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Gallic Acid Content and Antioxidant Activity of the Dried Fruit and the Processed Fruit of Amla - Emblica Officinalis L.

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ABSTRACT

Indian traditional health care system (Ayurveda) named Rasayana proposed Phyllanthus emblica Linn. (syn. Emblica officinalis), commonly known as Indian gooseberry or amla, family Euphorbiaceae, is an important herbal drug used in unani (Graceo - arab) and ayurvedic systems of medicine. The plant is used both as a medicine and as a functional food to promote vitality and vigor. E. Officinalis is highly nutritious and could be an important dietary source of vitamin C, amino acids, and minerals. Although, the E. Officinalis extract exhibits higher levels of antioxidant activity, the bioavailability of phenolic acids in the processed fruit is still poorly known. Therefore, in the present study, gallic acid efficacy as an antioxidant was evaluated in two different samples of E. Officinalis. Reliable and feasible HPLC and UFLP methods with diode array detection have been developed and compared for the determination of gallic acid in E. Officinalis dried fruit sample (DFS) and dried processed fruit sample (DPFS) prepared according to one of the Ayurveda principles. The antioxidant effects of both the extracts have also been compared with standard gallic acid by different antioxidant tests like DPPH and ABTS. The amount of gallic acid (20.16 mg/100g) in DPFS was higher in contrast to DFS (8.02 mg/100g) of E. Officinalis fruit which demonstrated higher antioxidant activity. Additionally, It has also been found that gallic acid accounts for approximately 35-71% of the antioxidant activity. UFLP system showed rapid and effiecint determination of gallic acid samples in comparison to HPLC system.

KEYWORDS : Emblica officinalis; Gallic acid; Liquid chromatography; Antioxidant activity

1. Introduction

Oxidants and antioxidants in humans are maintained in balance in a normal physiological state, and over production of oxidants in certain conditions such as smoking, hazardous environmental exposures or infectious diseases can cause oxidative stress leading to oxidative damage to biomolecules and cells (Temple, 2000; Willett, 2002; Ames, 1979; Thompson, 1994). Human diets constituting of fruits, vegetables and spices have been shown to contain beneficial components, including phenolic compounds with antioxidant properties (Croft, 1998). Phenolic components have been known to act as antioxidants not only because of their ability to donate electrons, but also because of their stable radical intermediates, which can effectively prevent the oxidation at cellular and physiological level (Cuvrelier *et al*., 1992). Amla (*Emblica officinalis*) have long been known in India and many other countries as important dietary sources in addition to their use in traditional medicine for wound healing, inflammation and stomach acidity (Srivasuki, 2012). Several investigators have determined the efficacy of Amla as antiatherosclerotic (Thakur *et al*., 1988), antidiabetic (Tripathi *et al*., 1979), antimutagenic (Nagabhushan and Bhide, 1986; Sharma *et al*., 2000a; Polasa *et al*., 1991) and anticancer agents (Aggarwal *et al*., 2003)

Large numbers of medicinal plants have been investigated for their antioxidant activities and the results illustrate that either their raw extracts or their individual chemical constituents are more effective antioxidants (*in vitro*) than the synthetic antioxidants, e.g.butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) or vitamin E (Gordon & Weng, 1992; Gu & Weng, 2001; Pyo, Lee, Logendrac, & Rosen, 2004). Moreover, these synthetic antioxidants, may have carcinogenic and other harmful effects on the lungs and livers (Branen, 1975) of human beings It has now been well established that oxidative stress plays an important role in these disorders. However, the precise phenolic acids and their antioxidant potency need to be determined in order to understand these health attributes. So, scientists are continuously busy in searching naturally occurring non-toxic antioxidants, which could prevent this free radical related disorders in human beings and also can replace the harmful synthetic antioxidants (Branen, 1975; Grice, 1986; Wichi, 1988). Traditionally used Indian medicinal plants like Amla, which are an integral part of Indian Ayurveda, can be the potential sources for various naturally occurring non-toxic antioxidants.

The present study aimed to determine the percentage potency of gallic acid content as antioxidant in *E. officinalis* fruit and what were latent effects of traditional Ayurvedic processing method "to increase the efficacy of dried fruit by treating it with its own juice". For this purpose, a simple and rapid liquid chromatographic HPLC/ UFLC method was developed to determine Gallic acid content in dried fruit sample (DFS) and dried processed fruit sample (DPFS) and their respective antioxidant activities were evaluated by means of 2,2_-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) tests in extracts having equal gallic acid levels.

2. Materials and methods

2.1. Materials

Dried pure Amla powder (Dried Amla fruit is crushed and powdered) (DFS) and processed amla fruit (DPFS) from Divya Pharmecy, Haridwar, India **was used**. The HPLC column used was Shimpack, C18 ODS (25cm x 4,6 mm x 5µm). The Acetonitrile (Merck) used as solvent was HPLC grade and the water MilliQ. The Folin-Ciocalteu reagent, DPPH radical, Gallic acid (99.5%, m/m) and ABTS were bought from Sigma Aldrich (St Louis, MO). The potassium phosphate (KH_2PO_4), the phosphoric acid, sodium persulfate and sodium carbonate (Na_2CO_3) were obtained of Dinamica Reagents.

2.2. Apparatus

The HPLC apparatus consisted of an LC 20A (Shimadzu Inc., Japan), with quaternary pump (model 20-AT), degasser (model DGU-20 A3), diode array detector (model SPD-M20A), auto sampler (model SIL-20A HT) and data controller (model CBM-20A).

The UFLC system consisted of an LC 20A (Shimadzu Inc., Japan), with binary pump (model 20-AT), degasser (model DGU-20 A3), diode array detector (model SPD-M20A), auto sampler (model SIL-20A HT) and data controller (model CBM-20A).

2.3. Standard solution and sample preparation

2.3.1. Extraction 1

The stock solution of gallic acid was 10 mg mL^{-1} in methanol. All dilutions subsequent were made in methanol. One gram of the finely powdered dried amla (DFS) or processed amla (DPFS), first extracted with 20 mL of methanol-water (1:1), sonicated for 30 minutes, filtered through whatman filter paper No 41. Residue is dissolved in 20 mL acetone, sonicated for 30 minutes, filtered through whatman filter paper No 41 again, mixed both the filterates and concentrated under vacuum (40°C) and the extract was purified.

2.3.1.1. Sample Purification

The extracts were purified in preparative column using silica active

at 800°C in muffle furnace for 1 hour and sodium sulfate (anhydrous) activated at 121°C for 30 minutes. The solvents used 8 mL of acetone and 22 mL of methanol. All samples were filtered through a 0.22 µm membrane filter from Millipore (Malsheim, France) before the injection.

2.3.2. Extraction 2

Five grams powdered dried or processed amla were extracted with 50 mL of ethanol-water (7:3) sonicated in shaker (300 rpm) for 15h and filtered through whatman filter paper No 41. The filter was washed with 50mL of ethanol. The extract was concentrated under vaccum (40°C) and injected in HPLC and UFLP system.

2.4. HPLC method

2.4.1. Chromatographic conditions

The DAD detector was set in the 254–370 nm range and quantitative analysis was performed at 254 nm. The mobile phase was constituted of a mixture 92% of potassium phosphate buffer 5mM, pH 2.2 and 8% of acetonitrile. The flow rate was 1.0 mL.min^{-1} and the injection loop was 20µL.

2.4.2. Calibration curve

The quantification of the galllic acid in samples is calculated based on the calibration curve with standard solutions of gallic acid, in the 0.006 - 0.015 mg.mL^{-1} concentration range, and injected in the HPLC and UFLC, comparing the spectrum of absortion and retention time in the same analytic condition.

2.4.3. Precision assays

The precision was determined by injecting five replicates of the standard gallic acid and measured by the standard deviation or coefficient of variation.

2.4.4. Recovery assays

Known amounts of standard solution of Gallic acid were added to known amounts of extracted and purified Amla sample. The proportion of recovery was evaluated accordingly by adding both the values of Gallic acid as well as the sample.

2.5. Total polyphenol content

Total polyphenol content of *Emblica* was determined by the adopted Folin–Ciocalteu colorimetric method (Ragazzini and Veronesi, 1973). The methanolic extracts DFS and DPFS (0.5 mL) were mixed with 2.0 mL of the 0.02 N Folin–Ciocalteu reagent and 2.0 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1h incubation at room temperature. Total polyphenols were expressed as gallic acid equivalents.

2.6. Scavenging activity on the DPPH radical

The antioxidant activity of the different *E. Officinalis* extracts was assayed by their scavenging effect on the DPPH radical (Wang *et al*., 1998). *Emblica* extracts were first analysed by HPLC for the quantitative determination of gallic acid content and then diluted in order to have exact concentrations of gallic acid in the range 0.01–0.5 mgmL^{-1}(corresponding to 0.001 – 0.05%). Dilution was also performed on pure gallic acid solution. Both *E. Officinalis* samples and pure gallic acid solution in the dark for 30 min. The absorbance of samples was measured with a spectrophotometer (UVmini-1240, Shimadzu) at 517 nm against a blank of DPPH. Positive controls consisted of gallic acid. The antioxidant activity of each extract, containing same concentration of gallic acid, was determined as follows, according to the percentage of DPPH decolouration:

$$\text{Decolouration}(\%) = \frac{(\text{Abs}_{\text{Blank}} - \text{Abs}_{\text{sample}}) \times 100}{\text{Abs}_{\text{Blank}}}$$

All tests were run tree times and averaged.

2.7. ABTS radical cation decolourization assay

The antioxidant capacity of each extract of *Emblica* was evaluated by studying its ability to bleach the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS•+) (Miller *et al*., 1993). Concentrations of gallic acid and dilutions for the assay were performed as described above. ABTS•+ was produced by the reaction of ABTS solution (2mM) with 70mM sodium persulfate (both solutions were prepared in bidistilled water) for 12–16 h, in the dark and at room temperature. The stock solution was diluted in phosphate buffered saline (PBS) so as to achieve an absorbance of 0.70±0.02 at 734 nm. Three thousand nine hundred ninety-six microliters of diluted ABTS solution was mixed with 4µL of sample. The absorbance at 734 nm was taken at 30◦C exactly 1 min after initial mixing. The percentage decrease of absorbance was calculated as follows:

$$\text{Inhibition}(\%) = \frac{(\text{Abs}_{\text{Blank}} - \text{Abs}_{\text{sample}}) \times 100}{\text{Abs}_{\text{Blank}}}$$

All tests were run tree times and averaged.

3. Results and discussion

3.1. Gallic acid conent through HPLC and UFLC method and total polyphenolic content:

Gallic acid content was evaluated in amla extracts using HPLC and UFLC systems in DAD detector in comparison of retention time. HPLC protocal was elaborated for separation of phenolic acids using two extration forms. The volume of methanol and acetone and the time required for optimal extraction were also investigated. The analysis was performed using a C18 reverse phase column, and the composition % acetonitrile, the pH, and the flow rate of the mobile phase were investigated. The best conditions were as follows: the mobile phase consisted of a mixture of 5mM phosphate buffer, pH 2.2 (92.0%), and acetonitrile (8.0%), with a flow rate of 1.0 mLmin^{-1} for HPLC and flow rate of 0.4 mLmin^{-1} for UFLC. The DAD detector was set in the 200–350 nm range, and the quantitative analyses were carried at 254 nm. Under these conditions, a neat peak of gallic acid was detected at 4.5 min to HPLC and 0.5 min to UFLC. The spectrum was recorded and maximum absorbance was found at 254 nm. A good linearity was found from 0.0005 - 0.015 mg.mL^{-1} gallic acid, and the linear regression equation was y = 2E+09x-84588 (r_c = 0.9978) for HPLC and y= 3E+08x-77286 for UFLC, where *y* is the peak height.

The chromatogram of dried E. officinalis fruit (DFS) or processed E. officinalis fruit (DPFS) at 254 nm showed prominent peak. A comparison between the spectra of these peaks with that of pure gallic acid confirmed that the retention time of the analyte was 4.5 min but, in UFLC system this value decreased for 0.5 min.

When we compared the extraction and purification between two methods used above, higher content of the gallic acid was observed when sample is extracted with methanol and purified with separative column than that amla extracted with ethanol for long time.

Limit of quantitation (LOQ) and limit of detection (LOD) values, calculated according Lanças (2004), repeatability and intermediate precision values, obtained at concentration values minimum, medium, and high (Table 1). The accuracy of the method, obtained by means of recovery assays, gave satisfactory results, always between 102 and 104%. Experimental tests were injected in quadruplicate and the results indicated that the analyte peak area show good repeatability and accuracy. So the method of extraction was easy, rapid, and reliable.

Above data shows that the processed *E. officinalis* fruit (DPFS) have higher content of gallic acid in comparision to that of *E. officinalis* fruit (DFS) in both the extractions (Table 2), the same observation was found in total phenolic compounds content. This could be due to the fact that processed *E. officinalis* fruit (DPFS), fresh fruit juice of Amla (*E. officinalis*) is added in the dried powder during treatment and dried again to enhance the potency of the powder.

Results indicate that the good quantitative extraction of gallic acid was obtained by eluting the extract with ethanol by long time of agitation, but, extraction and purification of the sample through sílica column (3 gms), acetone and methanol showed best results with identification and quantification of gallic acid. When compared the results of Gallic acid for HPLC and UFLC, it was observed that better repeatability (R.S.D.%), Intermediate precision (R.S.D.%), Limit of quantification (LOQ) and Limit of detection (LOD) was showed by UFLC equipment, verified high sensibility of the instrument, besides that elution of the compound requires very less time and consequently requires very less amount of solvents.

3.3. Antioxidant activity

The antioxidant activity of all extracts was evaluated with the ABTS and DPPH tests (fig. 2 and 3). These methods are rapid, sensitive, re-

producible, and require simple conventional laboratory equipment. They were selected for their different characteristics. In fact, the DPPH test is particularly suitable for the evaluation of antioxidant activity of crude extracts (Poli *et al*., 2003), while the ABTS one is usually applicable also to biological tissues (Re *et al*., 1999). The antioxidant assays were performed on extracts prepared as described above with five replicates per sample. S.D. ranged from 0.2 to 1.5%. DPPH test results (Fig. 2) indicate that, when the concentration of Gallic acid in the samples was higher than 0.25 mgmL−1 (corresponding to 0.025%), the bleaching power of the solutions was too high; for this reason, it is not possible to estimate the antioxidant role of Gallic acid versus the other compounds.

A comparison between antioxidant activity of Gallic acid standard solution and those of the two different *E.officinalis* extracts, measured at the same concentration of Gallic acid, shows that the processed fruit extract (DPFS) has the highest antioxidant activity. The results of both the tests (DPPH and ABTS) show that the antioxidant activity of processed and dried Amla does not depend only upon Gallic acid content. The extracts of processed amla sample shows antoxidant activity 51-86% whereas dried fruit, 30–70% of the antioxidant activity confirm that the Ayurvedic method of processing increases the amount of antioxidant compounds.

4. Conclusion

In the present study, the HPLC and UFLP method described here showed that the methods adopted here are easy, reliable and reproducible in *E.officinalis* samples. The selectivity was increased in order to get good separation between Gallic acid and interference peaks (tannins possible) becoming a rapid and accurate determination.

Out of HPLC and UFLC it is found that UFLC is accurate, shows better repeatability requires very less time and less amount of solvent.

The antioxidant activity of Gallic acid in two different *E.officinalis* samples (DFS and DPFS) have been investigated, and the results indicate higher amount of gallic acid was present in processed fruit extract (DPFS) in comparison to dried fruit sampledue to higher amount of gallic acid in DPFS. Moreover, these results concluded that the traditional processing method for obtaining the *E. officinalis* powder undergoes less degradation as compared to dried Amla powder.

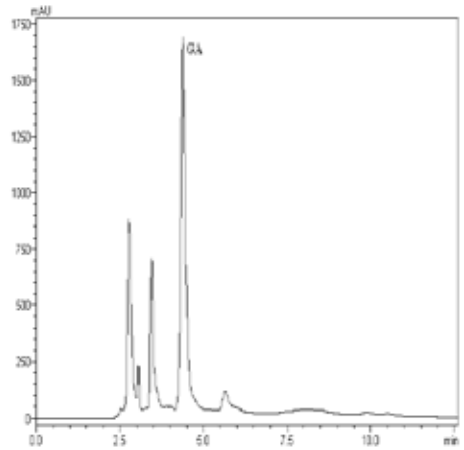
Table 1

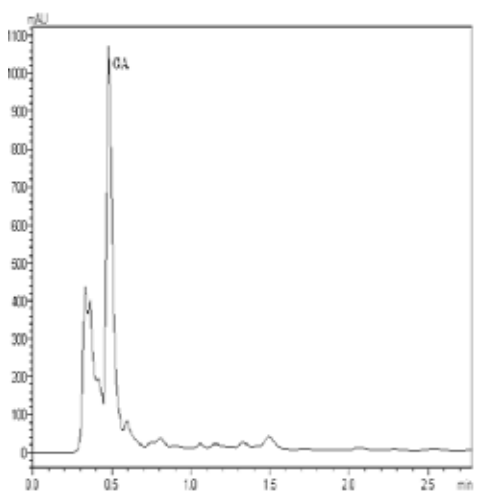
Parameter	HPLC Gallic Acid (μgmL^{-1})			UFLC Gallic Acid (μgmL^{-1})		
Linearity range	0.5 - 15			0.5 -	15	
Regression equation	y = 2E+09x-84588			y= 3E+08x-77286		
Correlation coefficient(rc)	0.9978			0.9988		
Analyte concentration	0.5	5	15	0.5	5	15
Repeatability (R.S.D.%)[b]	2.36	0.98	1.1	1.7	0.66	1.52
Intermediate precision (R.S.D.%)[b]	3.20	1.90	1.48	4.0	1.4	1.68
Limit of detection (LOD)	0.01			0.01		
Limit of quantification (LOQ)	0.04			0.04		

Analytical parameters of the HPLC/UFLC procedure for the gallic acid quantitation

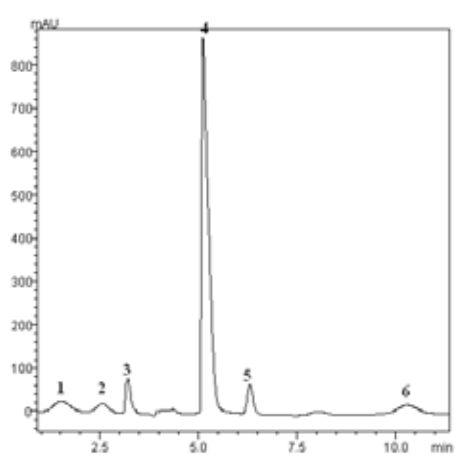
Table 2 Content of Gallic Acid in Dried and Processed Fruit Amla

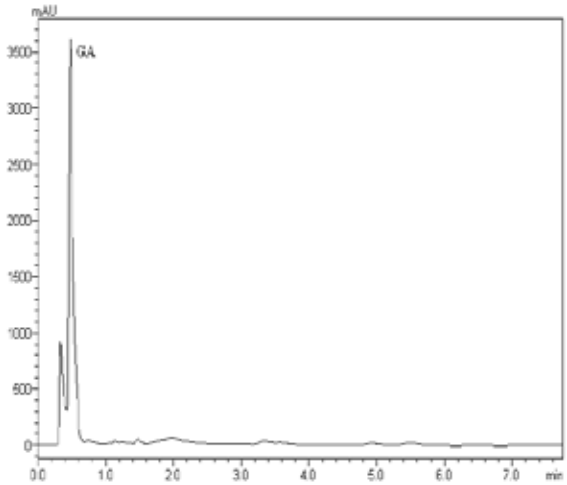
	Dried Fruit	Processed Fruit
HPLC (mg/100g gallic acid) – Ethanol extract	8.02±0.001	20.16±0.001
UFLC (mg/100g gallic acid) – Ethanol extract	10.05±0.0006	29.08±0.0005
HPLC (mg/100g gallic acid) – Methanol/ acetone	8.08±0.001	20.36±0.001
UFLC (mg/100g gallic acid) – Methanol/ acetone	10.06±0.0006	29.05±0.0005
Total polyphenols content(mg/100g gallic acid)	178.0±0.02	205.6±0.02





A **B**





D

Figure 1.
A: Dried Fruit in HPLC; B: Dried Fruit in UFLC; C: Processed Fruit in HPLC; D: Processed Fruit in UFLC

Figure 2. Antioxidant activity assayed by DPPH test (expressed as % bleaching) of extracts from different matrices of Emblica, containing known amount of gallic acid (◆) pure ascorbic acid; (▲) processed fruit; (■) dried fruit.

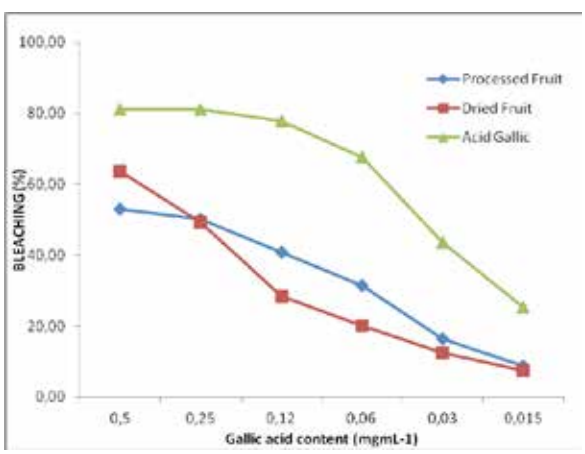
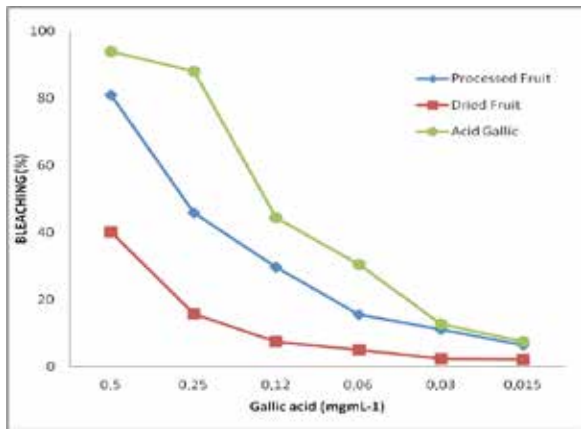


Figure 3. Antioxidant activity assayed by ABTS test (expressed as % bleaching) of extracts from different matrices of Emblica, containing known amount of gallic acid (◆) pure ascorbic acid; (●) processed fruit; (■) dried fruit.



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