 Collection of plant materials

Twenty Pomegranates were collected randomly from the local market of Loni, Maharashtra during November 2015. Aarakta is a dark-red fruit with dark-red colored seeds having sweet taste **(**Fig. 1a, b, c, d and e**)**.

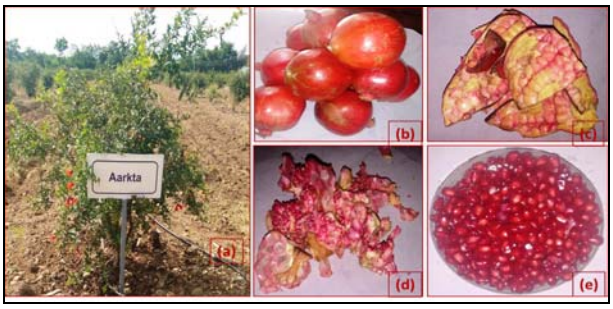

Fig 1: (a) The plant Aarakta, (b) The fruit, (c) Exocarp, (d) Mesocarp and (e) Seeds.

The fruits were brought in wooden boxes to the laboratory and kept at room temperature. The fruits were washed thrice with running tap water and later washed with distilled water once, before starting analysis of characters. The exocarp, mesocarp and seeds were separated manually. These parts were dried in hot air oven at 50ºC to become suitable material for further study. The dried plant materials were separately grinded by using a house-hold mixer grinder to collect the powder **(**Fig. 2a, b and c**)**.

Fig 2

2.2 Preparation of plant extracts

Two extraction procedures were carried out to process the phytochemical compounds i.e. Methanol and Aqueous extract methods. The methanol extract was prepared by using 12 g of three types of powders separately weighed through a digital balance and the same was packed in a filter paper by rapping with a thread. HPLC-grade Methanol was used as a solvent for the extraction of seed powder. Extracted samples of exocarp (A), mesocarp (B) and seeds (C) were dried separately at room temperature. Here after, the extraction sample indicate samples A, B and C used separately. These extracted samples were used for preliminary phytochemical screening and HPLC analysis. The aqueous extract was prepared by boiling the powders in distilled water for 15-20 minutes and left at room temperature. Later, it was filtered and the filtrate was evaporated by keeping in hot air oven. The sample was stored in refrigerator. These are the samples used for preliminary screening of phytochemicals in triplicates. However, the further analyses were conducted by using methanol extractions.

2.3 Phytochemical analysis

Each extract was screened for the presence of key phytochemicals as follows:

2.3.1 Test for saponins: 1 mL of extraction samples were taken in separate test tubes containing 5 mL of distilled water and shaken.

2.3.2 Test for flavonoids: 1 ml of extraction samples were taken in separate test tubes. 1mL of 10% $FeCl_3$ solution was added and mixed well.

2.3.3 Test for glycosides: Two to three drops of glacial acetic acid and 10% ferric chloride solution were added to 1 mL of extraction samples and mixed well. Then, 1mL of conc. sulphuric acid was added.

2.3.4 Test for proteins: 1 mL of extraction samples were taken in separate test tubes. One mL of 10%sodium hydroxide solution and two to three drops of 0.1% copper sulphate solution were added.

2.3.5 Test for carbohydrates: To one ml of extraction samples, 2-3 drops of Benedict's reagent was added and boiled in water bath for 5-10 minutes.

2.3.6 Test for alkaloids: 1mL of extraction samples were taken and added 5 mL of diluted 1% HCl. The test tubes were placed on boiling water bath for 5 min. The mixture in test tube was then filtered. To the filtrate 1mL of Wagner's reagent was added.

2.3.7 Test for amino acids: To 1 mL of extraction samples, 1 mL of 0.2% Ninhydrin solution was added.

2.3.8 Test for tannins: 2 mL of extraction samples were taken and added few drops of 5% $FeCl_3$ solution.

2.3.9 Test for anthraquinone: To 1 mL of extraction samples, 10 mL of ethanol was added and stirred for 5 min. The mixture was filtered. To 2mL of filtrate, 2ml of chloroform was added and shaken thoroughly. The chloroform layer was taken and 5mL distilled water, aqueous ammonia were added and shaken thoroughly.

2.3.10 Test for phytobilin: 1 mL of extraction samples was boiled in 2mL of 1% aqueous HCl.

2.3.11 Test for vitamin C: 1 mL of extraction samples was treated with dinitrophenyl hydrazine dissolved in concentrated sulphuric acid.

2.4 HPLC of vitamin-C

The liquid chromatographic system consisted of a Shimadzu LC-6A model Shimadzu, fitted with a Waters í-Bond pack (Waters Corp., Milford, MA) C18 column (250 x 4.6 mm i.d.) and an SCL-6A system controller. The extraction samples were dissolved in a mixture of methanol and water (6:4 v/v) and the sample size was 20μL.

3. Results

The phytochemical constituents found in Aarakta were summarized (table 1). The presence of saponins, carbohydrates, alkaloids, phycobilins and Vitamin-C were confirmed through various phytochemical tests. The other constituents, glycosides, proteins, amino acids and anthraquinones were found absent in this fruit. The presence of flavonoids in exocarp and mesocarp were confirmed, but found absent in seeds. Gallotannins were found only in the seeds and absent in both exocarp and mesocarp.

Table 1: Phytochemical analysis of the pomegranate variety Aarakta

S. No.	Phytochemical constituent	Aarakta		
		Exocarp	Mesocarp	Seeds
1	Saponins	+	+	+
2	Flavonoids	+	+	-
3	Glycosides	-	-	-
4	Proteins	-	-	-
5	Carbohydrates	+	+	+
6	Alkaloids	+	+	+
7	Amino acids	-	-	-
8	Gallotannins	-	-	+
9	Anthraquinones	-	-	-
10	Phycobilins	+	+	+
11	Vitamin-C	+	+	+

The HPLC analysis for ascorbic acid realized in the given sample of exocarp, mesocarp and seed extracts. The peaks obtained were compared with the standard to compare and confirm the Vitamin-C quantity. The chromatogram for exocarp samples showed peaks indicating the presence of ascorbic acid (Fig. 3a). The maximum peak area was found as 156113.7969 mV*s with a retention time (RT) of 4.4667 minutes. This point has an area of 91.73%. This point has a number of Theoretical Plates (TP) of 290.9 and Tailing Factor (TF) of 0.6591. The total area of ascorbic acid was found to 170187.6250 mV*s. The mesocarp extract had a maximum peak area of 121335.0703 mV*s with a retention time (RT) of 4.1833 minutes. This point has an area of 88.93 %. This point has a (TP) of 320.8 and (TF) of 0.9400 (Fig. 3b). The total area of ascorbic acid was found to be 136438.0781 mV*s. In the case of seeds, the maximum peak area was found to 2858.2646 mV*s with a retention time (RT) of 4.1833 minutes. This point has an area of 30.97%. This point has a number of Theoretical Plates (TP) of 415.4 and Tailing Factor (TF) of 0.9063 (Fig. 3c). The total area of ascorbic acid was found to be 9228.3750 mV*s.

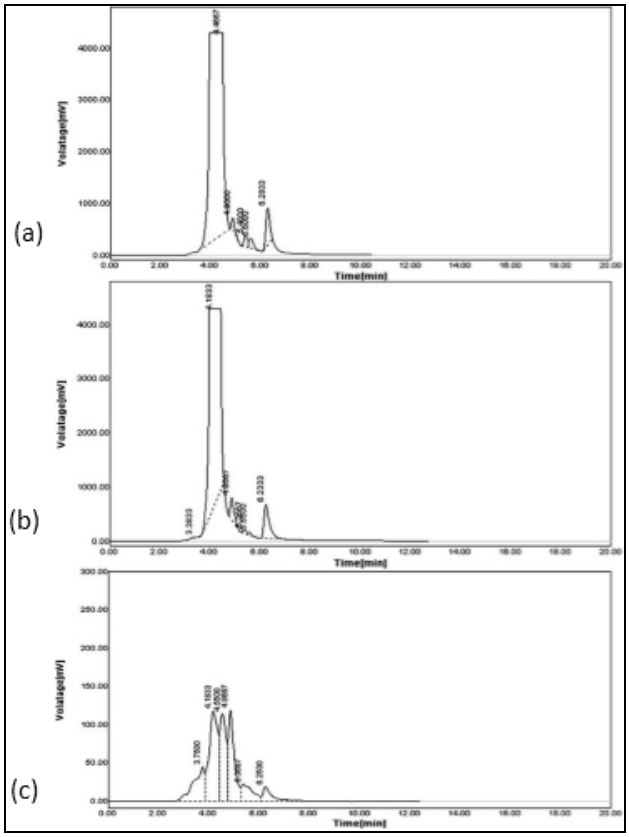

Fig 3: HPLC chromatograms for ascorbic acid obtained from the methanol extracts of Aarakta (a) exocarp, (b) mesocarp and (c) seeds.

4. Discussion

In the current work, phytochemical constituents of exocarp, mesocarp and seed of Aarakta variety were identified along with the HPLC study of Vitamin-C. Even though there are many investigations performed by using ethanol extraction procedure [12, 13] our current study with Aarakta adapted methanol extraction procedure, due to high output of extraction samples. Previous studies [6] found the presence of Vitamin C, Glycosides, Flavonoid, tannin, carbohydrates. Another study [7] of performed phytochemical analysis of pomegranate plant extracts and declared the presence of phytochemicals such as tannins, phenols, flavonoids, glycosides, saponins, terpenoids, steroids, and alkaloids, where as our preliminary phytochemical evaluation resulted in the presence of flavonoids, saponins, carbohydrates, alkaloid, vitamin C, phycobilins. Thus, it is useful to have the preliminary phytochemical tests, which are helpful in finding chemical constituents that are present in the plant material, which may lead to their quantitative estimation and also in locating the source of pharmacologically active chemical compounds. The presence of different phytochemical constituents in the exocarp, mesocarp and seed extracts further can be used in extracting for the therapeutic properties of pomegranate. The presence of gallotannins in seeds supported the study of antioxidant capacity of pomegranate, which can act as primary antioxidants or free radical scavengers [13]. The presence of saponins is confirmed across all three parts of Aarakta which mainly have cardio-depressant property.

HPLC analysis of vitamin C from exocarp, mesocarp and seed extraction samples was performed for the quantification. A previous study [12] measured vitamins composition in pomegranate by HPLC and result showed that pomegranate peel could be a complimentary source of vitamin C and A. In acceptance with these authors, our exocarp, mesocarp and seed extracts also showed a very good amount of Vitamin C. Out of these parts of the fruit selected, the exocarp showed a maximum area (156113.7969 mV*s) of Vitamin-C and the seeds have very less Vitamin-C (2858.26 mV*s) content. This information can be useful during further investigations of Vitamin-C.

5. Conclusion

The role of pomegranate in alternate medicine is of great importance due to its high phytochemical constituents. It is possible to find out the important chemical constituents, which can be in later stages converted into useful products in medicine. The high impact of Aarakta variety of India in medicine can be enhanced with further studies. The phytochemical constituents identified in this current study have confirmed previous studies. HPLC analysis of Vitamin–C confirmed that, out of the three parts used in this study, the exocarp has high content of Vitamin-C, which can be further used in treating scurvy.

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