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QUANTITATIVE ANALYSIS OF FLAVONOID 'NARINGIN' A NATURAL PRODUCT AND ITS CORRELATION WITH ANTIOXIDANT ACTIVITY IN DIFFERENT FRUIT FRACTIONS OF GRAPEFRUIT, *CITRUS DECUMANA* VAR. *PARADISI* (MACFAD.) H.H.A NICHOLLS]: USE OF INDUSTRIAL WASTE

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ABSTRACT

Grapefruit [*Citrus decumana* var. *paradisi* (Macfad.) H.H.A Nicholls] is a subtropical citrus tree. It is an 18th century hybrid of *Citrus maxima* × *Citrus sinensis,* was first bred in Barbados (West Indies). Among the various phytochemicals detected in grapefruit, the flavonoid, naringin, which contributes to the distinctive bitterness of citrus fruits, exerts a variety of pharmacological effects The methanolic extract of different parts of the fruit such as flavedo, albedo, segment membranes and seeds which are the main by products in citrus processing industry were subjected to HPLC analysis for their naringin content with mobile phase containing acetonitrile and 2%acetic acid (25:75) at wavelength 280 nm while antioxidant activity was determined by DPPH free radical scavenging assay. Highest naringin content was detected in albedo, flavedo and juice followed by segment membranes and seeds. Antioxidant activity determined by DPPH free radical scavenging activity revealed the fruit peel containing albedo and flavedo parts with highest antioxidant activity which could be correlated with their flavonoid content.

KEY WORDS: ***Grapefruit, naringin, flavedo, albedo, acetonitrile, antioxidant, DPPH.***

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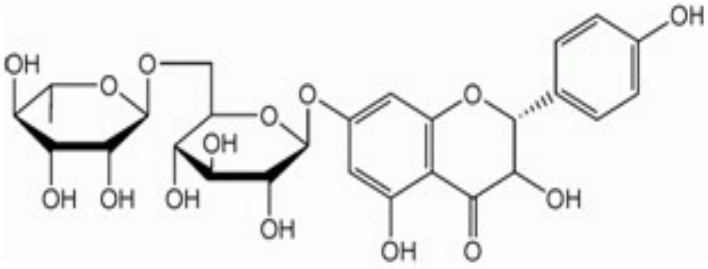
INTRODUCTION

The beneficial effect of a diet supplemented with fruits and vegetables have enhanced interest in their bioactive compounds. It has been shown that positive effects of these natural products is usually linked with their antioxidant compounds such as carotenoids, flavonoids, vitamin C etc. Until the last 2 decades, it was assumed that vitamin C and carotenoids were the only chemopreventive agents in citrus because of its free radical scavenging ability.[1] Recent accumulative evidences suggest that citrus contains several possible anti-cancer agents such as flavonoids and limonoids.[2] Citrus flavonoids have shown a wide range of therapeutic properties for medical and clinical applications such as anti-inflammatory, antihypertensive, diuretic, analgesic, and hypolipidemic effects[2,3]. Citrus juice flavonoids have also shown activity against acute myeloid leukemia.[4] A flavonoid glycoside, naringin, which contributes to the distinctive bitterness of citrus fruits, was first found in grapefruit exerts a variety of pharmacological effects such as antioxidant, anticancer, anti-atherogenic and blood lipid lowering activity.[5] To increase the amount of nutrients absorbed by the body the activity of several enzymes within the digestive tract is decreased by naringin. This prevents the breakdown of nutrients allowing more to be absorbed into the blood stream. Naringin supplements are consumed along with other nutritional supplements to increase their absorption. Naringin, when treated with potassium hydroxide or another strong base, then catalytically hydrogenated, it becomes naringin dihydrochalcone, a compound roughly 300-1800 times sweeter than sugar at threshold concentrations [5,6]. Solid residues consisting of grapefruit peel, segment membranes and seeds of grapefruit processing industry cause great environment havoc in term of its disposal. It was opportune if this residue can be exploited to produce higher value added products for use in food, pharmaceutical and cosmetic industry. Commercial brands of grapefruit juice have been analyzed for their flavonoid content by HPLC and naringin was identified in all grapefruit juices[7,8]. In this study different parts of grapefruit fruit such as albedo, flavedo, segment membranes and seeds in addition to juice were subjected to HPLC analysis to determine their naringin content and antioxidant activity was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay.

Naringin profile

Chemical name of naringin: Naringenine-7-rhamnoside-glucoside;4',5,7-Trihydroxyflavone-7-rhamnoglucoside
Chemical formula of naringin: $C_{27}H_{32}O_{14}$
Molecular weight: 580.53 in its purest form.
Molar mass: 580.54 g/mol
Melting point: 166°C
Molecular structure of naringin



MATERIAL AND METHODS

Material

Fully mature fruits of grapefruit were obtained in the month of January from the plant growing in the college campus. The fruit was identified by the Head Department of Botany of our college (specimen #: br p 190111) and deposited in the college herbarium.

HPLC analysis of flavonoid naringin
Reagents and chemicals

Glacial acetic acid was purchased from S.D. Fine Chemicals, Mumbai and methanol, acetonitrile, deionised water from Merck Limited, Mumbai. All HPLC grade, standard naringin were obtained from Hi Media Laboratories Pvt. Limited, Mumbai.

Instrumentation

HPLC system consists of Shimadzu Prominence HPLC (gradient) system with two hydraulic pumps, 6-valved Rheodyne injector, Thermoscientific Synchronis C18 column (250 × 4.6 mm, pore size 5µm) and a diode array detector. Data was processed by acquisition and integration LC software solutions.

Preparation of samples

Fruit was thoroughly washed with running tap water and divided into five different parts: flavedo, albedo, segment membranes, fruit juice and seeds as shown in Fig.1. All these tissue except for the juice were homogenously blended with the help of methanol by using a blender to make fine slurry. The juice was typically obtained by use of mechanical juice maker. Both juice and slurry of different tissues were stored frozen at −80°C for future analysis.

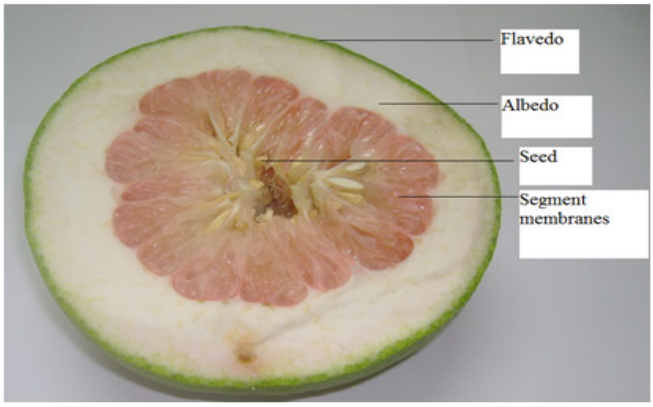


Figure 1
Cut fruit of grapefruit to show diferent parts used for naringin analysis

Extraction of naringin from fruit juice
A sample of 1-2 ml of the juice was extracted with 4 ml of methanol by shaking for 1 min using a Vortex Mixer and then followed by centrifuging at 3000 rpm for 10 min. The residue was re-extracted twice under the same condition to ensure complete extraction. The extract was passed through a 0.22 µm nylon filter prior to the HPLC analysis [9].

Extraction of naringin from fruit parts
Sample of 5 gm slurry of each was weighed and then extracted with 20 ml of methanol by shaking for 1 min with a Vortex Mixer, followed by centrifuging at 3000 rpm for 10 min. The extract was passed through 0.22 µm nylon filter prior to the HPLC analysis. Albedo, flavedo and segment membrane samples were diluted with methanol to 1:50, 1:25 and 1:25 respectively. Seed and juice samples were used without any dilution [10].

Standard naringin solution
100 mg / 100 mL (1 mg / mL). 100 mg or 0.1 gm standard naringin was dissolved in 100 ml of mobile phase consisting of 2% acetic acid: acetonitrile (75:30) to prepare 1000 ppm stock solution of naringin. Standard solutions of naringin were prepared by diluting the stock solution to 60, 80, 100, 120 and 160 ppm with the mobile phase.

Determination of naringin by HPLC
For determination of naringin mobile phase consisted of (2% acetic acid:acetonitrile, 75:30) with flow rate of 1 ml/min. The injection volume of sample and detection wavelength was 20 µl. and 280 nm respectively. Experiment was carried out at room temperature and pressure 134 kgf (Kilogram force).

Antioxidant activity by DPPH free radical scavenging assay
The radical scavenging ability of flavedo, albedo, segment membranes, seeds and juice was assessed on the basis of the radical scavenging effect on the DPPH free radical. The fruit juice and fruit fractions sample as prepared for naringin analysis were prepared in methanol (10-200 µg /ml). In clean and labeled test tubes, 2 mL of DPPH solution (0.002% in methanol) was mixed with 2 ml of each sample extract. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was measured at 517 nm using UV-Vis Spectrophotometer. The absorbance of the DPPH control was also noted. The scavenging activity of all fruit fractions and juice was calculated using the formula: Scavenging activity (%) = [(A – B) / A] x 100, where A is absorbance of DPPH control and B is absorbance of DPPH and sample combination [10, 11]. Experiment was repeated thrice for each sample and results were expressed as mean±SD.

Hydrogen peroxide scavenging activity
Hydrogen peroxide scavenging activity of various samples prepared from different fruit parts (albedo, flavedo, segment membranes, seeds and fruit juice) was determined according to the method of Yen and Chen (1995)[11]. A solution of hydrogen peroxide (45 mM) was prepared in 0.1M phosphate buffer (pH 7.4). Different concentrations (10–200 µg/ml) of different samples prepared in methanol were added to 0.6 ml of hydrogen peroxide solution (45 mM). Absorbance of reaction mixture was recorded at 230 nm in a spectrophotometer (Shimadzu UV- 1201; Shimadzu, Kyoto, Japan) against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of different samples was calculated using the following equation: Scavenging activity (%) = [(A – B) / A] x 100, where A is absorbance of hydrogen peroxide control and B is absorbance of hydrogen peroxide and sample combination [12, 13, 14].

RESULTS AND DISCUSSION

HPLC analysis of flavonoid naringin
Each fruit part - flavedo, albedo, segment membranes, seeds and juice was analyzed for naringin content. Chromatogram of standard naringin (Fig. 2) showed the RT (retention time) equal to 6.646 min. and the peak areas detected at wavelength 280 nm of all standard concentrations of naringin were taken to construct a calibration curve [12]. Calibration curve (Fig.7) obtained was linear with regression coefficient R^2 = 0.9953. By simply determining the peak area for naringin from the chromatograms of all samples (Fig.,3,4,5 and 6) a line was drawn on the standard naringin graph at that area. When the calibration curve is reached, a line was dropped down to the x-axis which gave the concentration of the naringin in sample (Fig.8) [15, 16, 17].

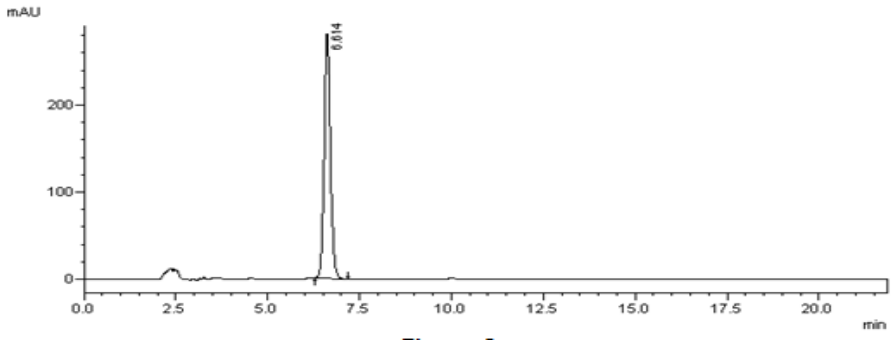


Figure 2
Chromatogram of standard naringin (100 ppm)

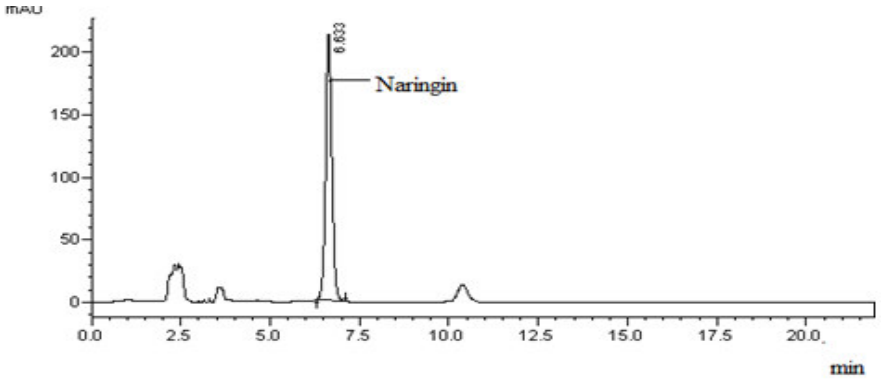


Figure 3
Chromatogram of naringin in grapefruit albedo

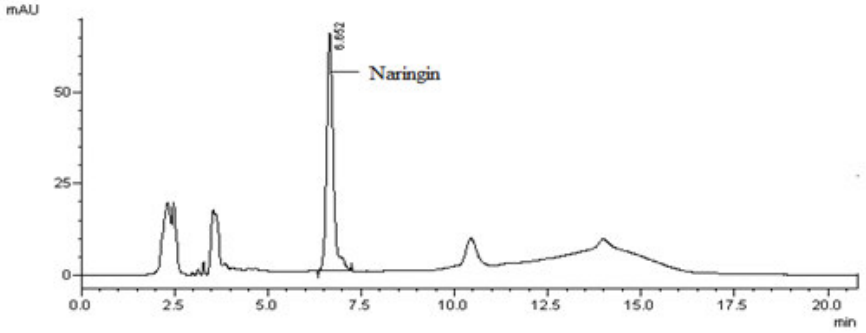


Figure 4
Chromatogram of naringin in grapefruit flavedo

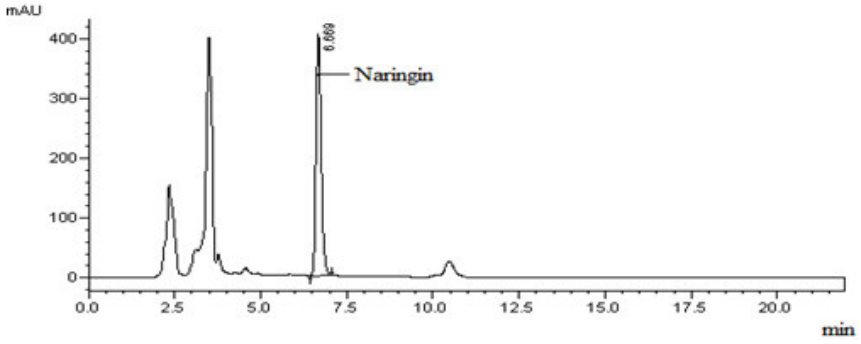


Figure 5
Chromatogram of naringin in grapefruit juice

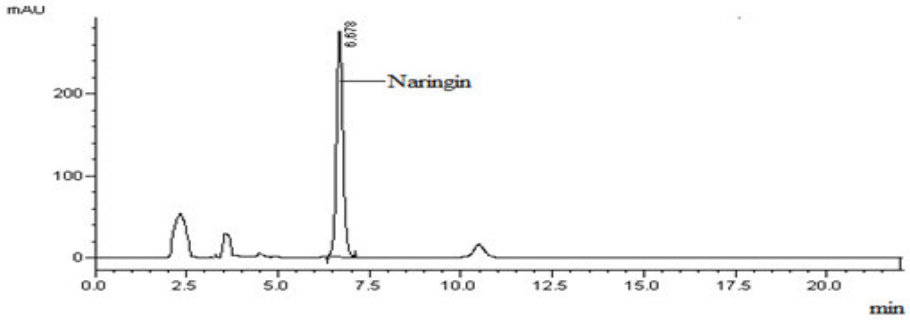


Figure 6
Chromatogram of naringin in grapefruit segment membranes

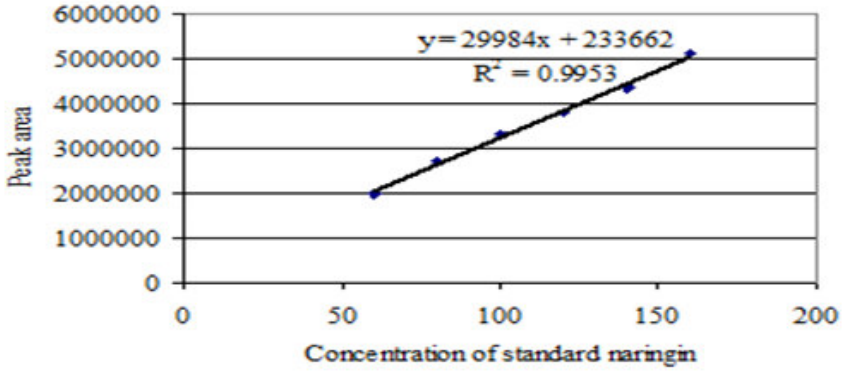


Figure 7
Calibration graph of naringin

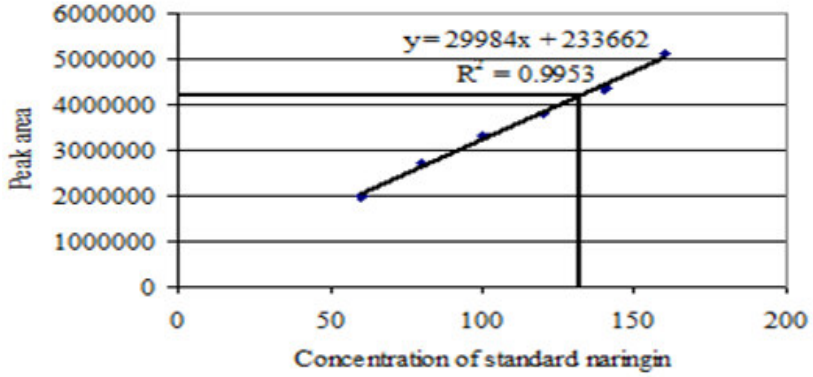


Figure 8
Concentration of naringin in grapefruit juice shown with drop line

Flavonoid composition appears to vary greatly in the different parts of the fruit used. The major source of naringin was the albedo, while seed and segment membranes contained a minor amount. Fruit parts that contained naringin in a decreasing order were: albedo (3924.50±385.43ppm), flavedo (2575.50 ± 318.67ppm), juice (1872.34 ± 259.33ppm), seeds (1478.53 ± 93.78 ppm) and segment membranes (524.70 ± 87.90 ppm).

Antioxidant activity by DPPH free radical scavenging assay

The antioxidant activity increases with increase in concentration of sample solution. The highest antioxidant activity in term of % inhibition (Table1) was detected in juice (98.85±1.78), followed by flavedo (79.87±0.24), albedo (69.80±0.45), segment membranes (39.55±1.15) and seeds (35.42±1.25) [18].

Antioxidant activity by hydrogen peroxide scavenging assay

Antioxidant activity of methanolic fruit fraction samples is also dose dependent as highest antioxidant activity was shown at concentration 200 µg/ml for all samples. The same results were obtained by hydrogen scavenging assay although the overall percentage inhibition is less as compared to DPPH scavenging assay but again highest antioxidant activity is detected in fruit peel (albedo 59.89±0.75, flavedo 67.31±0.65) next to fruit juice (89.90±0.90) as in Table 2.

Table 1
DPPH free radical scavenging assay of different fruit fractions of Citrus paradisi

Concentration µg/ml	% inhibition of albedo	% inhibition of flavedo	% inhibition of segment membranes	% inhibition of seeds	% inhibition of fruit juice
10	23.31±0.23	30.23 ±0.42	7.25±0.21	5.66±0.19	57.72±0.33
20	25.45±1.2	32.57 ± 0.35	8.12±0.23	6.65±0.20	60.50±0.45
30	28.18±1.34	39.65 ±0.26	12.87±0.35	8.43±0.32	68.34±0.35
40	35.76±0.89	45.67 ± 0.41	15.48±0.67	11.30±0.12	76.71±1.62
50	46.32±0.34	56.12 ±0.32	19.40±0.78	15.40±0.15	80.15±1.32
100	54.15±0.32	64.67 ± 0.13	24.12±0.43	20.42±0.89	92.50±1.85
150	63.78±1.45	72.73 ±0.32	28.16±1.30	25.86±0.56	97.72±2.30
200	69.80±0.45	79.87 ±0.24	39.55±1.15	35.42±1.25	98.85±1.78

Table 2
Hydrogen peroxide scavenging activity of different fruit fractions of Citrus paradisi

Concentration µg/ml	% inhibition of albedo	% inhibition of flavedo	% inhibition of segment membranes	% inhibition of seeds	% inhibition of fruit juice
10	20.21±0.29	24.35±0.40	6.21±0.12	4.21±0.13	43.67±0.67
20	23.37±0.34	27.21±0.21	7.19±0.15	4.79±0.14	50.40±0.29
30	25.17±0.31	31.75±0.32	10.15±0.13	5.80±0.09	55.32±0.90
40	30.32±0.54	39.45± 0.29	12.34±0.42	8.40±0.12	61.78±0.47
50	39.10±0.89	44.15±0.90	15.43±0.48	10.42±0.12	69.50±1.39
100	45.12±1.38	57.50±1.49	17.23±0.31	14.12±0.36	77.70±1.35
150	54.76±1.95	61.45±1.29	22.15±0.21	19.40±0.48	82.56±1.85
200	59.89±0.75	67.31±0.65	26.30±1.15	28.15±0.25	89.90±0.90

The results indicate that there is no direct correlation between flavonoid naringin content of grapefruit fruit fractions and antioxidant activity except the naringin content and antioxidant activity of peel (albedo and flavedo) and segment membranes. Thus many other phytochemicals such as carotenoids, phenolics and vitamin C reported in grapefruit may also playing a pivotal role in scavenging free radicals[19,20]. Study conducted by AK Hadi Al- Anbari et al, on antioxidant activity of leaves and seeds of *Citrus paradisi* Macfad showed scavenging of Hydrogen peroxide 95.1% [21]. The high phenol and flavonoids contents of peels and tissue of *C. reticulata* var. Ponkan has been attributed to high antioxidant activity of this plant[22]. Toh JJ et al. (2013) reported that the peels of pommelo, *Citrus grandis* (L) Osbeck] fruit had higher antioxidant content and was positively high correlated with total phenolic content (r = 0.978) and total flavonoid content (r = 0.959)[23]. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Lee et al., 2003)[24]. It was found that the radical- scavenging activities of all the extracts increased with increasing concentration. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Van Acker et al., 1996)[25]. In the present study, the correlation between total flavonoids contents and radical scavenging activity of grapefruit fruit fractions were analyzed. In general, extracts or fractions with a high radical scavenging activity showed a high flavonoid content as well, but good correlations could not be found among them. Lack of relationship is in agreement with other literature (Anagnostopoulou et al., 2006; Nickavar et al., 2007)[26,27]. It is known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity (Mensor et al., 2001; Hou et al., 2003)[28,29]. Furthermore, the extracts are very complex mixtures of many different compounds with distinct activities (Mensor et al., 2001; Hou et al., 2003).

CONCLUSION

The higher amount of flavonoid naringin in grapefruit peel, flavedo, albedo and segment membranes makes these fruit parts a valuable secondary raw material. The higher antioxidant activity of fruit peel and segment membranes next to fruit juice offers insight into how to explore further the benefits of by- products of citrus juice industry for use as a rich source of flavonoids which are the compounds of considerable importance to the food, pharmaceuticals and cosmetic industry.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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