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Modulation of oxidative damage by natural products

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Abstract

Using rat liver membrane as a model system, modulation of oxidative damage induced by pathophysiological agents such as photosensitization and radiation was examined with two medicinal plant extracts, namely *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). Results showed that simultaneous addition of both the extracts (50 μg/ml) in independent experiments during generation of reactive oxygen species (ROS) could significantly prevent increased levels of products of lipid peroxidation, such as conjugated dienes, lipid hydroperoxide, TBARS and 4-hydroxylnonenals. The oxidative damage observed with depletion of major endogenous antioxidants, such as GSH, as well as enhanced formation of protein oxidation, was effectively reduced by these extracts. Similarly, degradation of mitochondrial proteins by ROS, induced during photosensitization, was effectively prevented by both the extracts (SDS–PAGE experiments). The antioxidative property of these extracts could be attributed to their scavenging ability with superoxide, hydroxyl radicals and singlet oxygen species, the major species generated during photosensitization and γ-radiation. The high scavenging ability of the extracts may be due to high phenolic contents, flavonoid constituents and considerable reducing equivalents. The pulse radiolysis studies showed high reactivity with $ABTS^{-}$. The reaction of the extracts of Ap and Sc with dimethyl *p*-phenylene diamine dihydrochloride (DMPD), one of the important stable synthetic radicals gave >30% inhibition at 50 μg/ml. In view of these observations, termination of the free radical reaction, and quenching of reactive oxygen are suggested to be, in part, responsible for the antioxidant activity of *Andrographis paniculata* and *Swertia chirata* extracts. Therefore, *Andrographis paniculata* and *Swertia chirata* extracts may emerge as effective antioxidative agents, protecting cells from pathophysiological oxidants, generated during UV–vis photosensitization/radiation-induced injury, and may be useful in the food industry as effective synthetic antioxidants.

Keywords: Antioxidant activity; Lipid peroxidation; Free radical scavenging ability; Photosensitization; Radiation; *Andrographis paniculata* and *Swertia chirata* extracts

1. Introduction

In recent years, the studies on "oxidative stress" and its adverse effects on human health have become a subject of considerable interest. It is a well-documented fact that exposure of organisms to exogenous and endogenous factors generates a wide range of reactive oxygen species (ROS), resulting in homeostatic imbalance (Bonnefont, Bastard, Jandon, & Delattre, 2000; Halliwell & Gutteridge, 1999; Sies, 1997; Thomas & Kalyanaraman, 1998). Polyunsaturated fatty acids of cell membranes are the critical components, susceptible to such insult (Esterbauer, 1996; Halliwell & Gutteridge, 1999). ROS induce alterations and loss of structural/functional architecture in the cell, leading directly to cytotoxicity and/or indirectly to genotoxicity, with numerous serious anomalies favouring disharmony and diseases (Esterbauer, 1996; Girotti, 1994; Halliwell & Gutteridge, 1999; Sies, 1997). Therefore, the factors that shift the physiological process,

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in the homeostatic balance are of considerable interest (Sies, 1997).

Antioxidant principles from herbal resources are multifaceted in their effects and provide enormous scope in correcting the imbalance through regular intake of a proper diet. It has been assumed that nutritional intervention to increase intake of phyto-antioxidants may reduce threat of free radicals (Arora, Kaur, & Kaur, 2003; Ng, Liu, & Wang, 2000). Plants play a significant role in maintaining human health and improving the quality of human life. They serve humans well as valuable components of food, such as seasonings and beverages as well as in cosmetics, dyes, and medicines. The World Health Organization estimated that <80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components (Winston, 1999).

Lipid peroxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavours and potentially toxic reaction products (Maillard, Soum, Meydani, & Berset, 1996). Many synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, *t*-butylhydroquinone and propyl gallate, are used to retard lipid peroxidation (Wanita & Lorenz, 1996). However, the use of synthetic antioxidants is under strict regulation due to the potential health hazards caused by such compound (Hettiarachchy, Glenn, Gnanasambandam, & Johnson, 1996; Park, Jung, Nam, Shahidi, & Kim, 2001). In view of the beneficial role of herbs in the food industry and the present understanding about the role of oxidative stress in pathogenesis of multiple diseases, attempts have been made to examine the antioxidant status of some herbal products, *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc), (Banerjee, Sur, Mandal, Das, & Sikdar, 2000; Ghosal, Sharma, & Jaswal, 1978; Saha & Das, 2003; Saha, Manadal, Das, Das, & Das, 2004), commonly known as Kalmegh (Sanskrit), and chirayata (Hindi) of the families, *Acanthaceae* and *Gentianaceae*, respectively. These herbs are found in many Asian countries and make significant contributions in ayurvedic preparations against a variety of diseases (Poolsup, Suthisisang, Prathanturarug, Asawamekin, & Chanchareon, 2004; Zhang & Tan, 2000). Hence, present investigations were carried out on antioxidant properties of these two important herbs against photosensitization (endogenous pigments, riboflavin act as sensitizers in presence of light and induced ROS) (Paillous & Fery-Forgues, 1994), and ionizing radiation (present in environmental flares) (Von Sonntag, 1987), the ROS-generating agents to which humans are frequently exposed. Oxidative damage was studied in rat liver mitochondria, one of the crucial sub-cellular organelles and the major site of oxidative reactions. No studies have so far been conducted in these plant extracts against such agents. The results demonstrated that the extracts of Ap and Sc have significant antioxidant activity against various reactive oxidants.

2. Materials and methods

2.1. Plant extracts

Aqueous extracts of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc) were obtained as a gift from Zandoo Research Laboratory, Mumbai.

2.2. Animals

The rats were bred in the BARC Laboratory Animal House Facility and obtained after getting clearance from the BARC Animal Ethics Committee. All the experiments were conducted with the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India on the use of animals in scientific research.

Female Wistar rats (weighing 250 ± 20 g, 10–12 weeks old), maintained, under controlled laboratory conditions (25 ± 2 °C; RH 60 ± 5%; 12 h photoperiod), fed standard animal food and tap water ad libitum were used for oxidative studies.

2.3. Chemicals

2-Thiobarbituric acid, vitamin C, GSH, xylem orange, 2-deoxyribose, mannitol, SOD, FAD, methionine, ABTS, dinitrophenyl hydrazine, *N*,*N*-dimethyl *p*-phenylene diamine dihydrochloride, methylene blue and hydrogen peroxide were purchased from Sigma Chemical Co. Tetraethoxypropane was used as the standard for estimating malonaldehyde equivalents. All other chemicals used in the study were of the highest purity commercially available.

2.4. Preparation of rat liver mitochondria

Rats were fasted overnight, sacrificed by cervical dislocation. Livers were removed, and homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 3000*g* for 10 min to remove cell debris and the nuclear fraction. The resulting supernatant was centrifuged at 10,000*g* and for 10 min to sediment mitochondria in a Sorvall RC5C centrifuge. The mitochondrial pellets thus obtained were washed thrice with 5 mM potassium phosphate buffer pH 7.4 to remove sucrose and were suspended in the same buffer at a concentration of 10 mg protein/ml (Kamat, Sarma, Devasagayam, Nesaretnam, & Basiron, 1997).

2.5. Photosensitization as a source of ROS generation

The system for exposing mitochondria to photosensitization was very simple. The mitochondria (final concentration 0.5 mg protein/ml) were suspended in 50 mM phosphate buffer (pH 7.4) and kept in a 'trap' maintained at 37 °C, with or without the sensitizer and constant

bubbling of O_2. The light source used, with intensity of 100 W, was sufficient to induce significant peroxidation. The distance between light source and the trap was 15 cm. The light intensity, as measured by potassium ferrioxalate at 665 nm, in which methylene blue absorbs maximum light was 0.15 J cm^{-2} $s.^{-1}$ After photosensitization, oxidative damage was measured in terms of various lipid peroxidation products (Kamat & Devasagayam, 1996).

2.6. γ-Radiation as a source of ROS generation

The suspension of mitochondria (2 mg protein/ml) in 5 mM potassium phosphate buffer, pH 7.4, was exposed to gamma radiation from a ^{60}Co source (Atomic Energy of Canada Limited) at a dose rate of 15 Gy/min. In our earlier studies, the oxidative damage to rat liver mitochondria was examined in doses ranging from 45 to 600 Gy and the dose of 450 Gy demonstrated significant damage to mitochondrial membrane lipids (Kamat, Boloor, Devasagayam, Jayashree, & Kesavan, 2000); hence, this dose was selected for radiation exposure with and without extracts (Kamat & Devasagayam, 1996). Mitochondria were then subjected to oxidative studies related to lipid damage.

2.7. Assessment of oxidative damage in mitochondrial membrane lipids

Oxidative damage to lipid was assessed in terms of various lipid peroxidation products, such as conjugated dienes (CD), lipid hydroperoxide (LOOH), thiobarbituric acid-reactive substances (TBARS) and 4-hydroxynonenal (4HNE) from liver mitochondria, unexposed and exposed to radiation with or without Ap or Sc extracts independently. CD (Kamat, Boloor, Devasagayam, Jayashree, & Kesavan, 2000; Kamat et al., 1997) was measured by mixing the samples with chloroform:methanol (2:1) and evaporating the chloroform layer under a stream of nitrogen and lipid residue was dissolved in cyclohexane and absorbance was measured at 233 nm. The formation of LOOH was measured by FOX II reagent, based on the concept that hydroperoxide oxidizes ferrous to ferric ion selectively in dilute acid medium, and the resultant ferric ions can be determined using ferric sensitive dyes as an indirect measurement of hydroperoxide concentration. Absorbance was measured at 560 nm (Nourooz-Zadeh, Tajaddini-Sarmadi, Eddie Ling, & Wolff, 1996). TBARS were isolated by boiling mitochondrial samples for 15 min at 100 °C with thiobarbituric acid reagent (0.5% 2-thiobarbituric acid/10% trichloroacetic acid/0.63 M hydrochloric acid) and measuring the absorbance at 532 nm against a blank unexposed to radiation (Kamat et al., 1997). The malonaldehyde equivalents of the sample were calculated using tetraethoxypropane as standard. Formation of HNE was measured by derivatization of HNE with DNPH in the dark for 1 h. Underivatized DNPH was removed by extracting with hexane. The hexane phase was evaporated completely and dissolved in 5 mM DNPH and absorbance was measured spectrophotometrically at 350 nm (Esterbauer, 1996).

2.8. Measurement of protein oxidation and GSH

Protein oxidation was monitored by measuring carbonyl contents of the protein by derivatization with phenylhydrazine (Levine, Williams, Stadtman, & Shacter, 1994). In general, mitochondrial proteins in 50 mM potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2 N HCl). Blank samples were mixed with 2 N HCl incubated at 1 h in the dark; protein was precipitated with 20 % TCA. Underivatized proteins were washed with an ethanol:ethyl acetate mixture (1:1). Final pellets of protein were dissolved in 6 N guanidine hydrochloride and absorbance was measured at 370 nm. The levels of mitochondrial GSH were measured spectophotometrically by using Ellman's reagent (DTNB). The absorbance was measured at 412 nm. Mitochondrial suspension in 0.15 M. Tris–HCl buffer, pH 7.4, was treated with Ellman's reagent (pH 8), incubated at room temperature for 10 min and absorbance was read. Standard GSH was used as positive control (Moron, Depierre, & Mannervick, 1979).

2.9. Measurement of scavenging activity of Ap and Sc extracts

2.9.1. 2-Deoxyribose assay and detection of hydroxyl radical

The hydroxyl radical scavenging activities of Ap and Sc were determined (Maulik et al., 1997). Briefly, the reaction mixture (1 ml) contained 28 mM 2-deoxyribose, 10 mM Tris–HCl buffer (pH 7.4), 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2 and extracts. The reaction was initiated by adding ascorbic acid (300 μM). After incubating the mixture for 1 h at 37 °C, it was treated with 30% ice-cold HCl containing 0.75% TBA in sodium acetate. The mixture was boiled for 30 min, cooled and the absorbance of the supernatant at 532 nm was read. Mannitol was used as a positive control.

2.10. Measurement of reducing power

The estimation of reducing power was carried out by treating plant extract (5–100 μg/ml in methanol) with 1% potassium fericyanide at 50 °C for 20 min, followed by precipitation of protein with 10% TCA. After centrifugation, clear supernatant was treated with 0.1% $FeCl_3$ and absorbance was measured at 700 nm. The total phenolic content was estimated by treating plant extract with 80% Na_2CO_3 followed by addition of Folin–Clocalteu reagent and measuring the absorbance at 700 nm (Ng et al., 2000).

2.11. Superoxide radical assay

In the present study superoxide radical generation by photo-reduction of riboflavin was combined with nitrite formation from hydroxylamine hydrochloride. In brief, superoxide radicals were allowed to react with hydroxyl-

amine hydrochloride to produce nitrite. The nitrite in turn reacted with sulphanilic acid to produce diazonium compound that subsequently reacted with napthylamine to produce a red azo compound with maximum absorbance at 543 nm. The assay mixture contained 50 mM phosphate buffer, pH 6.6, 20 mM methionine, 1% Triton X-100, 10 mM hydroxylamine, 50 μM EDTA and an appropriate amount of plant extract. All the components of the mixture were incubated at 37 °C for 5 min and the reaction was started with riboflavin (50 μM). The mixture was exposed to light for 10 min, followed by addition of Geriss reagent. The formation of superoxide radical was measured at 543 nm (Das, Samanta, & Chainy, 2000). Superoxide dismutase (SOD) was used as positive control.

2.12. Estimation of phenolic and flavonoid contents

The measurement of total phenolic contents is based on the reduction of Folin–Ciocalteu reagent by reducing equivalents from phenols. Briefly, Folin–Ciocalteu reagent was mixed equally with sample and the mixture was treated with 80% Na_2CO_3 and absorbance was measured at 730 nm (Fukumto & Mazza, 2000). Measurement of total flavonoid content of the extracts was carried out by the standard method (Jia, Tang, & Wu, 1999). $NaNO_2$ (5%) was added to the extract mixture, followed by the addition of $AlCl_3 \cdot 6H_2O$ (10%). Absorbance was taken at 510 nm, following the addition of NaOH. Quercetin was used as positive control.

2.13. SDS–PAGE to examine mitochondrial protein profiles following exposure to photosensitization

Mitochondrial proteins were exposed to photosensitization at various time intervals (5–120 min) with and without Ap and Sc extracts (1 h photosensitization) and subjected to SDS–PAGE (Lammelli, 1970). A Bio-Rad-gel documentation (Discovery series) system was used for measurement of band intensity.

2.14. Pulse radiolysis and examination of antioxidant activity of Ap and Sc extracts with ABTS radical

The pulse radiolysis experiments were carried out with high electron energy pulses (7 MeV, 50 ns). $ABTS^{-\cdot}$ was generated by pulse radiolysis of a N_2O-saturated aqueous solution of N_3^- (0.05 M) and $ABTS^{2-}$ (2 mM) and decay of the $ABTS^-$ was measured at 600 nm (Scotl, Chen, Bakac, & Espenson, 1993). The decay was followed with known concentrations of extract. From the measured rate constant value, the ascorbic acid equivalent present in the extract was calculated.

2.15. Statistical analysis

The results obtained were expressed as means ± SEM. A student's *t*-test was used to make a statistical comparison between the groups. A statistical comparison was done with the radiation/photosensitization alone group vs. the Ap or Sc and radiation/photosensitization combined group. The significance levels were set at $^{+}p < 0.05$, $^{\#}p < 0.01$, and $^{*}p < 0.001$.

3. Results and discussion

3.1. Inhibition of photosensitization-induced lipid peroxidation by Ap and Sc extracts

Photosensitization is a widely occurring phenomenon in biological systems. It is induced due to the ubiquitous nature of visible light and presence of number of endogenous/exogenous pigments (Kanofsy, 1989). Methylene blue is a typical sensitizer, widely used in a several test systems (Floyd, West, Eneff, & Schneider, 1990), and shown to generate $^1O_2$ (singlet oxygen), an electronically excited highly reactive molecule. Due to its relatively long half-life, in the range of 10–50 μs, it is capable of travelling an appreciable distance in the cellular milieu and being implicated in various human disorders (Kanofsy, 1989; Sies, 1997).

The results on photosensitization-induced lipid peroxidation products, such as lipid hydroperoxide (LOOH) and HNE formation and their modulation with extracts, are given in Fig. 1. Photosensitization exposure (for 15 min) resulted in significant damage, showing 90% ($p < 0.001$) and 54% ($p < 0.01$) inhibition of LOOH by Ap and Sc extracts (50 μg/ml) respectively. Similarly, these extracts could also prevent enhanced formation of HNE to some extent (inhibition by Ap is 30% and that by Sc is 19%).

Fig. 2 demonstrates oxidative damage measured in terms of TBARS, the final stable aldehydic product of peroxidation, generated by breakdown of LOOH. TBARS and HNE, which are the crucial indicators of cellular damage.

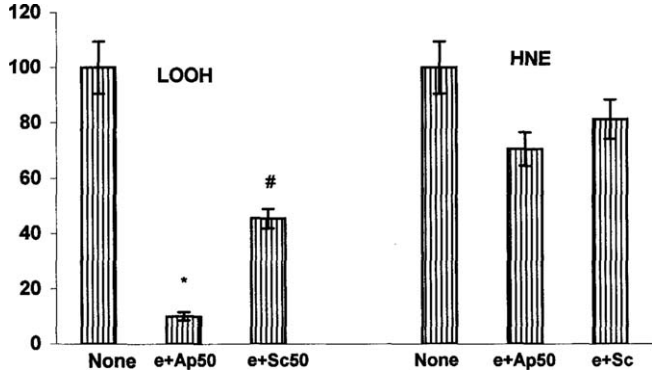

Fig. 1. Photosensitization-induced formation of lipid hydroperoxide (LOOH) and hydroxylnonenal (HNE) in rat liver mitochondria and prevention by aqueous extracts of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). Mitochondrial protein (0.5 mg/ml) was exposed to photosensitization as described in *methods* with or without Ap and Sc, independently, and estimations of LOOH and HNE were carried out. Ap and Sc were used at 50 μg/ml, independently, during photosensitization. The values are means ± SEM from five experiments, $^{*}p < 0.001$, $^{\#}p$ compared with photosensitization alone.

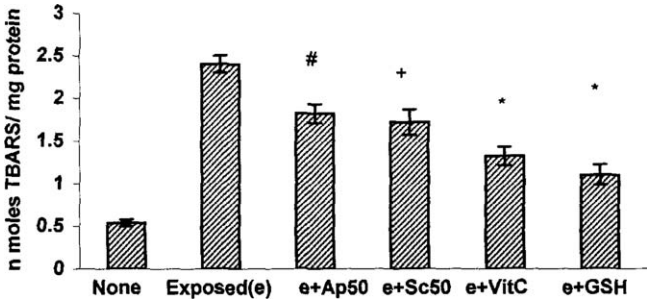

Fig. 2. Photosensitization-induced formation of TBARS in rat liver mitochondria and prevention by aqueous extracts of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). The legends are the same as given in Fig. 1. After exposing of mitochondria to photosensitization, the formation of TBARS was measured. None – unexposed control mitochondria, exposed (e) – mitochondria exposed to photosensitization, e + Ap50, e + Sc50, e + Vit C, e + GSH – mitochondria exposed to photosensitization with Ap and Sc at 50 μg/ml, vit. C and GSH (1 mM), independently, during photosensitization. Formation of TBARS was measured. The values are means ± SEM from five experiments, $^{*}p < 0.001$, $^{\#}p$, $^{+}p < 0.05$ compared with photosensitization alone.

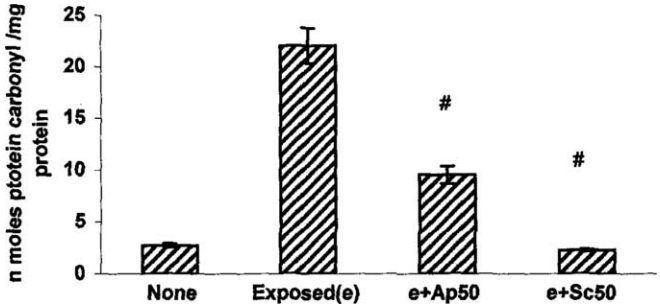

Fig. 3. Photosensitization-induced formation of protein carbonyl in rat liver mitochondria and prevention by aqueous extracts of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). The legends are the same as given in Fig. 1. After exposing mitochondria to photosensitization, the formation of protein carbonyl was measured. None – unexposed control mitochondria, exposed (e) – mitochondria exposed to photosensitization, e + Ap50, e + Sc50 extracts with mitochondria exposed, independently, to photosensitization. Formation of TBARS was measured. The values are means ± SEM from five experiments $^{\#}p < 0.01$ compared to photosensitization alone.

They form adducts with various biomolecules and show many adverse effects on the organisms (Esterbauer, 1996; Halliwell & Gutteridge, 1999). Photosensitization showed significant induction in TBARS ($p < 0.001$). However simultaneous addition of the extracts independently prevented the damage (24% by Ap; $^{\#}p < 0.01$ and 28% by Sc; $^{+}p < 0.05$). This effect was more pronounced than standard antioxidants, GSH (1 mM) and vit. C (1 mM) which required much higher concentration than the extracts. These experiments indicated potent antioxidant ability of the extracts. (vit. C requires >3 times more than Ap or Sc, GSH > 6 times more; GSH = 307 lg/ml and vit. C = 175 lg/ml).

3.2. Preventive effects of Ap and Sc extracts on photosensitization-induced protein oxidation

Protein oxidation is an important process occurring in the cell. It generates a wide range of ROS by oxidation of several cellular proteins and is implicated in various physiological disorders (Stadman, 1992). Fig. 3 demonstrates the enhanced formation of protein carbonyls, a measuring index of protein oxidation following photosensitization ($p < 0.001$). Addition of Ap and Sc extracts during photosensitization showed 56% ($p < 0.001$) and 87% ($p < 0.001$) inhibition in protein carbonyl, respectively.

3.3. Radioprotective ability of Ap and Sc extract

It is a well-established fact that ionizing radiation, especially that involving low linear energy transfer (LET), e.g., γ-rays, generates large numbers of ROS through the radiolysis cleavage of water, exerting highly damaging events in the cell components and loss of the structural-functional integrity of the cell (Esterbauer, 1996; Von Sonntag, 1987; Wallace, 1988). The persistent oxidative stress induced by radiation impairs the comprehensive/integrated network of the antioxidant repair system. Our studies demonstrated radiation-induced oxidative damage to lipid and possible protection by Ap and Sc extracts. The results are incorporated in following few Figures.

The radioprotective ability of Ap and Sc against γ-ray-induced peroxidation of rat liver mitochondria was studied by examining the formation of CD, LOOH, TBARS and 4-HNE. Fig. 4 demonstrates prevention of radiation-induced formation of CD and LOOH by Ap and Sc extracts. Exposure of mitochondria to radiation at 450 Gy showed increase in CD formation and the inhibitory effect was found to be 35% and 50%, respectively, at 25 and 50 μg/ml. The effects of Sc at corresponding concentrations are 31% and 45.2%, respectively. The enhanced formation of

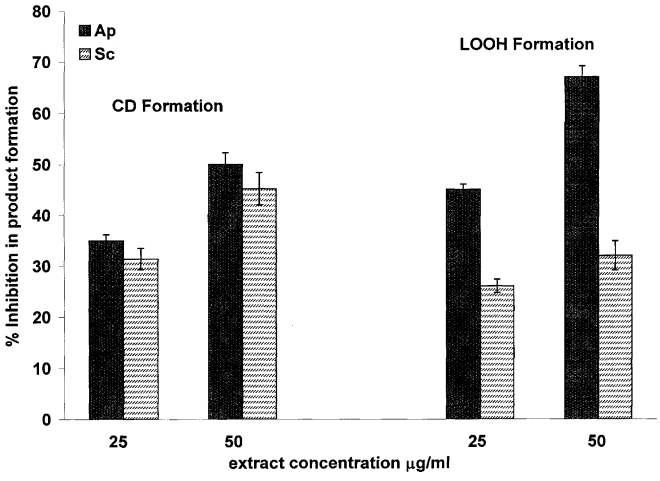

Fig. 4. Photosensitization-induced formation of conjugated dienes (CD) and lipid hydroperoxide in rat liver mitochondria and prevention by aqueous extracts of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). Mitochondrial protein (2 mg/ml) was exposed to radiation at 450 Gy with or without Ap and Sc, independently, and estimations of CD and LOOH were carried out. Ap and Sc were used at 25 and 50 μg/ml, independently, during radiation. The values are means ± SEM from five experiments.

LOOH due to radiation was also reduced by 45% and 67% by Ap at 25 and 50 μg/ml, respectively. Sc at the same concentration resulted 26% and 32% inhibition, respectively.

Radiation-induced TBARS were 2.45 ± 0.1 nmol/mg protein as against the control values, 0.54 ± 0.041 nmol/mg protein without exposure. Simultaneous exposure of liver mitochondria to γ-rays at the same dose, with Ap and Sc at 50 μg/ml, independently demonstrated significant protection ($^{*}p < 0.001$) in the formation of TBARS (Fig. 5(a)). The results were compared with standard antioxidants, vit. C and GSH. Though these antioxidants showed more (>50%) protection than Ap and Sc, the dose required to get the protection was comparatively higher than that of Ap and Sc. Radiation-induced formation of 4-hydroxylnonenal (4-HNE) in rat liver mitochondria was also decreased by extracts of Ap and Sc. A significant damage to HNE

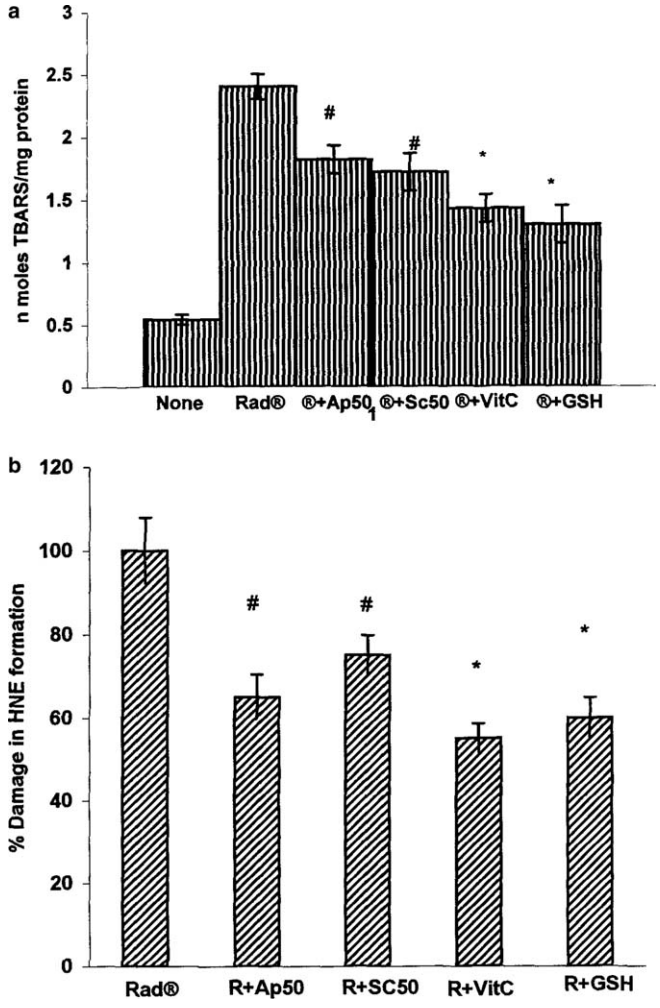

Fig. 5. Radiation-induced formation of TBARS (a) and HNE (b) in rat liver mitochondria and prevention by aqueous extracts of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). The legends are the same as given in Fig. 4. After exposing mitochondria to radiation, the formations of TBARS and HNE were measured. None – unexposed control mitochondria, Rad® – mitochondria exposed to 450 Gy, Ap50, Sc50, ®+ vit. C, ®+ GSH – mitochondria exposed to 450 Gy with Ap and Sc at 50 μg/ml, vit. C and GSH (1 mM), independently, during radiation. Formation of TBARS was measured. The values are means ± SEM from five experiments, $^{*}p < 0.001$, $^{\#}p$ compared with radiation alone.

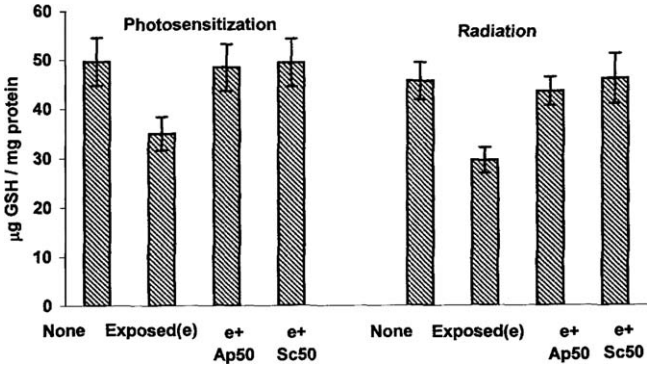

Fig. 6. Photosensitization and radiation-induced formation of GSH in rat liver mitochondria and prevention by aqueous extracts of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). The legends are the same as given in Figs. 1 and 4. After exposing mitochondria to radiation/photosensitization, GSH was measured. None – unexposed control mitochondria, exposed (e) – mitochondria exposed, e + Ap50, e + Sc50, – mitochondria exposed with Ap and Sc at 50 μg/ml. The values are means ± SEM from four experiments

formation was observed following γ-radiation ($^{*}p < 0.001$). Simultaneous addition of the extracts of Ap and Sc independently resulted in 35% and 28% protection, respectively ($^{\#}p < 0.01$). Vit. C and GSH showed about 40% protection in the HNE contents ($^{*}p < 0.001$) (Fig. 5(b)).

3.4. Photosensitization and radiation-induced depletion of GSH and effect of extracts

Photosensitization and radiation, the major sources of ROS generation, significantly depleted endogenous antioxidant GSH and Ap and Sc extracts showed effective restoration (Fig. 6).

3.5. Scavenging capacity of extracts for hydroxyl and superoxide radicals

Fig. 7 demonstrates the scavenging capacity of the extracts for superoxide and hydroxyl radicals. It is clear that both the extracts at 25 and 50 μg/ml have significant potential for scavenging superoxide and hydroxyl radicals. Ap, at 25 and 50 μg, showed 58% and 75% inhibition of the

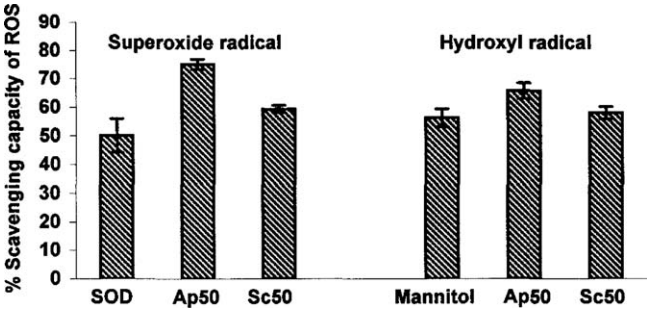

Fig. 7. Estimation of scavenging capacity of Ap and Sc extracts with ROS. Superoxide and hydroxyl radicals are generated as given in methods. The values are means ± SEM from four experiments

formation of superoxide radicals and that with Sc at the corresponding concentration was 45% and 59%, respectively. The positive control, SOD (35 ng), showed 50% scavenging of superoxide radical. The scavenging ability of Ap and Sc with hydroxyl radicals at corresponding concentration was 35% and 65% and that for Sc was 25% and 58%, respectively, and positive control, mannitol showed 56% scavenging with hydroxyl radical. These experiments indicated excellent scavenging capacity of the extracts as compared to standard antioxidants. Mannitol was required 3.6 times higher concentration to scavenge hydroxy radical at 56% than was Ap and Sc. Being an enzyme, present in pure form, SOD was required at only 36 ng to get 50% inhibition of superoxide.

3.6. Measurement of reducing ability, phenolic and flavonoid contents of extracts

The reducing ability (Fig. 8(a)) and phenolic and flavonoid contents (Fig. 8(b)) of the extracts were measured at various concentrations. Both the extracts had high reducing ability, as indicated by increased absorbance as a function of extract concentration. Extracts also showed high phenolic and flavonoid contents. At 50 μg, Ap and Sc showed phenolic contents of 5.3 and 4.9 μg equivalents of gallic acid, respectively, and flavonoid contents at corresponding concentrations were 8.6 and 10.6 μg equivalents of quercetin, respectively. The total phenolic contents were found to be higher in Ap extract whereas Sc extract showed higher contents of flavonoids.

3.7. Photosensitization-induced degradation of proteins and prevention by extracts

A drastic time-dependent degradation (as shown by diminished intensity in the protein bands) was observed following exposure of mitochondrial proteins to photosensitization (Fig. 9(a)) and simultaneous addition of Ap and Sc extracts during photosensitization (1 h) showed remarkable reversal in the band intensities of the proteins (Fig. 9(b)).

3.8. Pulse radiolysis studies of the extracts

The antioxidant capacity of both the extracts was examined by pulse radiolysis in the presence of $ABTS^{-\cdot}$. The ABTS radicals were generated by pulse radiolysis in the presence of N_2O-saturated aqueous solution of N_3 and ABTS. Comparison was done with ascorbic acid at various concentrations (10–40 μg) and ascorbic acid equivalents present in the extracts were calculated (Fig. 10). Sc had 3.5 μg of ascorbic acid equivalents and Ap 1.5 μg of ascorbic acid.

The scavenging assay for the stable synthetic radical, DMPD, was carried out with both the extracts (25, 50 and 100 μg/ml). Ap and Sc extracts at 25 μg showed about 20% inhibition and that at 50 μg was around 34%. The comparative analysis of the results with standard antioxidants GSH and vit. C demonstrated about 50% inhibition in DMPD formation, even at 5 μg/ml. The potency of the extracts was shown to be relatively less for DMPD radical inhibition (data not given).

The exact mechanism of action for the radioprotective/antioxidant effect is not known. The possible antioxidant action of Ap can be explained on the basis of an earlier report that the extract contains several active components, such as andrographolide (0.6%), 14-deoxy-11-oxoandrographolide (0.12%), 14-deoxyandrographolide (0.02%), and neoandrographolide (0.005%). Extracts also showed the presence of two flavonoids, namely 5,7,tetramethoxyflavanone and 5-hydroxy-7, trimethoxyflavone (Poolsup et al., 2004; Rao, Vimalamma, Rao, Rao, & Yew-Min, 2004; Shen, Chen, & Chiou, 2002). Pharmacological studies have

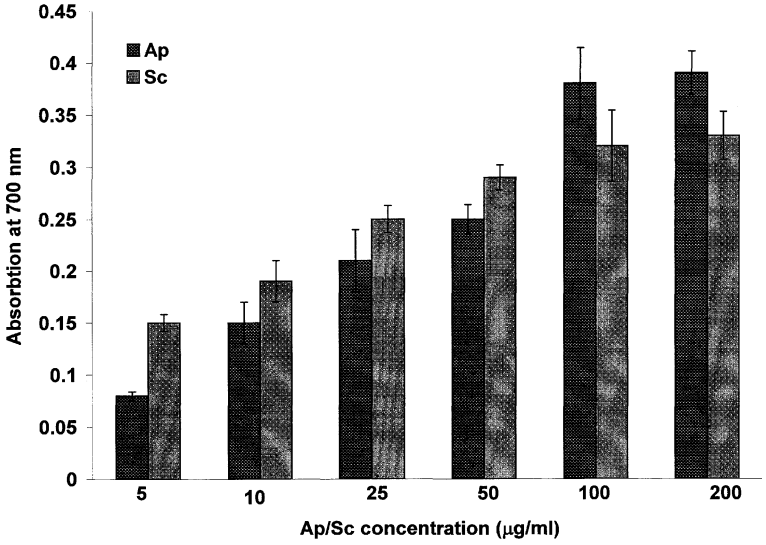

Fig. 8. Estimation of reducing power (a), phenolic and flavonoid contents of *Andrographis paniculata* (Ap) and *Swertia chirata* (Sc). The values are means ± SEM from five experiments.

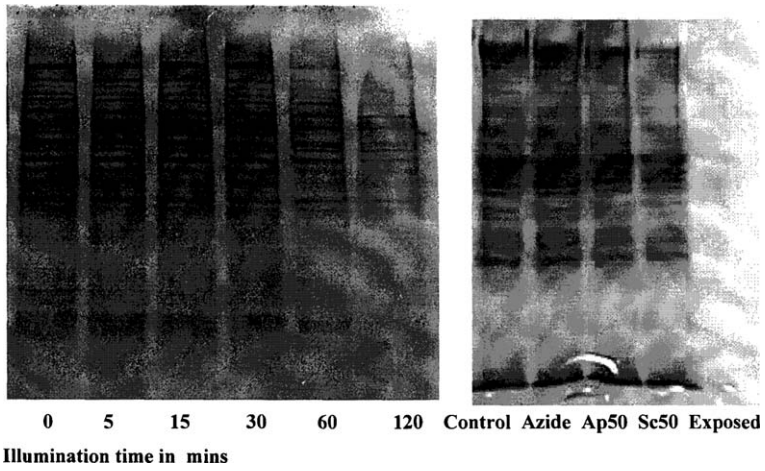

Fig. 9. Photosensitization-induced degradation of total mitochondrial proteins at various times from 5 to 120 min (a) and preventive effects of aqueous extracts of *Andrographis paniculata* (Ap) *Swertia chirata* (Sc) (b). Mitochondria were exposed to photosensitization for 1 h, with and without Ap and Sc extract and intensities protein bands were examined by SDS–PAGE. Sodium azide (10 mM) was used as a standard quencher of singlet oxygen. Ap50 and Sc50 extracts were used at 50 μg/ml during photosensitization.

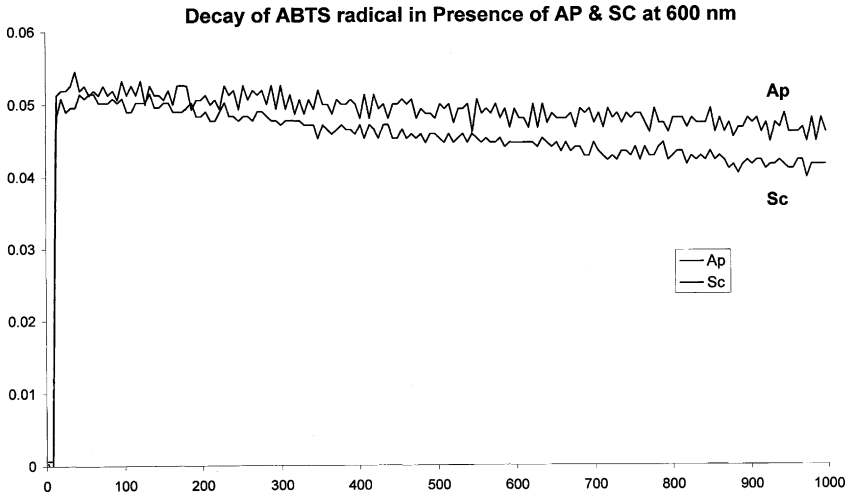

Fig. 10. Decay of ABTS radical at 600 nm in the presence of Ap and Sc extracts.

suggested that some of the active components function as chain-breaking antioxidants. Structural perspective chain-breaking antioxidants are hydrogen donors and/or possess an extensive system of conjugated double bonds that stabilizes reaction transients by resonance. The above active components do not have such structural features. However, some reports have demonstrated ability have of Ap extract to scavenge superoxide radical (Frimer, Tova, & Aljadeff, 1986). Our results showed significant scavenging effect of Ap with $O_2^{-\bullet}$, $^{\cdot}OH$, and $^1O_2$. The studies dealing with Sc have demonstrated that xanthones (swertanone, episwertiol, chiratenol, gammacer-16-en-3β-ol, ursolic acid, swerta-7-9(11)-diene-3β-01, pichierenol) are powerful, life-enhancing substances with crucial medicinal/pharmaceutical properties and highly involved in the inhibition of initial lipid peroxidation steps (Banerjee et al., 2000; Ghosal et al., 1978). Hence, antioxidative effect is explained. Sc also contains and swerchirin and amarogentin (Ghosal et al., 1978), in addition to some alkaloids, e.g., gentianine, gentiocrucme and enicoflavine. Both the extracts have excellent physiological significance, They are used in cancer therapy (Singh, Banerjee, & Rao, 2001), immunostimulation, antihepatotoxic agents (Kumar, Sridevi, Kumar, Nanduri, & Rajagopal, 2004; Trivedi & Rawal, 2001; Zhang & Tan, 2000), fever and many other human disorders. In India, *Swertia* is present as the main ingredient in Mahasudarshana churna, a remedy containing more than 50 herbs with immunostimulatory and antihepatotoxic effects (Kumar et al., 2004). Besides, the considerable phenolic and flavonoid contents, a well-established index of antioxidant function (Maulik et al., 1997; Ng et al., 2000) found in the extract, is additional support. Biodistribution experiments in experimental animals have demonstrated that radioactively

labeled active components of the extracts appear to be widely distributed in the body. High concentrations are noted in the central nervous system (brain and spinal cord) and other organs with high blood flow, including the colon, spleen, heart, lungs and kidneys. Andrographolide appears to have a relatively short half-life of approximately 2 h. Andrographolides from Ap are excreted fairly rapidly from the body via the urine and gastrointestinal tract. In some studies, 80% of the administered dose of andrographolide is removed from the body within 8 h, with excretion rates of more than 90% of the compound within 48 h (Jean Barilla, 1999). The wide tissue and organ distribution and the immune-stimulating and regulatory actions of AP make it an ideal candidate in the prevention and treatment of many diseases and conditions.

Therefore, such molecules may exhibit radioprotection by scavenging free radical species and are benificial to patients undergoing radiotherapy and over-exposure to ROS (Ames, 1983; Hasan & Khan, 1986). Radioprotective/antioxidative effects of various natural products e.g., *Asparagus racemosus* (Kamat & Venkatachalam, 2004, chapter 4) caffeine (Kamat, Boloor, Devasagayam, Jayashree, & Kesavan, 2000a), chlorophyllin (Kamat, Boloor, & Devasagayam, 2000b), *phyllanthus amarus* (Hari Kumar & Kuttan, 2004), ocimum (UmaDevi & Ganasoundari, 1995), piper betle (Bhattacharya et al., 2005), nicotinamide (Kamat & Devasagayam, 1996), and tocotrienols, (Kamat et al., 1997), are known. Ap and Sc also may be useful new candidates in this direction.

In conclusion, the aqueous extracts of Ap and Sc significantly protect against oxidative damage induced by various oxidants. These results may shed light on an important strategy of biological homeostasis involving pro-oxidant and antioxidant profiles in the cell. These drugs are popular in ayurvedic formulations. They may have significant potential applications in the food industry for the preservation of food products and advantages over synthetic food antioxidants.

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