Characterizing the antioxidant activity of amla (*Phyllanthus emblica*) extract

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Amla is well-known for its rich vitamin C (ascorbic acid) and polyphenol contents. To assess its antioxidant activity, we examined aqueous amla extract for its ability to inhibit γ-radiation-induced lipid peroxidation (LPO) in rat liver microsomes and superoxide dismutase (SOD) damage in rat liver mitochondria. For the LPO experiment, amla extract was added as its aqueous solution; and irradiation was carried out at different time intervals. The extent of LPO was measured in terms of thiobarbituric acid reactive substances. It was observed that the amla extract acts as a very good antioxidant against γ-radiation-induced LPO. Similarly, it was found to inhibit the damage to antioxidant enzyme SOD. The antioxidant activity of the amla extract was found to be both dose- and concentration-dependent. The amount of ascorbic acid in amla was standardized by HPLC and titrimetric methods and was found to be 3.25 to 4.5% w/w. However in microsomes containing this composition of pure ascorbic acid alone, no inhibition in LPO was observed. Cyclic voltammetry of the amla extract was carried out to estimate the ascorbic acid equivalents, which was found to be 9.4% w/w of amla. This value was found to be in agreement when compared with the reactivity of both amla and ascorbic acid towards ABTS[‒] radical, a stable free-radical. Based on these results it is concluded that amla is a more potent antioxidant than vitamin C.

PLANT and plant products are being used as a source of medicine since long. Among the most important constituents of edible plant products, low molecular weight antioxidants are the most important species. It is known that consumption of fruits and vegetables is essential for normal health of human beings. Vegetarian diet can reduce the risk of cancer, atherosclerosis, etc. *Phyllanthus emblica*, also known as amla, has been used in Ayurveda, the ancient Indian system of medicine. It has been used for treatment of several disorders such as common cold, scurvy, cancer and heart diseases[1–4]. It is

believed that the major constituent responsible for these activities is vitamin C (ascorbic acid). Ascorbic acid shows antioxidant, anti-inflammatory and antimutagenic properties[5–7]. It is a very effective free-radical scavenger. However, there are some *in vivo* studies indicating that antioxidant activities of amla cannot be attributed to ascorbic acid alone and that the overall effect is due to other polyphenols such as ellagic acid, gallic acid, tannins, etc.[8–10]. It is in fact reported that autoxidation of ascorbic acid can actually increase free-radical production[11]. In the present paper, we studied the effect of aqueous amla extract on the γ-radiation-induced lipid peroxidation (LPO) in rat liver microsomes and inhibition of superoxide dismutase (SOD) enzyme. Attempts have also been made to understand the role of ascorbic acid and the antioxidant equivalents in its activity.

Materials and methods

Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), ascorbic acid and epinephrine were obtained from Sigma Chemicals. All the other reagents were of analytical reagent grade. Nitrous oxide (N_2O) gas, obtained from Indian Oxygen Ltd, Mumbai was of IOLAR grade purity.

Preparation of amla extract

Fresh fruits were freed from foreign matter like dust or other organic matter. The cleaned raw material was then commuted to reduce its size. The commuted raw material was extracted with the extraction medium and converted into powder form. Since the extract is hygroscopic, enough care was taken while handling the sample.

Method of estimation of ascorbic acid

Sample solution equivalent to 0.2 mg ascorbic acid/ml was prepared in water containing 3% w/v metaphos-

phoric acid, added to increase the stability of ascorbic acid. It was titrated against standard 2,6-dichlorophenol indophenol (2,6-DCPIP) solution of concentration 0.5 mg/ml, until the pink colour developed completely. The operation was repeated with a blank solution omitting the sample being examined. From the difference, the ascorbic acid in each mg of sample was calculated from the ascorbic acid equivalent of the standard DCPIP solution[12]. The ascorbic acid content in the amla extract was determined to be 4.465% or 44.65 mg/g of amla.

Ascorbic acid content of amla extract was also estimated by HPLC (Spectra Series, P 100) with UV detector at 265 nm, C-18 column and 5% v/v methanol in 0.01 M KH_2PO_4 as the mobile phase, at a flow rate of 1 ml/min. Sample solution (0.2%) was prepared in the mobile phase. Chromatogram of the sample showed a peak at 2.60 min retention time and was assigned to ascorbic acid, as standard solution of ascorbic acid also gave a peak at 2.64 min (chromatogram not shown). From the peak area the ascorbic acid content was calculated to be 3.25% or 32.5 mg/g of amla.

Isolation of microsomes and mitochondria

Rat liver mitochondria and microsomes were isolated from liver of male albino wistar strain rats (180–200 g) as described earlier[13,14]. Animals were killed by decapitation, livers were quickly removed and washed with isolation medium (ice-cold 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4). A 10% liver homogenate was made in isolation medium. Mitochondria were isolated by differential centrifugation, washed twice with 10 mM phosphate buffer at pH 7.4 and suspended in the same buffer. Microsomes were isolated from mitochondria-free supernatant by differential centrifugation[15,16]. They were washed twice with 10 mM phosphate buffer (pH 7.4) and suspended in the same buffer. All operations were carried out at 0–4°C. The protein was estimated by the Lowry method[17]. During the experiments, microsomes/mitochondria were diluted with pH 7.4 phosphate buffer. For studying the effect of amla extract or ascorbic acid, aqueous solutions at pH 7.4 were prepared just before the experiment added, to the microsomes/mitochondria and diluted to get the required concentration expressed as μg/ml of the microsomal solution.

γ-Radiolysis

Steady state γ-radiolysis was carried out using ^{60}Co source with a dose rate of 7.4 Gy/min, measured by standard Fricke dosimetry[18]. LPO was studied in N_2O-purged microsomal solution at pH 7.4. γ-radiolysis of aqueous solution generates primary radicals as given in eq. (1). Under N_2O saturated condition, e^-_{aq} gets con-

verted to hydroxyl radical ('OH) as shown in eq. (2), which can induce LPO in microsomes.

$$H_2O \xrightarrow{\gamma\text{-ray}} e^-_{aq}, \ ^{\cdot}OH, \ H^{\cdot} \tag{1}$$

$$N_2O + e^-_{aq} \rightarrow N_2 + {}^{\cdot}OH + OH^- \tag{2}$$
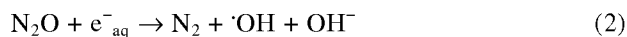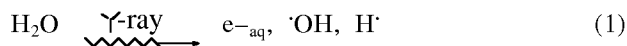
Lipid peroxidation in the presence and the absence of amla extract or ascorbic acid was studied as follows: Two sets of sealed vials, one containing normal microsomes diluted to 2 ml at a protein concentration of 0.4–0.6 mg per ml and the other containing microsomes with amla extract/ascorbic acid with the same dilution and protein concentration were prepared and vortexed. Dilution was done with pH 7.4 phosphate buffer. N_2O purging was done by passing N_2O gas through the microsomal suspension for two or three minutes, in such a way that some dissolved oxygen would still remain inside. Both sets were irradiated for different time intervals. For blank correction, identical sets were prepared to see the extent of LPO in absence of irradiation. The extent of LPO was estimated in terms of thiobarbituric acid reactive substances (TBARS) as follows: At regular intervals, 0.5 ml of microsomal suspension from the respective vial of both the sets was removed and added to the TBA reagent (TBA reagent: 15% w/v trichloroacetic acid, 0.375% w/v TBA, 0.25 N hydrochloric acid, 0.05% w/v BHT) and heated for 20 min at 80°C in a water bath. After centrifuging, the precipitate was removed and the absorbance of the supernatant was measured at 532 nm $(\varepsilon_{532} = 1.56 \times 10^5 \, M^{-1} \, cm^{-1})$[19] to calculate TBARS.

Estimation of superoxide dismutase enzyme activity

Effect of amla on protection of γ-radiation-induced damage to SOD was studied in rat liver mitochondria. Mitochondria suspended in oxygenated phosphate buffer equivalent to 2 mg protein/ml were taken in glass vials and exposed to a total dose of 570 Gy, both in the presence and absence of amla extract. For control experiment, identical glass vials were prepared and the activity was calculated in the absence of radiation. SOD levels in control and irradiated samples were estimated[20]. Briefly, 1 ml solution contains sodium carbonate buffer (50 mM, pH 10), 5 mM epinephrine and 40 μg mitochondrial protein. The rate of auto-oxidation of only epinephrine standard was initially followed by monitoring its absorbance at 320 nm, spectrophotometrically. Similarly, the absorbance at 320 nm was also monitored in unirradiated and irradiated mitochondria samples under identical conditions. The difference in the absorbance of epinephrine standard and that in mitochondria sample was used to calculate the enzyme activity. A difference in the

absorbance of 0.033 at 320 nm is defined as 1 unit of SOD[20].

Estimation of antioxidant capacity of amla extract and its components by cyclic voltammetry and pulse radiolysis

Antioxidant capacity of amla extract was estimated in terms of mg equivalents of ascorbic acid per gm of amla using cyclic voltammetry methodology[21–23]. The cyclic voltameter used for these studies was obtained from Ecochemie Autolab, model PGSTAT 20. Three-electrode system was employed with Ag/AgCl as the reference electrode, a glassy carbon electrode as working electrode and a platinum wire as a counter electrode. The cell contains 25 ml of sample solution and 0.1 M KCl. pH was adjusted to 7 using phosphate buffer. Cyclic voltammetry tracings were recorded from – 0.25 V to 1.2 V at a scan rate of 50 mV/s.

The antioxidant capacity with respect to pure ascorbic acid was also estimated by determining the reactivity towards ABTS[.-]. These studies were carried out using pulse radiolysis technique, the details of which are described elsewhere[24]. Typically 50 ns electron pulses from a 7 MeV linear electron accelerator were used for the pulse radiolysis studies and the reaction was monitored by the kinetic spectrophotometry.

Results and discussion

Figure 1, curves a and b shows the change in TBARS formation as a result of LPO in microsomes in the
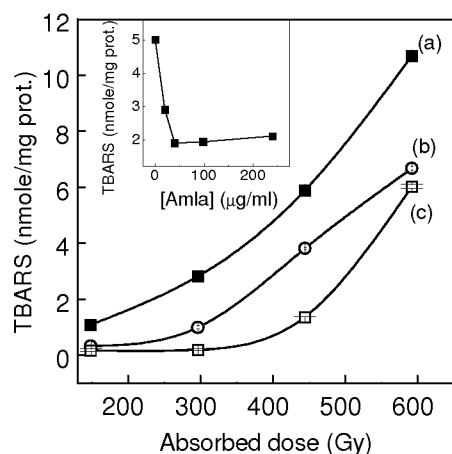

Figure 1. Effect of amla extract on γ-radiation-induced lipid peroxidation. Microsomes in N_2O-purged buffer (pH 7.4) were exposed to different doses using ^{60}Co source and lipid peroxidation was assayed in terms of TBARS; (a) normal microsomes, (b) 24 μg/ml amla extract, (c) 192 μg/ml amla extract. (Inset) Effect of concentration of amla on [.]OH-induced lipid peroxidation at an absorbed dose of 444 Gy. Error bars indicate mean variation of two independent experiments.

absence and presence of 24 μg of amla extract/ml of microsomal solution, after exposing it to γ-radiation for different time intervals corresponding to the total absorbed doses of 148, 296, 444 and 592 Gy. It can be seen that the peroxidation increased with increasing dose absorbed, but in the presence of amla extract it decreased, suggesting inhibition of [.]OH radical-induced LPO by the amla extract. The effect is more pronounced at a low dose than at a high dose. At a dose of 296 Gy, the protection by amla extract is 65%, while at 592 Gy the protection is only 40%. This experiment was repeated by increasing the amla content significantly almost by 8 times (Figure 1, curve c), which gives 93% protection at 296 Gy and 40% at 592 Gy. These results suggested that the membrane-protecting ability of amla extract is dependent on the concentration or the amount of the extract given. To determine the IC_{50} value (the amount of amla extract required to inhibit LPO by 50%) for the amla extract, we followed the LPO at constant dose of γ-radiation, changing the amount of amla extract in the range 20–240 μg/ml of microsomal solution. The inset of Figure 1 shows the effect of varying content of amla extract on LPO at an absorbed dose of 444 Gy. It can be seen that the protection rendered by amla extract increased with increasing amount up to 100 μg/ml. Further increase in amla extract did not show any significant protection. From this figure, the IC_{50} value was estimated to be 30 μg/ml.

Since the ascorbic acid content in amla is about 4.5% (the highest value estimated by titration was used), we felt it was necessary to know the role of ascorbic acid on LPO. Thus, 4.5% of 24 μg/ml of amla corresponds to 1.1 μg/ml of ascorbic acid. At this concentration of ascorbic acid, the LPO in microsomes was studied at different γ-radiation doses under the conditions as seen with amla extract and no inhibition in LPO was observed. However, only very high concentration of ascorbic acid showed significant protection. Thus, at an ascorbic acid concentration of 6.2 μg/ml of microsomal solution, which corresponds to 25.4% of amla extract, the TBARS were inhibited down to 28% at 444 Gy (Figure 2). Other known important constituents of amla extract are polyphenolic substances such as gallic acid and ellagic acid[25]. It was not possible to determine their composition as it was not easy to separate them from the amla extract.

Protection of superoxide dismutase enzyme by amla extract

Superoxide radicals ($O_2^{.-}$) have been implicated in several pathological disorders and are responsible for elevated oxidative stress[26]. SOD catalyses the decomposition of $O_2^{.-}$ to give H_2O_2 and O_2 and therefore acts as one of the important antioxidant enzymes[27].
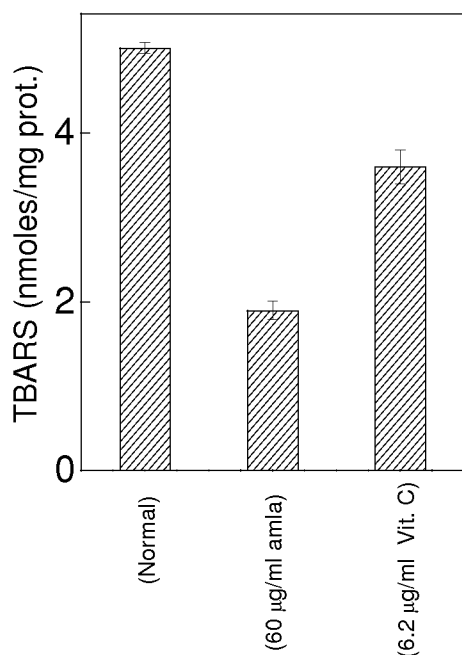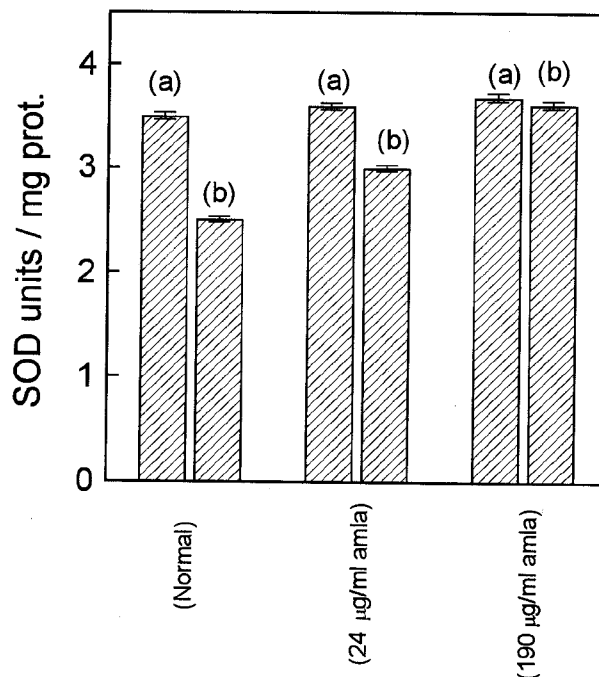
Figure 2. Inhibition of γ-radiation-induced lipid peroxidation assessed in terms of TBARS in the absence and presence of amla extract and ascorbic acid corresponding to 10% of amla extract. Error bars indicate mean variation of two independent experiments.

Figure 3. Protection of superoxide dismutase activity by amla extract. SOD level of mitochondria (a) without irradiation, (b) exposed to 570 Gy dose of γ-radiation. Loss in SOD activity was measured in the absence and presence of two different concentrations of amla extract. Error bars indicate mean variation of two independent experiments.

During irradiation, SOD activity initially increases to combat oxidative stress and starts decreasing at very high doses, either due to the direct damage of the enzyme protein or its increased consumption by the excessive generation of reactive oxygen species[28]. We have tested the SOD activity in rat liver mitochondria under irradiation conditions, both in the presence and absence of amla extract. Figure 3 shows the bar chart indicating the initial level of SOD in the control, unexposed to irradiation and after exposure to an absorbed dose of 570 Gy. Compared to the control, upon irradiation, there is a reduction in the SOD activity by 72%. In the same figure is given the bar chart for SOD levels in mitochondria containing 24 and 192 μg of amla extract/ml of mitochondria solution and exposed to the radiation. At low levels of amla extract, the protection in SOD is less; however at 192 μg/ml, the SOD level is equivalent to that of the control. This experiment shows that amla extract acts as a very good antioxidant by scavenging the reactive oxygen species and protects the antioxidant enzymes like SOD required for the cellular defence.

Cyclic voltammetric estimation of the antioxidant capacity

Cyclic voltammetry method as suggested by Chevion et al.[21–23] was used to estimate the total antioxidant capacity. Here, the potential of the working electrode is scanned from an initial value of – 0.25 V to a final value 1.2 V. Initially, the voltammograms were recorded for aqueous solutions of ascorbic acid at varying concentration from 0.1 to 1.2×10^{-3} M at pH 7, which (Figure 4, inset, a) shows a peak at 350 mV corresponding to the oxidation potential of ascorbic acid. The area under the curve and the peak current were measured at different concentrations of ascorbic acid and were found to increase linearly with increasing ascorbic acid concentration. Figure 4 shows the linear plot of peak area against concentration of ascorbic acid, which is used as a calibration curve to estimate antioxidant capacity of amla extract. The inset of Figure 4 shows the voltammogram for the aqueous solution of amla extract (408 μg/ml) and ascorbic acid (0.25 mM). The cyclic voltammetry signal for amla extract shows a peak at 317 mV (Figure 4, inset, b). The peak is shifted by ~ 30 mV compared to ascorbic acid. Such shifts were also noticed by Chevion et al.[21] in several natural tissues and formulations and were attributed to the presence of other low molecular weight antioxidants. From the area and the peak current and using the linear plot, the oxidizable equivalents were found to be 94 ± 6 mg/g of amla extract. This suggested that the total antioxidant capacity in terms of the ascorbic acid equivalents is 94 mg/g of amla extract, which is ~ 9.4%. This value appears very different from that estimated by the HPLC and titrimetric methods, indicating that the
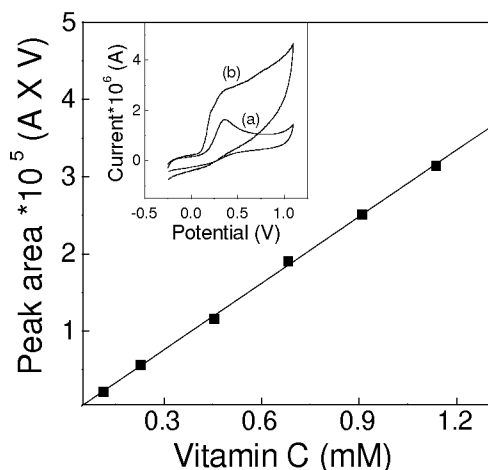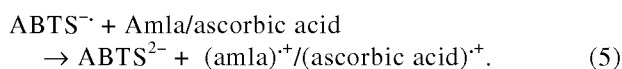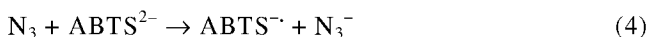
Figure 4. Peak area of cyclic voltammogram was plotted against corresponding concentrations of vitamin C (ascorbic acid). 0.1 M phosphate buffer was used for adjusting the pH to 7 and 0.1 M KCl was used as a supporting electrolyte. (Inset) Cyclic voltammetry traces of (a) 0.25 mM of vitamin C corresponding to 40 μg/ml, and (b) 408 μg/ml of amla extract. Signals were recorded from − 0.25 to 1.2 V with the scan rate of 50 mV/s.

antioxidant capacity is not only due to ascorbic acid, but that other components such as polyphenols are also responsible.

Estimation of oxidizing equivalents by the reactivity towards ABTS⁻

Reactivity with ABTS⁻ radicals can also be used to estimate the antioxidant activity of natural compounds[29]. For this, ABTS⁻ radicals were generated after the radiolysis of N_2O-saturated aqueous solutions containing 2 mM $ABTS^{2-}$, 0.05 M NaN_3 at pH 7.

$$N_3^- + {}^{\cdot}OH \rightarrow N_3^{\cdot} + OH^-, \tag{3}$$

$$N_3 + ABTS^{2-} \rightarrow ABTS^{-\cdot} + N_3^- \tag{4}$$

$$ABTS^{-\cdot} + \text{Amla/ascorbic acid}$$
$$\rightarrow ABTS^{2-} + (\text{amla})^{\cdot+}/(\text{ascorbic acid})^{\cdot+}. \tag{5}$$

Here, the ˙OH radicals produced by water radiolysis (eqs (1) and (2)) react with N_3^- to produce $N_3^{\cdot}$ radicals (eq. (3)), which in turn oxidize $ABTS^{2-}$ to produce $ABTS^{-\cdot}$ (eq. (4)), absorbing at 600 nm. In the absence of any additive, it does not show any decay even in the time-scale of seconds (Figure 5 a), but increased in the presence of both ascorbic acid and amla (Figure 5 b and c), respectively. In presence of 1×10^{-4} M ascorbic acid (19.2 μg/ml), ABTS⁻ radical decays with the rate constant of $6.15 \pm 0.15 \times 10^3$ s⁻¹. This pseudo first-order decay constant is indicative of the total reactivity of ABTS⁻ towards the substrate. The reactivity is the product of the bimolecular rate constant for the reaction
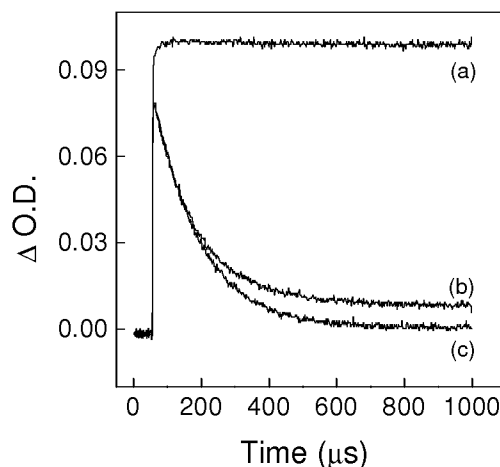
Figure 5. Absorption-time profiles showing the decay of ABTS⁻ radical at 600 nm (a) in the absence of any additive, (b) in the presence of 204 μg/ml of amla extract, and (c) in the presence of 19.2 μg/ml of ascorbic acid.

and the concentration of the substrate. Earlier, from cyclic voltammetry, we estimated that ascorbic acid equivalents as 94 mg/g of amla. For the ascorbic acid concentration of 1×10^{-4} M, equivalent amount of amla extract containing 9.4% ascorbic acid therefore corresponds to 204 μg/ml. At this amla content, we tested reactivity of amla extract with ABTS⁻ under similar conditions, which also showed a total reactivity of $6.23 \pm 0.15 \times 10^3$ s⁻¹. This further confirmed that the ascorbic acid equivalents determined by cyclic voltammetry are in very good agreement with the reactivity parameter for ABTS⁻.

Conclusions

Amla or *Phyllanthus emblica* is known since ancient times for its medicinal value and is commonly used in Ayurvedic medicine. It is also believed to be a rich source of vitamin C and is being considered as a good replacement for vitamin C. However, in the medical field amla is not as popular as ascorbic acid. In this paper, our efforts are to show that amla is a more powerful antioxidant than ascorbic acid. Our results showed that amla extract inhibits radiation-induced lipid peroxidation in microsomes and SOD in mitochondria. Amla extract being water-soluble, may scavenge the free radicals responsible for initiating LPO. However, ascorbic acid alone does not account for all these antioxidant activities. The ascorbic acid content estimated by titrimetry and HPLC gives 4.5% and 3.25% respectively, whereas the total ascorbic acid equivalents estimated by cyclic voltammetry and reactivity to ABTS⁻ radical indicate a value around 9.4%. This suggests that other polyphenols, which are also present in amla and which are capable of scavenging oxidizing radicals are respon-

sible for its enhanced antioxidant activity. Even at elevated ascorbic acid concentration of 9.4%, ascorbic acid alone does not show as much protection as amla extract, which is shown as bar graphs in Figure 2 giving the extent of inhibition of LPO. This suggests that ascorbic acid and other polyphenols present in the natural formulation of amla show much superior antioxidant activity compared to their equivalent amounts in pure isolated form.

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