

Correlation between scavenging property and antioxidant activity in the extracts of *Emblca officinalis* Gaertn., syn. *Phyllanthus emblica* L. Fruit

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Abstract

The present study investigates a concentration-dependent assessment of scavenging property and antioxidant activity in the extracts of *Emblca officinalis* Gaertn. syn. *Phyllanthus emblica* L. fruit using various *in vitro* assays. A correlation of aqueous and ethanolic extract of *Emblca* fruit along with different known standards for its antioxidative properties was made by performing assays like TPC, RP, TAC, DPPH FRAP and HRSA. Both the aqueous and ethanolic extract of fruit showed high phenolic content beside strong reducing power and free radical scavenging capacity. The hydroxyl radical scavenging potential of aqueous extract was higher compared to ethanolic extract. A high positive correlation was observed among the *in vitro* assays for the antioxidative properties except for HRS assay which showed negative correlation. Our finding not only tops up the results related to antioxidant asset of amla fruit but also emphasizes the fact of its usefulness as chemopreventive and/or chemotherapeutic importance. Further studies are warranted to explore the constituents responsible for the antioxidant property and other pharmacological activities.

Key words: *Emblca officinalis* Gaertn., *Phyllanthus emblica*, DPPH, FRAP, antioxidant

Introduction

Indian gooseberry (*Emblca officinalis* Gaertn. synonymously known as *Phyllanthus emblica* L. (Family: Euphorbiaceae), popularly known as amla, is widely distributed plant in subtropical and tropical areas of India, China, Thailand and Indonesia (Liu *et al.*, 2008). It is a well-known Ayurvedic plant used in many traditional systems of medicine, believed to be created first in the universe (Khan, 2009). Fruits of amla together with varied parts of the plant are used to treat a number of diseases and disorders including fever, cold, stomachache, dyspepsia *etc.* It is also used as an anti-

inflammatory, antipyretic, diuretic, hair tonic, laxative, liver tonic and prevent peptic ulcer (Baliga and Dsouza, 2011). The fruit contains tannins, alkaloids, and phenolic compounds, but flavonoids exhibits maximum benefit (Chatterjee *et al.*, 2011). Reported as one of the highly active plant for various ailments, amla is considered useful as an antimalarial (Pinmai *et al.*, 2010 and Bagavan *et al.*, 2011), antiatherosclerotic, anti-inflammatory (Dang *et al.*, 2011), antimutagenic, immunomodulatory (Baliga and Dsouza, 2011), antitussive (Nosalova *et al.*, 2003), free radical scavenging, antioxidant, antidiabetic (Nampoothiri *et al.*, 2011; Hazra *et al.*, 2010), antibacterial (Ghosh *et al.* 2008 and Saeed and Tariq, 2007), anticancer, antidiarrheal (Nagamkitidechakul *et al.*, 2010; Baliga and Dsouza, 2011), adaptogenic, liver protective (Tasduq *et al.*, 2005; Reddy *et al.*, 2010), wound healing (Sumitra *et al.*, 2009), cardioprotective, gastro-protective, cerebral and intestinal tonic (Aslokar *et al.*, 1992; Rajarama Rao and Siddiqui, 1964), antipyretic and analgesic. It is also used in cure of anaemia, dyspepsia, jaundice,

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haemorrhage, diarrhoea (Perianayagama, 2004; Baliga and Dsouza, 2011). In some studies, amla is reported to reduce the cadmium (Swapna *et al.*, 2010), arsenic (Sharma *et al.*, 2009), chromium toxicity induced by oxidative stress (Sai Ram *et al.*, 2003), showed radiomodulatory, chemomodulatory, chemopreventive effects (Baliga and Dsouza, 2011) and inhibits the UV B-induced photoaging in human skin fibroblast through ROS scavenging property (Adil *et al.*, 2010). The fruit of amla is a rich source of vitamin C and the crude extract was reported to antagonize the toxic effects of metals on liver and kidney due to its antioxidant properties (Halliwell and Gutteridge, 1985; Roy *et al.*, 1991; Baliga and Dsouza, 2011). In appurtenance, previously reported experimental stipulations have shown that phytoconstituents of amla like corilagin, gallic acid, elaeocarpusin, ellagic acid, geraniin, norsesquiterpenoids, pyrogallol, and prodelphinidins B₁ and B₂ also possess antitumor effects. In our present study, we investigate, assess and correlate the scavenging property and antioxidant activity in the aqueous and ethanolic extracts of Indian gooseberry fruit with known antioxidants by measuring the performance in different assays.

Materials and Methods

Chemicals

DPPH, DMSO, Folin-Ciocalteu Reagent, Ascorbic acid, Sodium phosphate, Thiobarbituric acid, Ammonium molybdate, Ethyldimine tetraacetic acid (EDTA), Sodium acetate trihydrate, Sodium hydroxide, Tricarboxylic acid, Tripyridyl-s-triazine (TPTZ) and Sodium citrate were purchased from Sigma-Aldrich Chemical Co. Other chemicals such as Gallic acid, Tris-HCl, Sodium carbonate, Sodium chloride, Potassium ferricyanide, Ferric chloride, Potassium dihydrogen phosphate, Disodium hydrogen phosphate, were purchased from Himedia, India. Solvents like Methanol, Ethanol, Glacial acetic acid, Sulphuric acid and Hydrochloric acid were obtained from Merck Chemicals Ltd., India. All the chemicals used were of analytical grade or higher.

Collection of plant material and extract preparation

The fruits of *Emblica officinalis* Gaertn., syn. *Phyllanthus emblica* L. were obtained from the research farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow. The plant material was authenticated by Dr. S.C. Singh of our institute and a voucher specimen was deposited in Gyan Surabhi (No. 9071). Extracts were prepared in similar manner as described previously (Luqman *et al.*, 2005; Luqman *et al.*, 2009a; Luqman *et al.*, 2009b). In brief, the fruits were washed and dried at 40°C followed by extraction with absolute ethanol (99.5%, Merck Specialities, Mumbai, India) and double-distilled water. Three step extractions were done and extracts were filtered and concentrated in a rotary evaporator (Buchi, Flavel, Switzerland) at 45°C. All the extracts were dried at 40°C

in a hot air oven and stored at 4°C. Stocks of the extracts were prepared by dissolving 100mg per mL of dimethyl sulfoxide (DMSO 99.5%, Sigma-Aldrich). The final concentration of DMSO was always <0.05% in the reaction cocktail.

Determination of total phenolic content

The total phenolic content of aqueous and ethanolic extract of *Emblica* fruits and reference standards were determined using Folin-Ciocalteu reagent as described by Singleton and Rossi (1965) reported previously (Luqman *et al.*, 2009). Aliquots of samples were maintained to 50µl, followed by the addition of 250µl pre-diluted Folin's reagent (1:9 with distilled water) and mixed thoroughly. After 5 min., 200 µl of 7.5% Na₂CO₃ added and incubated at 37°C for 90 min. The absorbance was measured at 765nm. Standard curve of gallic acid was made similarly and results were expressed in terms of gallic acid equivalence.

Estimation of reducing power (RP)

The reducing power of aqueous and ethanolic extract of *Emblica* fruits along with different reference standards were determined by following the method described by Yen and Chen (1995), published earlier (Luqman *et al.*, 2009). Aliquots of samples were maintained to 100µl, followed by the addition of 250µl phosphate buffer (0.2M, pH 6.6) and 250µl of 1 % potassium ferricyanide in each reaction mixture and incubated at 50°C for 20 min. After the incubation, reaction was terminated by the addition of 250µl of 10% tri chloro acetic acid and centrifuged at 5000 rpm for 10 min. From each reaction mixture, 250µl of supernatant was diluted with equal amount of distilled water. Aliquot of 50µl FeCl₃ (0.1%) was added in each and absorbance was recorded at 700 nm. Increased absorbance in terms of optical density of the reaction mixture indicates high reducing power.

Evaluation of free radical scavenging activity using DPPH assay

The DPPH (2,2-diphenyl-1-picryl hydrazyl) radical scavenging activity of aqueous and ethanolic extract of *Emblica* fruits and reference standards were measured according to Luqman *et al.* (2009) based on method of Chung *et al.* (2002). Different concentrations of each extracts were added to 100mM Tris-HCl buffer (pH 7.4) and 100µM of methanolic solution of DPPH. The reaction mixture was shaken vigorously incubated for 30 minutes in the dark at room temperature and changes in absorbance of samples were measured at 517nm, against a blank. All measurements were performed in triplicate.

Total antioxidant capacity estimation

The total antioxidant capacity of aqueous and ethanolic extract of *Emblica* fruits along with different reference standards were evaluated by the method of Preito (1999) reported previously (Luqman *et al.*, 2009). In brief, aqueous

and ethanolic extract of *Emblica* fruits (10, 50, 100, 250, and 500 μg) were mixed with phosphomolybdenum using ascorbic acid as the standard in 1 ml of TAC reagent (3.3ml sulphuric acid, 335mg sodium phosphate and 78.416 mg ammonium molybdate in 100ml of distilled water). The sample mixtures were incubated in a boiling water bath at 95°C for 90 min. The absorbance of the samples was measured at 695 nm. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) measures the antioxidant capacity to reduce the Fe^{3+} /tripyridyl- s-triazine (TPTZ) complex, to the ferrous form (Benzie and Strain, 1996 and Luqman *et al.*, 2009). The FRAP reagent was freshly prepared by mixing 10 mM TPTZ with 20 mM ferric chloride in 300 mM acetate buffer, pH 3.6 in ratio 1:1:10. Different concentrations (10, 50, 100, 250, and 500 μg) of aqueous and ethanolic extract of *Emblica* fruits were added and the decrease in the absorption by the complex was measured after 5 min at 593 nm at room temperature and the standard curve was prepared by using different concentration of ferrous sulphate. The activities were calculated by comparing the concentration of each extract with the concentration of Fe^{++} required to give the same absorbance change using FeSO_4 as standard. All the tests were done in triplicate.

Hydroxyl Radical Scavenging (HRS) assay

Potassium dihydrogen phosphate (272.2 mg) and Disodium hydrogen phosphate (356 mg) were mixed and dissolved in 100 ml distilled water with adjustment of pH to 7.4. The procedure for preparation of dissimilar reaction cocktail (A, B, C & D) for HRS assay reported previously (Luqman *et al.*, 2008; Luqman and Kumar, 2011) are described. (A): 3.75 mM Deoxyribose, 1 mM H_2O_2 , 100 μM FeCl_3 , 100 μM EDTA, 100 μM Ascorbic acid dissolved in potassium phosphate buffer; (B): 3.75 mM Deoxyribose, 1 mM H_2O_2 , 100 μM FeCl_3 , 100 μM Ascorbic acid dissolved in potassium phosphate buffer; (C): 3.75 mM Deoxyribose, 1 mM H_2O_2 , 100 μM FeCl_3 , 100 μM EDTA dissolved in potassium phosphate buffer and (D): 3.75 mM Deoxyribose, 1 mM H_2O_2 , 100 μM FeCl_3 dissolved in potassium phosphate buffer. In addition, TBA (1%) and TCA (2%) acid solution was also prepared in double distilled water. The experiment of HRS assay was performed according to the procedure reported earlier (Halliwell and Gutteridge, 1987; Aruoma, 1994; Paya *et al.*, 1999; Luqman *et al.*, 2008; Luqman and Kumar, 2011). Aliquots of sample (10 μg , 50 μg , 100 μg , 250 μg) were mixed with 200 μl of reaction cocktail (A, B, C & D) and incubated at 37°C for 60 min. 100 μl each of TCA (2%) and TBA (1%) were added and the reaction cocktail was heated at 100°C for 15 min. Similarly, appropriate control (DMSO) was also run parallel and the absorbance was recorded at 532 nm.

All the determinations were performed in duplicates and values plotted were mean \pm SE (SEM) of three independent analyses. Percent scavenging activity was calculated by using the formula: Control-Experimental/Control \times 100.

Statistical analysis

All the experiments were carried out in triplicates and results were expressed as Mean \pm Standard Error. The difference in the percent scavenging activity of DPPH and HRSA experimental values was found significant ($p < 0.05$) compared to control in student's *t* test. The Pearson coefficients were also calculated for TPC, RP, DPPH, TAC, FRAP and HRSA (Table 1).

Results and Discussion

A concentration-dependent *in vitro* comparative examination of aqueous and ethanolic extract of *Emblica* fruit along with different known standards for its antioxidative properties was done and their correlation were investigated by performing assays like TPC, RP, TAC, DPPH FRAP and HRSA. The observations obtained are presented in Figures 1-9.

Indian gooseberry, an ancient and substantial Ayurvedic medicine ascribed to a number of medicinal properties has a distinguished history in Indian System of Medicine. It also act as a dietary supplement and serves as a potent antioxidant due to its high content of ascorbic acid, flavonoids, polyphenols and tannins (Naik *et al.*, 2005; Malar and Bai, 2009). Phenols are the major plant metabolites with an immense biological importance (Ani and Naidu, 2011). They show extreme diversity in their amount, type and availability in plants (Hakkinen *et al.*, 1999 and Gorinstein *et al.*, 2001). The total phenol content was estimated using Folin-Ciocalteu reagent and the values are expressed as gallic acid. The aqueous and ethanolic extract of *Emblica* fruits along with known standard antioxidant were found to show significant increase in phenol contents in concentration-dependent manner (Figure 1) and the value of total phenols in both the extracts (aqueous and ethanolic) were found to be 336 ± 33.94 and 318 ± 45.25 $\mu\text{gGAE}/\text{mg}$ of extract respectively. Phenolic are one of the important constituent of plants responsible for their high antioxidant activity (Povichi *et al.*, 2010). Phenolic content of both the extracts (aqueous and ethanolic) exhibit a very high correlation with reducing power (0.91, 0.86), DPPH (0.94, 0.91), Antioxidant capacity (0.83, 0.81) and FRAP value (0.98, 0.96). Comparative data suggests that aqueous extract shows higher phenolic content than ethanolic extract, tocopherol and BHT, but lower than ascorbic acid and quercetin (Ascorbic acid > Quercetin > Aqueous > Ethanolic > Tocopherol > BHT).

Reducing power measures the antioxidant activity of extracts by converting Fe^{+++} to Fe^{++} which is due to the reductones present in it, responsible for the termination of free radical

generation by donating the H atom (Ramesh *et al.*, 2011). The conversion of Fe⁺⁺⁺ to Fe⁺⁺ in the presence of *Emblica* extracts and known standards were found to be concentration dependent. The reductive capabilities of aqueous and ethanolic extracts (Figure 2) were found to be higher than quercetin, tocopherol and BHT, whereas ascorbic acid exhibit highest reducing power (Ascorbic acid > Aqueous = Ethanolic > Quercetin > Tocopherol > BHT). The reducing power of both extract of *Emblica* fruits (aqueous and ethanolic) display strong correlation with DPPH (0.99, 0.99), antioxidant capacity (0.57, 0.51) and FRAP (0.97, 0.72) values.

The scavenging property of stable DPPH free radical by natural compounds is considered as a valid and easy method to determine the trapping activity of antioxidants (Ozturk *et al.*, 2007; Suhaj, 2006). To act as an antioxidant the polyphenols either captures the free radicals or donates the hydrogen atom as a result the purple colour of 2, 2-diphenyl-1-picryl hydrazyl (DPPH) will reduce to α , α -diphenyl- β -picrylhydrazine (yellow coloured compound, Akowuah *et al.*, 2005). The scavenging activity of aqueous and ethanolic extract of *Emblica* fruits and known standards showed a significant increase in inhibition as the concentrations increases (Figure 3). Ascorbic acid shows the highest inhibition followed by quercetin, BHT, ethanolic extract, aqueous extract and tocopherol. Significant correlations of DPPH inhibition of both extracts of *Emblica* fruits (aqueous and ethanolic) were found with antioxidant capacity (0.68, 0.63) and FRAP value (0.99, 0.80).

The basis of formation of green colour phosphate/Mo(V) complex at acidic pH is due to the reduction of Mo(VI) to Mo(V) in the presence of extracts. The antioxidant capacity was expressed in μ g of ascorbic acid equivalent and a concentration-dependent increase was observed in both the extracts. Aqueous and ethanolic extract of *Emblica* fruit showed higher antioxidant capacity compared to BHT but lower than tocopherol and quercetin (Figure 4). A positive correlation was found for both extracts of *Emblica* fruit (aqueous and ethanolic) among antioxidant capacity and FRAP value (0.76, 0.93).

A concentration-dependent steep increase in FRAP value was determined in both extract of *Emblica* fruit (aqueous and ethanolic) and known standards (Figure 5). The aqueous extract showed a slightly higher value than ethanolic extract, tocopherol and BHT, but both extracts were exhibiting lower FRAP value than quercetin and ascorbic acid (Quercetin > Ascorbic acid > Aqueous > Ethanolic > Tocopherol > BHT).

In HRS assay, a mixture of Fe⁺⁺⁺-EDTA, H₂O₂ and ascorbic acid generates hydroxyl radical detected by deoxyribose degradation, a reaction useful for studying free radical initiated oxidative stress and antioxidant activity of various test compounds (Gutteridge, 1984; Gutteridge and Halliwell, 1990; Halliwell *et al.*, 1991; Luqman and Kumar, 2011). The ethanolic and aqueous extracts of *Emblica* fruit exhibit a potent scavenging activity for hydroxyl radical in a concentration-dependent manner. The exclusion of ascorbic acid from the reaction mixture promotes generation of hydroxyl radicals and causes degradation of deoxyribose (Figures 6 and 7). The scavenging effect of amla extracts may either be due to metal chelation or through trapping of hydroxyl radical. Both aqueous and ethanolic extract showed scavenging potential upto a concentration of 250 μ g except suppression of activity by aqueous extract at higher tested concentration when ascorbic acid was omitted from the reaction system (Figure 6). However, in absence of both EDTA and Ascorbic acid, aqueous extract (100 μ g) showed maximum scavenging effect (Figure 7). The utilization of EDTA is essential because inhibition of iron dependent deoxyribose degradation in the absence of EDTA depends not only on the aptitude of a scavenger to react with \bullet OH but also on its ability to form complexes with iron (Figures 7 and 8). The hydroxyl radical scavenging effect of aqueous and ethanolic extracts of *Emblica* fruit in presence of EDTA and Ascorbic acid showed maximum scavenging potential at a tested concentration of 50 μ g (Figure 9).

In earlier published reports, methanolic extract of amla were found to inhibit lipid peroxidation, scavenge hydroxyl and superoxide radicals, reduces the blood sugar level in normal and alloxan-induced diabetic rats (Sabu and Kuttan, 2002; Poltanov *et al.*, 2009) and inhibits LDL oxidation and key enzymes linked to type 2 diabetes (Nampoothiri *et al.*, 2011). The extract also reduces oxidative stress in streptozotocin-induced diabetic rats (Rao *et al.*, 2005). Amla fruit extract administered to chronically alcohol-fed rats (250 mg/kg of body weight/day) offers protection by lowering the carbonyl content and lipid peroxidation simultaneously elevating activities of antioxidant enzyme (SDH, NADH dehydrogenase, and cytochrome c oxidase) in hepatic mitochondria (Hazra *et al.*, 2010). It also ameliorates alcohol-induced brain mitochondrial dysfunction in rats (Reddy *et al.*, 2011), attenuates N-nitrosodiethylamine-induced apoptosis, autophagy, and inflammation in rat livers (Chen *et al.*, 2011) and exert protective effect on isoproterenol-induced

cardiotoxicity (Ojha *et al.*, 2011). Five compounds (Gallic acid, Methyl gallate, Corilagin, Furosin, and Geraniin) of ethyl acetate phase showed strong NO scavenging activity *in vitro* (Kumaran and Karunakaran, 2006) and were also found capable of scavenging DPPH radical (Emblicanin B > Emblicanin A > Gallic Acid > Ellagic Acid > Ascorbic Acid) and the antioxidant property was relative to their polyphenol content. The potent antioxidant activity of tannoids is mainly due to the presence of emblicanin A, emblicanin B, punigluconin and pedunculagin. These tannoids exert their protective effects on free radical scavenging enzymes (SOD, CAT, GPx) and lipid peroxidation of rat brain (Bhattacharya *et al.*, 1999; Bhattacharya *et al.*, 2000; Pozharitskaya *et al.*, 2007).

Based on our observations, the aqueous and ethanolic extract of *Emblica* fruits along with known standard antioxidant were found to show significant change in TPC, RP, TAC, DPPH, FRAP and HRSA values in a concentration-dependent manner. The aqueous extract of fruit showed highest phenolic content along with strong reducing power and free radical scavenging capacity. A high positive correlation was observed among the *in vitro* assays for the antioxidative properties. Our findings not only top up the results related to antioxidant asset of amla fruit but also emphasize the fact of its usefulness as chemopreventive and/or chemotherapeutic appurtenance. Further investigations on isolation, characterization, and identification of novel constituents responsible for the antioxidant and other pharmacological activity are considered necessary.

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Figure 1. Total Phenolic content of *Emblica* extracts and known antioxidants expressed as μg of Gallic Acid Equivalence

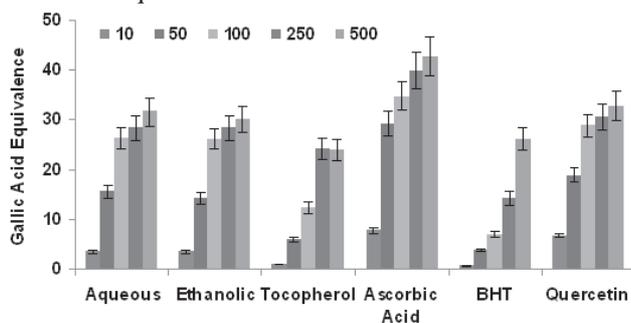


Figure 2. Reducing power of *Emblica* extracts and known antioxidants

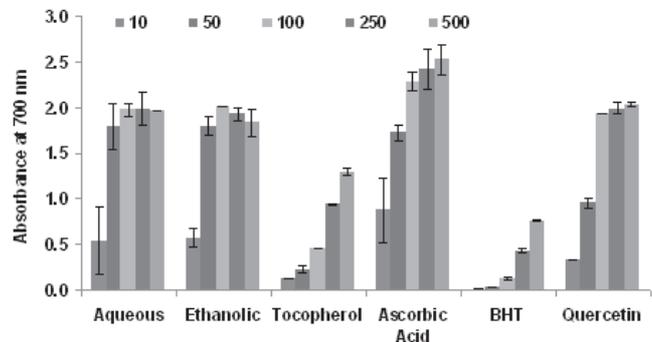


Figure 3. DPPH radical scavenging activity of *Emblica* extracts and known antioxidants

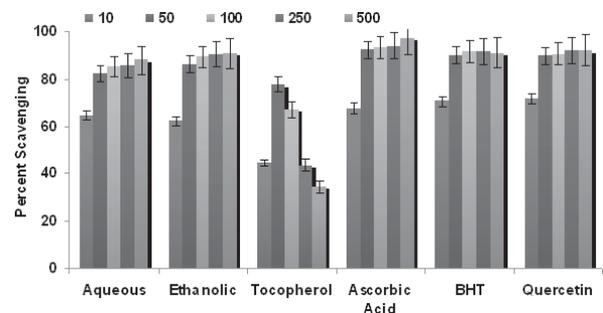


Figure 4. Total Antioxidant Capacity of *Emblica* extracts and known antioxidants expressed as μg of Ascorbic Acid Equivalence

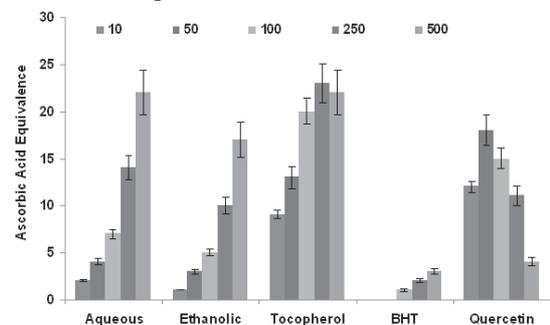


Figure 5. Ferrous reducing antioxidant power of *Emblica* extracts and known antioxidants expressed as μmol of ferrous sulphate

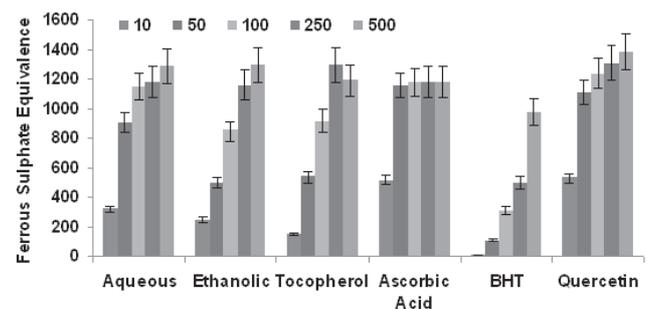
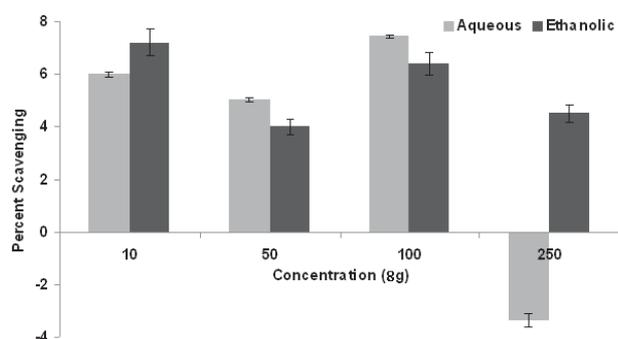
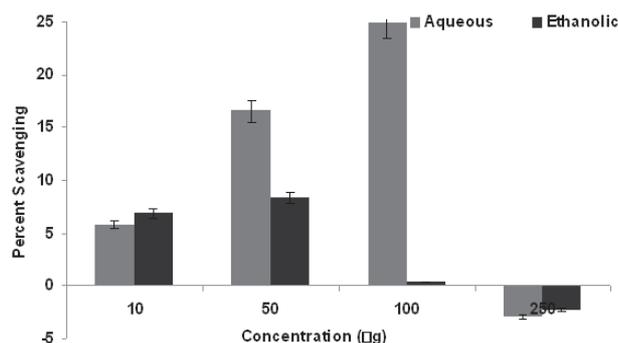


Figure 6. Concentration-dependent hydroxyl radical scavenging effect of aqueous and ethanolic extract of *Emblica* fruit in presence of EDTA but absence of Ascorbic acid



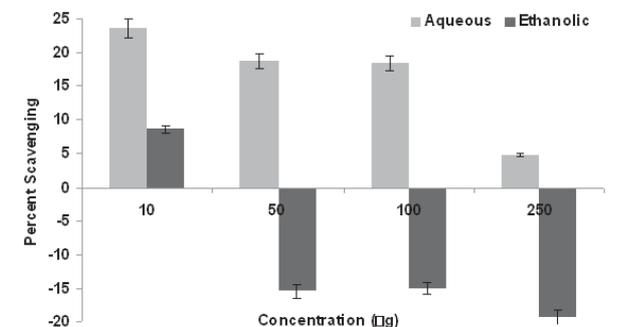
Percent Scavenging Values (Y-Axis) are mean \pm SE of three independent experiments in duplicates at each concentration (X-Axis)

Figure 7. Concentration-dependent hydroxyl radical scavenging effect of aqueous and ethanolic extract of *Emblica* fruit in absence of both EDTA and Ascorbic acid.



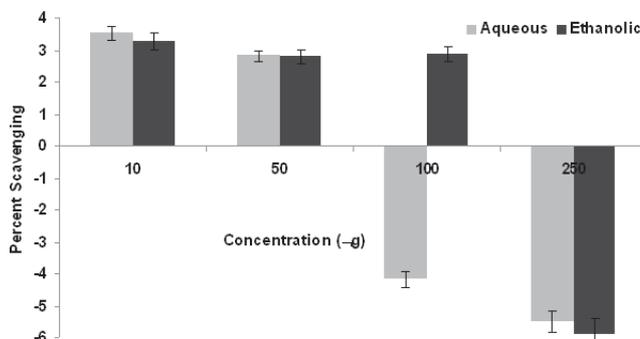
Percent Scavenging Values (Y-Axis) are mean \pm SE of three independent experiments in duplicates at each concentration (X-Axis)

Figure 8. Concentration-dependent hydroxyl radical scavenging effect of aqueous and ethanolic extract of *Emblica* fruit in presence of Ascorbic acid but absence of EDTA



Percent Scavenging Values (Y-Axis) are mean \pm SE of three independent experiments in duplicates at each concentration (X-Axis).

Figure 9. Concentration-dependent hydroxyl radical scavenging effect of aqueous and ethanolic extract of *Emblica* fruit in presence of both EDTA and Ascorbic acid.



Percent Scavenging Values (Y-Axis) are mean \pm SE of three independent experiments in duplicates at each concentration (X-Axis).

Table 1. Pearson Coefficient values of *Emblica* aqueous and ethanol extract for different assays

Aqueous Extract	PEARSON COEFFICIENT	Total Phenolics	Reducing Power	DPPH Assay	Total Antioxidant	FRAP Assay	HRS Assay
		1.0	0.91	0.94	0.83	0.98	0.04
E t h a n o l i c E x t r a c t	PEARSON COEFFICIENT	Total Phenolics	Reducing Power	DPPH Assay	Total Antioxidant	FRAP Assay	HRS Assay
		0.86	1.0	0.99	0.59	0.97	0.42
		0.91	1.0	1.0	0.68	0.99	-0.18
		0.81	0.63	1.0	1.0	0.76	-0.33
		0.96	0.80	0.93	1.0	1.0	-0.05
		-0.65	-0.72	-0.93	-0.86	1.0	1.0

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