Identification and quantification of polyphenolic compounds in underutilized fruits (Star fruit and Egg fruit) using HPLC

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The study was aimed to investigate the antioxidant capacity, total phenolics, total flavonoids and polyphenolic compounds of two common underutilized fruits namely, star fruit and egg fruit. Results showed that, the star fruit extract had higher total phenolics content (161.56±9.24 mg GAE/100 gm FWB) and DPPH radical scavenging ability (91.77±8.68 mg ascorbic acid equivalent antioxidant activity (AAEAA) /100 gm fresh weight basis (FWB) with low IC50 value = 0.6 mg/ml compared to egg fruit extract which had a low phenolics content (152.45±8.75 mg gallic acid equivalent (GAE) /100 gm FWB) as well as lesser DPPH radical scavenging activity (87.21±6.32 mg AAEAA/100 gm FWB) with the IC50 value = 0.88 mg/ml. Total flavonoids content was 72±2.69 and 109.73±5.91 mg quercetin equivalent (QE) /100 gm FWB for star fruit and egg fruit, respectively. Polyphenolic compounds were identified and quantified using HPLC and the data revealed the abundance of gallic acid; quercetin; ferulic acid and epi-catechin in star fruit and egg fruit extracts as well. Overall, the higher potency of star fruit in terms of radical scavenging effects is due to higher content of total phenolics that of egg fruit. In conclusion, the results obtained from our study indicates the star fruit and egg fruit are a good source of phytochemical properties, which are nutritionally prominent for their antioxidant activities and defensive functions against diseases caused by oxidative stress, therefore can be used in functional food formulations.

Keywords: Underutilized fruits, DPPH, Flavonoids, Polyphenolics, HPLC

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Fruits and vegetables are important constituents of the human diet and provide significant quantities of nutrients, especially carbohydrates, minerals, vitamins and fibre. Generally fruits are known to have plentiful amounts of different antioxidant compounds including polyphenolics, flavonoids, carotenoids, tocopherols, ascorbic acid and glutathione1. A number of epidemiological studies have found that daily consumption of fruits and vegetables are associated with lowering the incidence of many degenerative diseases such as heart diseases, cancer, premature aging, stress, inflammation, brain dysfunction, cataract and fatigue. These protective effects are mainly due to the integrated action of oxygen radical terminators (antioxidants) present in the fruits2-3. India is one of the most favorable countries for growing diversity of underutilized fruits. But these fruits are rarely eaten since the health and nutritional benefits of the fruits not familiar to the peoples. Underutilized fruit crops are the plant species that are conventionally used for food, fodder, fiber, oil or medicinal properties4. Recently many researchers have concentrated their attention on underutilized fruits as a natural source of treatment for curing various diseases and ailments5. The star fruit and egg fruit are some of the important underutilized fruits attracting the global markets currently as a natural source of spectrum of nutritional and phytochemical components6-8. Star fruit (Averrhoa carambola L.) belongs to the family Oxalidaceae and is commonly called as Carambola or Five finger fruit. It is a juicy fruit with mainly golden yellow to greenish in appearance with a succulent pulp. The star fruit is available as sweet and sour tastes9. Egg fruit (Pouteria campechiana Baehni) is one of the important tropical fruit belonging to the family Sapotaceae. It is frequently called ‘Canistel’ in English8. The flesh of the ripe egg fruit has the consistency of a hard-boiled egg yolk. So it is repeatedly referred to colloquially as Egg fruit10.

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Traditionally, almost every part of star fruit plant can be used for remedy to certain ill-health symptoms\(^\text{11}\). In India, the fresh star fruit and their juice are recommended to counteract fever. In Brazil, the star fruit tonic is used to relieve eye afflictions and as a diuretic for kidney and bladder complaints. The star fruits are used in Indian Ayurveda medicine as an anti-helminth, anti-malarial, antipyretic, digestive tonic, febrifuge, anti-scrobutic and antidote for poison\(^\text{12}\). Egg fruits are used as antipyretic in Mexico as well as to treat skin eruptions in Cuba. The egg fruit seed extracts has also been employed for treating ulcers. Many authors indicated that the egg fruit contains group of compounds, which exhibits an extensive range of biological activity including hepatoprotective action, antimicrobial, anti-inflammatory\(^\text{13}\), anti- HIV\(^\text{14}\) and anti-tumor activity\(^\text{15}\). The aforementioned medicinal properties are mainly due to different phytochemicals found in these fruits, acting singly or in concert. Therefore, the purpose of this study was to probe antioxidant potential and also quantify the major polyphenolic compounds present in star fruit and egg fruit using HPLC.

**Materials and methods**

**Plant material**

The ripened star fruit and egg fruit were acquired from Horticultural Research Station, Tamil Nadu Agricultural University, Thadiyankudisai, Kodaikanal, Tamil Nadu, India. Immediately after collecting the fruits were brought to the laboratory and kept in 4 °C before further analysis.

**Sample preparation and extraction**

The star fruit and egg fruit were sliced into small pieces (50 gm), soaked with (80% v/v) methanol at the sample to solvent ratio of 1:3 (w/v) and placed in rotary shaker for continuous stirring in 24 hrs at ambient temperature. The extracts were filtered and the filtrates were centrifuged at 8000 rpm for 15 min. The supernatant was collected and used for determination of total antioxidant activity, total phenolic and flavonoids contents\(^\text{16}\). For HPLC analysis, the supernatant was filtered through a 0.45 µm membrane filter and then the sample was injected manually into the HPLC system for analysis of polyphenolic compounds\(^\text{17-18}\).

**Determination of total phenolics content**

Total phenolics content of the methanolic extracts was determined with Folin-Ciocalteu colorimetric method\(^\text{19}\) Briefly, 0.2 ml extract was mixed with 0.5 ml of the Folin-Ciocalteu reagent previously diluted with 3 ml of de-ionised water. The content was mixed by manual shaking for 15–20 seconds. After 3 min (25 °C), 2 ml of 20 % saturated sodium carbonate solution was added. The reaction mixture was placed in water bath for one minute and cooled. The absorbance was measured at 650 nm against reagent blank using a dual beam recording UV-vis spectrophotometer (2201, Systronics, India). Gallic acid was used as a standard for the calibration curve. The total phenolics content was expressed as milligrams of gallic acid equivalents (GAE) per 100 gm of sample in fresh weight basis (FWB).

**Determination of total flavonoids content**

Total flavonoids content of the samples were measured according to the method of\(^\text{20}\) with slight modification. One ml of extract was added with 0.3 ml of 5 % sodium nitrite and well mixed. After 5 min of incubation, 0.3 ml of 10 % aluminium chloride (AlCl\(_3\)) solution was added. Then after 6 min 2 ml of 1M NaOH was added to the mixture and made up the volume to 10 ml using distilled water. The absorbance was measured at 510 nm against reagent blank using UV-vis spectrophotometer. A standard curve was prepared using different concentration (20-100 µg / ml) of Quercetin. The total flavonoids contents were expressed in milligrams quercetin equivalent (QE) per 100 gm of sample in fresh weight basis (FWB).

**Determination of radical scavenging activity**

The radical scavenging activity of the fruit extracts against stable DPPH radical was determined according to the method of Lim \textit{et al.}\(^\text{21}\). The L-ascorbic acid was used as the reference compounds. In this method, sample extracts were prepared in methanol was mixed with 3 ml (0.1mM) of DPPH and incubated in dark for 30 min. After 30 min, the absorbance was measured at 517 nm using UV-vis spectrophotometer. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound. The percentage of radical scavenging activity (% RSA) or percentage inhibitions of DPPH of the methanolic extract of the samples were calculated by the following formula:
% RSA = \frac{[A_{(C)} - A_{(S)}]}{A_{(C)}} \times 100

Where, \( A_{(C)} \) = absorbance of negative control, \( A_{(S)} \) = absorbance of sample

Inhibition concentration 50 % (IC \(_{50}\)) is defined as the amount of antioxidant required to inhibit 50 % of DPPH free radicals under the experimental conditions. Antioxidant activity is inversely proportional to IC \(_{50}\) value. Ascorbic acid was used as a standard. The graph was plotted between the percentage of radical scavenging activity and the different concentrations of standards. The IC \(_{50}\) values for the standards were calculated and compared with IC \(_{50}\) values of samples. Then the slopes of the standard graph were calculated and the radical scavenging activity of the samples was expressed as milligrams of ascorbic acid equivalent antioxidant activity (AAEAA) per 100 gm of sample in fresh weight basis (FWB). This was calculated by means of the equation given below\(^{22}\):

\[
\text{RSA} = \frac{(SA - BA)}{\text{Slope}} \times \frac{T}{U} \times \frac{100}{(1 - M/100) \times 1000}
\]

SA : Sample absorbance
Slope : Slope of the standard curve
BA : Blank absorbance
T : Total volume of the extract (ml)
U : Used volume of the extract (ml)
W : Weight of used sample (gm)
M : Moisture (%) content of the sample
1000: Factor for changing µg to mg

HPLC analysis of polyphenolic compounds

Simultaneously, 6 polyphenolic compounds such as Quercetin, Gallic acid, Ferulic acid, Caffeic acid, Catechin and Epi-catechin by HPLC method in methanol extracts of fresh star fruit and egg fruit were determined based on the procedure reported by Sun et al.\(^{23}\). The separation of phenolic compounds was performed on a HPLC system Jasco Modal 1500 equipped with Jasco PU-1580 intelligent pump and Jasco FP-2010 plus multiwavelength UV-VIS detector. Polyphenolic compounds were separated by using 250 mm x 4 mm id., 5 µm diameter, waters sunfire C18 column. Chromatograms were analyzed with the Jasco BORWIN software. The mobile phase used for the study was methanol, water and formic acid in the ratio of 80: 20: 1 and flow rate was 0.8 ml min\(^{-1}\) in isocratic mode. The column temperature was maintained at 30 °C. Detection wavelength was 280 nm. Injection quantity of the standard and extracts were 20µL. Identification of polyphenolic compounds was performed by comparing their retention times and UV spectral matching with those of their corresponding reference standards. Quantification was made from peak area of the each component against the standard curve obtained specifically for the reference solution containing that compounds. The standards, gallic acid, caffeic acid, ferulic acid, (+)-catechin, (-)- epi-catechin and quercetin were obtained from Sigma (St. Louis, MO, USA) and determined in the methanolic extracts of the samples.

Statistical analysis

All the experiments were conducted in triplicate and the mean and standard deviation (± SD) were calculated using MS Excel software. Differences are estimated by the analysis of variance (ANOVA). Differences were considered to be significant at P<0.05.

Results and discussion

Total phenolics content

The phenolics constitute one of the important groups of compounds acting as primary antioxidants or free radical terminators in plant spices, which are produced by plants as secondary metabolites. It has stronger free radical scavenging effects than those of vitamin C & E. It is also widely distributed in all fruits and vegetables. So, it must reasonable to determine their total amount in the fruit extracts\(^{24}\). Tanaka et al.\(^{25}\) suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1gm daily from a diet rich in fruits and vegetables. In the present study, a variation in total phenolics content was shown in Table 1. The highest total phenolic content was observed for star fruit (161.56 mg GAE / 100 gm FWB) in the current investigation may be due to high content of polyphenolic and flavonoids compounds especially quercetin, catechin, epi-catechin and ferulic acid. Whereas the egg fruit contained 152.45 mg GAE of total phenolics content / 100 gm FWB, it is slightly lower compared to star fruit. The lowest phenolic content of the egg fruit is mainly due to presence of low polyphenolic and flavonoids compounds. The results of the present study agreed with that of\(^{26}\) who reported that the star fruit had considerable amount of total phenolics content.
Table 1—Phytochemical properties of star fruit and egg fruit

<table>
<thead>
<tr>
<th>Phytochemical properties</th>
<th>Star fruit</th>
<th>Egg fruit</th>
<th>Mean</th>
<th>CD at 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total antioxidant activity (mg AAEAA / 100 gm FWB)</td>
<td>91.77±8.68</td>
<td>87.21±6.32</td>
<td>89.49±7.50</td>
<td>1.680</td>
</tr>
<tr>
<td>Total phenolic content (mg GAE / 100gm FWB)</td>
<td>161.56±9.24</td>
<td>152.45±8.75</td>
<td>157.01±8.99</td>
<td>3.561</td>
</tr>
<tr>
<td>Total flavonoids content (mg QE / 100gm FWB)</td>
<td>72.00±2.69</td>
<td>109.73±5.91</td>
<td>90.87±4.30</td>
<td>2.108</td>
</tr>
</tbody>
</table>

AAEAA-Ascorbic Acid Equivalent Antioxidant Activity, GAE- Gallic Acid Equivalent, QE- Quercetin Equivalent, FWB- Fresh Weight Basis

Zainudin et al.\textsuperscript{17} found significant amount of total phenolics content (117.72 mg GAE / 100 gm FWB) in fresh star fruit. Yan et al.\textsuperscript{27} identified highest amount of total phenolics content in \textit{Averrhoa carambola} L. (1296.25 ± 14.74 mg GAE / 100 gm DWB) compared to \textit{Averrhoa bilimbi} (629.17 ± 14.38 mg GAE / 100 gm DWB). Kong et al.\textsuperscript{8} reported that the 70 % methanol extracts of egg fruit pulp had extensive amount of total phenolics content (1209.96 mg GAE / gm DWB).

**Total flavonoids content**

Flavonoids are colouring pigments, which mainly present in plants in the form of diphenylpropanes (C6-C3-C6) and are an integral part of the human diet. It act as powerful antioxidants at various levels and could protect membrane lipids from oxidation\textsuperscript{27-28}. The total flavonoids content in methanolic extract of these fruits varied from 72 to 109.73 mg QE / 100 gm FWB (Table 1). The present investigation showed higher amount of total flavonoids content (109.73 mg QE / 100 gm FWB) in egg fruit extract while lesser amount was found in star fruit extract (72 mg QE / 100 gm FWB). This might be due to presence of high total carotenoids. This was confirmed by\textsuperscript{29} who quantified the important compounds of carotenoids pigment like β-cryptoxanthin (1106 µg / gm FWB) and neoxanthin (19270 µg / gm FWB) in egg fruits using HPLC. They also acknowledged both compounds were playing major role in antioxidant activity of egg fruit. However Kong \textit{et al.}\textsuperscript{8} also found 6414.03 mg rutin equivalent of total flavonoids content per 100 gm egg fruit pulp in dry weight basis (DWB).

**Radical scavenging activity**

The DPPH IC\textsubscript{50} values (IC\textsubscript{50} value is the concentration of the sample required to inhibit 50 % of radical) of the star fruit and egg fruit extracts were calculated and are presented in Figs. 1 & 2. The lesser IC\textsubscript{50} value showed that the extracts had superior free radical scavenging activity. In the present study, the low IC\textsubscript{50} value (0.6 mg / ml) was found in star fruit followed by egg fruit (0.88 mg / ml). The differences in the radical scavenging activity among the fruits could be accredited to their differences in phenolic and non-phenolic content and composition. The data obtained in this study revealed that (Table 1) the potent radical scavenging activity (91.77 mg AAEAA / 100 gm FWB) of the star fruit is attributed to its high total phenolics content. These results are accordance with the findings of several authors\textsuperscript{30}. Whereas the egg fruit had slightly lower radical scavenging potential (87.21 mg AAEAA / 100 gm FWB) which may be due to low content of total phenolics and
polyphenolic compounds. The correlation of IC$_{50}$ with potential radical scavenging activity was confirmed that the star fruit was a better radical scavenger followed by egg fruit. However, the overall radical scavenging activity does not follow the order of total phenolics and flavonoids content. It was an agreement with a study by Lim et al.$^{21}$ whereby reported that the star fruit was a very strong radical scavenger (98 mg AEAC / 100 gm FWB). On the other hand, Mahattanatawee et al.$^{31}$ studied antioxidant activity of select Florida grown tropical fruits where they found carambola (star fruit) and red guava had the highest antioxidant activity compared to sapodilla and green papaya. Zainudin et al.$^{17}$ cited that the star fruit had significant amount of IC$_{50}$ value (1.31 ± 0.53 mg / ml) using DPPH assay. Shofian et al.$^{26}$ reported that the fresh star fruit extract at the concentration of 5 mg / ml had 92 % of total antioxidant activity in DPPH method. Kubola et al.$^{4}$ reported that the freeze dried egg fruit powder contained 1.0 mg ascorbic acid equivalent antioxidant activity/gm sample. Which indicating the star fruit and egg fruit had significant free radical scavenging activity.

**HPLC results**

In the present study, 6 polyphenolic compounds have been investigated by HPLC in methanolic extract of star fruit and egg fruit. All the 6 polyphenolic compounds were found significant amount in the methanolic extracts of star fruit and egg fruit. The detected polyphenolics compounds were enumerated in the order of their retention time. The quantitative determination was performed using chromatogram (peak) of corresponding standards. The HPLC chromatogram of the mixture of 6 standards (gallic acid; ferulic acid; caffeic acid; epi-catechin; catechin; quercetin) shown in Fig 3. Among the 6 polyphenolic compounds, the quercetin, ferulic acid and epi-catechin was found to be the predominant (38.01 mg, 17.42 mg and 16.32 mg / 100 gm FWB) polyphenolic compounds in star fruit. Gallic acid (3.78 mg / 100 gm FWB) and caffeic acid (5.01 mg / 100 gm FWB) were found in significant amounts. Also the catechin (1.33 mg / 100 gm FWB) was identified, but the concentration was too low to be quantified (Fig. 4 & Table 2). However, Shui & Leong$^{32}$ reported that star fruit juice was superior source of antioxidant activities, which it is attributed to L-ascorbic acid (80.5 ppm), (-)-epi-catechin (73.1 ppm) and gallic acid (19.5 ppm) in gallotannin forms.

![Fig. 3—HPLC chromatogram at 280nm of the mixture of six standards](image3.png)

![Fig. 4—HPLC chromatogram at 280nm of the star fruit (Averrhoa carambola L.)](image4.png)

In the extract of egg fruit, the great amount of gallic acid and quercetin (15.35 mg and 14.78 mg / 100 gm FWB) were identified, both compounds functioning as a chief polyphenolic compounds in the egg fruit. The other polyphenolic compounds such as ferulic acid (6.76 mg / 100 gm FWB), caffeic acid (4.62 mg / 100 gm FWB), catechin (3.62 mg / 100 gm FWB) and epi-catechin (3.57 mg / 100 gm FWB) were also found at quantified levels (Fig. 5 & Table 2). Ma et al.$^{18}$ were determined the polyphenolic compounds from the fruits of three Pouteria species by HPLC and LC-MS. They reported that the gallic acid (16.85 ± 0.09 ppm), (+)-gallocatechin (5.62 ± 0.13 ppm) and (+)-catechin (1.04 ± 0.05 ppm) were the major polyphenolic compounds found in the three Pouteria species. Kubola et al.$^{4}$ also found myricetin (55.67 mg / gm DWB), quercetin (23.15 mg / gm DWB) and rutin (20.64 mg / gm DWB) were the prominent polyphenolic compounds in egg fruit. Same trend was also obtained in the present investigation too. Among the star fruit and egg fruit, the star fruit had virtually highest amount of
Table 2—Composition of polyphenolic compounds (mg / 100gm FWB)

<table>
<thead>
<tr>
<th>Underutilized fruits</th>
<th>Gallic acid</th>
<th>Ferulic acid</th>
<th>Caffeic acid</th>
<th>Epi-catechin</th>
<th>Catechin</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Star fruit</td>
<td>03.78±0.31</td>
<td>16.32±1.56</td>
<td>05.01±0.42</td>
<td>17.42±1.75</td>
<td>01.33±0.04</td>
<td>38.01±2.16</td>
</tr>
<tr>
<td>Egg fruit</td>
<td>15.35±1.43</td>
<td>6.76±0.65</td>
<td>4.62±0.37</td>
<td>3.57±0.28</td>
<td>3.62±0.16</td>
<td>14.78±1.32</td>
</tr>
<tr>
<td>CD at 5%</td>
<td>0.249</td>
<td>0.280</td>
<td>0.113</td>
<td>0.278</td>
<td>0.660</td>
<td>0.642</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>5.16</td>
<td>8.84</td>
<td>11.09</td>
<td>10.64</td>
<td>16.09</td>
<td>23.94</td>
</tr>
</tbody>
</table>

The retention times indicate the differences in the complexity of the polyphenolic profile between the star fruit and egg fruit, with the star fruit having a higher retention time for many compounds, suggesting a more complex profile. The CD values at 5% further suggest that the compounds are well separated and identifiable.

Fig. 5—HPLC chromatogram at 280nm of the egg fruit (Pouteria campechiana Baehni)

6 polyphenolic compounds (gallic acid; ferulic acid; caffeic acid; catechin; epi-catechin; quercetin) compared to egg fruit which may be due to the synergistic effects of total phenolics and polyphenolic compounds of the extract.

Conclusion
It can be concluded from this investigation that the selected star fruit and egg fruit have potentially good antioxidant efficacy. Among the methanolic extracts examined, the star fruit extract had the higher total phenolics content and radical scavenging ability with lower IC_{50} value. The free radical neutralizing and scavenging effect of star fruit was attributed to its high content of total phenolics and polyphenolic compounds. The radical scavenging activity of egg fruit was also found significant with highest amount of total flavonoids content. The HPLC results indicated that the star fruit and egg fruit contains polyphenolic compounds such as, gallic acid, caffeic acid, ferulic acid, catechin, epi-catechin and quercetin. Nevertheless when see in the line of collective polyphenolic capacity, the star fruit had highest amount than egg fruit. The correlation between radical scavenging activity and polyphenolic compounds indicates that the cumulative polyphenolic compounds are responsible for strengthening antioxidant capacity in star fruit. The present study has set a platform for the futuristic research towards the mode of action of these polyphenolic compounds at cellular and molecular levels. Overall, results of our study indicates that these fruits have wide scope for being utilized as functional foods in the development of various food formulation towards combating oxidative-stress relates health problems.

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Reference