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INVESTIGATION OF THE MODULATORY ROLE OF *BACOPA MONNIERRI* (L.) PENNEL. EXTRACTS AGAINST RADIATION-INDUCED OXIDATIVE STRESS AND ANTIOXIDANT STATUS *IN VITRO*

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ABSTRACT

Radiation-induced Reactive Oxygen Species (ROS) can cause extensive oxidative damage to the tissues and protection from such damage is usually provided by endogenous and exogenous antioxidants. The antioxidative phytochemicals present in herbs have received increasing attention for their potential role in prevention of oxidative damage inflicted due to radiation. Phyto-based antioxidants are preferred due to their multiple mechanisms of actions on various target organs. Extracts from *Bacopa monnieri* have long been used in traditional medicine due to their anti-inflammatory, memory booster and anti-depressant properties. The present study was conducted to evaluate the radioprotective ability of aqueous (BMA) and alcoholic (BME) extracts of *Bacopa monnieri.* The extracts were comparatively evaluated to assess their free radical scavenging porperties. The results indicate that the aqueous extract of Bacopa (BMA) has significatn scavenging action and reducing power as compared to alcoholic extract of Bacopa (BME). These extracts have the potential for use for the management of nuclear and radiological emergencies and in the prevention and treatment of diseases in which oxidants and free radicals have been implicated. Further, *in vivo* studies, along with detailed investigation of the protective effects of *Bacopa monnieri* in alleviating radiation effects on the central nervous system (CNS) are continuing

Key words: Reactive oxygen species [ROS], Antioxidants, Polyphenols, *Bacopa monnieri,* nuclear and radiological emergencies, free radical-mediated diseases

INTRODUCTION

Widespread evidences indicate that exposure to ionizing radiation results in significant changes in biological systems caused due to increase in free radicals levels (1). The role of free radical mediated reactions in disease pathology is well established. These reactions are necessary for normal metabolism also, however, they can be detrimental to health after a certain threshold resulting in outcome in the form of various diseases like diabetes, immunosuppression, neurodegenerative problems. Radiation-induced reactive oxygen species (ROS), e.g. superoxide anion and hydroxyl radicals, hydrogen peroxide and singlet oxygen securely coupled at their site of generation or are detoxified by endogenous antioxidant defences so as to preserve optimal cellular function (2). In pathological conditions, however, the detoxifying mechanisms are often inadequate as excessive quantities of ROS can be generated during radiation exposure. This resulting pro-oxidant shift, a process known as oxidative stress, can result in the degradation of cellular components, viz., DNA, carbohydrates, polyunsaturated lipids and proteins, or precipitate

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enzyme inactivation causingirreversible cellular dysfunction and ultimately cell death if the pro-oxidant-antioxidant balance is not restored. (3) They also contribute to the deterioration of foodstuffs (4), cosmetics and pharmaceutical preparations. Antioxidants are compounds that help to inhibit the effect of oxidation reactions caused by radiation-induced free radicals such as singlet oxygen, superoxide, hydroxyl radicals and peroxynitrite by preventing or delaying damage to the system. Their mechanisms of action include scavenging reactive oxygen and nitrogen species, decreasing the localised oxygen concentration, thereby reducing molecular oxygen's oxidation potential, metabolising lipid peroxides to non-radical products and chelating metal ions to prevent the generation of free radicals (5).

Bacopa monnieri Linn. (Scrophulariaceae) is a creeping, glabrous, succulent herb, rooting at nodes, distributed throughout India in all plain districts, ascending to an altitude of 1.320 m . According to Ayurveda, this plant has been traditionally used for treatment of insanity, epilepsy and hysteria and also reported as a sedative and anti-inflammatory, anthelmintic activity (6). The plant has been reported to possess a plethora of constituents like tetracyclic tripterpenoid, saponins, bacosides A and B, hersaponin, alkaloids viz. herpestine and brahmine and flavonoids, all of which possess antioxidant properties (7).

In the present study, we investigated the protective effects of the ethanolic fraction (BME) and aqueous fraction (BMA) of an indigenous Ayurvedic medicinal plant in India viz. *Bacopa monnieri*, against radiation-induced oxidative stress.

MATERIAL AND METHODS

Collection of plant material

Fresh plant material of *Bacopa monnieri* was collected from plants growing in Bhopal, Madhya Pradesh (India). The plant specimen was authenticated by a Botanist. A voucher specimen (Specimen No. INM-2010-1) has been deposited at the Institute of Nuclear Medicine and Allied Sciences (INMAS), Delhi, India.

Preparation of plant extract

The air-dried material was ground into a coarse powder. Five hundred gram of the material was suspended in 2l of distilled water or 2l of 50% ethanol then both fractions are subjected to cold maceration for 24 h. Thereafter, the extracts were filtered through muslin cloth, and the filtrate was evaporated under reduced pressure and vacuum-dried. The yield of the extracts (BME and BMA) was 10 - 15% of the dry weight of the material.

Irradiation

Radiation dose (250Gy) was delivered from a ^{60}Co gamma chamber (Gamma cell 5000, Board of Radiation and Isotope Technology, Mumbai) at a dose rate of 1.3kGy/h. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's Chemical Dosimetry Method

Estimation of total polyphenolic content

The polyphenolic content was estimated using standard method(8) with some modification as described previously (9). Folin-Ciocalteu reagent (FCR) and 20% sodium carbonate solution were made to react with BME and BMA (stock solution = 1 mg/ml). Absorbance was recorded at 700 nm after 30 min, using a μQuant^{TM}microplate spectrophotometer (BioTek Instruments, Inc. Highland Park, Whinooski, VT, USA).

Estimation of total flavonoid content

The total flavonoid content was determined with aluminium chloride ($AlCl_3$) according to the method (10) using quercetin as a standard. BMA & BME (100μl) were added to 300μl of distilled water followed by $NaNO_2$ (300μl, 5%). After 5 min at 25°C, $AlCl_3$ (300μl, 10%) was added. After a further 5 min, the reaction mixture was treated with 200μl of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. The flavonoid content was calculated using quercetin as standard.

DPPH radical scavenging activity

Radical scavenging activity of BMA and BME (25 – 1000 μg/ml) against the stable DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical was determined spectrophotometrically (11). The assay was carried out in triplicate and percent inhibition was calculated using the following formula: % inhibition = [(O.D. control - O.D. sample) / O.D. control) · 100, where O.D. sample is the absorbance of sample and O.D. control is theabsorbance of control at 517 nm.

ABTS radical decolourization assay

ABTS diammonium salt radical cation decolourization test was performed using spectrophotometric method (12) with slight modifications. The principle of the ABTS (2,2-azinobis (3-ethylbenzothiazoline–6–sulfonicacid) diammonium salt cation radical decolourization assay is that the antioxidants react with ABTS

resulting in the decolourization of the ABTS radical in aqueous phase. The ABTS stock reagent mixture (1.8mM) was prepared by diluting it 1:4 and make working solution of 0.45mM was made. The ABTS working solution with equal volume of PBS served as control. The reaction mixtures were incubated at room temperature (28^0C) for 30 min and the absorbance was measured at 734 nm.

ANTIOXIDANT ACTIVITY IN AQUEOUS PHASE

Reducing Power Assay

The reducing power potential of BMA and BME was determined by the method of Oyaizu,1986. Different concentrations (0-2000µl) of silymarin (50µl) were mixed with 200µl of phosphate buffer (0.2M, pH 6.5) and 200µl of potassium ferricyanide (1%), followed by the incubation at 50^0c for 20 minutes in a water bath. After incubation, 200µl of TCA (10%, freshly prepared) was added to terminate the reaction. Then the mixture was centrifuged at 6000 rpm and supernatant was taken out, then 500µl of distilled water and 0.1ml of $FeCl_3$ solution (0.01%) was added. The reaction mixture was left for 10 minutes at room temperature and the absorbance was measured at 700nm. A higher absorbance of the reaction mixture indicated greater reducing power.

ANTIOXIDANT ACTIVITY IN LIPID PHASE

Linoleic acid degradation assay

Varied concentrations of BMA and BME (500 µl) mixed with 5 ml of pre-emulsion (three volumes of linoleic acid + equal volume of Tween-20 in 200 volumes of 30% (v/v) ethanol) and then exposed to 0.25KGy radiation dose followed by incubation at 37°C. The study is performed to evaluate the antioxidant activity of drug in lipid media (both in the presence/absence of radiation stress). Aliquots would be taken for assaying the end products of peroxidation using ammonium thiocyanate assay (Asamariet.al.,1996). The assay mixture contains 2.5ml of 75 % ethanol, 50 µl of $FeCl_2$ (0.1%w/v) along with 50µl of aliquot of the respective sample. Development of color will be measured at 500nm against ethanol in a reference cell.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging potential (site-specific / non-site-specific) was measured using the deoxyribose degradation assay (13). Different concentrations of BMA & BME (5 – 1000 µg/ml) were mixed with reaction mixture as described previously (14) and the percentage inhibition of degradation of deoxyribose or hydroxyl radical scavenging potential was evaluated as described above.

Nitric oxide ion scavenging potential

The nitric oxide ion scavenging potential of BMA and BME (2.5 – 1000 µg/ml) was evaluated using the methodology described by Kim (15). 5 mM aqueous sodium nitroprusside used as a source that generates nitric oxide at physiological pH which interacts with oxygen to generate nitrite ions is made to react with Griess reagent (1% sulfanilamide in 5% phosphoric acid, 0.1% N-1-napthylethylenediamine dihydrochloride in water). The nitric oxide scavenging activity was evaluated as decrease in percent absorbance of the chromogen formed by diazotiation of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine recorded at 546 nm with reference at 630nm.

Antioxidant activity (aqueous phase)

The antioxidant activity in the aqueous phase or reducing power of BMA and BME (2.5 – 500 µg/ml) was determined using the potassium ferricyanide reduction assay (Oyaziu, 1986) as described previously (Chawla *et al*. 2005). The antioxidant activity (aqueous phase) of BMA and BME, ascorbic acid (used as positive control) was compared.

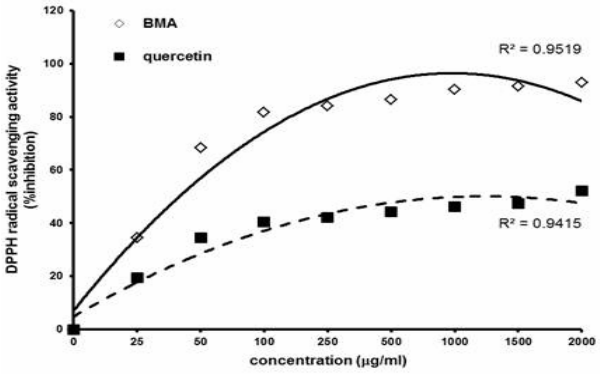
Protection of membrane against radiation damage (membrane protection index)

Soylecithin (phospholipid) and cholesterol (1:1 molar ratio) were suspended in an appropriate amount of chloroform. A thin film was developed by complete evaporation of chloroform in a rotary evaporator (Buchi, New Castle, USA) at 40^oC. The film was subjected to hydration in PBS (0.1 M, pH 7.4) and incubated in a shaking water bath (40^oC) for 4 h. The stock solution was then diluted with PBS (0.1 M, pH 7.4) to the final concentration in terms of phospholipid content (16). Different treated samples *i.e.*, liposome only (untreated), radiation only (0.25 kGy), liposome + BMA and BME and liposome + BMA or BME + 0.25 kGy were evaluated for the levels of malondialdehyde, an end product of membrane degeneration. A radiation dose of 0.25 kGy at a dose rate of 1.3 kGy/h was used and after exposure the samples were incubated for 1h at 37^oC. The equivalent volume of 10 % TCA and 0.5% thiobarbituric acid in 0.025 M NaOH were added. The resultant mixture was then heated at 80ºC for 1 h in water bath. A pink coloured chromogen complex thus formed was read at 535 nm (17).

RESULTS AND DISCUSSION

A plethora of synthetic and herbal extracts have been extensively screened to minimize radiation induced deterministic and stochastic effects developed after radiotherapy and other unplanned radiation exposures (1, 18). In principle, free radicals generated during radiation exposure initiate adverse changes which, in due course, lead to the generation of immediate and late effects depending on the quantum of radicals (18). The phytochemical analysis revealed that BMA and BME have high content of phenolic compounds and flavonoids. BMA phenolics: 31.23 ± 0.32 mg gallic acid/g and flavoids 20.05 ± 0.57 mg quercetin/g, BME phenolics 26.28 ± 0.62 mg gallic acid/g and flavonoids 18.05 ± 0.39 mg quercetin/g. These phenolics and flavonoids are known to have antioxidant activity (19, 20).

Total phenolics constitute groups of compounds acting as primary antioxidants, hence it was reasonable to detect their amount in herbal preparation (20); flavonoids are the most important natural phenolics. They possess a broad spectrum of chemical and biological activities including radical scavenging properties. Antioxidant activity has been reported to be concomitant with the development of reducing power (21). Free radical scavenging potential of BMA and BME was primarily evaluated by estimating electro donation potential. **Figure 1** shows that BMA has significantly higher electron donation potential as compared to BME and is equivalent to ascorbic acid - a natural antioxidant used as standard.



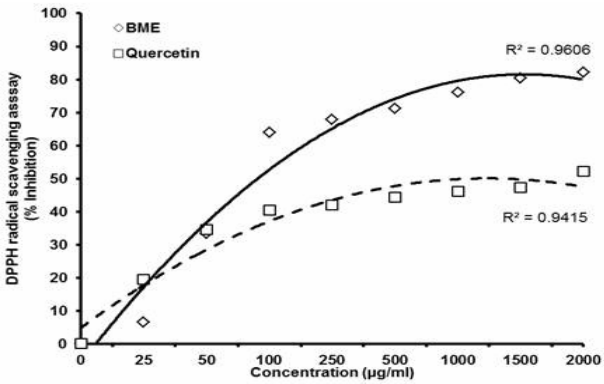
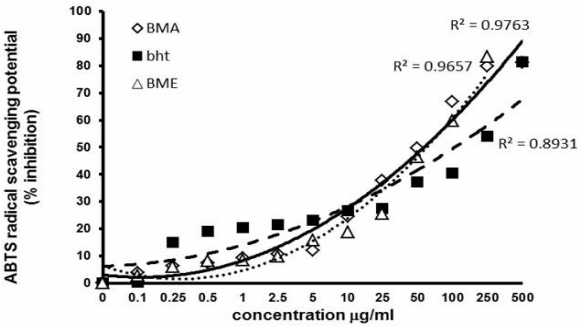


Fig. 1.



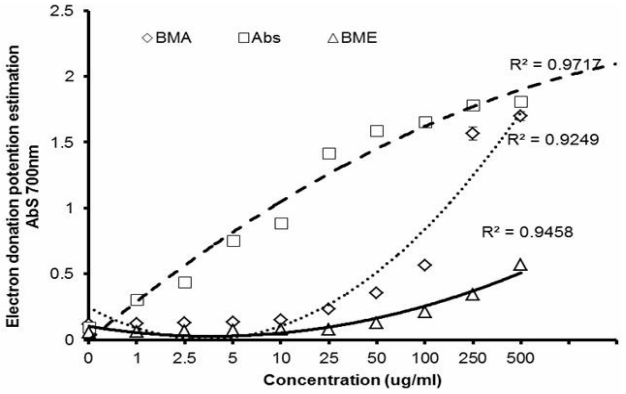


Fig. 2. **Fig. 3.**

Figure 1 and **Figure 7** indicate that *in vitro* DPPH and ABTS radical inhibitory effect was found to be strongest in BMA (82% DPPH and 91% ABTS) with slight difference in case of BME (78% DPPH and 89% ABTS) at a concentration of 1000µg/ml. The results indicate that both BMA and BME have the ability to scavenge primary free radicals. Nitric oxide and superoxide anion cause ischemic renal injury separately and these radicals work together to bring about further damage. The toxicity and damage caused by $NO^{\cdot}$ and $O_2^{\cdot}$ is multiplied as they react to produce reactive peroxynitrite (ONOO), which leads to serous toxic reaction with biomolecules, like protein, lipids, nucleic acid (22). Supression of $NO^{\cdot}$ could be partially attributed to direct $NO^{\cdot}$ scavenging, as both BMA and BME decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. These result showed the hydrophilic antioxidants are abundantly present in BMA which is more than natural antioxidant ascorbic and BME showed different chemical properties.

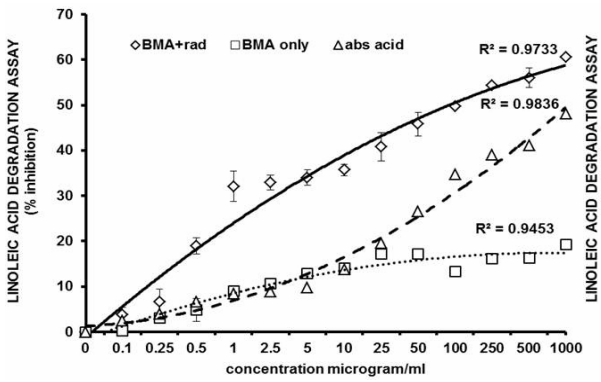


Fig. 4.

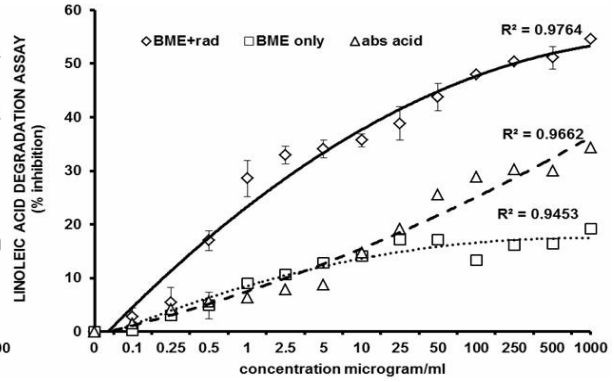


Fig. 5

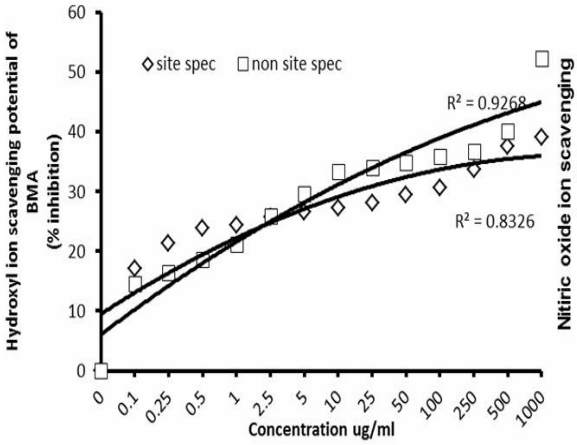


Fig. 6.

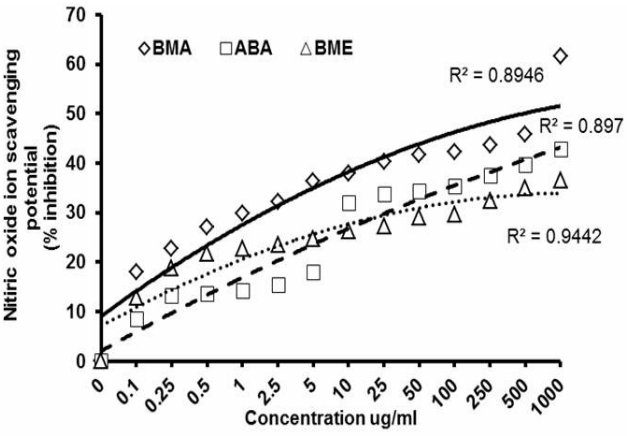


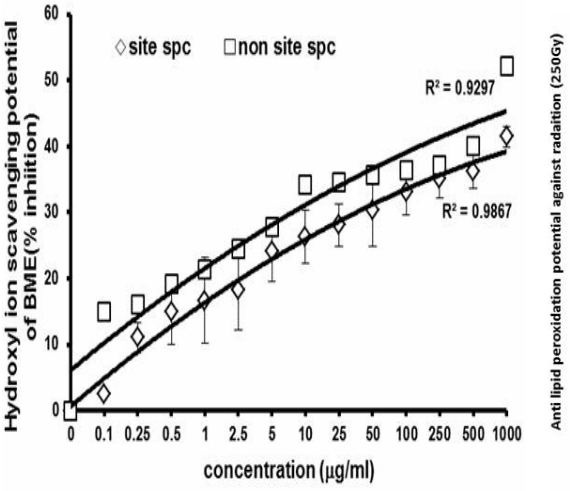
Fig. 7.

As shown in **figure 7** nitric oxide radical scavenging activity was found to increase in a concentration-dependent manner (R^2= 0.896) with maximal activity of 55% in case of BMA and 48% in case of ascorbic acid and 39% in case of BEM at 500µg/ml. On the other hand, a dose-dependent site-specific hydroxyl radical scavenging (R^2=0.9297) was also observed with significant 55.82% and non-site-specific hydroxyl radical scavenging activity (R^2= 0.9894) was observed (48.95%), inhibition in case of BMA and similar effect was observed in case of BME (Figure-6) (46% in site specific and 39% in case of non-site specific) inhibition of the formation of MDA at 1000 µg/ml. Hydrogen peroxide is converted into singlet oxygen and hydroxyl radicals, which then become very powerful oxidising agents. O_2, HO, and H_2O_2 can cross membranes and can oxidise a number of compounds. Significant H_2O_2 scavenging activity indicates the potential of hydrophilic total phenolics. Hydroxyl radical is the most reactive among ROS and it bears the shortest half-life compared with other ROS. Significant hydroxyl radical scavenging activity of BMA has been reported. According to these results, hydrophilic phenolics are predominant in BMA and the radical scavenging properties can be attributed to the presence of such compounds (23, 24).

Linoleic acid degradation assay: the ability of different concentrations of BMA and BME to restrict Fe^{2+} and Cu^{+} induced lipid oxidation is depicted in figure 4. Maximum inhibition was found at a concentration of 1000µg/ml viz., 58% in case of BMA and 55% in case of BME, which is significantly higher as compared with ascorbic acid.

Lipid peroxidation mechanism was studied by using artificial membrane system of liposome prepared from Soylecithin and cholesterol. During lipid peroxidation, low molecular weight end productslike malonaldehyde are formed by oxidation of polyunsaturated fatty acids that react with two molecules of thiobarbituric acid to give a pinkish red chromogen. The inhibitory effect on TBARS formation by BMA was significant when compared with MDA formed in radiation and drug-treated groups of liposome (artificial membrane sytem) (25). Figure 9 shows

the anti lipid peroxidation in membrane, which was evaluated using liposomal system at a radiation dose of 200Gy (dose rate = 1.24 Kgy/hr). Both BMA and BME exhibited significantly ($P<0.05$) higher membrane protection potential at all the concentrations tested (0-1000µg/ml) with maximal activity of 55% and 58% observed at 1mg/ml.



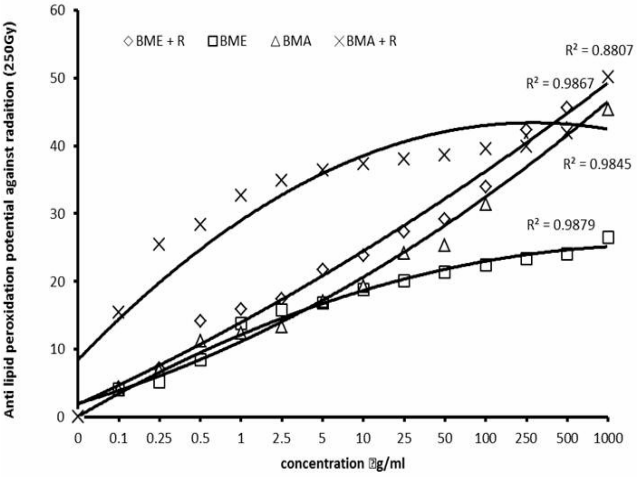


Fig. 8. **Fig. 9.**

CONCLUSION

The present studies on *Bacopa monnieri* extracts to ameliorate radiation-induced oxidative stress suggest the potential role of *Bacopa monnieri* in the management of nuclear and radiological emergencies (NREs) and also in the treatment of the radiation-induced free-radical mediated diseases. The ability of *Bacopa monnieri* to protect the Central Nervous System (CNS) against the deleterious effects of ionizing radiation is an area worth investigating since the taxon is known to improve memory capacity and cognitive ability. Studies are currently also underway to delineate the mechanism of protection of the CNS against ionizing radiation, since it would significantly improve the operational efficiency of first responders and other rescue personnel operating in radiation environment in the aftermath of a nuclear or radiological disaster.

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