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Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine

G.H. Naik[a], K.I. Priyadarsini[a,*], J.G. Satav[b], M.M. Banavalikar[c], D.P. Sohoni[c], M.K. Biyani[c], H. Mohan[a]

[a] *Radiation Chemistry and Chemical Dynamics Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India*
[b] *Radiation Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India*
[c] *Ajanta Pharma Ltd, Kandivili, Mumbai-400067, India*

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Abstract

Four aqueous extracts from different parts of medicinal plants used in Ayurveda (an ancient Indian Medicine) viz., *Momardica charantia* Linn (AP1), *Glycyrrhiza glabra* (AP2), *Acacia catechu* (AP3), and *Terminalia chebula* (AP4) were examined for their potential as antioxidants. The antioxidant activity of these extracts was tested by studying the inhibition of radiation induced lipid peroxidation in rat liver microsomes at different doses in the range of 100–600 Gy as estimated by thiobarbituric acid reactive substances (TBARS). Of all these extracts, AP4 showed maximum inhibition in the TBARS formation and hence is considered the best antioxidant among these four extracts. The extracts were found to restore antioxidant enzyme superoxide dismutase (SOD) from the radiation induced damage. The antioxidant capacities were also evaluated in terms of ascorbate equivalents by different methods such as cyclic voltammetry, decay of $ABTS^{\cdot-}$ radical by pulse radiolysis and decrease in the absorbance of DPPH radicals. The results were found to be in agreement with the lipid peroxidation data and AP4 showed maximum value of ascorbate equivalents. Therefore AP4, with high antioxidant activity, is considered as the best among these four extracts.

Keywords: Herbal extracts; Lipid peroxidation; Free radicals; Antioxidants

1. Introduction

Plants and plant products are part of the vegetarian diet and a number of them exhibit medicinal properties. Several Indian plants are also being used in Ayurvedic and Siddha medicines. The medicinal properties of several herbal plants have been documented in ancient Indian literature and the preparations have been found to be effective in the treatment of diseases (Chopra et al., 1956; Gupta, 1908; Handa et al., 1996). Recent reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases (Sies, 1993; Halliwell, 1997). Hence search for new synthetic and natural antioxidants is essentially important. Although initial research on antioxidants was mostly on isolated pure compounds, recent focus is more on natural formulations (Hagerman et al., 1998; Haramaki and Packer, 1995). It has been found that compounds in their natural formulations are more active than their isolated form (Khopde et al., 2001). Hence in this paper we have examined the antioxidant activity of four plant extracts commonly used in Ayurvedic medicine (Polva and Foo, 1994; Sham et al., 1984; Singh et al., 1976; Khavyal et al., 2001; Konovalova et al., 2000; Ji et al., 1998; Vaya et al., 1997, Grover et al., 2001; Scartezzini and Speroni, 2000; Jagtap and Karkera, 1999; Kaur et al., 1998). These plants are found in several parts of India and Asia. The plants are *Momardica charantia* Linn (Karela, AP1), *Glycyrrhiza glabra* (Yeshtimadhu, AP2), *Acacia catechu* (Khadira, AP3) and *Terminalia chebula* (Hirde; AP4). The details regarding the extracts, viz., habitat, constituents, pharmacological activity etc are listed in Table 1. From this table it can be seen that the active principles of these extracts are widely different, so their antioxidant potential is expected to be also significantly different. For this purpose, different methodologies were used to estimate their antioxidant activity. We studied

* Corresponding author. Tel.: +91-22-551-576; fax: +91-22-25505151.
E-mail address: kindira@apsara.barc.ernet.in (K.I. Priyadarsini).

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Table 1
Phytochmical details of the extracts

Extract	Popular name[a]	Botanical name	Habitat	Actions	Constituents
AP1	Karela (Bitter gourd)	*Momardica charantia* Linn	India	Tonic, stomachic, stimulant, emetic, antibilious	Glucosides, albuminoids, charantin, soluble carbohydrates
AP2	Yesthimadhu (Sweet wood)	*Glycyrrhiza glabra*	Arabia, Persia, Afghanistan, Turkistan, India	Tonic, cooling, demulcent, diuretic, emmenagogue laxative	Glycyrrhizin, asparagin, sugar, starch, acid resin, flavones, coumarins
AP3	Khadira (Black catechu)	*Acacia catechu*	India, Burma	Powerful astringent	Catechu tannic acid, catechin, red tannin, quarcetin
AP4	Hirde (Myrobalan)	*Terminalia chebula*	India.	Effective, purgative, astringent, blood purifier	Tannin, gallic acid, chebulinic acid

[a] Names in brackets are English names.

their effect on γ-radiation induced lipid peroxidation in rat liver microsomes and superoxide dismutase activity in rat liver mitochondria. The free radical scavenging ability of the extracts has been examined by DPPH assay. The total antioxidant potential of these extracts was also determined in terms of ascorbate equivalents using pulse radiolysis technique.

2. Results

2.1. Lipid peroxidation and SOD enzyme activity

Fig. 1a–e shows the change in TBARS formation as a result of LPO in microsomes in the absence and presence of different extracts, after exposing to γ-radiation for different time intervals corresponding to total absorbed doses of 100–600 Gy. In the normal microsomes, LPO (TBARS) increased with increasing absorbed dose, but in the presence of the extracts, there is a significant decrease in the extent of TBARS formation, indicating that the extracts protect the microsomes from the radiation induced LPO. The extent of inhibition of LPO was however, not uniform and varied for different extracts. It can be noticed that the extracts showed protection at all the doses and the effect is more significant at lower doses than at higher doses. AP4 (Fig. 1e) shows maximum inhibition followed by AP3 (Fig. 1d), AP2 (Fig. 1c) and AP1 (Fig. 1b). We also followed the effect of these extracts on LPO at different concentrations of the extracts (up to 60 μg/ml). Fig. 2 shows the effect of varying concentration of these extracts on the LPO in microsomes at an absorbed dose of 294 Gy. Within the range of concentrations

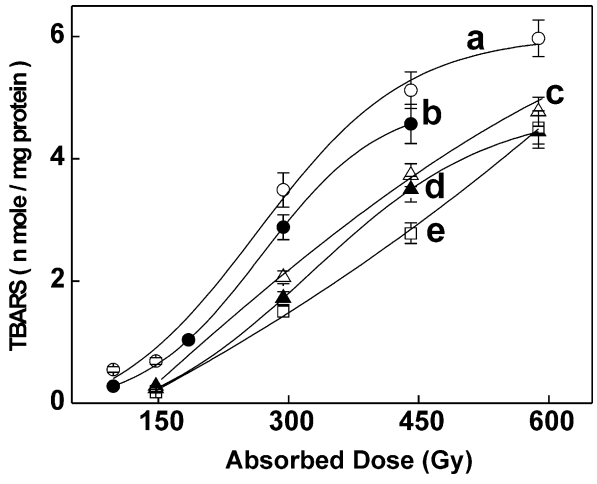

Fig. 1. Inhibition of γ-radiation induced lipid peroxidation as assessed in terms of TBARS in the absence and presence of different extracts. Microsomes in N_2O–O_2 purged buffer (pH = 7.4) were exposed to different doses using ^{60}Co γ-source. (a) Control microsomes; (b) AP1 (47.2 μg/ml); (c) AP2 (36 μg/ml); (d) AP3 (32.7 μg/ml) and (e) AP4 (23.6 μg/ml).

Table 2
Relative antioxidant activity parameters of the extracts

Extract	IC_{50} by TBARS (μg/ml)	IC_{50} by DPPH (μg/ml)	Ascorbate equivalents (%)
AP1	> 70	–	<1
AP2	> 70	–	<0.5
AP3	45	25	17.3
AP4	14.5	11.5	~60

employed, AP4 and AP3 showed significant concentration dependence on the inhibition of LPO. From this figure the IC_{50} values i.e. the concentration of the extract required to inhibit LPO by 50% were determined and the values are listed in Table 2. The remarkable protection by AP4 can be clearly seen from Fig. 2.

After studying their effect on LPO, we also looked for their effect on antioxidant enzyme super oxide dismutase (SOD). For this, rat liver mitochondria were employed and the mitochondrial samples were irradiated both in presence and absence of the extracts. As reported in our earlier studies (Khopde et al., 2001), irradiation of these samples with γ-radiation initially leads to increase in the SOD generation to combat oxidative stress, but further irradiation causes decrease in the enzyme due to its own damage by the radiation. Fig. 3 gives changes in SOD enzyme after irradiating to a dose of 546 Gy, in the presence and absence of the extracts. All the extracts protected the enzyme from the damage caused by irradiation. However, AP4 was more powerful and restored the enzyme completely as compared to the other extracts, followed by AP3, AP2 and AP1. These results are similar to those observed in case of LPO.

2.2. DPPH assay

DPPH is a stable free radical and is often used to evaluate the antioxidant activity of several natural compounds (Yokozawa et al., 1998; Fogliano et al., 1999). Antioxidants on interaction with DPPH, either transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character. DPPH shows strong absorption at 517 nm. Fig. 4 gives the changes in the absorbance at 517 nm, at different compositions of these extracts. It can be noticed that AP4 and AP3 showed significant decrease in the absorbance of DPPH, while AP1 and AP2 do not reduce the absorbance at 517 nm even at high concentration of the extract. From this

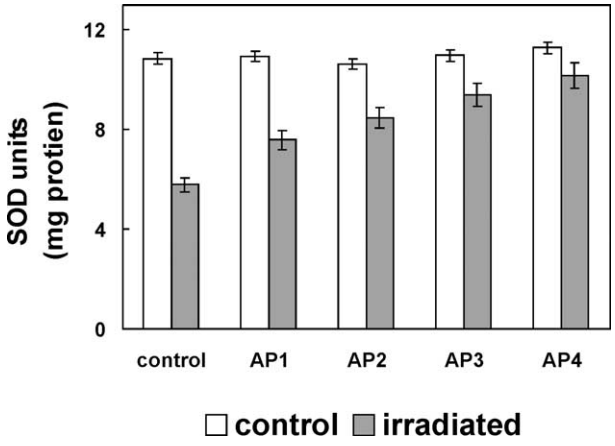

Fig. 3. Protection of radiation induced damage to super oxide dismutase (SOD) enzyme by different extracts. SOD level in mitochondria was compared in control and γ-irradiated (546 Gy) samples containing 20 μg/ml of the extracts.

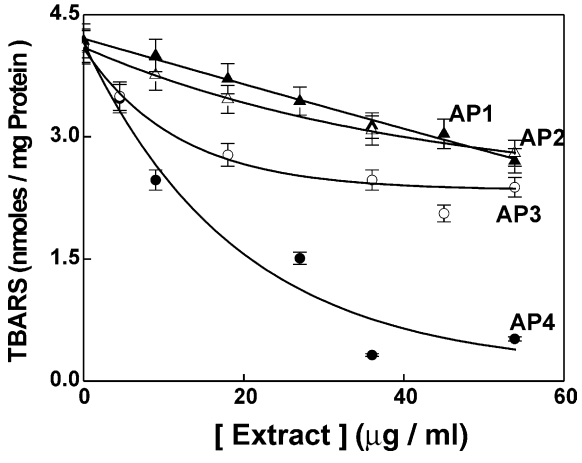

Fig. 2. Effect of the concentration of different extracts on γ-radiation induced lipid peroxidation as assessed in terms of TBARS at an absorbed dose of 294 Gy. (a) AP1, (b) AP2, (c) AP3 and (d) AP4.

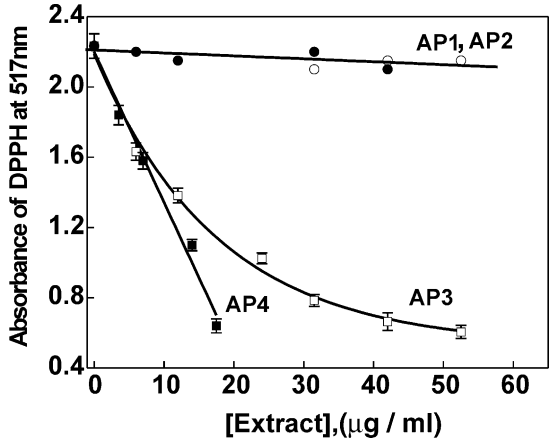

Fig. 4. Effect of the concentration of different extracts on DPPH (250 μM) absorption at 517 nm.

figure, the IC_{50} values for AP4 and AP3 were determined and are listed in Table 2. These results suggest that the antioxidant activity of AP4 and AP3 can be attributed to efficient free radical scavenging while AP1 and AP2 do not possess free radical scavenging ability.

2.3. $ABTS^{\cdot-}$ radical decay

The DPPH assay was useful in knowing the free radical scavenging ability of the extracts, $ABTS^{\cdot-}$ radical decay assay could be used to measure even the kinetics of free radical scavenging power of extracts. $ABTS^{\cdot-}$ radicals are more reactive than DPPH radicals and unlike the reactions with DPPH, the reactions with $ABTS^{\cdot-}$ radicals generally take place in less than a millisecond. In order to study this, we employed a fast kinetic technique known as pulse radiolysis. In this method, the $ABTS^{\cdot-}$ radicals were generated by the reaction of radiolytically produced azide radicals with $ABTS^{2-}$ [Eqs. (1) and (2)].

$$N_3^- + {}^{\cdot}OH \rightarrow N_3^{\cdot} + OH^- \quad (1)$$

$$N_3^{\cdot} + ABTS^{2-} \rightarrow N_3^- + ABTS^{\cdot-} \quad (2)$$

The $ABTS^{\cdot-}$ radicals were monitored by their absorption at 600 nm. In the absence of any extract, the radicals do not show any decay (Fig. 5a), however, in presence of oxidisable substrates (ascorbic acid) it decays in milliseconds (Fig. 5b). Initially we followed the decay of $ABTS^{\cdot-}$, in presence of ascorbic acid at pH 7. The decay followed pseudo first order kinetics and the first order rate constant (k_{obs}) increased with increasing ascorbic acid concentration in μg/ml (inset of Fig. 5) giving a bimolecular rate constant of $2.5\pm0.1\times10^7$ M^{-1} s^{-1}. This plot is used as a calibration curve to estimate the antioxidant capacity in terms of ascorbate equivalents of the above extracts. While AP4 and AP3 showed high reactivity, AP2 and AP1 showed very little reactivity towards $ABTS^{\cdot-}$. Fig. 6 shows the corresponding decay kinetic traces for $ABTS^{\cdot-}$ in presence of AP4 and AP3 extracts. The observed first-order decay rates were found to increase with increasing extract concentrations (Fig. 7). From the observed first order rate constant (Fig. 7), and the calibration curve (inset of Fig. 5), the ascorbate equivalents present in the extracts were calculated (Table 2). From this table it can be seen that the ascorbate equivalents agree with the other antioxidant properties of the extracts.

2.4. Cyclic voltammetry

Reaction of an antioxidant with $ABTS^{\cdot-}$ and DPPH radicals is possible only when it is capable of transferring an electron to either of these radicals and the reduction potential of the antioxidant is such that the reaction is favorable thermodynamically. Cyclic voltammetry can be used to estimate the reduction potential of the extracts (Kilmartin et al., 2001). Fig. 8 shows the voltammetric traces for aqueous solutions of different extracts at pH 7. None of them showed reversibility. From this figure it can be seen that extract AP1 did not show any oxidisable component in this potential range. AP2 shows a broad featureless and less intense voltammograms. The broad peaks are observed at the potential values of 0.645 and 0.9 V. AP3 shows well-defined peak at 0.646 V. This may correspond to the presence of catechin and its derivatives. AP4 shows a very high level of oxidisable components and the first peak is observed at 0.303 V and further it showed continuous increase without any prominent peak. The 0.303 V peak may correspond to ascorbic acid. Pure ascorbic acid solution

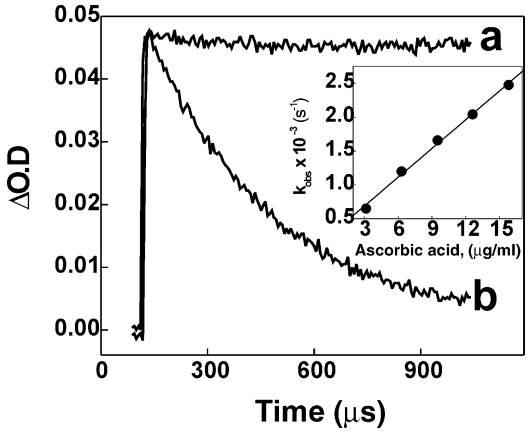

Fig. 5. Absorption-time profiles ($\lambda = 600$ nm) showing the decay of $ABTS^{\cdot-}$ radical anion produced on pulse radiolysis of N_2O-saturated aqueous solution of $ABTS^{2-}$ (2×10^{-3} mol dm^{-3}) (a) in the absence and (b) in the presence of ascorbic acid (15.75 μg/ml). Inset shows the variation of k_{obs} as a function of ascorbic acid concentration.

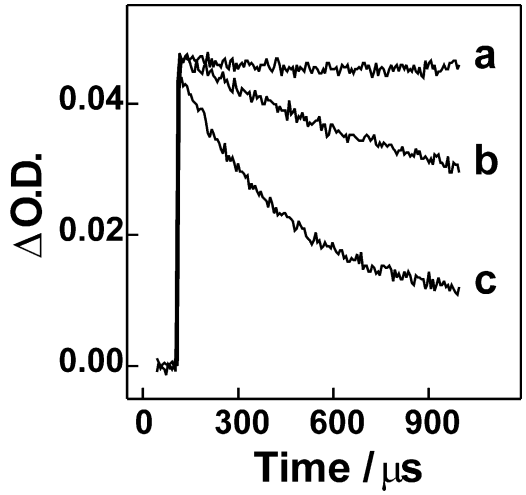

Fig. 6. Absorption-time profiles ($\lambda = 600$ nm) showing the decay of $ABTS^{\cdot-}$ (a) in the absence and (b) in the presence of AP3 (12 μg/ml) and (c) AP4 (14.5 μg/ml).

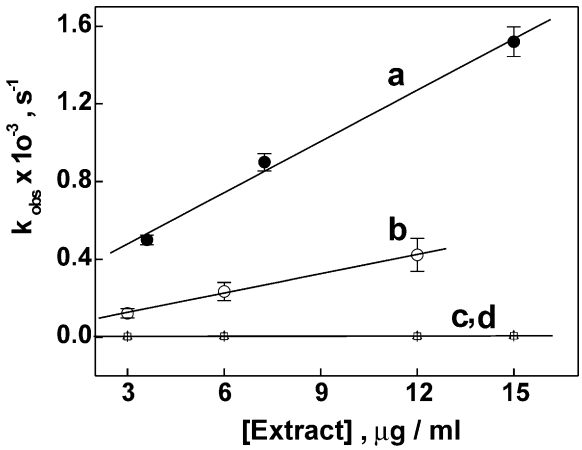

Fig. 7. Variation of k_{obs} for $ABTS^{\cdot-}$ decay at 600 nm as a function of concentration for different extracts (a) AP4, (b) AP3, (c) AP2 and (d) AP1.

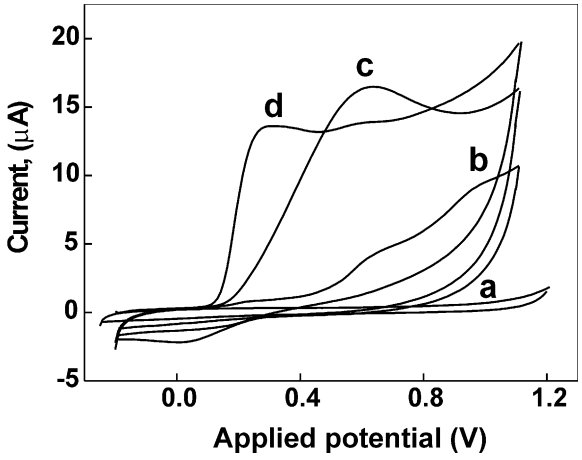

Fig. 8. Cyclic voltammetric traces for different extracts at pH 7. 0.1 M KCl was used as a supporting electrolyte and signals were recorded from −0.25 to 1.2 V with scan rate of 50 mV/s, (a) AP1, (b) AP2, (c) AP3 and (d) AP4.

gives a peak at 0.350 V, however shift in ascorbate peak potential values were observed in many systems containing mixtures of compounds. In our earlier report with *phyllanthus emblica* extract (Khopde et al., 2001). we used the peak area to estimate the ascorbate equivalents, but in the present case it was not possible to calculate this, as the peaks are not well defined. The cyclic voltammetric results are only used to indicate presence of large amount of oxidisable substrates in AP4 and AP3. AP2 showed much smaller level and AP1 showed none.

3. Discussion

Four different plant extracts used in ancient Indian medicine (Ayurveda) have been tested for their antioxidant activity by measuring their ability to inhibit LPO induced by γ-radiation in microsomes. The four extracts showed differential capacity to inhibit LPO. At a given concentration and radiation dose the inhibiting capacity for different extracts is in the order AP4 > AP3 > AP2 > AP1. Similarly effect of these extracts on radiation induced damage to the antioxidant enzyme SOD was studied in mitochondria. As observed with lipid peroxidation, the extent of protection to the enzyme was in the order AP4 > AP3 > AP2 > AP1. These two studies confirmed that all the extracts exhibit antioxidant potential but to different extents. There are several mechanisms by which antioxidants can act. One of them is by scavenging of reactive oxygen and nitrogen free radicals. There are many different experimental methods by which the free radical scavenging activity can be estimated. One such method, by which total free radical scavenging can be estimated, is by determining their efficiency to scavenge DPPH radicals. DPPH radical is a stable free radical, and any molecule that can donate an electron or hydrogen to DPPH can react with it and there by bleach the DPPH absorption. When the above extracts were tested for the DPPH scavenging ability, only AP4 and AP3 showed high activity with IC_{50} values of 11.5 and 25 µg/ml, respectively. AP2 and AP1 showed almost negligible reactivity. This suggested that AP4 and AP3 contain compounds such as polyphenols that can donate electron/hydrogen easily. To further confirm this we used reactivity of $ABTS^{\cdot-}$ radicals with the extracts by which total antioxidant activity in terms of ascorbate equivalents could be estimated. The results of these studies were in agreement with the LPO and SOD studies. Here the total reactivity of $ABTS^{\cdot-}$ radical in presence of extract is compared with that of known amount of ascorbic acid. Using this, the total antioxidants in terms of ascorbate equivalents were calculated for the extracts. Among these four extracts, AP4 showed very high level of ascorbate equivalents. AP3 showed moderate value and AP2 and AP1 showed negligible amounts. Ascorbate equivalents indicate a mixture of all the compounds that can be easily oxidised. These include ascorbic acid, polyphenols and tannins. The most important polyphenols in the natural products are gallic acid, catechins, ellagic acid, flavonoids etc. All these compounds have characteristic reduction potentials and hence can be detected by voltammetric methods. Cyclic voltammetry of AP4 shows presence of large amount of oxidisable compounds. However they were not resolvable easily. The potential values indicate that compounds like ascorbic acid, gallic acid, ellagic acid etc must be present in AP4. AP3 however shows a peak, which may correspond to the presence of catechins. No well-defined peaks could be detected in AP2 and AP1. From all these observations it can be concluded that the natural compounds with high level of polyphenolic compounds such as AP4 and AP3 show excellent antioxidant activity in vitro systems.

However compounds like bile acids, carbohydrates and other similar compounds present in many natural products such as AP2 and AP1 may also show antioxidant property but their efficiency is much less than that of phenolic constituents. Even if they exhibit such an activity, their mechanism is not by reacting with oxidant free radicals. Thus employing different methodologies, it was possible to estimate the antioxidant potential of different natural extracts.

4. Experimental

4.1. Chemicals

2,2′-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{2-}$) were purchased from Aldrich Chemicals, USA. Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), ascorbic acid and epinephrine were obtained from Sigma Chemicals, USA. All other reagents used were of the highest available purity. Nitrous oxide (N_2O) and oxygen (O_2) gases obtained from Indian Oxygen Ltd., Mumbai, were of IOLAR grade purity. Nanopure water, obtained from a Branstead nanopure purification system, was used for preparation of aqueous solutions. Freshly prepared solutions were used for each experiment. All the experiments were repeated at least twice and the errors correspond to mean ± deviations.

4.2. Isolation of microsomes and mitochondria

Rat liver mitochondria and microsomes were isolated from the liver of male albino wistar strain rats (180–200 g) as described earlier (Satav and Katyare, 1982; Satav et al., 1976). 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 were suspended in the same buffer. Microsomes were isolated from mitochondria free supernatant by differential centrifugation. 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 (Lowry et al., 1951). During the experiment microsomes/mitochondria were diluted with pH 7.4 phosphate buffer.

4.3. Method of extraction

The fine powder (mesh size 20) of the raw material was stirred with 8 parts of distilled water (70–80 °C) for 2 h. The liquid extract was filtered through sieve (mesh size 200). It was concentrated up to 2 parts on a rotary vacuum evaporator. The concentrated liquid was spray dried to get the dry powder of the extract. At the concentrations employed in this study, the solutions were clear without any suspensions indicating complete solubility in the buffer solution. Since it is not possible to express the concentrations in molarity, it was expressed as μg/ml.

For incorporation of plant extracts into the microsomes, aqueous solution of the extract at pH 7.4 was prepared just before the experiment. It was added to the microsomes/mitochondria and diluted to get the required concentration of the extract and its content is expressed as μg/ml.

4.4. γ-Radiolysis

Lipid peroxidation (LPO) was carried out by the γ-radiolysis of rat liver microsomes. Steady state γ-radiolysis was carried out using ^{60}Co γ-source with a dose rate of 9.1 Gy/min measured by standard Fricke dosimetry. γ-radiation induced lipid peroxidation at different doses was studied in N_2O-purged microsomal solution in absence and presence of the extract at physiological pH 7.4 (phosphate buffer). The detailed methodology used in the lipid peroxidation is given in our earlier references (Sreejayan et al., 1997; Khopde et al., 2000). The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) using 15% w/v trichloroacetic acid, 0.375% w/v TBA, 0.25 N hydrochloric acid, 0.05% w/v BHT as TBA reagent measuring the absorbance at 532 nm ($\varepsilon_{532} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

4.5. Estimation of superoxide dismutase enzyme activity

Protection of γ-radiation induced damage to superoxide dismutase (SOD) was studied in rat liver mitochondria which were isolated as given above. Mitochondria suspended in oxygenated phosphate buffer equivalent to 2 mg protein/ml were taken in glass vial and exposed to a total dose of 570 Gy both in presence and absence of the extract. For control experiment identical glass vials were prepared and the activity was calculated in absence of radiation. SOD levels in control and irradiated samples were estimated according to the procedure reported in the literature (Sum and Zigman, 1978; Khopde et al., 2001; Kamat et al., 1999). Briefly, 1 ml solution containing sodium carbonate buffer (50 mM, pH 10), mitochondria sample with 40 μg protein and 5 mM epinephrine was taken in a spectrophotometric cell. The change in its absorbance was monitored at 320 nm for 6 min against the blank containing identical solution except epinephrine. SOD units/mg protein was computed from the difference between the absorbance of standard and the sample.

4.6. Estimation of antioxidant capacity of the extracts by cyclic voltammetry and pulse radiolysis

Cyclic voltammetric traces of the extract solutions were recorded from −0.25 to 1.2 V at a scan rate of 50 mV/s (Ecochemie Autolab, PGSTAT 20 Model). Three-electrode system was employed with Ag/AgCl as the reference electrode, a glassy carbon electrode as working electrode and 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. The working electrode was repeatedly cleaned to avoid any deposition.

The antioxidant capacity with respect to ascorbic acid was also estimated by determining the reactivity towards $ABTS^{\cdot-}$ radical. These studies were carried out using pulse radiolysis technique, the details of which are described elsewhere (Guha et al., 1987). 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.

4.7. DPPH assay

One milliliter of 500 μM DPPH in methanol was mixed with equal volume of extract solution in phosphate buffer (pH = 7.4), mixed well and kept in dark for 20 min. The absorbance at 517 nm was monitored in presence of different concentrations of extracts. Blank experiment is also carried out to determine the absorbance of DPPH before interacting with the extract. The amount of extract in μg/ml at which the absorbance at 517 nm decreases to half its initial value is used as the IC_{50} value for the extract.

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