Quality assessment and evaluation of in-vitro antioxidant potential of *Phyllanthus emblica* L.

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*Phyllanthus emblica* L. syn. *Emblica officinalis* Gaertn. commonly known as *amla* is a well known drug in India having nutritional value with various health related benefits and employed in various herbal formulations due to the presence of high levels of vitamin C along with valuable phenolic constituents and amino acids. In the present study, quality assessment of *amla* fruit was carried out by studying macro and microscopic characters along with physicochemical tests for the identification of drug. Chemoprofiling was achieved with thin layer chromatography by optimizing the mobile phase for different extracts. Total phenolic contents and in-vitro antioxidant efficacy was also determined as the drug is well known for protective role in human body as antioxidant. The contaminant evaluation was carried out by analyzing the samples for the determination of heavy metals, pesticides and aflatoxins. The results of quality control and anatomical studies found useful for its identification with significant antioxidant efficacy. The drug was found free of contaminant when analyzed for pesticides and aflatoxins whereas heavy metals were found in safe limits. The work embodied in the present research can be utilized for the quality control and for the identification of the drug samples.

**Keywords:** *Phyllanthus emblica* L., Physicochemical, TLC fingerprint, Antioxidant, Quality control

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Amla (*Phyllanthus emblica* L.) belongs to family Euphorbiaceae, commonly used in Indian systems of medicine, is a nutritionally rich food with high therapeutic value. It is a well known drug containing high levels of vitamin C, which is resistant to storage and damaged by heating. Apart from vitamin C, the fruit also contains minerals and amino acids. Literature survey revealed various pharmacological activities of *amla* fruits such as antinociceptive, antimicrobial, antioxidant, gastroprotective, hepatoprotective, antiulcerogenic, antiabetic, anti-inflammatory, antipyretic and analgesic. A *amla* is one of the most popular drugs in Ayurvedic and Unani systems of medicine and is one of the major ingredients of *Chyawanprash, Triphala, Itrifals* and *Khamiras*. Different types of herbal formulations containing *amla* as an ingredient are used in Unani system such as solid (*Qurs, Habbs, Safoof*), semisolid (*Khamira, Itrifal, Majoon*) and liquid (*Sharbat*) preparations. *Itrifals* (Unani formulations) and *Triphala* (Ayurvedic formulations) are multi component compound formulations containing *amla* as one of the major ingredient and commonly used for several disorders. Hence, it was thought that it would be worthwhile to develop the quality standards of *amla* for its identification and standardization. The drug is well known for its antioxidant properties due to presence of ascorbic acid and various phenolic constituents, therefore the quality of the plant can be assessed by evaluating its antioxidant potential. The assay for the total phenolic content was carried out along with in-vitro antioxidant activity by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Nitric oxide (NO) radical scavenging methods. The present research work will be helpful and serve as an important tool for the quality assessment of the *amla*.

**Materials and methods**

The *amla* sample was purchased from local market of Delhi, India and the specimen (Ref. NISCAIR/RHMD/Consult/-2008-09/1149/181/02/01-08) authenticated by Botanist Dr H B Singh, Head Raw Material Herbarium and Museum, NISCAIR, New Delhi. All the chemicals used for the analysis and solvents were of analytical grade and obtained

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from Merck India. Standard catechin and ascorbic acid were purchased from Sigma Aldrich (USA).

**Morphology of amla**

Drug was evaluated for its physical appearance, shape and size, taste, colour and odour.

**Microscopy of amla**

**Preparation of specimens**

The drug specimen for the proposed study was collected from local market of Delhi. Care was taken to select healthy fruits. The required samples of amla fruits were cut and seeds were removed, fixed, prepared and cast in to paraffin blocks. The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was kept to 10-12 µm cleared and mounted. Microscopic descriptions of tissues were observed with micrographs. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic Unit (Nikon, Canada).

**Physicochemical evaluation of amla**

The physicochemical studies were carried out as mentioned in Indian Pharmacopoeia (IP, 1996). The different parameters like determination of foreign matter, loss on drying (LOD), moisture content by Karl Fischer titration, ash values with total ash, acid insoluble ash and water soluble ash, determination of pH using 1% and 10% solution of amla, extractive value by successive extraction as well as extractive values in ether, alcohol and water, respectively.

**Total phenolic contents**

Accurately weighed 10 mg of amla fruit powder was sonicated with 50 mL of double distilled water for 10 min and the volume was made upto 100 mL. Estimation of total phenolic content was carried out according to the Folin–Ciocalteau (FC) method using catechin as a standard phenolic constituent. The concentration of phenolic content in sample was calculated by using standard calibration curve.

**DPPH free radical scavenging method**

The free radical scavenging capacity of methanolic extract of amla was determined using stable radical DPPH method. DPPH solution (0.004%, w/v) was prepared in methanol. The methanolic extract was mixed with methanol to prepare stock solution (1.0 mg mL\(^{-1}\)). The percent scavenging of the DPPH free radical was measured using the following equation:

\[
\% \text{ Inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where, A\(_0\) was the absorbance of the control (blank, without extract) and A\(_1\) was the absorbance of the extract or standard.

**Nitric oxide scavenging method**

Nitric oxide radical scavenging activity of amla extract was determined according to the reported method. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethlenediamine was measured at 540 nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Greiss reagent as a positive control. The nitric oxide radicals scavenging activity was calculated as similar to DPPH method.

**HPTLC chemo profiling of amla**

The HPTLC fingerprints of different extracts were established by developing the solvent systems for their separation by thin layer chromatography. The solvent system in which maximum and well resolved spots were found, was selected for HPTLC. The samples were applied on precoated silica gel 60 F\(_{254}\) plates (E. Merck, 0.20 mm thickness) using Linomat V (HPTLC sample applicator) and developed in the solvent system. The chromatograms were scanned at 254 and 366 nm wavelength followed by spectral analysis. Reprostar Chromatography Documentation Apparatus was used for taking photographs of the HPTLC plates. Plates were also scanned at visual range after spraying visualizing reagent.

**Sample application and development**

Samples were applied on precoated silica gel 60 F\(_{254}\) TLC plates (5 X 10 cm) by linomat V sample applicator. The samples were applied in triplicate 8.0 µL each. The width of the track was kept to 5.0 mm and distance between tracks was 13 mm. After sample application, the plates were developed up to 80 mm in development chamber saturated with the specific solvent system. Then, plates were scanned at 254, 366 nm and in visible range after spraying with anisaldehyde sulphuric acid.
Evaluation of contaminants

Heavy or toxic metal analysis

The heavy metal analysis for the presence of lead, cadmium, mercury and arsenic was performed for the dried *amla* powder. It was carried out by using atomic absorption spectrometer. Standard linear calibration curve was prepared with absorbance against concentration and concentration of respective metal was calculated in the samples.

Aflatoxins determination

The AOAC official method of analysis was followed for the determination of aflatoxins. Prepared dried extract and standard aflatoxins were derivatized and analysed on a Waters Alliance e2695 separating module (Waters, USA) HPLC. The peaks of aflatoxin in drug samples were compared with peak of standards (B1, G1, B2 and G2).

Pesticide determination

The 50 mg of sample was dissolved into methanol and 1.0 gm of sodium oxalate was added followed by addition of 50mL each of diethyl ether and petroleum ether. It was shaken for 1.0 min. Organic layer was transferred into separating funnel and 600 mL of water saturated with sodium chloride was added. Aqueous layer was discarded and the process was repeated for 2-3 times. Organic layer was then passed through sodium sulphate solution and the solution was collected and evaporated up to 2-5 mL. This concentrated solution was again mixed with acetonitrile (30 mL) and petroleum ether (30 mL) and it was eluted with diethyl ether by passing through the column. The solution was concentrated up to 5.0 mL by using rotavapor (Buchi, R-215, Switzerland) and analysed in GC-MS (Agilent 7890A GC system, USA) by established AOAC official method.

Microscopy

Transverse section of fruits consisted of a thin, continuous membranous layer of epidermis comprising of less conspicuous cells smaller in size. Inner epidermis was fairly wider made up of hypodermal layer made up of parenchymatous cells rectangular in shape followed by large compact cells. The cells were variable in shape and size. The parenchymatous region represented the mesocarp of the fruit and the thin epidermal layer was the epicarp (Fig. 1). The mesocarp was well vascularised. Small vascular strands were wide spread in the mesocarp (Figs. 1b & c). The vascular strands varied in size; some were well developed with collateral xylem and phloem (Fig. 2d), whereas others were smaller and less distinct. Xylem consisted of narrow, thick walled with lignified clusters. Phloem included a prominent mass of sieve elements. Bundle sheath cells were not evident. Sections stained with neutral red exhibited some of the mesocarp cells being filled with reddish amorphous substance, lipoidal in nature, distributed randomly (Fig. 2). Calcium oxalate crystals were clear when viewed under polarised light interspersed with mesocarp cells. Raphides observed in parenchymatous cells consisted of numerous thin pointed needles packed within the bundle. The needles were loosely packed and break with ease (Figs. 2c, d & e).

Physicochemical evaluation

Different physicochemical parameters were assessed in triplicate and are represented with standard deviation. The determination of water is important to check the product quality and to assure even the chemical and physical properties of the

![Fig. 1—TS of the amla fruit (A), TS of the fruit showing the epidermis, mesophyll tissue, xylem and phloem (B), vascular bundle spread in the mesocarp (C), the xylem and phloem enlarged (D). [EP- epidermis; Hd- hypodermis; MC- mesocarp; Ph- phloem; VB- vascular budle; X- xylem]]
product. It was observed that in the drug 10.36 % loss on drying and 6.09 % moisture was found. Total ash can be used for the determination of inorganic materials, such as carbonates, silicates, oxalates and phosphates. The total ash in the amla sample was found 3.76 %w/w, whereas acid insoluble and water soluble ash were found 0.71 and 3.01%w/w, respectively. The drug is showing acidic nature when pH of 1.0 and 10.0 % solutions was measured which was found to be 3.93 and 4.67, respectively. In different extractive values, the maximum yield was found in methanol extract when successive extraction was carried out whereas minimum yield was found in petroleum ether. Similarly, in soluble extraction the methanolic extract was found maximum. All the results of physicochemical evaluation are depicted in Table 1.

Assessment of antioxidant potential of amla

Antioxidant efficacy of the plants is mainly due to presence of phenolic constituents, which may contribute directly to the antioxidant properties. Recently, many reports suggest that phenolic compounds have inhibitory effect on carcinogenesis and mutagenesis in humans and upon ingestion of high fruits and vegetables rich diet\(^\text{18}\). The total phenolic content in the amla was measured by Folin-Ciocalteu method and found to be 20.82 %w/w.

The amla extract showed a concentration-dependent antioxidant activity by inhibiting DPPH radical with an IC\(_{50}\) value of 22.07 µg mL\(^{-1}\) whereas, IC\(_{50}\) value of ascorbic acid was found to be 16.41 µg mL\(^{-1}\) used as standard and itself is a main marker constituent of amla (Fig. 3). The extract has the efficacy to reduce the stable radical DPPH to the yellow-coloured diphenyl picrylhydrazine comparable to standard ascorbic acid. The scavenging effect increased with increasing concentration of the extract and ascorbic acid (5 - 50 µg mL\(^{-1}\)). The results of nitric oxide scavenging activity assessment show that amla extract has dose dependent moderate activity between 10 -50 µg mL\(^{-1}\) with 32.6 µg mL\(^{-1}\) as IC\(_{50}\) value (Fig. 3) whereas IC\(_{50}\) value of ascorbic acid was found to be 15.9 µg mL\(^{-1}\).

HPTLC chemoprofiling

Different solvent systems were tried by trial and error method for the different extracts. Satisfactory separation of constituents was obtained in solvent systems - benzene: chloroform (80:20, v/v), chloroform: methanol (95:5, v/v) and

![Table 1—Summary of results of physicochemical evaluation of amla (n=3)](Image)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameters</th>
<th>% w/w (mean ± SD*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Foreign matter</td>
<td>4.65 ± 0.39</td>
</tr>
<tr>
<td>2</td>
<td>LOD</td>
<td>6.76 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>Moisture content</td>
<td>6.09 ± 0.18</td>
</tr>
<tr>
<td>4</td>
<td>Ash values</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total Ash</td>
<td>3.76 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>Acid insoluble Ash</td>
<td>0.71 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>Water soluble Ash</td>
<td>3.01 ± 0.22</td>
</tr>
<tr>
<td>5</td>
<td>Successive extractive values</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) Petroleum ether</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>2) Chloroform</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>3) Acetone</td>
<td>10.82 ± 0.77</td>
</tr>
<tr>
<td></td>
<td>4) Methanol</td>
<td>46.02 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>5) Water</td>
<td>12.22 ± 1.40</td>
</tr>
<tr>
<td>6</td>
<td>Extractive values</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ether soluble extractives</td>
<td>0.33 ± 0.07</td>
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<td></td>
<td>Water soluble extractives</td>
<td>45.43 ± 1.90</td>
</tr>
<tr>
<td></td>
<td>Alcohol soluble extractives</td>
<td>50.96 ± 3.86</td>
</tr>
</tbody>
</table>

*SD=Standard Deviation

Fig. 2—Mesocarp tissues stained with neutral red (A), cells with reddish amorphous substance and lipid (B), mesocarp with crystals under polarized light (C), crystals in the mesocarp (D), needle-crystals in the cells and raphide bundle (E); [Li- lipid; MC-mesocarp tissue; Cr-crystals; Ra-raphide]
toluene: ethyl acetate: formic acid (50:40:10, v/v/v) for petroleum ether, chloroform and methanol extract, respectively. The samples were applied and chromatograms were developed in respective solvents. The petroleum ether and chloroform extracts gave good results after spraying with anisaldehyde sulphuric acid as visualising agent. The chromatograms were air dried and kept in an oven at 110°C for 10 min (or until spot visibility). The chromatograms were then scanned at different wavelength in visible range and the best one (530 nm for petroleum ether and 610 nm for chloroform extract) was selected on the basis of maximum absorbance of the peaks. The methanolic extract was scanned at wavelength 254 nm and results were found satisfactory. The HPTLC fingerprints of petroleum ether, chloroform and methanol extract showed the presence of thirteen, nine and seven constituents, respectively (Fig. 4).

**Evaluation of contaminants**

While using herbs, it is essential to control the level of contaminants in medicinal raw materials. Therefore, lead, cadmium, arsenic and mercury were analysed in amla powder by using atomic absorption spectrometry and found within the limits (Table 2).

Aflatoxins are mycotoxins produced by some fungi of the genus *Aspergillus* that can grow on various foods, spices and medicinal herbs. There are four major classes of aflatoxins, namely, B1, B2, G1 and G2 which can cause acute structural and functional damage to the vital organs of the human body.

The HPLC chromatogram of mixed standard aflatoxins and sample showed that no aflatoxins were present in amla sample.

The pesticides have been shown to be hazardous to humans by causing cancer and neurological disorders and infertility in male, so several countries have set their own maximum residue limits of pesticides for medicinal plants. The literature shows different analytical procedures for the determination of pesticides in medicinal plants. Gas chromatography is a suitable technique for such a purpose so pesticide estimation was carried out by GC-MS and it was observed that the test sample did not contain any pesticide.

**Conclusion**

The present study is very much useful to develop the quality standards of *amla* for its identification and standardization. The drug contains antioxidant properties due to high source of ascorbic acid and various phenolic constituents; hence quality of the drug has been assessed by checking its antioxidant potential. The assay for the total phenolic content was carried out along with in-vitro antioxidant activity by using DPPH and Nitric oxide radical scavenging methods. The anatomical evaluation carried out can also be used as authentic source of information for its proper identification along with physicochemical tests. Conventionally, the monograph of the drug contains only TLC profiling of methanolic extract but here chemoprofilinig of nonpolar (Petroleum ether), medium polar (Chloroform) and polar (Methanol)
Fig. 4A-C: 4a—Developed TLC plates and HPTLC chromatograms of petroleum ether extract; 4B—Developed TLC plates and HPTLC chromatograms of chloroform extract of *amla* & 4C—Developed TLC plates and HPTLC chromatograms of methanol extract of *amla*
extracts was achieved by optimizing the suitable solvent systems.

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References

