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Exploring the total flavonoid content of peels of *Citrus auriantum, Citrus maxima and Citrus sinensis* using different solvents and HPLC- analysis of flavonones - Naringin and Naringenin in peels of *Citrus maxima*

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Abstract
The peels of citrus fruits contribute 30% of the total fruit weight and yet they have been dumped without recognizing the possible nutritional value of the peels. The present study has been carried out to identify the flavonoid content of the peels of 3 different citrus species, *Citrus auriantum, Citrus maxima and Citrus sinensis.* The total content of flavonoids was quantitatively determined by using spectrophometric analysis. The quantification of the flavonoid composition in each citrus variety were evaluated and allowed the selection of the best solvent for the extraction of flavonoids. Optimization of flavonoid compounds was conducted using methanolic extract of *Citrus maxima* peel. It showed the highest concentration of flavonoids ie. 4.3 mg quercetin equivavent/g of extract. An Isocratic reversed-phase HPLC was employed for the quantitative analysis. The solvents used were selective for flavonoid extraction, and depending on their polarity, glycoside or aglycone flavonoids were extracted. The most abundant flavonoids in *Citrus maxima* peels as shown by HPLC analysis were naringin and naringenin. Naringenin was found to be in higher concentration when compared to naringin in *Citrus maxima* peel. Since large amount of citrus peels are thrown as waste, the present study suggests their potential use as prophylactic agent in medical field.

Keywords: *Citrus maxima, Citrus auriantum, Citrus sinensis, spectrophotometer, Soxhlet extracts,* HPLC

1. Introduction
Citrus fruits, which belong to the family of rutaceae, have long been valued as most important horticultural crops, because of their nutritional value and special flavour. Bark, root and fruits of Citrus plants are found to contain many curative properties and have been used in indigenous system of medicine. Citrus fruits are well known for their fragrance, partly due to flavonoids in the rind. They have been shown to reduce the risk and progression of diseases like cancer, cardiovascular, neurodegenerative diseases etc (Liu *et al*., 2014) [16]. Rind is widely used as antiasthmatic, sedative in nervous affection, brain tonic and useful in vomiting, griping of abdomen, diarrhea, headache and eye troubles (Vijaylakshmi, 2015) [25]. According to Anagnostopoulou *et al*., (2006) [3], Manthey *et al*., (1996 and 2001) [18, 19], peel represents almost one half of the fruit mass and contains the highest concentrations of flavonoids in the Citrus fruits. The pharmacological actions of citrus peels were attributed to the presence of polyphenolic compounds such as flavonoids including hesperidin, naringin, naringenin, caffeic acid, p-coumaric acid, ferulic acid, and vanillic acid (Kharjul *et al*., 2012) [13]. These flavonoids are found to have various therapeutic properties, like antiviral, anticancer, antimicrobial and anti-inflammatory activities (Kumar *et al*., 2013) [14]. Hesperidin and naringin are found to improve hyperlipidemia and hyperglycemia in type 2 diabetic animals by partly regulating the fatty acid and cholesterol metabolism (Jung *et al*., 2006) [12]. Naringin and Naringenin are found to prevent the hypertension and obesity related cardiovascular complications (Alam *et al*., 2013) [1]. *Citrus maxima* is an extensively distributed native plant found in Indian subcontinent. It has a long use in traditional medicine. The peels of *Citrus maxima* is found to contain flavonones (Xu *et al*., 2008) [26]. In line with the above therapeutic functions, Naringin and Naringenin found to prevent hepatic steatosis, inflammation and fibrosis by improving oxidative stress (Alam *et al*., 2013) [1]. Following claims for cure of several diseases, efforts have been made by researchers to verify the efficiency of the plant through scientific biological screening.

Hence the main objective of this study is to unveil the total flavonoid content of peels of *Citrus maxima*, *Citrus auriantum* and *Citrus sinensis* by spectrophotometry and HPLC analysis of flavonones, Naringin and Narngenin in *Citrus maxima* peels.

For this, methanolic extract of the peels were obtained and the crude extract was analysed to detect total flavonoids and flavonones- Naringin and Naringenin by comparing the standards. Spectrophotometric assay based on aluminum complex formation is the procedure used for total flavonoid determination. Concentrations were calculated using a standard curve. An isocratic reversed-phase HPLC was employed for the quantitative analysis of a flavonoid, naringin, and its metabolite, naringenin.

2. Materials and Methods

2.1. Chemicals

Quercetin, Naringin and Naringenin standards were purchased from Sigma- Aldrich, USA. The solvents, Orthophosphoric acid Acetonitrile, employed were of HPLC-grade and ultrapure. All other chemicals and solvents used in the present study were of analytical grade and purchased from Himedia.

2.2. Collection of plant material

Healthy disease-free fruits of *Citrus maxima, Citrus sinensis* and *Citrus auriantum*, were collected from the forest areas of Shimoga and Chikkamagalur districts of Karnataka, India. The fruits were washed well using tap water and rinsed thrice using distilled water. Then peel and pulp of the fruits were separated. The peels were cut into small pieces and dried in sunlight for 3-4 days. The dried samples were grinded using mortar and pestle and further reduced to powder using electric blender. The powder was stored in air tight containers.

2.3. Preparation of extracts

2.3.1 Aqueous extract

15g of the dried powder was mixed with 200ml distilled water. Boiled for 10 min. on water bath. Filtered through Whatman filter paper No.1. The extract was cooled and kept in refrigerator at 4°C. This was used for further tests (Lin *et al*. 1999) [15].

2.3.2 Soxhlet extract

15g of the dried powder was taken for solvent extraction using 80% methanol, 100% ethyl acetate and 80% hexane separately in a standard soxhlet apparatus set-up for 2-3 hours at a temperature not exceeding the boiling point of the solvent (Lin *et al*. 1999) [15]. Each extract were transferred to sterile glass vials and kept at 4°C before use.

2.4. Preparation of Standard Solution for colorimetric method

1g of Quercetin was dissolved in 100 ml of methanol. This gives 1% solution of Quercetin (10mg/ml), termed as standard solution (Shirazi *et al*., 2014) [22].

2.5. Estimation of TFC (Total Flavonoid Content)

TFC in crude extract was determined by aluminum chloride colorimetric method ((Ebrahimzaded *et al*., 2008, Nabavi *et al*., 2008) [5, 20]. Quercetin was used as standard and flavonoid content was determined as quercetin equivalent. A calibration curve of standard Quercetin was drawn (Madaan *et al*., 2011). 10 mg of quercetin was dissolved in 100% Methanol and then diluted to 20, 40, 60, 80 and 100 µg/ml.) 0.5 ml of the diluted standard solutions of quercetin and plant extract were taken and were separately mixed with 1.5 ml of methanol. To this 0.1 ml of

10% aluminum chloride and 0.1 ml of 1 mol/L potassium acetate were added. The volume was made to 5 ml by adding 2.8 ml of distilled water in a test tube. The test tubes were incubated for 30 min at room temperature to complete the reaction. The absorbance of the reaction mixture was measured at 415 nm with double beam UV-Vis spectrophotometer against blank. A blank solution contained all reagents except aluminium chloride which is replaced by the same amount of distilled water. The amount of flavonoid was calculated from linear regression equation obtained from the quercetin calibration curve. The flavonoid content was calculated as mean, SD (n=3) and expressed as mg/g of quercetin equivalent (QE) of dry extract (Al-Owaisi *et al*., 2014) [2].

2.6. Analysis of flavonoids by HPLC

HPLC method was used for determining the amount of flavonoids, naringin, and its metabolite, naringenin. Isocratic reversed-phase HPLC was employed for the quantitative analysis, using naringin and naringenin as internal standards. A solid-phase extraction method was employed using an Inertsil ODS-3V column (250x4.6 mm I.D., 5 µm particle size). The mobile phases were 0.1% Orthophosphoric acid and acetonitrile in the ratio (70:30). The flow-rate was 1 ml min−1. The analyses were performed by monitoring the wavelength of maximum UV absorbance at 289 nm for both naringin and naringenin. The detection limits on-column were about 0.2 ng for the two flavonoids. Concentrations of the flavonoids in samples were determined by application of the obtained standard curve.

3. Results and Discussion

The total flavonoid content of peels of *Citrus auriantum*, *Citrus maxima* and *Citrus sinensis* using different solvents like methanol, ethyl acetate, hexane and aqueous extracts, as determined by aluminum chloride colorimetric method was expressed as quercetin equivalents by reference to standard curve. Standard curve is obtained from series of different quercetin concentrations. Quercetin standard curve for solvent methanol is shown in Fig.1. Samples were analysed in triplicates. Table.1 represents analytical data for flavonoid content of the methanolic extract of *Citrus auriantum, Citrus maxima and Citrus sinensis.*

Table 1: Total flavonoids content of methanolic extracts of different citrus fruits.

Plant sample	OD value	Total flavonoid content (mg/g)
Citrus auriantum	0.16	1.92
Citrus maxima	0.36	4.32
Citrus sinensis	0.35	4.20

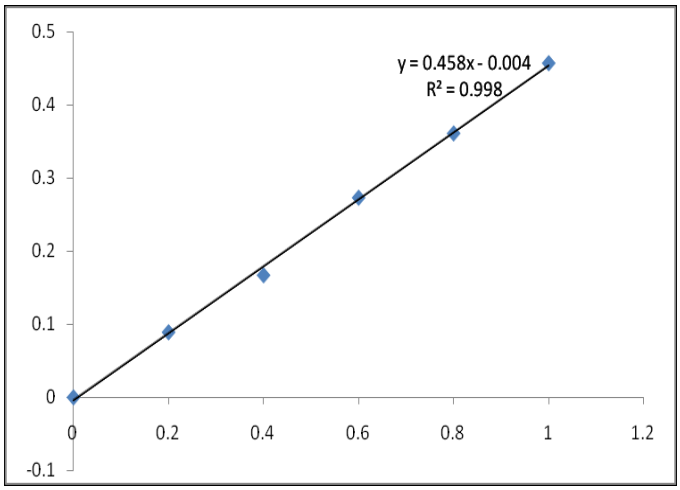

Fig 1: Calibration curve of Quercetin in methanol each point represents the mean of three experiments.

Quercetin standard curve for solvent Ethyl acetate is shown in Fig.2. Each point represents the mean of three experiments. Analytical data for flavonoid content of the methanolic extract of *Citrus auriantum*, *Citrus maxima* and *Citrus sinensis* is reported in Table.2.

Table 2: Total flavonoids content of Ethyl acetate extracts of *Citrus auriantum*, *Citrus maxima* and *Citrus sinensis*

Plant sample	OD value	Total flavonoid content (mg/g)
Citrus auriantum	0.001	0.012
Citrus maxima	0.002	0.024
Citrus sinensis	0.006	0.070

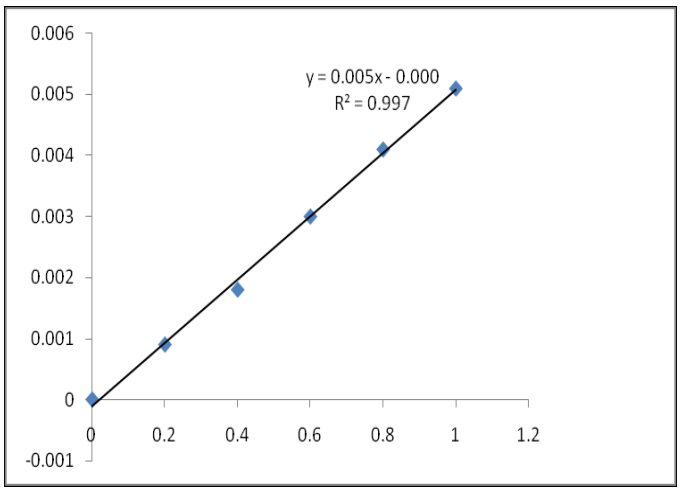

Fig 2: Calibration curve of Quercetin in Ethyl acetate. Each point represents the mean of three experiments.

Standard curve of Quercetin, for solvent Hexane and Water is shown in Fig.3. and Fig.4 respectively. Samples were analysed in triplicates. Table.3 and Table.4 represents analytical data for flavonoid content of the Hexane and Aqueous extracts of *Citrus auriantum*, *Citrus maxima* and *Citrus sinensis* respectively.

Table 3: Total flavonoids content of Hexane extracts of different citrus fruits.

Plant sample	OD value	Total flavonoid content (mg/g)
Citrus auriantum	0.009	0.108
Citrus maxima	0.020	0.240
Citrus sinensis	0.010	0.120

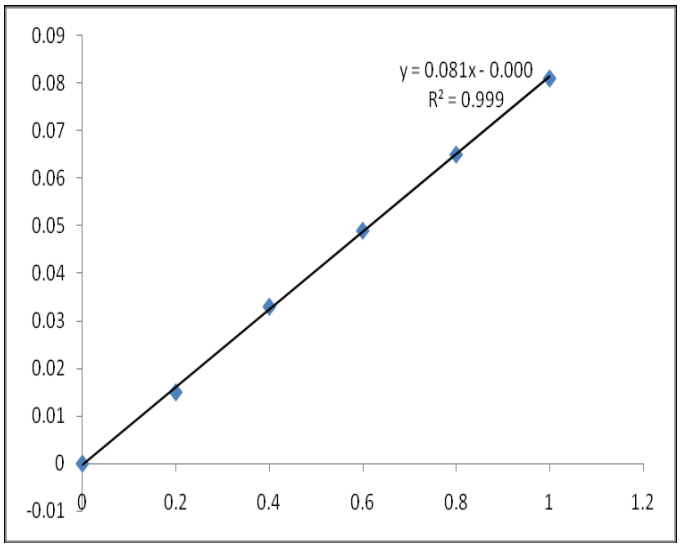

Fig 3: Calibration curve of Quercetin in Hexane. Each point represents the mean of three experiments.

Table 4: Total flavonoids content of Aqueous extracts of *Citrus auriantum*, *Citrus maxima* and *Citrus sinensis*

Plant sample	OD value	Total flavonoid content (mg/g)
Citrus auriantum	0.21	2.5
Citrus maxima	0.23	2.76
Citrus sinensis	0.20	2.4

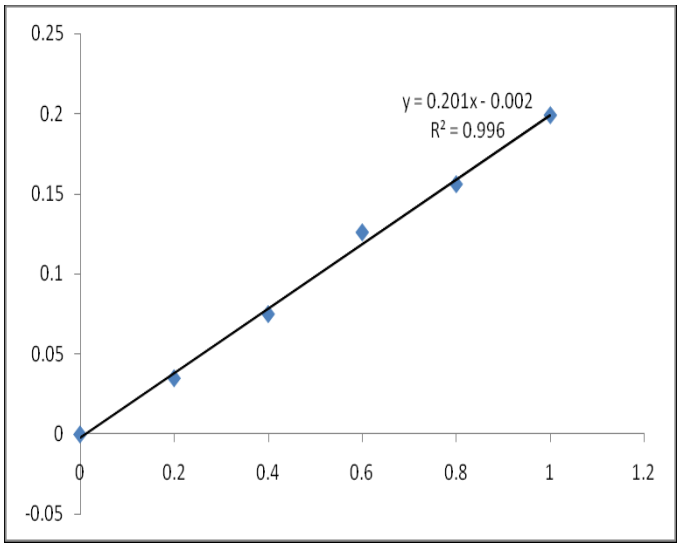

Fig 4: Calibration curve of Quercetin in Aqueous extract. Each point represents the mean of three experiments.

By observing the above results, the methanolic extract of peels of *Citrus maxima* showed the highest concentration of flavonoids, followed by the peels of *Citrus sinensis* and the least by that of *Citrus auriantum*. The flavonoid content in the peels of *Citrus sinensis* was found more in methanolic extract, less in aqueous, followed by hexane extract. It was found to be very low in the extract of ethyl acetate. On the other hand, total flavonoid content in the peels of *Citrus auriantum* was found to be very less among the three citrus species. However aqueous extract showed relatively higher concentration of flavonoids among the four solvents. Hence according to this study, total flavonoid content was found to be significantly more in methanolic extract of peels of *Citrus maxima*. This is in accordance with the studies of Al-Owaisi *et al*., (2014) [2], which says that methanol, the most polar extract, was found to

contain the highest content of total flavonoids as compared to ethyl acetate and chloroform extracts. The present study is also supported by the work of Goristein *et al*., (2004) [9], which says the total flavonoid content of peels of citrus fruits was always substantially high compared to the pulp. The results of this study demonstrated the presence of high flavonoid content in methanolic extracts, which is in conflict with the studies of Fattahi *et al*., (2014) [7], reporting more of their presence in aqueous extracts. Though several studies conducted have reported the presence of total flavonoids in hexane extract Ifason *et al*, (2013) [11], this study showed the divergent result of less flavonoids in hexane extract. Based on the above results, it can be suggested that biological activity of these citrus species could be due to the presence of flavonoids in them (Al-Owaisi *et al*., 2014) [2]. In overall, the total flavonoid content was more preferable and efficient when extracted using methanol in *Citrus maxima*.

Since *Citrus maxima* contains a wide range of flavonoids, their diversity was further examined by means of HPLC. The work of Goulas *et al*., (2012) [10] suggested that the chromatographic profiles of grapefruit revealed the presence of naringin as a major flavonoid in grapefruits. This was supported by the work of Silva *et al*., (2014) [23], who determined the presence of naringin and naringenin in different citrus fruits by HPLC analysis. Hence as in line with these results, the present study is focused on the determination of flavonones- naringin and naringenin in the peels of *Citrus maxima* by HPLC analysis.

HPLC analysis is a versatile and widely used technique for identification and isolation of natural products (Cannel, 1998) [4]. Evaluation of crude extracts can be done by using this method in order to characterize the active entity.

Usually biologically active entity will be present in minor quantities in plant extracts, and the resolving power of HPLC is best suited to the rapid processing of such multicomponent samples.

For the quantification of flavonoids, Naringin and Naringenin an Isocratic reversed-phase HPLC was employed. Representative HPLC profiles of the methanolic extract of *Citrus maxima* are given in Figures 5 and 6. Identification of the compounds was based on retention times in comparison with authentic standards. Fig.5 and 6 shows the Chromatogram of Naringin with standard along with the respective retention time. Similarly, the chromatogram of Naringenin is sown in Fig.7 and 8 with standard, along with the respective retention time. The values were determined using calibration plots of peak area vs. concentration of the pure compounds. The quantitative estimation of naringin in peels of *Citrus maxima* was found to be 2.36% and that of naringenin was 3.40%. The above result shows that naringenin was found in highest level in *Citrus maxima* peel when compared to naringin. This is in line with the results of Ramful *et al*., (2010) [21], showing the presence of naingin, and naringenin in Mandarin fruit. But several reports highlight the absence of these flavoinoids in some species of citrus fruits (Tomas *et al*., 2000) [24]. In overall, the present study represents the good level of flavonones- naringin and naringenin in methanolic extracts of *Citrus maxima* peels.

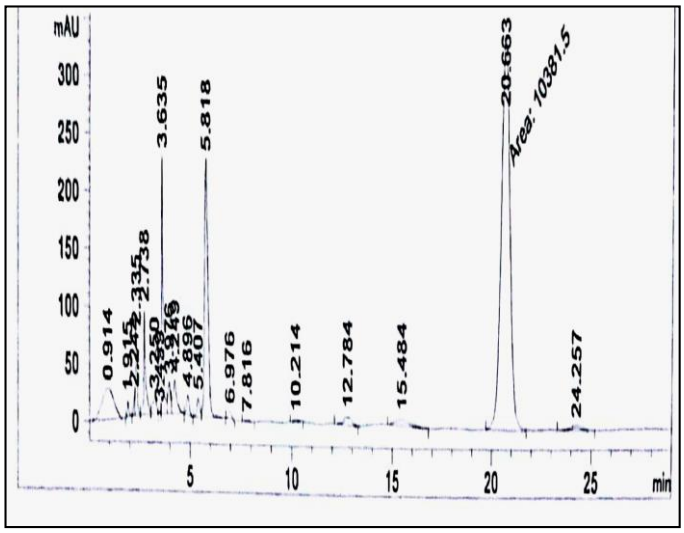

Fig 5: Chromatogram of Naringenin in sample

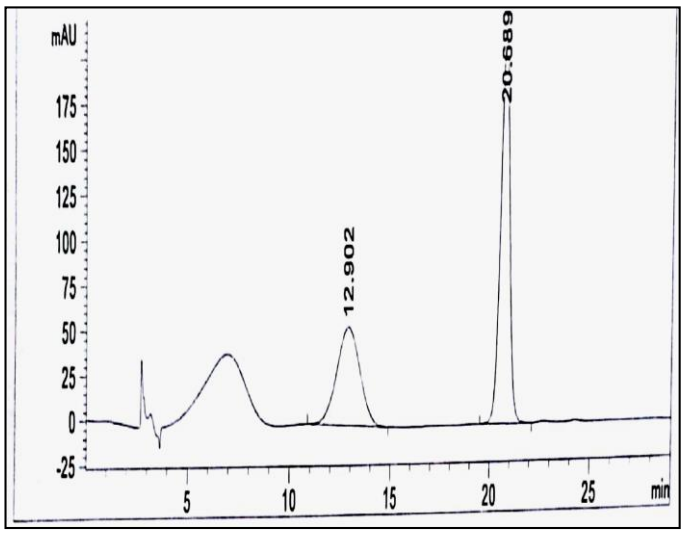

Fig 6: Chromatogram of standard Naringenin

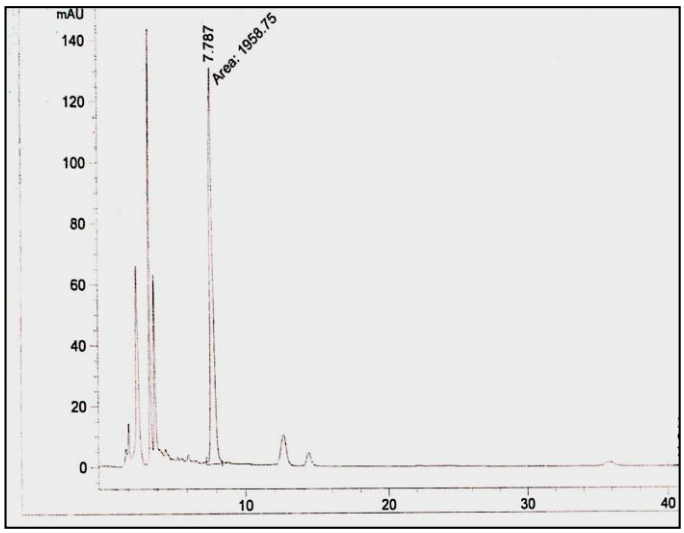

Fig 7: Chromatogram of Naringin in sample

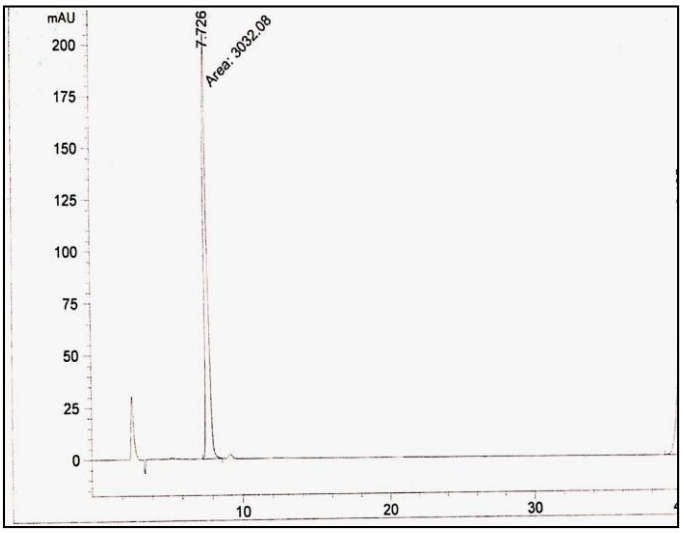

Fig 8: Chromatogram of standard Naringin

4. Conclusions

It is found that citrus fruits have abundant source of flavonoids, which can be exploited as anticancer, antibacterial and anti-inflammatory agents, indicating their essential role in the health benefits. In the present study, the flavonoid content of the peels of three different citrus fruits were determined and clear diversity was monitored. The quantitative analysis of flavonoids using spectrophotometer and HPLC, highlights the presence of good amounts of flavonones - naringin and naringenin (4.32g) in *Citrus maxima*. Overall this study provides superiority of *Citrus maxima* fruit peel as an excellent source of flavonones. Further studies on effective mechanisms by which these flavonones could protect against disease development are highly recommended.

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