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Vitamin C content and antioxidant activity of the fruit and of the Ayurvedic preparation of *Emblica officinalis* Gaertn.

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Abstract

Emblica officinalis Gaertn. is one of the most important plants of Āyurved, the traditional Indian medicine. In this ancient medicine, the fruit of *Emblica officinalis* is processed according to a method named "*Svaras Bhavana*", whereby the therapeutic potential of the plant is enhanced by treating the main herb with its own juice. For many years, the activity of the fruits was attributed to the high content of ascorbic acid; however, this has recently been questioned. The aim of the paper is to clarify this matter. A reliable and feasible HPLC method with diode array detection has been developed for the determination of ascorbic acid in *Emblica* fruit and particularly in *Emblica* fruit processed according to the Ayurvedic method. The antioxidant effects have also been evaluated in comparison to the real levels of Vitamin C by different antioxidant tests. The data obtained show that the *Emblica* fruit contains ascorbic acid (0.40%, w/w), and that the Ayurvedic method of processing increases the healthy characteristics of the fruit thanks to a higher antioxidant activity and a higher content of ascorbic acid (1.28%, w/w). It has also been found that Vitamin C accounts for approximately 45–70% of the antioxidant activity.

Keywords: *Emblica officinalis*; Āyurveda; Ascorbic acid; Liquid chromatography; Antioxidant activity

1. Introduction

Emblica officinalis Gaertn. (*Phyllanthus emblica* L.; *Dichelactina nodicaulis* Hance; *Emblica arborea* Raf.; *Phyllanthus glomeratus* Wall.; *Cicca emblica* Kurz.; *Diasperus emblica* Kuntze) is a medium to large deciduous tree belonging to a small subgenus of trees of the Euphorbiaceae growing in India, Sri Lanka, Pakistan, Uzbekistan, S.E. Asia, and China. *Emblica* (Amla in Hindi) grows wild and is cultivated up to 1400 m a.s.l.; in India, the most common cultivars are "Chakaiya", "Banarsi", and "Francis" (Scartezzini and Speroni, 2000). The previous name, *Phyllanthus emblica* L., was attributed by Linneaus with reference to a peculiar characteristic of this plant. The branches of this tree are oddly flattened in the manner of a leaf; the flowers bloom from the edges of these leaf-like branches, thus the name *Phyllanthus*, from the Greek words "*phyllon*" (leaf) and "*anthòs*" (flower). The name *Emblica* derives almost certainly from the "corruption" of the Sanskrit name "*Amlika*", although some authors believe that it could derive from the "corruption" of the Arabic word "*Embelgi*" used by Arabic physicians to name its fruit. In Sanskrit, *Emblica* has many synonyms: Amalaki (pure, clean), Dhatriphala (nurse fruit), Amritaphala (fruit of immortality), and others. All of these synonyms show how important this plant is in traditional Indian medicine. In Malaysia, this plant is so renowned that a city and a river bear its name: Malacca. The fruits of *Emblica* are widely consumed raw, cooked, or pickled, but they are also principal constituents of many Ayurvedic preparations (Scartezzini and Speroni, 2000). Indeed, *Emblica* is one of the most important plants of Āyurved, the Indian Traditional Medicine. According to the two main classic texts on Āyurved, *Charak Samhita* and *Sushrut Samhita*, Amalaki is regarded as "the best among rejuvenative herbs", "useful in relieving cough and skin disease", and "the best among the sour fruits". There are two historically ascertained events in which this plant was used for medical purposes: the famine of 1939–1940 and the cases of scurry in the Indian army in Nassirdab, today known as Rajasthan, in 1837 (Srinivasan, 1944). For many years, the therapeutic potential

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of the fruits was attributed to their high ascorbic acid content, about 1 g Vitamin C per 100 mL of fresh juice (Kapoor, 1990). Because of the presence of tannins, the ascorbic acid does not oxidise even in dried fruit, thus maintaining its antiscurvy property unchanged. Many studies have been published on the comparison between the effects of ascorbic acid and *Emblica*. The fruit extract has many pharmacological activities: it inhibits micronuclei formation, sister chromatid exchanges, clastogenicity and mutagenicity induced by metals such as lead, aluminum, cadmium, nickel, and caesium; it preserves against radiations (Scartezzini and Speroni, 2000), possesses antidiabetic activity (Sabu and Kuttan, 2002), inhibits clastogenicity of benzopyrene and cyclophosphamide (Sharma et al., 2000; Haque et al., 2001), is gastroprotective (Al-Rehaily et al., 2002), cytoprotective, and immunomodulating (Sai Ram et al., 2002). In recent years, new pharmacological activities have been found for *Emblica*: it has cytoprotective activity against chromium (Sai Ram et al., 2003), protects against oxidative stress in ischemic-reperfusion injury (Rajak et al., 2004), shows antivenom capacity (Alam and Gomes, 2003), ameliorates hyperthyroidism and hepatic lipid peroxidation (Panda and Kar, 2003), displays antiproliferative activity on MCF7 and MDA-MB-231 breast cancer cell lines (Lambertini et al., 2003), shows antitussive activity (Nosal'ova et al., 2003), and induces apoptosis in Dalton's Lymphoma Ascites and CeHa cell lines (Rajeshkumar et al., 2003).

In contrast with previous authors, Ghosal et al. (1996) asserted that *Emblica* fruits do not contain ascorbic acid, either in the free or in the conjugated form, but contain two new hydrolysable tannins of low molecular weight, namely emblicanin A (2,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-δ-lactone) and emblicanin B (2,3,4,6-bis-(*S*)-hexahydroxydiphenoyl-2-keto-glucono-δ-lactone), and other tannins, such as punigluconin (2,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoylgluconic acid) and pedunclagin (2,3,4,6-bis-(*S*)-hexahydroxydiphenoyl-D-glucose). The two emblicanins exhibited a very strong antioxidant action; moreover, they improved the efficacy of Vitamin C in reducing dehydroascorbic acid to ascorbic acid (Ghosal et al., 1996; Bhattacharya et al., 2000a). Some studies published later examined the pharmacological activities of these tannins and reported that they exhibit antioxidant activity in vitro and in vivo. In particular, they preserve erythrocytes against oxidative stress induced by asbestos (Ghosal et al., 1996), show antioxidant effects in rat brain (Bhattacharya et al., 2000a), exert a prophylactive effect against neuroleptic-induced tardive dyskinesia (Bhattacharya et al., 2000b), show antioxidant activity against ischemia reperfusion (Bhattacharya et al., 2002), antiulcerogenic effects (Sairam et al., 2002), and anticataractogenic effects in vitro (Suryanarayana et al., 2004).

The goal of the present paper was to evaluate the extent to which Vitamin C content determines the antioxidant activity of the fruit, and to what extent the traditional Ayurvedic processing method (called *Svaras Bhavana*) might influence the biological activity of the Ayurvedic preparation. In Ayurvedic terminology, *Svaras Bhavana* means "to increase the potency by treating the main herb with its own juice". For this purpose, a simple and rapid liquid chromatographic (HPLC) method was developed to determine Vitamin C content in dried and frozen *Emblica* fruit, in *Emblica* fruit processed according to the Ayurvedic method, and in an industrial extract (Merck). 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 ascorbic acid levels.

2. Materials and methods

2.1. Materials

The wild fruit of *Emblica* were authenticated by Dr. M.R. Uniyal, Maharishi Āyurveda Product Ltd., Noida, India. The commercial Ayurvedic samples of *Emblica* analysed in this study (Emblimap—MA-390), batch no. April 02 and April 01, were bought from Maharishi Āyurveda Product Italy (VR, Italy). The dry extract of *Emblica* was supplied by Dr. Phyllis Carter, External Corporate Communications of Merck KGaA (Darmstadt, Germany), batch no. K4908265 (titre in total small hydrolysable tannoids 69%). Waters C18 Spherisorb 5 μm ODS2 HPLC columns (Ireland) was used. Solvents (water and methanol) were of HPLC grade and purchased from Carlo Erba Reagenti S.p.a. (Milan, Italy). The Folin–Ciocalteu reagent and the DPPH radical and ABTS were bought from Sigma (Milan, Italy). Ascorbic acid (85%, m/m), phosphoric acid, dipotassium peroxidisulfate ($K_2S_2O_8$) and NaOH were obtained from Carlo Erba Reagenti S.p.a. Ultrapure water (18.2 MΩ cm^{-1}) was obtained by means of a Millipore (Milford, MA, USA) MilliQ apparatus.

2.2. Apparatus

The HPLC apparatus consisted of an Agilent (Waldbronn, Germany) 1100 series isocratic pump and an Agilent 1100 series diode array (DAD) detector. The data system consisted of an HP CORE Chemstation LC 3D (Waldbronn, Germany).

2.3. Standard solution and sample preparation

The stock solution of ascorbic acid was 10 mg mL^{-1} in water. All dilutions subsequent were made in water.

Five hundred milligrams of the finely powdered fruit material or Ayurvedic preparation was extracted once with 50 mL of water for 30 min. All samples were filtered through a 0.22 μm membrane filter from Millipore (Malsheim, France).

2.4. HPLC method

2.4.1. Chromatographic conditions

The DAD detector was set in the 200–350 nm range and quantitative analysis was performed at 243 nm with reference on at 308 nm. Separation was achieved using a Waters C18 Spherisorb 5 μm ODS2 Column. The mobile phase was constituted of a mixture (97.5:2.5, v/v) of phosphate buffer 100 mM, pH 2.5,

and methanol. The flow rate was 0.4 mL min^{-1} and the injection loop was 20 μL.

2.4.2. Calibration curves

Standard solutions of ascorbic acid, in the 1–50 μg mL^{-1} concentration range, were prepared and injected in the HPLC system.

The analyte peak area values were plotted against the corresponding concentrations of the analyte (expressed as μg mL^{-1}), and the calibration curve was constructed by means of the least-square method.

2.4.3. Sample analysis

An aliquot of the sample, after suitable dilution with water, was injected into the HPLC.

Every sample solution was injected in triplicate. The area value of ascorbic acid peaks obtained injecting the extract of *Emblica* into the HPLC was interpolated on the calibration curve.

2.4.4. Precision assays

A single sample of *Emblica* was analysed six times. Percentage standard deviations (R.S.D.%) were obtained for repeatability (intra-day precision) and intermediate precision (inter-day precision).

2.4.5. Accuracy assays

Known amounts of ascorbic acid standard solution were added to known amounts of *Emblica* sample. The method accuracy was evaluated by calculating the difference between the spiked sample peak areas and the original sample peak areas, then comparing these differences with the peak area obtained by injecting standard solutions having the same concentration as the sample spiking.

2.5. Total polyphenol content

Total polyphenol content of *Emblica* was determined by the Folin–Ciocalteu colorimetric method (Ragazzini and Veronesi, 1973). The methanolic *Emblica* extract was mixed with 0.5 mL of the Folin–Ciocalteu reagent and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h 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 *Emblica* 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 ascorbic acid content and then diluted in order to have exact concentrations of ascorbic acid in the range 0.01–0.5 mg mL^{-1} (corresponding to 0.001–0.05%). Dilution was also performed on pure ascorbic acid solution. Both *Emblica* samples and pure Vitamin C solution were added to a methanol solution of DPPH (1×10^{-4} M), shaken vigorously, and kept in the dark for 30 min. The absorbance of samples was measured with a spectrophotometer (Perkin-Elmer 554) at 517 nm against a blank of methanol. Positive controls consisted of ascorbic acid. The antioxidant activity of each extract, containing same concentration of ascorbic 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 five 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 ($ABTS^{\bullet+}$) (Miller et al., 1993). Concentrations of ascorbic acid and dilutions for the assay were performed as described above. $ABTS^{\bullet+}$ was produced by the reaction of ABTS solution (2 mM) with 70 mM potassium persulfate ($K_2S_2O_8$) (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. Nine hundred and ninety microliters of diluted ABTS solution was mixed with 10 μL 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:

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

All test were run five times and averaged.

3. Results and discussion

3.1.1. HPLC method

Attempts were made to evaluate Vitamin C content in *Emblica* extracts using previously published methods (Finley and Duang, 1981; Kim et al., 2002; Valls et al., 2002). In our experimental conditions, separation of the ascorbic acid peak from interference of the matrix was unsatisfactory; therefore, a new HPLC procedure was developed. The analysis was performed using a C18 reverse phase column, and the composition (% methanol), 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 100 mM phosphate buffer, pH 2.5 (97.5%), and methanol (2.5%), with a flow rate of 0.4 mL min^{-1}. The DAD detector was set in the 200–350 nm range, and the quantitative analyses were carried at 243 nm. Under these conditions, a neat peak of ascorbic acid was detected at 9.9 min. The spectrum was recorded and maximum absorbance was found at 243 nm (data not shown).

A good linearity was found from 1 to 50 μg mL^{-1} ascorbic acid, and the linear regression equation was $y = 145.44x - 68.938$ ($r_c = 0.9996$), where y is the peak height, expressed as arbitrary units, and x is the ascorbic acid concentration,

Table 1
Analytical parameters of the HPLC procedure for the ascorbic acid quantitation

Parameter	Ascorbic acid ($\mu g\,mL^{-1}$)		
Linearity range	1–50		
Regression equation[a]	$y = 145.44x - 68.938$		
Correlation coefficient (r_c)	0.9996		
Analyte concentration	10	25	50
Repeatability (R.S.D.%)[b]	2.36	0.98	1.1
Intermediate precision (R.S.D.%)[b]	3.20	1.90	1.48
Limit of detection (LOD)	0.01		
Limit of quantification (LOQ)	0.04		

[a] y: peak area and x: concentration ($\mu g\,mL^{-1}$).
[b] $n = 6$.

expressed as $\mu g\,mL^{-1}$. Limit of quantitation (LOQ) and limit of detection (LOD) values, calculated according to the USP XXV Edition Guidelines (United States Pharmacopoeia, 2003), were 0.04 and 0.01 $\mu g\,mL^{-1}$, respectively.

Repeatability and intermediate precision values, obtained at concentration values minimum, medium, and high, were in the range 0.98–3.2%. The validation parameters are reported in Table 1.

The volume of water and the time required for optimal extraction (50–200 mL and 0.5–24 h, respectively) were also investigated. Results indicate that the best quantitative extraction of Vitamin C was obtained by treating 500 mg of matrix with 50 mL of water for 30 min. The extract was injected into the HPLC after suitable dilutions with water.

To confirm that the extraction procedure under these conditions was quantitative, two successive extracts of the same sample of *Emblica* fruit were injected separately into the HPLC apparatus. In the second extraction step, the analyte peak area was 1% of that obtained in the first extraction step. These results indicate that the extraction procedure is easy, rapid, and reliable.

The chromatogram of an extract of processed Emblica recorded at 243 nm with reference off showed two peaks very close to each other (Fig. 1a). A comparison between the spectra of these peaks with that of pure ascorbic acid confirmed that the retention time of the analyte was 9.9 min. Moreover, it is possible to eliminate the interference peak by setting the reference of the detector (which is automatically subtracted from the signal at 243 nm) at 308 nm (Fig. 1b, reference on). In fact, the absorbance values of the interference spectrum at 243 and 308 nm were the same, while the absorbance of ascorbic acid at 308 nm is practically equal to zero.

3.2. Application to Emblica extracts

The above-described procedure was used to analyse different products of *Emblica*, and in particular frozen fruit, dried fruit, processed (Ayurvedic preparation) fruit, and a commercial Merck extract. As shown in Table 2, ascorbic acid is present in all the examined products, ranging from 0.37% in dried fruit to 2.00% in the Merck extract. The precision was calculated for processed *Emblica*, and gave good repeatability and inter-day precision values, lower than 3%.

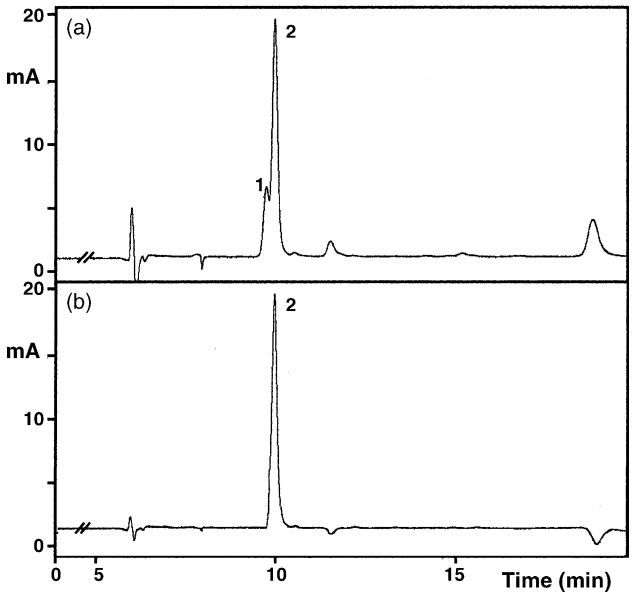

Fig. 1. Chromatograms of an extract of processed fruit recorded at 243 nm with detector reference: (a) off and (b) at 308 nm.

The accuracy of the method, obtained by means of recovery assays, gave satisfactory results, always between 102 and 104%.

The dried and frozen fruits contain about the same amount of Vitamin C (0.4%), while a higher level of Vitamin C was found in the processed fruit (*Ayurvedic preparation*). In fact, the Ayurvedic preparation (called "Svaras Bhavana") consists in mixing a specific quantity of the dried fruit powder with its own fresh juice for a few hours; then, it is dried and powdered again (fruit processed once). This powder is again mixed with the juice of fresh *Emblica*, dried, and powdered again; this is the second processing. The procedure is repeated for up to 21 times. The Ayurvedic procedure aims to increase the health efficiency of the product (The Ayurvedic Formulary of India, 2003). Indeed, processed fruit contained up to three times more ascorbic acid than the dried unprocessed ones; also, the total content of polyphenolic compounds was higher (Table 2), suggesting that the Ayurvedic method of processing the fruit increased its beneficial properties. The difference between dried and frozen fruit is also worth noting. Both of them have a very similar ascorbic acid content, and this suggests that the tannins present in the fruit may prevent the oxidation of Vitamin C during the drying

Table 2
Ascorbic acid and total polyphenol content in different products of *Emblica officinalis*

	Frozen fruit	Dried fruit	Processed fruit	Merck extract
Ascorbic acid (%)	0.40 ± 0.01	0.37 ± 0.01	1.28 ± 0.01	2.00 ± 0.01
Total polyphenols content (GAE[a])	201.8 ± 0.8	206.9 ± 0.9	255.2 ± 1.2	200.1 ± 1.3

[a] Expressed as gallic acid equivalents.

process, thereby maintaining the nutritional quality of the fruit unaltered.

The amount of total polyphenols in the matrices analysed was performed with the aim of verifying if the increase in ascorbic acid was accompanied by a similar increase in the content of these compounds, which are also involved in antioxidant activity. Results showed that processed *Emblica* fruits had a higher level of total polyphenols (about 25%) than the other matrices (Table 2).

3.3. Antioxidant activity

The antioxidant activity of all extracts was evaluated with the ABTS and DPPH tests. These methods are rapid, sensitive, reproducible, 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 aqueous 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 ascorbic acid in the samples was higher than 0.25 mg mL^{-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 Vitamin C versus the other compounds.

The results of the ABTS test are plotted in Fig. 3. Also, this test cannot be applied to samples with an ascorbic acid concentration higher than 0.25 mg mL^{-1} for the same reason described above.

In any case, all extracts exhibited an antioxidant activity even at the lowest concentration tested, i.e. 0.01 mg mL^{-1}.

A comparison between antioxidant activity of ascorbic acid standard solution and those of the three different *Emblica* products, measured at the same concentration of ascorbic acid, shows that the processed fruit has the highest antioxidant activity. The results of both tests also show that the antioxidant activity of processed and dried *Emblica* does not depend only upon Vitamin C content. In fact, in aqueous extracts of processed and dried fruit, Vitamin C accounts for 45–70% of the antioxidant activity. In particular, the antioxidant activity of processed fruit is due to ascorbic acid for only 60% or less, while in the other products this percentage increases. These results are in agreement with those indicating that the presence of emblicanins and rutin favours the conversion of dehydroascorbic acid to ascorbic acid (Ghosal et al., 1996), and confirm that the Ayurvedic method of processing increases the amount of antioxidant compounds present in the final product.

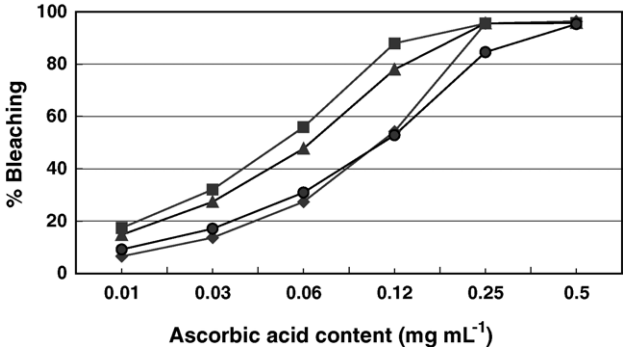

Fig. 2. Antioxidant activity assayed by DPPH test (expressed as % bleaching) of extracts from different matrices of *Emblica*, containing known amount of ascorbic acid ((♦) pure ascorbic acid; (■) processed fruit; (▲) dried fruit; (●) Merck extract).

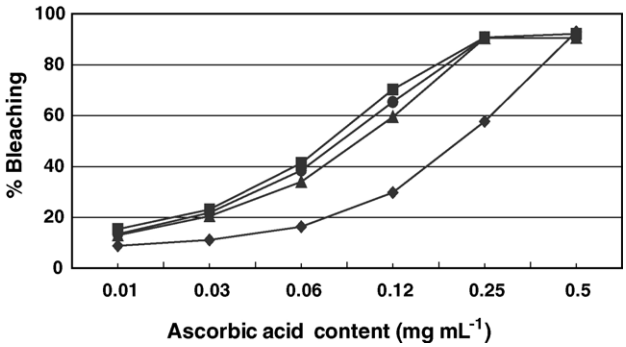

Fig. 3. Antioxidant activity assayed by ABTS test (expressed as % bleaching) of extracts from different matrices of *Emblica*, containing known amount of ascorbic acid ((♦) pure ascorbic acid; (■) processed fruit; (▲) dried fruit; (●) Merck extract).

4. Conclusions

Until the 1990s, it was believed that *Emblica officinalis* contained a large amount of Vitamin C. Ghosal et al. (1996) published a paper where he asserted that the fruit of this plant does not contain any ascorbic acid, but contains emblicanins which are responsible for the biological activity of the fruit. Our results indicate that these fruits do indeed contain ascorbic acid, albeit in a smaller quantity than previously reported (Kapoor, 1990). The evaluation of antioxidant activity shows that it is due in a large percentage to presence of ascorbic acid. It is however possible that the tannins identified by Ghosal et al. (1996) favour the recycling of ascorbic acid, thereby increasing the antioxidant activity of the *Emblica* fruit.

The HPLC method described here represents a reliable procedure for analysis of ascorbic acid in *Emblica officinalis*: indeed, the selectivity was increased in order to obtain a good separation between ascorbic acid and interference peaks, allowing a rapid and accurate determination, which could be helpful for scientific as well as commercial applications.

The results of this paper also indicate that the Ayurvedic traditional method of processing the fruit (*Svaras Bhavana*) increases its beneficial characteristics. In fact, the amount of ascorbic acid and polyphenols found in processed fruit as well as the antioxidant activity was higher than in dried fruit.

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