Chapter 2
Antioxidant Activity of Brahma Rasayana, In vitro and In vivo
2.1. Introduction

Free Radicals (FR) are highly reactive chemical species produced as a result of incomplete reduction of oxygen during the course of various biochemical reaction. FRs are believed to play a fundamental role in many physiological reactions such as respiration at the level of mitochondria, transport of oxygen by haemoglobin, for the activity of cytochrome p450 enzymes etc. FRs also play an important role in phagocytosis or the removal of the “irritant” that is causing inflammation (Sinclair et al., 1991). Living organisms are equipped with FR scavengers. FR scavengers are enzyme systems and non enzyme systems. These FR scavengers which are called as antioxidants intercept the FR mediated reactions that cause tissue damage and thereby protect the cause tissue from the oxidative damage of FRs.

Indigenous medicines in India have several preparations which are implicated in preventive medicines. Brahma Rasayana (BR) is a group of non-toxic polyherbal drug preparation, which are immunostimulatory and thereby prevent the causation of diseases (Singh, 1990). One of the main ingredients in BR, Emblica officinalis has high antioxidant potency (Jose & Kuttan, 1995). In the present chapter we evaluated the in vitro and in vivo antioxidant activity of BR.

2.2. Materials And Methods

a. Drug

Brahma Rasayana (BR) was purchased from Vaidyaratnam Oushadhasala, Ollur, Kerala, India. For in vitro studies aqueous and methanolic extracts were used. Aqueous extract was prepared by freshly stirring BR in water for one hour, filtered and centrifuged and supernatant was used for assay. Methanolic extract of BR was prepared by dissolving 10 gm of material in 100 ml of 70 % methanol twice, by stirring overnight, filtered and centrifuged. The supernatant was collected and evaporated to dryness. The dried methanolic extract (70 % yield) was thoroughly mixed with double distilled water and was used for the assay. Water suspension of BR was used for all in vivo experiments.
b. Animals

Inbred strains of Bal b/c mice (4-5 weeks old, 20-25 g) were purchased from National Center for Laboratory Animal Sciences, Hyderabad. They were housed in well ventilated cages in air controlled room and fed with normal mouse chow (Saifeeds, Bangalore) and water ad libitum.

2.2.1. Determination of antioxidant activity of Brahma Rasayana (BR) *in vitro*

2.2.1.a. Effect of BR on inhibition of lipid peroxide formation induced by ferrous Fe$^{2+}$ - ascorbate system

Lipid peroxidation (LP) was stimulated by ferrous (Fe$^{2+}$) or ferric (Fe$^{3+}$) ions was determined by thiobarbituric acid (TBA) method of Ohkawa *et al.* (1979).

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

2.2.1. b. Effect of BR on inhibition of lipid peroxide formation induced by ferric Fe$^{3+}$- adenosine diphosphate (ADP) - ascorbate system

Lipid peroxidation was also induced by iron – ADP complex in presence of ascorbic acid. Iron – ADP chelate promotes redistribution of iron from water into the lipid phase and also helps to keep the iron in solution, thereby rendering its redox active.
The incubation mixture contained 10% rat liver homogenate (0.5mL), ferric iron (0.1mM), ADP(1.7mM), ascorbic acid(0.5mM) and the final volume was made upto 1.5mL with KCl (0.15M). Mixture was incubated for 20min. at 37°C in presence and absence of different concentration of BR. After incubation 0.6mL 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, 1979 as given above.

2.2.1. c. Hydroxyl radical scavenging activity of BR

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_2O_2 (Fenton reaction,Halliwell,1990). The hydroxyl radicals attack deoxyribose and set off a series of reactions that eventually result in TBARS formation.

The reaction mixture contained deoxyribose(2.8mM), FeCl_3(0.1mM), EDTA(0.1mM), H_2O_2(1mM), ascorbic acid(0.1mM), KH_2PO_4-KOH buffer(20mM, pH 7.4) and various concentrations of BR in a final volume of 1mL. The reaction mixture was incubated for 1hr at 37°C. Deoxyribose degradation was measured as TBARS by the method of Ohkawa et al. The percentage inhibition was determined by comparing the results of test compounds and control.

2.2.1. d. Effect of BR on inhibition of superoxide radical generation

Superoxide scavenging was determined by the NBT reduction method of McCord and Fridovich,1969. The reaction mixture contained EDTA(6.6mM) containing 3µg NaCN, riboflavin(2µM), NBT(50µM), various concentrations of aqueous extract of BR or methanolic extract of BR prepared as given above and phosphate buffer (67mM, pH 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.
**2.2.1.e. 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 (Green et al 1982; Marcocci et al 1994a). 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 150 minutes. 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% sulphanilamide, 2% H₂PO₄ 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.

**2.2.1.f. Effect of BR on inhibition of nitrite production in mice 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 using a spectrophotometric assay of Ding et al (1988), based on Griess reaction. 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 1 mL of untreated and treated culture supernatant of each concentrations (in triplicate) were 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 10 minute and at 4°C for 5 minute. The optical density was measured at 546 nm, 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 subtracted from the corresponding normal values.

2.2.2. Determination of antioxidant activity of Brahma Rasayana *in vivo*

2.2.2.a. Effect of BR on nitrite production in mice peritoneal macrophages

Inbred strains of female Balb/c mice (20-25 g, 4-5 weeks old) were used for this study. They were divided into three groups (3 animals/group). Peritoneal macrophages were elicited in all animals by injecting a 5% solution of sodium caesinate (0.2 mL) intraperitoneally. Group I served as untreated control. Group II was treated with daily single dose of BR (10 mg/animal for 5 days, po). Group III was treated with daily single dose of BR (50 mg/animal, po) for 5 days. Peritoneal macrophages were harvested on fifth day after drug administration and cells were cultured (1 x 10⁶ cells/well/0.25 mL) in 96 well flat bottom titre plates in RPMI-1640 supplemented with 5% FCS for 24 hr. The concentration of nitrite ions formed in the cell free culture supernatant was determined by the method of Ding et al., 1988 as given above.

2.2.2. b. Effect of BR on PMA induced superoxide generation in mice peritoneal macrophages

Inbred strains of female Balb/c mice (20-25 g, 4-5 weeks old) were divided into three groups (3 animals/group). All the animals were injected (ip) with 0.2 mL of sodium caesinate (5%) to elicit macrophages. Group I kept as untreated control. Group II was treated with daily single dose of BR (50 mg/animal for 5 days, po). Group III was treated with single dose of BR (50 mg/animal on day 1). Peritoneal macrophages elicited by sodium caesinate were activated *in vivo* on 5th day by injecting PMA (100 mg/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 (1992). The reaction mixture (1.0mL) contained NBT (0.2% in PBS, pH 7.4), dextrose (5%) and Hank's balanced salt solution (pH 7.4) in a ratio of 6:2:1 was mixed with 0.5mL of peritoneal macrophages (1x10^6 cells/mL) and incubated for 45 minutes at room temperature. The mixture was centrifuged and cell pellet was boiled with 2mL of pyridine for 10 minutes. The optical density of the supernatant was measured at 515nm, 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.

2.2.3. Antiinflammatory Activity of Brahma Rasayana (BR)

Antiinflammatory activity was determined by the carrageenan induced mice paw oedema method of Langrange et al (1974). Inbred strains of female Bal b/c mice weighing 20 - 25 g were divided into control and treated groups (3 animals/group). Administration of BR (50 mg/dose/mouse) was done orally everyday for 5 days. Paw oedema was induced by injecting carrageenan (200 μg/20 μL) into the subplanter region of left paw on day 5. The thickness of paw was measured before and after injection and continued up to 4 hours (3 min interval) and at 24 hour. The inhibition of paw oedema was calculated by comparing the difference in paw thickness of control and treated group.

2.3. Statistical Analysis

Data was expressed as mean ± standard deviation (SD). Significance levels for comparison of differences were determined using student’s t test.

2.4. Results

2.4.1. Antioxidant activity of Brahma Rasayana (BR) In vitro

2.4.1.a. Inhibition of lipid peroxidation induced by Fe^{2+}-ascorbate system by BR

Figure 6 represents the effect of BR on lipid peroxidation induced by Fe^{2+}-ascorbate system. The addition of water suspension of BR was found to inhibit lipid peroxides generation after induction of Fe^{2+}-ascorbate in rat liver.
homogenate. The concentration of BR needed for 50% inhibition was found to 2600 µg/mL.

Time course of lipid peroxidation induced by Fe$^{2+}$- ascorbate system as seen by TBARS formation in the absence and presence of BR (5 µg/mL) is shown in figure 7. Initial absorbance value of without BR treated (control) group was 0.42 at 532 nm and absorbance value of BR treated group was 0.19. In BR treated group the absorbance value was gradually decreased and at 60-90 min and the value was 0.09. But in control group, the absorbance value was increased after 15 min and at 90 min the value reaches to 0.77. This indicated that BR could not only inhibit lipid peroxidation but can reduce the preformed lipid peroxides in the homogenate.

2.4.1.b. Inhibition of lipid peroxidation induced by Fe$^{3+}$- ADP- ascorbate system by BR

Aqueous suspension of BR was also found to inhibit the lipid peroxide present in the rat liver homogenate and inhibit the generation of lipid peroxide after induction of Fe$^{3+}$- ADP- ascorbate in rat liver homogenate (Fig.6). Concentration of BR needed for 50% inhibition of lipid peroxides already present in rat liver homogenate was found to be 700 µg/mL. In the case of Fe$^{3+}$- ADP- ascorbate system, concentration of BR needed for 50% inhibition was found to be 1200 µg/mL.
Figure 6
Effect of Brahma Rasayana (BR) on Lipid Peroxidation

- Ferrous (Fe2+) - ascorbate
- Ferric (Fe3+)-ADP-ascorbate
- Without any induction
Figure 7
Time Course of Inhibition of Brahma Rasayana (BR) on Lipid Peroxide Formation Induced by Fe$^{2+}$ - Ascorbate System
2.4.1.c. Inhibition of hydroxyl radical by BR

Figure 8 represents the effect of BR on hydroxyl radical generation by Fenton reaction. 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 7400µg/mL.

2.4.1.d. Inhibition of superoxide radical by BR

Addition of BR was found to scavenge the superoxide radical generated by photoreduction of riboflavin. Concentration of BR needed for 50% inhibition was found to be 180µg/mL (Fig.9). When methanolic extract of BR dissolved in water was used, concentration needed for 50% inhibition was found to be 4 µg/mL (Fig.10) indicating that original BR may have several insoluble matter which increased the concentration needed to scavenge superoxide radicals.

2.4.1.e. Inhibition of nitric oxide radical by BR

Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by BR. Concentration of BR needed for 50% inhibition was found to be 5.5µg/mL (Fig.11).

2.4.1.f. Inhibition of nitrite production in mice peritoneal macrophages by BR

The nitrite concentration of cell free culture supernatant of mice peritoneal macrophages were also found to be reduced by treatment with methanolic extract of BR (24 hrs). Concentration needed for 50% inhibition was found to be 4µg/mL (Fig.12).

2.4.2. Antioxidant activity of Brahma Rasayana (BR) in vivo

2.4.2.a. Inhibition of nitrite production in mice peritoneal macrophages by BR

Table 6 represent the effect of BR on inhibition of nitrite production in mice peritoneal macrophage. Oral administration of BR (10mg/dose/mouse for 5 days) was found to scavenge the nitrite ions; the percent inhibition observed was found to be 25.2. In the case of mice treated with BR 50 mg per dose per mouse for 5 days, percent inhibition of nitrite ions was found to be 37.8.
Figure 8
Effect of Brahma Rasayana (BR) on Inhibition of Hydroxyl Radical Generation Induced by Fenton Reaction
Figure 9
Effect of Brahma Rasayana (BR) on Inhibition of Superoxide Radical Generation

![Graph showing the effect of Brahma Rasayana (BR) on inhibition of superoxide radical generation. The x-axis represents concentration of BR (µg/mL), and the y-axis represents percent inhibition.]
Figure 10
Effect of Methanolic Extract of Brahma Rasayana (BR) on Inhibition of Superoxide Radical Generation
Figure 11
Effect of Brahma Rasayana (BR) on Inhibition of Nitric Oxide Radical Generation

![Graph showing the effect of Brahma Rasayana (BR) on inhibition of Nitric Oxide Radical Generation. The x-axis represents the concentration of BR (μg/mL) ranging from 0 to 18, and the y-axis represents percent inhibition ranging from 0 to 60. The graph shows an increase in percent inhibition as the concentration of BR increases, with a plateau at higher concentrations.](image-url)
Figure 12
Effect of Methanolic Extract of Brahma Rasayana (BR) on Inhibition of Nitrite Production in Mice Peritoneal Macrophages
Table-6
Effect of Brahma Rasayana (BR) on nitrite production in mice peritoneal macrophages.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without drug)</td>
<td>--</td>
</tr>
<tr>
<td>BR treated (5 x 10 mg)</td>
<td>25.2 ± 2.5</td>
</tr>
<tr>
<td>BR treated (5 x 50 mg)</td>
<td>37.8 ± 9.6</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triplicates.

Percent inhibition was calculated from untreated control.
2.4.2.b. Inhibition of PMA induced superoxide generation in mice peritoneal macrophages by BR

BR was found to scavenge the superoxide generation in vivo in the macrophages as seen from the inhibition in the reduction of NBT to formazan (Table 7). Percent inhibition of superoxide generated in macrophages from mice treated orally with BR (50 mg/dose/mouse for 5 days) was found to be 44.4. In animals treated with single dose of BR (50 mg/mouse), the percent inhibition was 21.

2.4.3. Antiinflammatory activity of Brahma Rasayana (BR)

Development of paw oedema was measured at various intervals in both control and treated group after carageenan injection. The thickness of mice paw oedema was found to be increased from 60 min and reached a maximum at 210 min in control group. The difference in the thickness in the paw oedema was found to be 0.07 cm at 210 min in control group. In the treated group paw oedema developed only after 180 min and reached a maximum of 0.01 cm at 240 min. After 24 hour, paw oedema was decreased considerably to attain the normal status in both groups (Fig.13).

2.5. Discussion

Present chapter reveals the in vitro and in vivo antioxidant property of BR. BR was found to inhibit lipid peroxidation induced by Fe²⁺-ascorbate or Fe³⁺-ADP-ascorbate in rat liver homogenate. It was also found to scavenge the lipid peroxide already present in rat liver tissue. Apart from inhibiting lipid peroxidation, BR was also capable of scavenging free radicals such as hydroxyl radical, superoxide radical and nitric oxide radical. When compared to the aqueous extract of BR which needed higher concentration for the inhibition of superoxide radical (IC₅₀ 180µg/mL), concentration needed by an alcoholic extract of BR was significantly low (IC₅₀ 4µg/mL). This result indicate that original BR may have several insoluble matter. BR was also found to scavenge superoxide radical and nitrite ions (in vivo) produced in mice peritoneal macrophages. Moreover BR was found to be anti-inflammatory activity in mice against
**Table 7**

Effect of Brahma Rasayana (BR) on PMA induced superoxide generation (*in vivo*) in mice peritoneal macrophages.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without drug)</td>
<td>--</td>
</tr>
<tr>
<td>BR treated (5 x 10 mg)</td>
<td>21.0 ± 2.8</td>
</tr>
<tr>
<td>BR treated (5 x 50 mg)</td>
<td>44.4 ± 4.2</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triplicates.

Percent inhibition was calculated from superoxide generated in mice peritoneal macrophages, which were activated with PMA (100 ng/mouse) on day 5.
Figure 13
Effect of Brahma Rasayana (BR) on Antiinflammatory Activity
carageenan induced mice paw oedema. In addition to anti-inflammatory screening, carageenan induced paw oedema can be used as simple, economical and routine animal model for evaluation of pain at the site of inflammation without any injury or damage to the inflamed paw (Winter et al, 1962). Present studies clearly reveals that BR could reduce the oxygen free radical mediated injuries (in vivo).

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 drug 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 Ayurveda have withstood the test of time and is being practiced in India along with the modern medicine. Herbal preparations in India are especially useful against of auto immune diseases and many are immunorestorative. BR is a poly herbal preparation with nearly 35 plant extracts of various concentrations being used as a medicine to combat immunodeficiency. A systemic study of BR has shown that it could improve the cell mediated and humoral immunity in mice. (Praveen Kumar et al, 1999). Conventional therapy of cancer always produce side effect and the most important being myelosuppression, which at times produce life threatening consequences. Radioprotectors can protect the tissues from the undesirable side effects of radiation induced free oxygen radicals, especially hydroxyl radicals which are produced by the interaction of gamma rays with water.