Chapter-4

Extraction, identification and antioxidant properties of bioactive components of Amla (*Emblica officinalis*) pomace powder
4.1. Introduction

Oxidative stress, induced by oxygen radicals is one of the major causative factors of various degenerative diseases, such as cancer,$^{1}$ atherosclerosis,$^{2}$ gastric ulcer,$^{3}$ and other conditions.$^{4,5}$

The strong antioxidant activity of the phenolics and their ability to protect cells against oxidative damage caused by free radicals are well established.$^{6}$ Due to the presence of conjugated ring structures the hydroxyl group of many phenolic compounds has the potential to function as singlet oxygen$^{7}$ and as antioxidants by scavenging superoxide anions$^{8}$ and lipid peroxy radicals$^{9}$.

Phenolics are the major chemical constituents of Amla$^{10}$ and these substances have strong antioxidant property and might contribute to the healthy affects of Amla. Several active compounds like ellagic acid, gallic acid, 1-O-galloyl-D glucose, chebulinic acid, quercetin, chebulagic acid, kaempferol, mucic acid, 1,4-lactone 3-O-gallate, isocorilagin, chebulanin, mallotusinin, acylated apignin glucoside compounds etc. have been isolated from the aqueous extract of Amla$^{11-14}$. Amla pomace is a major processing waste of Amla juice industries has never been investigated for its bioactive properties. The objective of present investigation is to characterize the polyphenols present in Amla pomace by analytical HPLC, GC-MS and FTIR.

4.2. Materials and methods

4.2.1. Raw material

*Chakaiya* variety of Amla was procured from local market of Allahabad, India. Grated Amla shreds were pulped in laboratory mixer and the juice was extracted by using double fold muslin cloth. Pomace left after the extraction of the juice was dried at 40°C and ground into powder in a laboratory mill by passing 0.5 mm sieve. The pomace powder was stored at refrigerated temperature for further extraction and purification of phenolics. On an average 0.58±0.02 kg of pomace powder was obtained from 5 kg of fresh Amla fruit. Image of Amla pomace powder is given in Fig. 4.1.

4.2.2. Solvents and reagents

Ethyl acetate, ethanol, butanol, Folin-Ciocalteu reagent, sodium carbonate, HPLC grade methanol and orthophosphoric acid were purchased from Merck.
Standards of caffeic acid, gallic acid, catechin, quercetin, syringic acid, chlorogenic acid, vanillic and p-coumaric acid, BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) and 5% DMDCS (dimethyldichlorosilane) in toluene were procured from Sigma Aldrich.

4.2.3. Purification and extraction of total phenolics from Amla pomace powder

The phenolics in 30g of Amla pomace powder was extracted (under optimised conditions of extraction from Amla powder as reported in Chapter-3) in 300 mL of ethanol (78%) by shaking in an incubator shaker for 4 h at 30.50°C and 4.5 pH. The image of ethanol extract of Amla pomace powder is shown in Fig 4.2a. The extract was centrifuged in refrigerated centrifuge at 4°C and 5687 g for 15 min and supernatant was collected. The residue was re-extracted and then further centrifuged. Both the collected supernatants were combined and evaporated in a rotary evaporator under reduced pressure followed by freeze drying under vacuum. Ethanolic extract of pomace powder (8.3 g) was dissolved in 40 mL of water and was partitioned with ethyl ether (100 mLx3), ethyl acetate (100 mLx3) and butanol (100 mLx3) successively. All four fractions were evaporated separately under reduced pressure at 45°C for ethyl acetate and ethyl ether fraction and at 65°C for butanol and aqueous
fraction as per the method of Liu et al. (2012)\(^\text{15}\) and freeze dried under vacuum. Images of ethyl acetate extracted Amla pomace powder and butanol extracted powder are shown in Fig 4.2b & c. Ethyl acetate extract powder was light golden yellow color while butanolic extract powder was of light reddish yellow in color. The yields of ethyl ether, ethyl acetate, butanol and aqueous fractions were 0.25 g, 4.83 g, 3.45 g and 2.50 g, respectively. Ethyl acetate, butanol, aqueous and ethyl ether fractions were analyzed for their TPC content and %DPPH* scavenging activity. As ethyl acetate fraction showed highest DPPH* scavenging activity, it was further purified by fractionation on a Sephadex G-25 (25-100 µm, Sigma Aldrich) column (450 x 25 mm) with water/methanol (100:0-0:100) as the eluent and obtained six different fractions (I-VI). All six fractions were evaluated for characterization of the phenolics present by HPLC and FTIR.

4.2.4. Fourier Transform Infrared Spectroscopy (FTIR)

All extracted powders and different fractions obtained after column chromatography were scanned in the range of 4000-650 cm\(^{-1}\) with a resolution of 4/cm\(^{-1}\). Attenuated total reflection/Fourier transform infrared spectroscopic (ATR/FTIR) spectra was collected at room temperature by coupling ATR accessory to an FTIR spectrometer (Perkin Elmer, Spectrum 100). The time required to complete background was 20 s. Average of 3 scans were collected\(^{16}\).

4.2.5. Total phenolic content

Estimation of total phenolic content was performed by both Folin-Ciocalteu reagent (FCR) method and HPLC method. Estimation of FCR was conducted by the method of Liu et al. (2008)\(^\text{17}\) with some modifications. Briefly, 60µL of extract (in methanol), 300 µL of Folin-Ciocalteu reagent and 900 µL of 20% sodium carbonate were added to 4.75 mL of water. The mixture was allowed to stand for 30 min at 37°C. The absorbance was noted at 765 nm and the results were expressed as mg of gallic acid equivalent (GAE). For estimation of total phenolic content of extracted powder, 50 mg of extracted powder was mixed with 5 mL of methanol and the mixture was stirred for 30 min at 25°C. The stirred mixture was centrifuged and supernatant was analyzed for its total phenolic content as described above.
Extraction, identification and antioxidant properties of bioactive components of Amla (Emblica officinalis) pomace powder

Fig. 4.2 Images of extracted Amla pomace powder (a) ethanol extracted Amla pomace powder, (b) powder of Amla pomace powder partitioned with ethyl acetate, and (c) powder of Amla pomace powder partitioned with ethyl acetate.
Analytical HPLC was conducted on a Waters (Breeze-2) liquid chromatography fitted with a C-18, reversed phase (5 µm) column (7.2 x 300 mm) following the method of Seruga et al. (2012)\textsuperscript{18} with slight modifications. In short, orthophosphoric acid (0.1%) was taken as solvent A and 100% HPLC grade methanol was used as solvent B for the separation of phenolics. The elution conditions used for the identification of phenolics were as follows: 0-30 min from 5% B to 80% B; 30-33 min 80% B; 33-35 min from 80% B to 5% B; 35-40 min at 5% B; flow rate 0.8 mL/min. Column temperature and injection volume were kept constant at 20°C and 20µL, respectively. The standards of gallic acid, quercetin, catechin, caffeic acid, syringic acid, chlorogenic acid, and p-coumaric acid were estimated at two different wavelengths, i.e. at 280 nm and 360 nm. Total area under curve at 280 nm was calculated as total phenolic content of the analyzed sample.

For identification of isolated components, 0.1 mg of the sample was dissolved in one mL of HPLC grade methanol and 20 µL of the sample was injected in HPLC with above prescribed method and obtained peaks were compared with the peak of standard components.

4.2.6. DPPH\textsuperscript{*} scavenging activity

The DPPH\textsuperscript{*} scavenging activity of extract of powder was determined by the method of Luo et al. (2009)\textsuperscript{14} with slight modifications. Briefly, 50 mg of the extracted powder were dissolved in 5 mL of methanol solution and shaken in an incubator shaker at 150 rpm at 25°C for 30 min. The mixture was filtered and 2 mL of methanolic extract was mixed with 2 mL of methanolic solution containing 0.1 mM DPPH. The mixture was shaken vigorously and then left to stand for 30 min in the dark. The absorbance was taken at 517 nm. The absorbance of control was measured by replacing the sample with methanol.

\[
\text{DPPH}^* \text{ radical scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100
\]

4.2.7. Assay of ABTS\textsuperscript{+} scavenging activity

Antioxidant activity of extracted powder from Amla pomace was measured by the method of Cai et al. (2004)\textsuperscript{19} and Re et al. (1999)\textsuperscript{20}. The ABTS\textsuperscript{+} cation (ABTS\textsuperscript{+}) solution was prepared by the reaction of 7 mM ABTS and 2.45 mM sodium persulphate, and kept for incubation at room temperature for 12 h in dark. The ABTS\textsuperscript{+}
solution was then diluted with methanol to obtain an absorbance in the range of 0.75±0.03 at 734 nm. For the experiment 1.5 mg of extracted powder was dissolved in 10 mL of methanol before analysis, 2mL of the dissolved sample or trolox standard (2 mL) were added to 2 mL of ABTS$^+$ solution and mixed vigorously. The reaction mixture was kept at room temperature for 6 min and the absorbance at 734 nm was immediately recorded. A standard curve was obtained using trolox standard solution at various concentrations (ranging from 0 to 20 µg/mL) in methanol. Antioxidant properties of samples were expressed as trolox equivalent antioxidant capacity (TEAC). The results were compared with the standard curve for calculation of antioxidant activity$^{21}$.

4.2.8. Preparatory HPLC

Fraction V had maximum DPPH* scavenging activity but recovery was very less hence, fraction III the second highest DPPH* radical scavenging activity was selected for further purification. Fraction III (170 mg) was further purified by reverse-phase HPLC (Waters 600E, Breeze-2, Milford, USA) on a C$_{18}$ l-Bondapak column (300 x 7.8 mm, flow rate = 1.5 mL/min) with methanol/water (40:60) for 40 min to yield pure compounds E1–E3.

4.2.9. UV–Vis spectrophotometric analysis

Each of isolated compounds, E1-E3 (1 mg) was dissolved in 10 mL of methanol. The sample solution was scanned from 200 to 750 nm, using a UV–Vis spectrophotometer (Evolution, Thermofisher, USA).

4.2.10. Derivatization for GC-MS

Isolated component E-1 could not be determined by HPLC hence isolated component was further derivatized for identification through GC-MS. The derivatization and identification by GC-MS was carried out by the method given by Proestos et al. (2006)$^{22}$ with slight modification. For the silylation procedure, 100 µL of BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide) was added in 0.01 mg of sample (sensitivity of analytical balance 0.001 mg) and vortexed in screw-cap glass tubes (previously deactivated with 5% dimethylchlorosilane in toluene, and rinsed twice with toluene and thrice with methanol), and consecutively placed in a water bath, at 80°C for 45 min. The silylated samples were injected into a GC-MS system of Perkin Elmer, model Clarus 600 gas chromatograph coupled 600C mass spectrometer.
and the mass range scanned at m/z 25–500. A capillary column Elite 5 (30 m x 0.32 mm) was used for the identification. The injector and detector were set at 280°C and 290°C respectively. GC was performed in the split mode. The temperature programme was as follows: from 70° to 135°C at 10°C/min, from 135° to 220°C at 15°C/min, from 220° to 270°C at 10°C/ min and then held for 10 min. The flow rate of carrier gas (helium) was maintained at 1.9 mL/min. Identification of compound was achieved by comparing the spectral data obtained from the NIST libraries.

4.2.11. Statistical analysis

All experiments for extraction and purification were performed in duplicate however the analyses were performed in triplicates and the mean value and standard deviation were calculated using Excel version of 2003.

4.3. Results and discussion

4.3.1. Extraction of phenolics content

Fig 4.3 presents the HPLC chromatogram of Amla pomace powder at (a) 280 nm and (b) 360 nm which show the presence of significant amount of total phenolic content in pomace powder that were not extracted in juice. Some amount of gallic acid was found to be available in the pomace. The total phenolic content in Amla pomace powder was 95 mg/g and gallic acid was 9.77 mg/g (Fig 4.3a).

![HPLC chromatogram of Amla pomace powder](image)

(a)

(b)

Fig 4.3 HPLC chromatogram of Amla pomace powder (a) at 280 nm, and (b) at 360 nm. (1) Gallic acid.
Ethanol extracted Amla pomace powder weighing 8.32 g was dissolved in 40 mL of water and then partitioned with ethyl ether, ethyl acetate and butanol. All four fractions were evaluated for their DPPH\(^*\) scavenging activity. Diethyl ether fraction showed very poor scavenging activity and therefore was not taken for further studies. The present finding was in accordance with the observation of Roby et al. (2013)\(^{23}\) that diethyl ether had much lower ability in extracting the phenolic compounds.

**Table 4.1** presents the bioactive properties of ethyl acetate, butanol and aqueous fractions. The amount of total phenolic content varied in different extracts and ranged from 43.20 to 119.50 mg GAE/g of extracted powder (**Table 4.1**). One mg of ethyl acetate fraction showed 14.29% DPPH\(^*\) scavenging activity, whereas butanol and aqueous fractions had 8.85% and 4.55 %DPPH\(^*\) radical scavenging activities, respectively for the same amount of the powder (**Table 4.1**). The less potency of free radical scavenging activity of aqueous fraction of Amla pomace powder is also corroborated by the low amount of TPC as estimated by the HPLC method (**Fig 4.4a**) and FCR method (**Table 4.1**). The aqueous fraction had 43.20 mg/g GAE equivalent by HPLC method (**Fig. 4.4a**) and 121.32 mg/g GAE equivalent by FCR method (**Table 4.1**). On the other hand, ethyl acetate fraction of Amla pomace powder had 98.80 mg/g GAE equivalent by HPLC method (**Fig 4.4b**) and 389.65 mg/g GAE equivalent by FCR method (**Table 4.1**) and butanol fraction had 119.50 mg/g GAE equivalent by HPLC method (**Fig 4.4c**) and 264.33 mg/g GAE equivalent by FCR method (**Table 4.1**).

When analyzed by HPLC, butanolic fraction of Amla pomace powder had significantly higher amount of phenolic content than the ethyl acetate fraction and aqueous fraction but the reverse trend was observed in case of FCR method. FCR method used for determination of total phenolic content gave considerably high value of phenolics than determined by HPLC method (**Table 4.1**). FCR method was not found suitable for the total phenolics estimation because the FCR reagent reacts not only with phenolics but also with a number of non-phenolic reducing compounds such as tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents because of which the total phenolic content is overestimated\(^{24}\). Different phenolics can also react differently with the Folin-Ciocalteu’s reagent, which results in lower absorption that underestimates the concentration of various compounds\(^{25}\).
So, further modification and validation of FCR method is required to correct the error as it is widely used in routine analysis for the estimation of total phenolic contents.

Inspite of having high amount of total phenolic content as determined by HPLC, the DPPH* scavenging activity of the butanolic extracts was comparatively lower than ethyl acetate extract fraction. One probable reason may be the high boiling point of butanol. The extract was exposed to higher temperature to remove the solvent which may adversely affect the availability of functional groups to form complexes with phosphomolybdate and hence butanolic extract showed less % DPPH* radical scavenging activity.

### Table 4.1 Bioactive component and DPPH* scavenging activity of aqueous, ethyl acetate, and butanol fractions of Amla pomace powder

<table>
<thead>
<tr>
<th>Particular</th>
<th>Aqueous fraction</th>
<th>Ethyl acetate fraction</th>
<th>Butanol fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Yield</td>
<td>22.66±1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.78±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.27±1.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% DPPH* scavenging activity/mg of powder</td>
<td>4.55±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.29±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.85±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TPC by FCR (mg/g of GAE)</td>
<td>121.32±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>389.65±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>264.33±2.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TPC by HPLC (mg/g) GAE</td>
<td>43.20±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.80±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>119.50±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gallic acid (mg/g)</td>
<td>ND</td>
<td>8.81±0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Catechin (mg/g)</td>
<td>ND</td>
<td>9.70±0.2</td>
<td>ND</td>
</tr>
<tr>
<td>Caffeic acid (mg/g)</td>
<td>ND</td>
<td>0.52±0.1</td>
<td>ND</td>
</tr>
<tr>
<td>Quercetin (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>p-Coumaric acid (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Syringic acid (mg/g)</td>
<td>ND</td>
<td>3.60±0.1</td>
<td>15.60±0.2</td>
</tr>
<tr>
<td>Vanillic acid (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>3.74±0.2</td>
</tr>
<tr>
<td>Chlorogenic acid (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mean ± standard deviation

Observation with different superscript in the same row differs significantly at 5% probability level
Fig. 4.4. HPLC chromatograms of (a) aqueous fraction, (b) ethyl acetate fraction, and (c) butanol fraction. (1) Unidentified, (2) gallic acid, (3) catechin, (4) syringic acid, (5) caffeic acid, (6) vanillic acid, and (7) syringic acid.
The low free radical scavenging activity of the aqueous extract of Amla pomace powder may be because of the phenolics left behind in the pomace after juice extraction were either present in bound form or had better affinity to solvents like ethyl acetate and butanol than water. So, during partitioning higher fractions of the phenolics went either in the ethyl acetate or butanol fraction. The present results suggested that the phenolic compounds in ethyl acetate fraction had maximum DPPH* radical scavenging activity and therefore ethyl acetate fraction was subjected to further purification and identification.

**4.3.2. Identification of phenolics**

**4.3.2.1. Reverse phase high performance liquid chromatography (HPLC)**

Amla fruit is known to contain a range of secondary metabolites such as polyphenols and flavonoids but the availability of phenolic constituents in Amla pomace powder have never been investigated. Therefore ethyl acetate extract, butanolic extract, aqueous extract and the different fractions of Amla pomace powder obtained after column chromatography of ethyl acetate fraction were qualitatively and quantitatively analyzed by reverse phase HPLC. The bioactive properties of HPLC chromatograms of above three fractions are given in Fig 4.4. Ethyl acetate fraction gave maximum recovery i.e. 43.78% (of initial amount of ethanol extracted powder taken for the partition) whereas diethyl fraction powder gave the lowest recovery. The present findings were in agreement with the findings of Liu et al. (2008)\textsuperscript{17}. Table 4.1 shows that the major polyphenols contained in ethyl acetate fractions were gallic acid (8.81 mg/g, rt 10.09), catechin (9.70 mg/g, rt 15.94 min), caffeic acid (rt 19.48 min, 0.52 mg/g) and syringic acid (3.6 mg/g, rt 19.01). Butanol fraction had syringic acid (15.6 mg/g, rt 19.01,) and vanillic acid (3.74 mg/g, rt 18.51 ). The main phenolic compounds in ethyl acetate fraction of pomace powder were catechin (9.70 mg/g) and gallic acid (8.81 mg/g) (Table 4.1). p-Coumaric acid and quercetin could not be detected in any of the three fractions tested. Presence of major portion of the phenolics in ethyl acetate fraction confirms the finding of Liu et al. (2008)\textsuperscript{17} that the compounds with relatively high antioxidant activity and phenolics are present in ethyl acetate fraction. Six different ethyl acetate fractions obtained after column chromatography were further lyophilized before analysis. The % recovery of all six
fractions (in respect of initial weight of sample taken for Sephadex column chromatography) i.e. I, II, III, IV, V and VI were 20.59%, 40.37%, 27.04%, 12.20%, 1.48% and 1.09% respectively (Table 4.2). It could be noticed from the results that the highest extract yield was obtained in fraction II followed by III, I and IV, V and VI. Inspite of having good recovery of total phenolics in fraction II, the DPPH* scavenging activity of fraction II was significantly less than fraction III, IV and V suggesting the low availability of compounds having free radical scavenging activity low in that fraction (Fig 4.5). Fraction V showed exceptionally good free radical scavenging activity with a mean value of 149.86% but due to only 1.48% of recovery of powder it may not be economical for the extraction of the phenolics. Fig 4.6 presents the HPLC chromatograms of fraction III, IV and V of pomace powder. It was found that catechin with 424.92 mg GAE/g and gallic acid with 17.15 mg GAE/g concentration were eluted in fraction III (Table 4.2; Fig 4.6b) and this may explain the potency of this fraction to scavenge the DPPH*. Syringic acid (18.95 mg/g) and caffeic acid (10.19 mg/g) (Fig 4.6c) were eluted in fraction IV (Table 4.2) whereas 4.24 mg/g of syringic acid was eluted in fraction V. The high value of % DPPH* scavenging activity of fraction V indicates the presence of some phenolic compounds which have not been identified. Since the extraction and purification were carried out in duplicate hence further investigation may be carried out in future to reconfirm our findings.

4.3.2.2. FTIR analysis

Fig 4.7 presents the FTIR spectra of different fractions of Amla pomace powder. It could be seen from Fig 4.7 that peak intensity of aqueous fraction was less than ethyl acetate and butanol fractions. Significant drop in functional groups were also observed in aqueous fraction which may explain the low DPPH* scavenging activity of the aqueous fraction Noticeably the presence of gallic acid peak at 3377, 1703, 1517, 1254 cm<sup>-1</sup>, 1100 cm<sup>-1</sup> and 1025 cm<sup>-1</sup> (Nirmaladevi et al., 2010) were observed in ethyl acetate fraction at slightly different locations and confirms the presence of gallic acid in ethyl acetate fraction of Amla pomace powder. In aqueous and butanolic fractions the significant drop in peaks below 750 cm<sup>-1</sup> region were observed. Presence of peak below 750 cm<sup>-1</sup> region in ethyl acetate fraction suggested the presence of disubstituted, meta, para or ortho hydrocarbons in ethyl acetate fraction. Significant drop in functional groups of fraction I was observed from Fig 4.7 whereas fraction V showed comparatively higher peak intensity of the
functional groups which is substantiated by the potent ability of the fraction V to scavenge DPPH*. FTIR spectra also suggests the presence of some other phenolic compounds not yet detected in the present study. However, as the recovery of fraction V is very low it can not be recommended for further purification or extraction.

**Table 4.2 Characterization of phenolics and DPPH* scavenging activity of different fractions obtained after column chromatography**

<table>
<thead>
<tr>
<th>Particular</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Recovery</td>
<td>20.59±2.2^a</td>
<td>40.37±2.3^b</td>
<td>27.04±2.5^c</td>
<td>12.20±1.1^d</td>
<td>1.48±0.8^e</td>
<td>1.09±0.3^f</td>
</tr>
<tr>
<td>% DPPH* scavenging activity/mg of powder</td>
<td>32.77±0.5^a</td>
<td>17.27±0.3^b</td>
<td>44.61±0.8^c</td>
<td>29.91±1.1^d</td>
<td>149.86±0.9^e</td>
<td>13.1±0.7^f</td>
</tr>
<tr>
<td>Gallic acid (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>17.15±1.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Catechin (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>424.92±1.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Syringic acid (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>18.95±1.0</td>
<td>4.34±0.1</td>
<td>ND</td>
</tr>
<tr>
<td>Caffeic acid (mg/g)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10.19±0.8</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mean ± standard deviation
Observation with different superscript in same row differs significantly at 5% probability level

Fig. 4.5 % Yield and DPPH* scavenging activity of different fractions of Amla pomace powder.
Extraction, identification and antioxidant properties of bioactive components of Amla (Emblca officinalis) pomace powder

Fig. 4.6 HPLC chromatograms of (a) fraction II, (b) fraction III, and (c) fraction IV. (1) Unidentified, (2) gallic acid, (3) catechin, (4) syringic acid, and (5) caffeic acid.
Extraction, identification and antioxidant properties of bioactive components of Amla (Emblica officinalis) pomace powder

Fig. 4.7 FTIR spectra of different fractions of Amla pomace powder. REA: aqueous extract, REA: ethyl acetate extract, RB: butanolic extract, I: fraction I, II: fraction II, III: fraction III, and V: fraction V

4.3.2.3. Detection of isolated compounds

As seen from Fig 4.5, fraction V showed maximum DPPH* scavenging activity but its yield was low. On the other hand, fraction II with high yield showed poor DPPH* scavenging activity. Hence fraction III with moderate yield and moderate DPPH* scavenging activity was further purified by preparatory HPLC and three different components were isolated.

Isolated components, i.e E-1, E-2 and E-3 had maximum absorbance at 275 nm, 275 nm and 277 nm, respectively. Representative chromatograms of isolated components are shown in Fig 4.8. By comparing HPLC chromatograms of E-2 and E-3 with standards it was observed that isolated component E-2 was gallic acid whereas E-3 was catechin. Oliver et al. (2010) had also reported the maximum absorbance of catechin is 278 nm.

The present finding also supported the findings of Luo et al. (2009) for the presence of gallic acid in Amla. Due to nonavailability of the standards, the
component of E-1 could not be identified by HPLC. E-1 was identified through silylation process, which is an ideal procedure for the GC analysis of non-volatile and thermolabile compounds. By comparing the mass spectra of E-1 by NIST library the compound was detected as hydroxytyrosol. The molecular weight (m/Z) of TMS derivative of hydroxyl tyrosol was 370 and major characteristic fragments of isolated compound were obtained at 73, 267, 193 and 179. The present finding was also in accordance with Proestos et al (2006)\textsuperscript{22}.

### 4.3.3. DPPH\(^*\) scavenging activity

Ethyl acetate fraction showed significantly less total phenolic content than the butanolic fraction of the Amla pomace powder. Probably during evaporation of butanol solvent, some other phenolics may have been synthesized from the secondary metabolites which may increase the total phenolic content of the butanolic fraction. However inspite of having good amount of total phenolic content, the DPPH\(^*\) scavenging activity was significantly less than the ethyl acetate fraction. Our present findings contradicted Piljac et al. (2007)\textsuperscript{28} and Seruga et al. (2011)\textsuperscript{18} for their observation that the DPPH\(^*\) scavenging activity is directly proportional to the total phenolic content. The antioxidant activity and DPPH\(^*\) scavenging activity not only depends on the total phenolic content but also depends on the position and availability of the free hydroxyl groups to show antioxidant activity or to scavenge the free radicals. Exposure of high temperature to evaporate the solvents from butanolic extracts adversely affects the availability of hydroxyl groups that reduces the functional properties of the phenolic compound present and hence was not found suitable for the extraction of bioactive components. Fraction V (Table 4.2) showed the highest DPPH\(^*\) scavenging activity among all fractions tested but due to comparatively poor recovery of phenolics could not be recommended for the extraction of bioactive components. It can also be concluded from Fig 4.7 that FTIR spectra of fraction V had comparatively higher intensity of functional peaks as compared to others. Luo and Foo (2001)\textsuperscript{29} explained that such hydroxy-phenolic compounds can donate hydrogen atoms to DPPH\(^*\) and can scavenge it. Fraction III with significant recovery of the phenolics and compatible DPPH\(^*\) free radical scavenging activity may be suggested for the further extraction and purification of the components.
Fig. 4.8 HPLC chromatogram of (a) E-1 (hydroxytyrosol), (b) E-2 (gallic acid), and (c) E-3 (catechin) at 280 nm.
The DPPH* scavenging activities of compound E1-E3, and control are shown in Fig 4.9. It could be observed that all three compounds exhibited strong DPPH* scavenging activity in a dose dependent manner. The DPPH* scavenging activity was in decreasing order; gallic acid (E-2) > catechin (E-3) > hydroxytyrosol (E-1) > vitamin C > β-carotene. As shown in Fig 4.9, the DPPH* scavenging activities of the isolated compounds were significantly higher than those of vitamin C and β-carotene.

Fig 4.10 presents the ABTS+ scavenging activities of the isolated compounds and standards of vitamin C and β-carotene. Catechin, gallic acid and hydroxytyrosol exhibited strong antioxidant activities and these results were consistent with the report of Giedrius et al (2004) and Luo et al. (2009). Moreover, catechin, gallic acid and hydroxytyrosol exhibited higher antioxidant activities than the vitamin C and β-carotene standards. The ABTS+ scavenging activity in decreasing order was as follows: catechin > gallic acid > hydroxytyrosol > vitamin C > β-carotene. This result confirmed the idea that a high scavenging activity can be attributed to the availability of their free hydroxyl groups, and the phenolic hydroxyl structural group in benzene ring contributes much to the free radical scavenging activity.
Extraction, identification and antioxidant properties of bioactive components of Amla (*Emblica officinalis*) pomace powder

![Graph showing ABTS+ scavenging activity of isolated compounds (E-1 to E-3), standard vitamin C and standard β-carotene.](image)

**Fig. 4.10** ABTS$^+$ scavenging activity of isolated compounds (E-1 to E-3), standard vitamin C and standard β-carotene.

### 4.4. Conclusion

Amla pomace powder has never been investigated for optimization of extraction of total phenolic content. Major polyphenols contained in ethyl acetate fractions of pomace powder were gallic acid, catechin, caffeic acid and syringic acid. Ethyl acetate fraction extracted by methanol:water (80:20) showed the maximum DPPH$^*$ scavenging activity but due to very poor recovery it was not found suitable for the further purification. Catechin was the dominating acid present in fraction III. Isolated catechin, gallic acid and hydroxytyrosol exhibited stronger DPPH$^*$ free radical scavenging activity as compared to vitamin C and β-carotene. Present study revealed that most of the major fractions of the crucial bioactive components of Amla are present in the pomace powder which can be exploited after extraction of juice from the fruit.
References


27. Olivier, M., et al. Identification of catechin as one of the flavonoids from *Combretum albiflorum* bark extract that reduces the production of quorum-


