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Evaluation of antioxidant and radical-scavenging activities of certain radioprotective plant extracts

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Abstract

The extract of *Adhatoda vasica*, *Amaranthus paniculatus*, *Brassica compestris*, *Mentha piperita* and *Spirulina fusiformis* has radioprotective effects in animal model systems. In the present investigation, the extracts of *A. vasica*, *A. paniculatus*, *B. compestris*, *M. piperita* and *S. fusiformis* were further evaluated for their antioxidant (GSH&LPO) and radical-scavenging activities ($DPPH^{\cdot}$ and $ABTS^{\cdot +}$ assays). All these plant extracts showed antioxidant activity, as measured by estimating reduced glutathione and lipid peroxidation in liver, and showed radical-scavenging activity in both $DPPH^{\cdot}$ and $ABTS^{\cdot +}$ assays. The extracts of *M. piperita*, *A. vasica* and *B. compestris* showed very strong radical-scavenging activity in both the assays. However, extracts of *A. paniculatus* and *S. fusiformis* showed moderate radical-scavenging activity. The IC_{50} values of these plant extracts were: *M. piperita* – 273 μg/ml, *A. vasica* – 337 μg/ml, *B. compestris* – 398 μg/ml, *A. paniculatus* – 548 μg/ml and *S. fusiformis* – 620 μg/ml, respectively. The differential radioprotective and antioxidant activity of these plant extracts observed may be assigned to different chemical constituents present in the different plant extracts. The result of the present investigation indicates that the antioxidant mechanism of radioprotection and free-radical scavenging appear to be likely mechanisms of radiation protection by these plant extracts.
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Keywords: Radical scavenging activity; Radioprotection; GSH; LPO; $DPPH^{\cdot}$ and $ABTS^{\cdot +}$

1. Introduction

The essential oils and extracts of many plant species have become popular in recent years, and attempts to characterize their bioactive principles have gained momentum in many pharmaceutical and food processing applications (Cowan, 1999). Plants (fruits, vegetables, medicinal herbs) contain a wide variety of free radical-scavenging molecules, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and some other endogenous metabolites, that are rich in antioxidant activity (Cai, Sun, & Corke, 2003; Cotelle et al., 1996; Larson, 1988; Shahidi & Naczk, 1995; Velioglu, Mazza, Gao, & Oomah, 1998; Zheng & Wang, 2001).

In our laboratory, the extracts of *Adhatoda vasica*, *Amaranthus paniculatus*, *Brassica compestris*, *Mentha piperita* and *Spirulina fusiformis* were shown to have radioprotective effects in Swiss albino mice (Table 1). Pre-treatment with these plant extracts was shown to provide protection of mice against gamma radiation. For assessment of radioprotective effects, the most reliable procedure involves determination of a dose reduction factor [DRF] (Brown, Graham, Mackensie, Pittock, & Shaw, 1988; Yuhas & Storer, 1969). The dose reduction factor (DRF) values reported were *A. vasica* = 1.6 (Kumar, Ram, Samarth, & Kumar, 2005); *A. paniculatus* = 1.36 (Krishna & Kumar, 2005); *B. compestris* = 1.59 (Soni et al., 2006); *M. piperita* = 1.78 (Samarth & Kumar, 2003); *S. fusiformis* = 1.3 (Kumar, Verma, Kumar, & Kiefer, 2002). These plants contain various chemical constituents that have shown antioxidant properties (Table 1).

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Table 1
Plant extracts screened for antioxidant and radical-scavenging activities

Plant	Family	Voucher number	Major chemical constituents	Effective dose	End point	DRF value	Reference
Adhatoda vasica	Acanthaceae	RUBL-18822	Vesicine, vesicinone, betaine, vitamin C, β-carotene and vasakin	800 mg/kg b. wt.	30 day survival	1.6	Kumar et al. (2005)
Amaranthus paniculatus	Amaranthaceae	RUBL-19869	Proteins, vitamins (C and E), provitamin A, riboflavin	800 mg/kg b. wt.	30 day survival	1.36	Maharwal et al. (2003), Krishna and Kumar (2005)
Brassica compestris	Cruciferae	RUBL-19932	Allyl isothiocyanate, glucosinolates, indoles	800 mg/kg b. wt	30 day survival	1.59	Soni et al. (2006)
Mentha piperita	Labiatae	RUBL-19443	Caffeic acid, rosamarinic acid, eugenol and α-tocopherol	1 g/kg b. wt.	30 day survival	1.78	Samarth and Kumar (2003)
Spirulina fusiformis	Oscillatoriaceae	*	Proteins, natural biochelated vitamis (β-carotene) and SOD	800 mg/kg b. wt.	30 day survival	1.3	Kumar et al., 2002; Verma et al., 2006

A voucher specimen of plants has been preserved in the herbarium of the Botany Department, University of Rajasthan, Jaipur (India).
* The *Spirulina* extract in powder form was obtained gratis from M/s Recon Pharmaceuticals, Bangalore, India.

It is important to employ a stable and rapid method to assess the antioxidant activity of plant extracts. Several methods have been developed to assay radical-scavenging capacity and total antioxidant activity. The most common and reliable method involves the determination of the disappearance of free radicals (using a spectrophotometer), e.g. 2,2-azinobis (3-ethyl benzothiazolium-6-sulfonic) acid radical ($ABTS^{\cdot+}$) and 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$). Therefore, in the present investigation, the extracts of *A. vasica*, *A. paniculatus*, *Brassica compestris*, *M. piperita* and *S. fusiformis* were further evaluated for their radical-scavenging activity using $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays.

2. Materials and methods

2.1. Preparation of the extracts

2.1.1. Adhatoda vasica Nees

A. vasica plants were collected from Khetri, Rajasthan, India; their leaves were washed thoroughly and shade-dried; a known quantity of the material was Soxhlet-extracted using 80% ethanolic solvent (three changes). Finally, the extract was lyophilized, weighed and preserved at 4 °C and used as and when required (Kumar et al., 2005).

2.1.2. Amaranthus paniculatus

The extract of fresh, shade-dried and powdered leaves of *A. paniculatus* was prepared by refluxing with double-distilled water (DDW) for 36 h (12 × 3) at 40 °C and concentrated under vacuum (Krishna & Kumar, 2005; Maharwal, Samarth, & Saini, 2003).

2.1.3. Brassica compestris

The extract was prepared as described earlier. Briefly the extract was prepared by distilling the dried seed powder of *B. compestris* in a round-bottom flask using 95% ethanol at 60 °C. The procedure was repeated thrice for 12 h durations. The left over residue, after the third distillation, was filtered and the remaining alcohol was allowed to evaporate (Qiblawi & Kumar, 1999).

2.1.4. Mentha piperita Linn

Freshly collected leaves of *M. piperita* Linn. were air-dried, powdered and extracted with double-distilled water (DDW) by refluxing for 36 h (12 h × 3) at 80 °C. The extract thus obtained was vacuum-evaporated to a powder (Samarth & Kumar, 2003).

2.1.5. Spirulina fusiformis

The *S. fusiformis* extract in powder form was obtained gratis from M/s Recon Pharmaceuticals, Bangalore, India.

2.2. Chemicals

DPPH (1,1diphenyl-2-picryl hydrazyl), ABTS (2,2 azinobis)3-ethylbenzothiozoline-6-sulfonic acid, potassium persulphate was obtained from Sigma Chemicals Co. St. Louis, USA.

2.3. Evaluation of antioxidant activity

2.3.1. General

The mice were divided in two groups. Group-I animals were fed orally with 0.1 ml of double-distilled water once a day for 7 days before radiation and served as the control group, while animals of Group-II received plant extract in 0.1 ml of double-distilled water in a similar fashion.

Animals were sacrificed by cervical dislocation and liver was perfused in situ immediately with cold 0.9% NaCl and thereafter carefully removed and rinsed in chilled 0.15 M Tris KCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. Aliquots (0.5 ml) of this homogenate were used for assaying reduced glutathione and lipid peroxidation.

2.3.2. Reduced glutathione

Reduced glutathione (GSH) level was determined by the method described by Moron, Depierre, and Mannervick (1979). Homogenates were immediately precipitated with

0.1 ml of 25% TCA and the precipitate was removed after centrifugation. Free-SH groups were assayed in a total 3 ml volume by the addition of 2 ml of 0.6 mM DTNB and 0.9 ml 0.2 mM sodium phosphate buffer (pH 8.0) to 0.1 ml of the supernatant and the absorbance was read at 412 nm using a UV–VIS Systronics spectrophotometer. Glutathione was used as a standard to calculate μmole GSH/g tissue.

2.3.3. Lipid peroxidation

Lipid peroxidation in liver was estimated spectrophotometrically by thiobarbituric acid-reactive substances (TBARS) method of Ohkawa, Ohishi, and Yogi (1979) and was expressed in terms of malondialdehyde (MDA) formed per mg protein. In brief, 0.4 ml of microsomal sample was mixed with 1.6 ml of 0.15 M Tris KCl buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 52 mM TBA was added and the mixture placed in a water bath for 25 min 80 °C, cooled in ice and centrifuged at room temperature for 10 min at 3000 rpm. The absorbance of the clear supernatant was measured against a reference blank of distilled water at 531.8 nm.

2.4. Evaluation of radical-scavenging activity

2.4.1. DPPH radical-scavenging assay

Radical-scavenging activity of plant extracts against stable DPPH (1,1diphenyl-2-picryl hydrazyl radical) was determined spectrophotometrically. The DPPH assay was carried out as described by Cuendet, Hostettmann, and Potterat (1997). Stock solutions of crude extracts were prepared as 1 mg/ml in methanol. Fifty microlitres of different concentration samples were added to 5 ml of 0.004% methanol solution of DPPH$^{\cdot}$. After 30 min of incubation in the dark at room temperature, the absorbance was read against a blank at 517 nm. The assay was carried out in triplicate and percentage of inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{AB–AA})}{\text{AB}} \times 100$$

where AB = Absorbance of blank; AA = Absorbance of test.

2.4.2. ABTS radical cation decolorization assay

The ABTS, 2,2 azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay was carried out, using an improved ABTS decolorization assay of Re et al. (1999). ABTS$^{\cdot+}$ was generated by oxidation of ABTS with potassium persulphate. ABTS was dissolved in deionized water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12–16 h) in the dark before use for the study of plant extracts, the ABTS$^{\cdot+}$ solution was diluted with ethanol, to an absorbance of 0.700 ± 0.020 at 734 nm. After addition of 1 ml of diluted ABTS solution (A 734 nm = 0.700 ± 0.020) to 10 μl of plant extracts, the absorbance reading was taken at 30 °C exactly 1 min after initial mixing and up to 6 min. All determinations were carried out in triplicate.

3. Results

A significant increase in liver reduced glutathione (GSH) content and decrease in lipid peroxidation (LPO) level was observed in extract-treated animals. However, treatment with extracts of *M. piperita* and *A. vasica* showed highly significant increases in GSH contents ($p < 0.005$). *A. paniculatus*, *B. compestris* and *S. fusiformis* extract treatments showed comparatively less significant increase ($p < 0.05$) in GSH content over the normal (Fig. 1). Also, significant ($p < 0.05$) decrease in lipid peroxidation (LPO) level was observed in extract-treated animals (Fig. 2).

The results of DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ inhibition by extracts of *A. vasica*, *A. paniculatus*, *B. compestris*, *M. piperita* and *S. fusiformis* are summarized in Figs. 3 and 4. The IC_{50} value for each plant extract was calculated and defined as the concentration of extract causing 50% inhibition of absorbance. In the present investigation, extracts of the *M. piperita*, *A. vasica* and *B. compestris* showed excellent percent inhibition of DPPH$^{\cdot}$ activity (93.9 ± 1.68%,

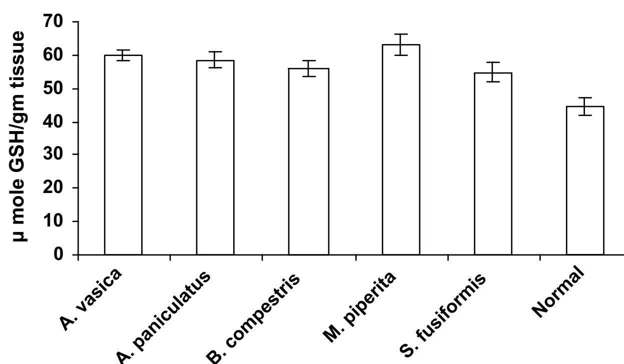

Fig. 1. Antioxidant influence of plant extracts on GSH content in liver of Swiss albino mice.

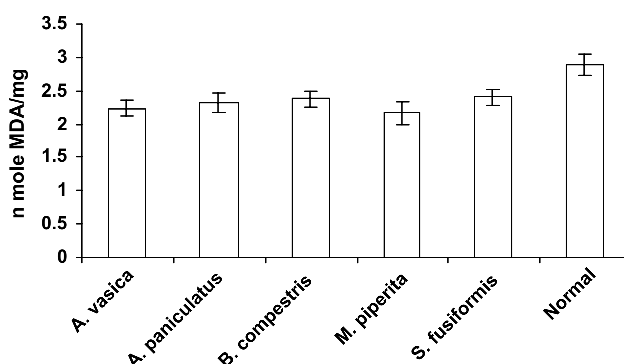

Fig. 2. Antioxidant influence of plant extracts on LPO levels in liver of Swiss albino mice.

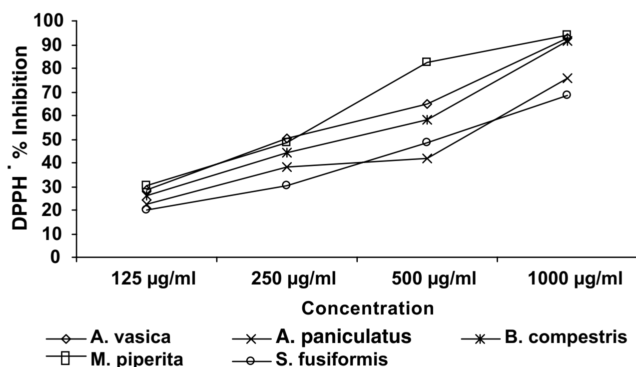

Fig. 3. DPPH·% inhibition at different concentrations of plant extracts. (Fifty microlitres of different concentration of samples were added to 5 ml of 0.004% methanol solution of DPPH·. After a 30 min incubation in the dark at room temperature, the change in colorization from violet to yellow and subsequent fall in absorbance of the stable radical DPPH was measured at 517 nm for various concentrations (1000, 500, 250 and 125 µg/ml).)

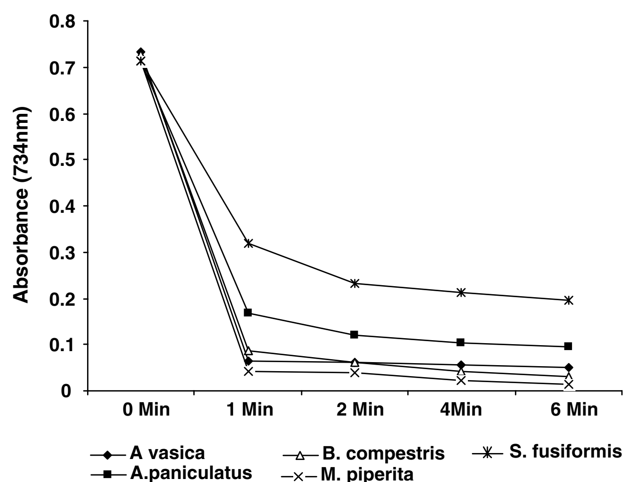

Fig. 4. ABTS activity at different time intervals by plant extracts. (After addition of 1 ml of diluted ABTS solution (A 734 nm = 0.700 ± 0.020) to 10 µl of plant extracts, the absorbance reading was taken at 300 °C exactly 1 min, after initial mixing and up to 6 min. All determinations were carried out in triplicate.)

92.9 ± 1.87%, and 91.7 ± 1.55%, respectively) and were the most effective DPPH radical scavengers (Table 2). These percentages can be considered as a full absorbance inhibition of DPPH· as, after completing the reaction, the final solution always possessed some yellowish colour and therefore, its absorbance inhibition compared to the colourless methanol solution could not reach 100%. (Miliauskas, Venskutonis, & Van Beek, 2004). For further DPPH·-scavenging assessment and determination of IC_{50} values, these plants extracts were further diluted and % inhibitions at different concentrations were evaluated (Fig. 3). These results demonstrated that the most active radical-scavengers were the extracts of *M. piperita* (IC_{50} = 273 µg/ml), *A. vasica* (IC_{50} = 337 µg/ml) and *B. compestris* (IC_{50} = 398 µg/ml).

Table 2
IC_{50} (µg/ml) values and % inhibition of DPPH· activity of plant extracts

Plant extract	IC_{50} (µg/ml)	DPPH inhibition (%)
Adhatoda vasica	337	92.9 ± 1.86
Amaranthus paniculatus	548	75.8 ± 1.62
Brassica compestris	398	91.7 ± 1.55
Mentha piperita	273	93.9 ± 1.68
Spirulina fusiformis	620	68.7 ± 0.41

Stock solutions of crude extracts were prepared as 1 mg/ml in methanol. Fifty microlitres of samples were added to 5 ml of 0.004% methanol solution of DPPH·. After 30 min of incubation in the dark at room temperature, the absorbance was read against a blank at 517 nm. IC_{50} values were calculated by linear regression analysis.

Another antioxidant activity screening method, applicable for both lipophilic and hydrophilic antioxidants, i.e., ABTS radical cation decolorization assay, showed results similar to those obtained by the DPPH· assay. The extracts of *M. piperita*, *A. vasica* and *B. compestris* were the most active as they nearly fully scavenged $ABTS^{\cdot+}$. The absorbances after 6 min were 0.014 ± 0.001, 0.050 ± 0.002, 0.032 ± 0.001, 0.094 ± 0.008 and 0.196 ± 0.010, respectively. It was noted that the reaction with $ABTS^{\cdot+}$ was fast and in almost all cases was completed within 1 min. During the remainder of the reaction time, the changes in absorbance were negligible. Thus, *M. piperita A. vasica* and *B. compestris* showed the highest radical-scavenging activities (Fig. 4).

4. Discussion

Several pathways of radioprotection have been suggested for the mechanism of protective action in mammalian cells against the damaging effects of ionizing radiation (Weiss & Landauer, 2000). The mechanisms implicated in the protection of cells by radioprotectors include free radical-scavenging, that protects against reactive oxygen species (ROS) generated by ionizing radiation or chemotherapeutic agents, and hydrogen atom donation to facilitate direct chemical repair at sites of DNA damage (Bump & Brown, 1990; Murray, 1998). ROS, generated by ionizing radiation, are scavenged by radioprotectors before they can interact with biochemical molecules, thus reducing the harmful effects of radiation.

Significant increase in liver reduced glutathione (GSH) contents and decrease in lipid peroxidation (LPO) levels were observed in extract-treated animals. GSH offers protection against oxygen-derived free radical cellular lethality following exposure to ionizing radiation (Biaglow, Varnes, Epp, & Clark, 1987). The radioprotective effects of these plant extracts observed may be attributed to the antioxidant properties.

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidant free radical terminators (Agrawal, 1989). Flavonoids, as one of the most diverse and widespread groups of natural compounds, are probably the most natural phenolics (Shimoi, Masuda, Shen, Furugori, & Kinae, 1996). These compounds possess a wide spectrum of chemical and biological activities

including radical-scavenging properties. A strong relationship between total phenolic content and antioxidant activity in fruits, vegetables and grain products has been reported (Dorman, Kosar, Kahlos, Holm, & Hiltunen, 2003; Velioglu et al., 1998). Both the $ABTS^{\cdot +}$ and $DPPH^{\cdot}$ assays measure the total antioxidant activity of the plant extracts. The results of both the assays are in agreement in that the extracts of *M. piperita*, *A. vasica* and *B. compestris* displayed the highest antioxidant activities. The methanolic solutions of these plant extracts were strong radical-scavengers, indicating that active compounds of different polarity could be present in these plants. The high antioxidant activities of these plants might be due to their flavonoid and phenolic contents.

As shown in Table 1, *A. vasica* contains vesicine, vesicinone, betaine, vitamin C, β-carotene and vasakin (The Wealth of India, 1989). *A. paniculatus* contains proteins, vitamins (C and E), provitamin A and minerals, such as Ca and Fe (Gopalan, Rama Sastri, & Balasubramanian, 1989). *B. compestris* contains allyl isothiocyanates, glucosinolates and indoles (Vorhoeven, Verhagen, Goldbohm, van den Brandt, & Poppel, 1997). *M. piperita* contains antioxidants, such as caffeic acid, rosmarinic acid, eugenol and α-tocopherol (Al-Sereiti, Abu-Amer, & Sen, 1999; Krishnaswamy & Raghuramulu, 1998; Rastogi & Mehrotra, 1991). *S. fusiformis* contains proteins, natural biochelated vitamins (β-carotene) and SOD (Prescott, 1978; Sheshadri, Umesh, & Manoharan, 1991). These chemical constituents in each plant extract may display significant variation in total antioxidant activity. Thus, different radioprotective and antioxidant activities of these plant extracts, observed in the present study, may be ascribed to different chemical constituents.

Shimoi et al. (1996) concluded that plant flavonoids which show antioxidant activity in vitro also function as antioxidants in vivo, and their radioprotective effect may be attributed to their radical-scavenging activity. ROS, generated by ionizing radiation, are scavenged by radioprotectors before they can interact with biochemical molecules, thus reducing the harmful effects of radiation. The antioxidant mechanisms of radioprotection and free radical-scavenging have been attributed to flavonoids, orientin and vicenin (Uma Devi, Ganasounadari, Vrinda, Srinivasan, & Unnikrishnan, 2000). Radioprotective/antioxidative effects of various other natural products have also been reported (Tripathi, Mohan, & Kamat, 2007).

The results of the present investigation suggest that the antioxidant and free radical-scavenging activities of the plant extracts constitute a likely mechanism for their radioprotective effect. The different radioprotective and antioxidant activities of these plant extracts may be assigned to different chemical constituents.

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