Antioxidant activity of brahma rasayana

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Free oxygen radical scavenging activity of brahma rasayana (BR) was studied by in vitro and in vivo models. Addition of aqueous extract of BR was found to scavenge the lipid peroxides already present in rat liver homogenate (IC₅₀ 700µg/ml) and inhibit the lipid peroxide generated by Fe²⁺—ascorbate (IC₅₀ 2600µg/ml) and Fe³⁺—ADP—ascorbate system (IC₅₀ 1200µg/ml). BR was found to scavenge the hydroxyl radical generated by Fenton reaction (IC₅₀ 7400µg/ml) and superoxide generated by photoreduction of riboflavin (IC₅₀ 180µg/ml). BR was also found to inhibit the nitric oxide radical generated in vitro from sodium nitroprusside (IC₅₀ 5.5µg/ml). Oral administration of BR (50mg/dose/animal) was found to inhibit the PMA induced superoxide generation in mice peritoneal macrophages. Oral administration of BR 10 and 50µg/dose/animal was also found to inhibit the nitric production in peritoneal macrophages and percentage inhibition was 25.2% and 37.8% respectively. These results indicate significant antioxidant activity of BR in vitro and in vivo.

Reactive oxygen species (ROS) and free radicals which are formed in the body as a consequence of normal metabolic reactions, exposure to ionising radiation and by the influence of many xenobiotics are indicated in the causation of several diseases. Antioxidants, which can scavenge free radicals have an important role in biological system and their use is implicated in the prevention of cancer, heart diseases, aging etc. Human body has an inherent mechanism to reduce the free radicals induced injury by enzymatic or non-enzymatic methods. When the normal level of antioxidant defense mechanism is not sufficient for the eradication of free radicals induced injury, administration of antioxidants have a protective role to play. Several antioxidants of plant origin are experimentally proved and used as effective protective agents against oxidative stress.

Indigenous medicines in India have several preparations which are implicated in preventive medicine. Rasayanas are a group of non-toxic polyherbal drug preparation, which are immunostimulatory and thereby prevent the causation of the diseases. We have earlier reported that rasayanas could reduce the side effects of radiation and chemotherapy and could stimulate the immune cells. It was also found to significantly reduce the methylcholanthrene induced sarcoma in mice and transplanted tumors in animals. In the present study we have evaluated the potency of brahma rasayana as an antioxidant.

Materials and Methods

Brahma rasayana (BR) was purchased from Vaidyaratnam Oushadhasala, Ollur, India (composition of BR and mode of preparation is given in Table 1). Nitroblue tetrazolium (NBT), adenosine diphosphate (ADP) and N(1-naphthylethylene diamine dihydrochloride were purchased from Sisco Research Laboratories Pvt. Ltd, Bombay. 2-deoxy-D-ribose was obtained from Sigma Chemical Co. St. Louis M.O. Phorbol 12-myristate-13-acetate (PMA) was a gift from Dr. Allan Conney, USA. Tissue culture medium RPMI-1640 was obtained from Hi-media Laboratories, Bombay. Foetal calf serum (FCS) was obtained from Biological Industries, Kibbutz Beit Haemek, Israel. All other chemicals and reagents used were of analytical grade. Aqueous extract of BR was prepared by freshly stirring BR in water for 1hr and centrifuged and the supernatant was used for the assay.

Inbred strains of Balb/c mice(20-25g, 4-5weeks old) were purchased from National Centre for Laboratory Animal Sciences, Hyderabad. They were housed in ventilated cages in air controlled rooms and fed with normal mouse chow (Lipton, India) and water ad libitum.

Determination of antioxidant activity of brahma rasayana in vitro

Effect of BR on inhibition of lipid peroxide formation induced by Fe²⁺-ascorbate system—
Reaction mixture (0.5 ml) containing 25% rat liver homogenate (0.1 ml) w/v in tris-HCl buffer (40 mM, pH 7.0), potassium chloride (30 mM), ferrous iron (0.16 mM) and ascorbic acid (0.06 mM) was incubated for 1 hr at 37°C in presence and absence of different concentrations of BR. The lipid peroxide formed was measured by the method of Ohkawa et al. For this 0.4 ml of reaction mixture was treated with sodium dodecyl sulphate (SDS-0.2 ml, 8.1%), thiobarbituric acid (TBA-1.5 ml, 0.8%) and acetic acid (1.5 ml, 2.5% of pH 3.5). The mixture (4 ml) was then kept in a water bath at 95°C for 1 hr. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and shaken vigorously. After centrifugation, the chromophore was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the result of control and test compounds.

Effect of BR on inhibition of lipid peroxide formation induced by Fe^{3+}-ADP-ascorbate system—

The incubation mixture contained 10% rat liver homogenate (0.5 ml), ferric iron (0.1 mM), ADP (1.7 mM), ascorbic acid (0.06 mM) and the final volume was made up to 1.5 ml with KCl (0.15 M). Mixture was incubated for 20 min at 37°C in presence and absence of different concentration of BR. After incubation 0.6 ml of reaction mixture was taken and inhibition of lipid peroxidation was determined by estimation of thiobarbituric acid reacting substances (TBARS) as described by Ohkawa et al. as given above.

Effect of BR on hydroxyl radical scavenging activity—Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compounds for hydroxyl radical generated by Fe^{2+}-ascorbate-EDTA-H_{2}O_{2} (Fenton reaction). The hydroxyl radicals attack deoxyribose that eventually result in TBARS formation.

The reaction mixture contained deoxyribose (2.8 mM), FeCl_{3} (0.1 mM), EDTA (0.1 mM),

<table>
<thead>
<tr>
<th>Items</th>
<th>Composition of brahma rasayana (BR) and mode of preparation</th>
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<tbody>
<tr>
<td>1. Emblica officinalis (20%)</td>
<td>19. Desmostachya bipinnata (0.4%)</td>
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<tr>
<td>2. Terminalia chebula (6.67%)</td>
<td>20. Saccharum officinarum (0.4%)</td>
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<tr>
<td>3. Uraria pico (0.4%)</td>
<td>21. Oryza malampurcis (0.4%)</td>
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<tr>
<td>4. Desmodium gangeticum (0.4%)</td>
<td>22. Cinnamomum iners (0.16%)</td>
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<tr>
<td>5. Guinean arbores (0.4%)</td>
<td>23. Elettaria cardamomum (0.16%)</td>
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<tr>
<td>6. Solanum nigrum (0.4%)</td>
<td>24. Cypers rotundus (0.16%)</td>
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<tr>
<td>7. Tribulus terrestris (0.4%)</td>
<td>25. Curcuma longa (0.16%)</td>
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<tr>
<td>8. Aegle marmelos (0.4%)</td>
<td>26. Piper longum (0.16%)</td>
</tr>
<tr>
<td>9. Premna tomentosa (0.4%)</td>
<td>27. Aquilaria agallocha (0.16%)</td>
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<tr>
<td>10. Stereospermum suaveolens (0.4%)</td>
<td>28. Santalum album (0.16%)</td>
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<tr>
<td>11. Sida rhombifolia (0.4%)</td>
<td>29. Centella asiatica (0.16%)</td>
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<tr>
<td>12. Boerhaavia diffusa (0.4%)</td>
<td>30. Mesua ferrea (0.16%)</td>
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<tr>
<td>13. Ricinus communis (0.4%)</td>
<td>31. Clitoria ternatea (0.16%)</td>
</tr>
<tr>
<td>14. Vigna vexillata (0.4%)</td>
<td>32. Acorns calamus (0.16%)</td>
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<tr>
<td>15. Phaseolus adzunatus (0.4%)</td>
<td>33. Scirpus crosus (0.16%)</td>
</tr>
<tr>
<td>16. Asparagus racemosus (0.4%)</td>
<td>34. Glycyrrhiza glabra (0.16%)</td>
</tr>
<tr>
<td>17. Holostemma annulare (0.4%)</td>
<td>35. Emblica ribes (0.16%)</td>
</tr>
<tr>
<td>18. Leptadenia reticulata (0.4%)</td>
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</table>

Items 1-21 were made into small pieces and washed it well. 16 part of water was added to the total quantity of drugs and allowed to boil to get one fourth of original volume. The seeds from fruit of Emblica officinalis and Terminalia chebula were removed and the pulp was roasted well by adding sufficient quantity of ghee and sesame oil. Then sufficient quantity of sugar was added to the above decoction to get a paste like formation. The plants 22-35 were cleaned and dried well and was made into fine powder and this powder was mixed with above paste and stirred well. When the preparation comes to normal temperature, sufficient quantity of honey was added, mixed well and stored at room temperature.
elicited in all animals by injecting a 5% solution of culture supernatant of peritoneal macrophages were compounds.

0.1% concentrations of water extract of BR was incubated macrophages-Nitric oxide generation-Aqueous sodium caesinate (0.2ml) intraperitoneally. Five days female Balb/c mice (20-25g, 4-5 weeks old) were used for the experiment. Peritoneal macrophages were elicited in all animals by injecting a single dose of BR (50mg/animal for 5 days, po). The reaction mixture contained EDTA (6.6mM) containing 3μg NaCN, riboflavin (2μM), NBT (50μM), various concentrations of BR and phosphate buffer (67mM, p H 7.8) in a final volume of 3ml. The tubes were uniformly illuminated with an incandescent lamp for 15min. and the optical density was measured at 530nm before and after the illumination. The percentage inhibition of superoxide generation was measured by comparing the absorbance values of control and those of test compounds.

Effect of BR on inhibition of nitric oxide radical generation—Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ions and which was measured colorimetrically.

3ml of reaction mixture containing sodium nitroprusside (10mM) in phosphate buffered saline (PBS) and various concentrations of water extract of BR was incubated at 25°C for 150min. Controls without test compound was kept in an identical manner. After incubation 0.5ml of reaction mixture was removed and 0.5ml of Griess reagent (1% sulfanilamide, 2% H3PO4 and 0.1% naphthylethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546nm. The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

Effect of BR on nitrite production in peritoneal macrophages—Nitric oxide(NO) produced by macrophages quickly reacts with oxygen to produce nitrite ions. The nitrite concentration in the cell free culture supernatant of peritoneal macrophages were measured spectrophotometrically. Inbred strains of female Balb/c mice (20-25g, 4-5 weeks old) were used for the experiment. Peritoneal macrophages were elicited in all animals by injecting a 5% solution of sodium caesinate (0.2ml) intraperitoneally. Five days after injection, macrophages were harvested by peritoneal lavage using sterile phosphate buffered saline (PBS). Cells were washed, centrifuged and 1x10⁶ cells were cultured in 96 well flat bottom titre plates in RPMI-1640 supplemented with 5% FCS for 24hr at 37°C in presence and absence of various concentrations of methanolic extract of BR. Culture plates were centrifuged and medium from five wells of treated and untreated wells were pooled. 1ml of medium (in triplicate) was mixed with sulphosalicylic acid (0.1ml, 70%), vortexed, centrifuged and the supernatant was mixed with 5% NH4Cl solution (0.8ml) containing sodium borate buffer pH9, NaOH (0.2ml, 10%) and Griess reagent (0.5ml). The reaction mixture was incubated at 60°C for 10min and at 4°C for 5min. The optical density was measured at 546nm, which is a measure of the amount of nitrite ions produced by the peritoneal macrophages. In all the experiments the nitrite content in the well containing medium without cells alone and with drug were determined as controls and substraction from the corresponding normal values.

Determination of antioxidant activity of brahma rasayana in vivo

Effect of BR on nitrite production in peritoneal macrophages—Inbred strains of female Balb/c mice (20-25g, 4-5weeks old) were used for this study. They were divided into three groups (3animals/group). Peritoneal macrophages were elicited in all animals by injecting a 5% solution of sodium caesinate (0.2ml) intraperitoneally. Group I served as untreated control. Group II was treated with daily single dose of BR (10mg/animal for 5 days, po). GroupIII was treated with daily single dose of BR (50mg/animal, po) for 5 days. Peritoneal macrophages were harvested on fifth day after drug administration and cells were cultured (1x10⁶ cells/well/0.25ml) in 96 well flat titre plates in RPMI-1640 supplemented with 5% FCS for 24hr. The concentration of nitrite ions formed in the cell free culture supernatant was determined by the method of Ding et al. as given above.

Effect of BR on PMA induced superoxide generation in peritoneal macrophages—Inbred strains of female Balb/c mice (20-25g, 4-5 weeks old) were divided into three groups (3animals/group). All the animals were injected(ip) with 0.2ml of sodium caesinate (5%) to elicit macrophages. Group I kept as untreated control. Group II was treated with daily single dose of BR (50mg/animal for 5 days, po).
Group III was treated with single dose of BR (50mg/animal on day 1). Peritoneal macrophages elicited by sodium caesinate were activated in vivo on 5th day by injecting PMA (100 ng/animal, ip). Three hour after activation, peritoneal macrophages were harvested. The effect of the test compounds on the inhibition of superoxide generation in the macrophages were measured by inhibition in the reduction of NBT to formazan by the method of Dwivedi et al\(^\text{17}\). The reaction mixture (1.0ml) contained in a ratio of 6:2:1 of NBT (0.2% in PBS, pH 7.4), dextrose (5%) and Hank's balanced salt solution (pH 7.4) was mixed with 0.5ml of peritoneal macrophages (1x10\(^6\) cells/ml) and incubated for 45minutes at room temperature. The mixture was centrifuged and cell pellet was boiled with 2ml of pyridine for 10minute. The optical density of the supernatant was measured at 515 nm, which is a measure of the superoxides produced by the activated peritoneal macrophages. The percentage inhibition was determined by comparing the absorbance values of untreated and treated animals.

**Results**

**In vitro study**

*Inhibition of lipid peroxidation by BR* — Addition of BR was found to inhibit peroxides generated by Fe\(^{2+}\)-ascorbate and Fe\(^{3+}\)-ADP-ascorbate in rat liver homogenate (Fig. 1A). The concentration of BR needed for 50% inhibition was found to be 2600 \(\mu\)g/ml, 1200 \(\mu\)g/ml respectively. Addition of BR was also found to scavenge lipid peroxides already present and the concentration needed for 50% inhibition was found to be 700 \(\mu\)g/ml (Fig 1A). Time course of lipid peroxidation induced by BR\(^{2+}\)-ascorbate as shown by TBARS in the absence and presence of BR (5mg/ml) is shown in Fig. 1B.

*Inhibition of hydroxyl radical by BR* — Degradation of deoxyribose by hydroxyl radical generated from Fe\(^{3+}\)-ascorbate-EDTA-H\(_2\)O\(_2\) system was found to be inhibited by BR. Concentration of BR needed for 50% inhibition was found to be 7400 \(\mu\)g/ml (Fig. 2A).

*Inhibition of superoxide radical by BR* — BR was found to scavenge the superoxide radical generated by photo reduction of riboflavin. Concentration of BR needed for 50% inhibition was found to be 180 \(\mu\)g/ml (Fig 2B).

*Inhibition of nitric oxide radical by BR* — Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by BR.

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**Table 2:** Effect of brahmagrāsana (BR) on nitrite production and PMA induced superoxide generation in mice peritoneal macrophages (in vivo).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrite production</th>
<th>Superoxide generation</th>
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</thead>
<tbody>
<tr>
<td>BR (5x10mg)</td>
<td>25.2 ± 2.5</td>
<td>-</td>
</tr>
<tr>
<td>BR (5x50mg)</td>
<td>37.8 ± 9.6</td>
<td>44.4 ± 4.2</td>
</tr>
<tr>
<td>BR (1x50mg)</td>
<td>-</td>
<td>21.0 ± 2.8</td>
</tr>
</tbody>
</table>

Percentage inhibition was calculated from control peritoneal macrophages on day 5.
Concentration of BR needed for 50% inhibition was found to be 5.5 µg/ml (Fig 2C). Methanolic extract of BR was found to scavenge the nitric oxide radical produced by peritoneal macrophages. Concentration needed for 50% inhibition was found to be 4µg/ml.

**In vivo study**

**Inhibition of nitric oxide radical production in peritoneal macrophages by BR** — *In vivo* treatment with BR was found to reduce the nitrite ions produced by macrophages (Table 2). Animals were treated with five doses of BR (10 and 50mg/dose/animal, po) was found to produce lesser nitrite ions; the percent inhibition observed was found to be 25.2 and 37.8 respectively.

**Inhibition of PMA induced superoxide generation in peritoneal macrophages by BR** — BR was found to scavenge the superoxide generation in the macrophages (Table 2). Percent inhibition of superoxide generated by macrophages from animals treated with 5 daily doses of BR was 44.4% and from animals treated with one dose of BR inhibition was 21%.

**Discussion**

Even though herbal drugs and products derived from plants are still being used in medical practice, the mechanism of action of many herbal drugs are unknown. Active principle in these drugs are seldom identified. In the case of polyherbal preparations this problem is more acute as many drugs may synergistically or antagonistically act together to give the final activity of the preparation. Herbal preparations in Indian medicines (Ayurveda) have withstood the test of time and are being practiced in India along with the modern medicine. Herbal preparations in India are especially useful against autoimmune diseases and many are immunorestorative.

Brahma rasayana (BR) is a polyherbal preparations with nearly 60 plant extracts of various concentrations being used as a medicine to combat immunodeficiency. A systemic administration of BR was found to improve the cell mediated humoral immunity in mice. Conventional therapy of cancer always produce side effect and the most important being myelosuppression, which at times produce life threatening consequences. Brahma rasayana has been shown to protect the tissues from the undesirable side effects of radiation and was found to reduce myelosuppression in cancer patients undergoing chemotherapy.

The present study indicate that BR could inhibit the oxygen radicals as seen from the inhibition of lipid peroxidation, scavenging of superoxide, hydroxyl radical and nitric oxide radical *in vitro* and *in vivo*. It was also found to scavenge the lipid peroxide already present in the tissue. BR was also found to scavenge both superoxide radical and nitrite ions (*in vitro* and *in vivo*) produced in mice peritoneal macrophages. Present studies indicates that BR could reduce the oxygen radicals and subsequently reduce the harmful effects produced by the oxygen free radicals mediated injuries.

**References**


