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Comparative Evaluation of Immune Protective Effect of Melatonin and *Boerhaavia Diffusa* in Lymphoid Tissues of Park's Strain Mice Against Antibiotic Induced Oxidative Stress

Running Title: Immune Protective Roles of Melatonin and *Boerhaavia diffusa* Against Oxidative Stress

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Abstract: ***Boerhaavia diffusa, a native plant of India, is an antioxidant. Aims were to compare, physiological efficacy of root and stem extracts of B. diffusa either alone or in combination with melatonin on improvement of immune and antioxidant status of mice under normal or antibiotic induced stress. Park's strain mice were grouped into control (Con), melatonin (Mel), ethanolic root (BRE) and stem (BSE) extract treated alone and in combination with melatonin (BRE+Mel and BSE+Mel). Further, mice were grouped into control (Con), ampicillin treated (AMP), and BRE, BSE and Mel pre-treated groups receiving AMP (AMP+BSE, AMP+BRE, AMP+Mel). Blood and spleens of all the groups were collected and processed for Total Leukocyte Count (TLC), % Stimulation Ratio (%SR) of splenocytes, estimations of Total Anti-oxidant Status (TAS), TBARS, Super Oxide Dismutase (SOD) and apoptosis analysis (AO-EB Assay). Significant increase in TLC, % SR of splenocytes, TAS level, SOD and significant decrease in apoptotic index of splenocytes and TBARS level in BSE+Mel, BRE+Mel, AMP+Mel than Con, Mel, BRE, BSE, AMP, AMP+BSE and AMP+BRE treatments were noted. Conclusively, melatonin in combination with root and stem extracts of B. diffusa improved immune and antioxidant status. Melatonin pre-treatment alone ameliorates ampicillin induced immune compromised condition and oxidative stress.***

Keywords: Antibiotic, Antioxidant, Boerhaavia, Melatonin, Mice.

1. Introduction

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are the main components of free radical biology. They may play some important role in regulation of physiological homeostasis in positive manner like regulation of smooth muscle tone, platelet activation and vascular cell signalling [1]. It is also well reported that ROS and RNS may also cause cancer and different chronic diseased conditions of the body [2]. Thus, in most of the cases of applied biology oxidative stress has become the most potent player to make to threat to body physiology in general and immunity in particular. Most of the oxidative stress caused in the body is mainly due to oxygen free radicals generated from different metabolic pathways going on inside the cell.

Antibiotics are an important class of medications that can save lives. The purpose of antibiotics is presumably to help the immune response. Some of the antibiotics (like adriamycin and daunorubicin) may also cause oxidative damage [3] however, such kind of reports are scanty and never tested for their oxidant roles in lymphoid tissues taking immunity a major concern in general.

Neurohormone, melatonin is multi-potent in nature as it maintains seasonality both in terms of reproductions [4] and other physiological functions is also having seasonal modulation particularly immune functions in different animal models [5]-[6]. In most of the cases it is reported to be immune enhancer in different rodents [7] even in humans [8]-[9]. It is also reported to ameliorate the stress induced immune suppression in rodents [10] even after irradiation [11] directly or via receptor mediated pathways [12]. It is reported to be more potent free radical scavenger and anti-oxidant than Vitamin-C [13]. But, till date no one has reported the free radical scavenging efficacy and potency in ameliorating immune suppressed conditions induced by antibiotic treatment.

Boerhaavia diffusa (Family: Nyctaginaceae) is plant which is extremely rich in different retinoids, flavins (Punarnavin) [14], boeravione A, Punarnavosides, vitamin A, B, C, E, lignans and sterols. In most of the countries of northern and southern hemispheres from the time unknown this is regarded as a potent and aboriginal herbal medicine in treatment of nephritic syndrome, urinary-tract and urinary bladder disorders, convulsions, abdominal pain, splenomegaly, and tissue inflammation in general [15]. The methanolic extract of the whole plant has been shown to have anti-estrogenic [16], hepatoprotective [17], immunomodulatory [18] and anti-inflammatory [19] activities. The leaf extract of the plant was reported to have anti-diabetic activity [20]. The root extract of this plant is having a very rich variety of flavins (boeravinones, like boeravinone A – J) [21]-[27]. Thus, as a basic property of any flavin it may be speculated that the root extract would be having very high free radical scavenging capacity and anti-oxidant effect. But, till date no one reported about the stress ameliorating activity of the root and stem extract under antibiotic treatment.

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Thus, the first aim of the present study was to compare the physiological efficacy of BRE, BSE and Mel either alone or in combination (like BRE + Mel and BSE + Mel) in improvement of general immune and anti-oxidant status of body. The second aim was to note the physiological efficiency of BRE, BSE and Mel in ameliorating the immune compromised condition and oxidative stress management induced due to ampicillin treatment which is a very potent anti-biotic for regular use.

2. Materials and Methods

2.1 Animals and Maintenance

Forty five (45) five weeks old male Park strain albino mice were used for the study. All male mice (*Peromyscus leucopus*), were obtained from Varanasi (latitude 25°89' N; longitude 83°19' E) from the colony of the mice maintained in our animal room. Mice weighing from 10 to 15 g was housed in polyvinyl cages in animal room with temperature maintained at 25 ± 2°C and photoperiod of 12 hour light and 12 hour dark cycle with lights on from 06:00 to 18:00 h. They were fed with standard commercial mice feed and water *ad libitum*. All the experiments were conducted in accordance with institutional practice and within the framework of revised animals (Scientific Procedures) Act of 2002 of the Govt. of India on Animal welfare.

2.2 Chemicals

All the chemicals were purchased from Sigma Aldrich Chemicals, St. Louis, MO, USA. The TUNEL assay kit for DNA fragmentation and Caspase-3 assay kits were purchased from R & D Systems, MN, USA.

2.3 Treatments

The ethanolic extract of roots and stems of *B. diffusa* were prepared and the minimal effective dose of root extract (25 mg/kg body wt.) and stem extract (20 mg/kg body wt.) was selected after standardization experiments. The dose of ampicillin was prepared in 0.9% normal saline and the minimal effective dose was 500mg/kg body wt. which was selected after standardization experiment as suggested by D. Chandra (13). The minimal effective dose of melatonin was standardized (0.25 mg/kg body wt.) in our laboratory and reported elsewhere [28].

2.4 Experimental protocol

1) **Experiment I. Effect of melatonin and *B. diffusa* root extract in lymphatic tissue of mice**

After acclimatization for two weeks to laboratory conditions, mice were divided into four groups having 5 mice each for following:

a) **Group A:** Control, treated with normal saline (0.9% NaCl) for 28 days.
b) **Group B:** Melatonin treatment (i. m.; 0.25 mg/kg body wt.) daily for 4 weeks, (total 28 days).
c) **Group C:** Oral administration of *B. diffusa* root extract (BRE=25 mg/kg body wt.) daily for 4 weeks in the morning hrs.
d) **Group D:** Oral administration of *B. diffusa* root extract (BRE=25 mg/kg body wt.) in the morning hrs followed by melatonin treatment (0.25 mg/kg body wt.) in the evening hrs for 4 weeks.

2) **Experiment II. Effect of melatonin and B. diffusa stem extract in lymphatic tissue of mice**

After acclimatization for two weeks to laboratory conditions, mice were divided into four groups having 5 mice each for following:

a) **Group A:** Control, treated with normal saline treatment (0.9% NaCl) for 28 days.
b) **Group B:** Melatonin treatment (0.25 mg/kg body wt.) daily for 4 weeks, (total 28 days).
c) **Group C:** Oral administration of B. diffusa stem extract (BRE=25 mg/kg body wt.) daily for 4 weeks.
d) **Group D:** Oral administration of B. diffusa stem extract (BRE=25 mg/kg body wt.) in the morning hrs. followed by melatonin treatment (0.25 mg/kg body wt.) in the evening hrs. for 4 weeks.

3) **Experiment III. Comparative effect of melatonin and B. diffusa root and stem extracts in lymphatic tissue (spleen) of mice against antibiotic (ampicillin) induced stress.**

After acclimatization for two weeks to laboratory conditions, mice were divided into five groups having 5 mice in each, respectively for following treatments:

a) **Group A:** Control, treated with normal saline (0.9% NaCl) for 28 days.
b) **Group B:** Mice were administered Ampicillin (i.m.; AMP= 500 mg/kg body wt.) daily for 15 days to saline treated animals.
c) **Group C:** Ampicillin (500 mg/kg body wt.) treatment to melatonin pre-treated mice (0.25mg/kg body wt.) daily for 2 weeks, i.e. 15 days.
d) **Group D:** Ampicillin (500 mg/kg body wt.) treatment to B. diffusa root extract pre-treated mice (BRE=25 mg/kg body wt.) daily for 2 weeks, i.e. 15 days.
e) **Group E:** Ampicillin (500 mg/kg body wt.) treatment to B. diffusa stem extract pre-treated mice (BSE=25 mg/kg body wt.) daily for 2 weeks, i.e. 15 days.

2.5 Collection of Samples:

All the animals were sacrificed (anesthetized to death) after 24 hrs of treatment and blood was collected from heart and was processed for TLC. Spleen tissues were collected on chilled PBS and were immediately processed for splenocyte culture. The blood and tissues were further processed for analyses of different immunological, apoptotic and oxidative stress parameters.

2.6 Parameters Studied

2.6.1 Immunological Parameters:

a) **Total Leukocyte Count (TLC):**
Peripheral blood was collected directly from the heart under anesthesia using a heparinised leukocyte pipette. Samples were used to evaluate the total leukocyte count (TLC) (number mm^{-3}) in Neubauer's counting chamber (Paul Marienfeld GmBH & Co. KG, Lauda-

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Ko¨nigshofen, Germany) under a Nikon microscope (Nikon, Kawasaki, Japan).

b) **% SR of splenocytes by MTT Assay:**
Cell-mediated immune function was assessed by measuring splenocyte proliferation in response to the T-cell specific mitogen, Con-A, using a colorimetric assay based on the reduction of 3-(4,5- Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) following protocol of Mossman [29]. In short spleens of mice were removed under sterile condition and a single-cell suspension was prepared with culture medium (RPMI-1640 supplemented with penicillin, streptomycin, L-glutamine, 2-mercaptoethanol and heat-inactivated fetal calf serum). Viability was determined with a hemocytometer and trypan blue exclusion method. Viable cells (which exceeded 95%) were adjusted to 1×10^7 cells/mL. 100µL aliquots of each cell suspension were added to the wells of sterile flat-bottom 96-well culture plates. Concanavalin-A (Sigma–Aldrich, St. Louis, USA) was added to the culture medium at the concentration of 5 µg/mL. Plates were incubated at 37^0C with 5% CO2 for 69 h prior to addition of 10 µL of MTT (SRL, Bombay, India; 5 mg/mL in phosphate-buffered saline) per well. Acidified propanol (0.04 mol/L HCl in isopropanol) was added and the optical density (OD) was determined with a microplate reader (ELx-800, Biotek Instruments, Winooski VT, USA) equipped with a 570 nm wavelength filter. Mean OD values for each set of triplicates were used in subsequent statistical analyses. Blastogenic response was calculated as %SR representing the ratio of absorbance of mitogen stimulated cultures to control cultures.

2.6.2 Apoptotic parameters:

Morphological Analysis of Apoptotic Cells:

Cell death was microscopically analyzed following Acridine Orange–Ethidium Bromide (AO–EB) double staining method as described elsewhere [11]. Acridine orange (AO) stained both apoptotic and viable cells emitting green fluorescence when bound to double strand DNA and red fluorescence when bound to single stranded RNA. Viable cells fluoresced uniform bright green nuclei with organized structure while early apoptotic cells showed intact membranes and chromatin condensation as bright green patches or fragments and late apoptotic cells showed orange to red nuclei with condensed or fragmented chromatin. Ethidium bromide (EB) is taken up only by dead cells and emitted red fluorescence when intercalated with DNA. Necrotic cells showed a uniformly orange to red nuclei with organized structure. AO–EB dye of volume 0.01 mL (1×) was admixed gently with 0.2 mL of the diluted sample (1×10^6 cells/mL in PBS). A drop of this mixture was placed underneath cover slip on a clean slide and cells were observed immediately under fluorescence microscope (Leitz MPV3, Wetzlar, Hesse, Germany) at 440–520 nm at 920× magnification. A minimum of 200 cells was counted in every sample.

2.6.3 Oxidative stress parameters:

a) **Lipid peroxidation assay by TBARS level estimation:**
All spleens were excised and weighed for preparing 10% tissue homogenates in 20 mM Tris-HCl buffer (pH 7.4). To prevent new lipid peroxidation during homogenisation, butylated hydroxytoluene (BHT, 2.8 mM) was added to the samples. Homogenates were centrifuged at 3000 g for 15 min at 4°C. The supernatant was subjected to thiobarbituric acid (TBA) assay by reacting an aliquot with 8.1% sodium dodecyl sulphate (SDS), 20% cold acetic acid, 0.8% TBA and distilled water in a boiling water bath for 1 h to yield a chromogenic product. The reaction mixture was immediately cooled in running water and vigorously shaken with n-butanol and pyridine reagent (15:1); the sample was then centrifuged for 10 min at 1500 g to extract thiobarbituric acid-reactive substances (TBARS). The absorbance of the upper phase was read at 534 nm at 25°C [30]. The product concentration was expressed as TBARS level in nmol/g tissue weight using 1,1,3,3-tetraethoxy propane (TEP) as the source for the standard curve.

b) **Superoxide dismutase (SOD) activity assay:**
Superoxide dismutase (SOD) activity measurement was based on the ability of the enzyme to inhibit nitrite formation by superoxide radicals. Tissues were washed with 0.9% NaCl and 10% tissue homogenates were prepared in 150 mM phosphate-buffered saline (PBS, pH 7.4) and centrifuged for 45 min at 12 000 g at 4°C. To 0.5 ml of homogenate, 1.4 ml of reaction mixture (50 mM phosphate buffer, 20 mM L-methionine, 1% Triton X-100, 10 mM hydroxylamine hydrochloride, 50 mM EDTA) was added followed by pre-incubation at 37°C for 5 min. After adding 0.8 ml of riboflavin to all mixtures, including a control containing only buffer, samples were exposed to a 24 W fluorescent lamp fitted in an aluminium foil coated wooden box. This exposure resulted in the photogeneration of superoxide anion upon illumination of riboflavin. After 10 min, 1 ml of freshly prepared Greiss reagent (1% sulphanilamide in 5% orthophosphoric acid, 0.1% N-1-naphthylethylenediamine dihydrochloride in distilled water) was added. The absorbance of diazo dye, formed as a function of nitrite concentration, was read at 543 nm at 25°C [31]. One unit (U) of SOD activity (defined as the amount of SOD inhibiting 50% nitrite formation under assay conditions) was expressed as SOD activity in U/g tissue weight.

c) **Total Antioxidant Activity Assay:**
ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cat-ion decolourisation test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. The radical scavenging activity of antioxidants for ABTS radical cat-ions was measured according to the method of Re et al. [32]. A stock solution of ABTS radical cat-ions was prepared one day before the assay by mixing 5 ml of 7 mM ABTS with 1 ml of 14.7 mM potassium persulfate, and kept in dark at room temperature for 16 h. The stock solution of ABTS radical cat-ions was then diluted with distilled water till its absorbance reached 0.7 at 734 nm. ABTS radical cat-ion was generated by oxidation of ABTS with potassium persulfate. 2.95 ml of ABTS radical cat-ion solution were mixed with 50 µl of 10% tissue (spleen) homogenate and the decrease in absorbance was monitored for 60 min at 10 min intervals at 734 nm.

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2.7 Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) of at least five animals per group. Data comparisons were statistically analyzed using one way ANOVA followed by Student's Newman–Keul's multiple range tests. Differences were considered to be statistically significant when $p<0.05$ [33].

3. Results

3.1 Experiment I. Effect of melatonin and *B. diffusa* root and stem extracts in peripheral blood and lymphatic tissue (spleen) of mice

3.1.1 TLC

Both Melatonin and root extract pre-administration for 28 days significantly increased the leukocyte count by 1.2 fold ($p<0.01$) and 1.14 fold ($p<0.05$) in group B and group C, respectively when compared with controls. Melatonin plus root extract group D significantly ($p<0.01$) increased TLC by 1.44 fold when compared with control group A and by 1.26 fold when compared with root extract group C. Stem extract group C showed non-significant increase in TLC of peripheral blood but significant ($p<0.01$) increase of 1.18 fold was noted in melatonin plus stem extract group D when compared with control group (Fig 1a).

3.1.2 Apoptotic Index

Root extract group C showed non-significant reduction in apoptotic index but melatonin administration in root extract group D significantly ($p<0.01$) lowered the percentage of apoptotic cells by 2.15 fold when compared with control group A and by 1.85 fold when compared with root extract group C. Melatonin plus stem extract group D showed significant ($p<0.01$) reduction in apoptotic index of splenocytes by 1.75 fold when compared with control group A and by 1.62 fold when compared with stem extract group C (Fig 1b).

3.1.3 TBARS level

Both Melatonin and root extract treatments for 28 days significantly lowered TBARS level production by 1.73 fold ($p<0.01$) and 1.28 fold ($p<0.05$) in group B and group C, respectively when compared with controls. Melatonin plus root extract group D significantly lowered TBARS level by 1.84 fold ($p<0.01$) when compared with control group A and by 1.44 fold ($p<0.05$) when compared with root extract group C. Melatonin plus stem extract group D significantly lowered TBARS level by 1.34 fold ($p<0.05$) when compared with control group A whereas no reduction was observed in stem extract group C (Fig 1c).

3.1.4 SOD Activity

SOD activity was found to be significantly increased in group B by 1.53 fold ($p<0.01$) and in group C by 1.3 fold ($p<0.05$) when compared with control group A. Melatonin administration in root extract group D significantly ($p<0.01$) increased SOD activity by 1.67 fold when compared with control group A and by 1.28 fold when compared with root extract group C. Melatonin administration in stem extract group D significantly increased SOD activity by 1.49 fold ($p<0.01$) when compared with control group A and by 1.28 fold ($p<0.05$) when compared with stem extract group C (Fig 1d).

3.1.5 Total antioxidant activity (TAA)- TAA which was measured in terms of ABTS radical cat-ion scavenging/inhibition/decolonization percentage was found to be significantly ($p<0.01$) increased in group B and group C by 1.6 fold and 1.54 fold, respectively when compared with control group A. We observed significant ($p<0.05$) increase in TAA of melatonin plus root extract group D by 1.87 fold ($p<0.01$) when compared with control and by 1.2 fold ($p<0.05$) when compared with root extract group C. TAA was found to be significantly ($p<0.01$) increased in group C by 1.42 fold when compared with control group A. We observed significant ($p<0.05$) increase in TAA of melatonin plus stem extract group D by 1.68 fold ($p<0.01$) when compared with control and by 1.2 fold ($p<0.05$) when compared with stem extract group C (Fig 1e).

3.1.6 Blastogenic Response: Splenocytes proliferation following T-cell mitogen challenge measured as stimulation index of cells were significantly ($p<0.01$) increased by melatonin pre-treatment by 1.67 folds when compared with control whereas non-significant increase was observed in root extract group. But melatonin plus root extract group showed significant increase in stimulation index of splenocytes by 1.70 fold ($p<0.01$) when compared with control and 1.47 fold for splenocytes ($p<0.05$) when compared with root extract group C. Melatonin plus stem extract group showed significant increase in stimulation index of splenocytes by 1.6 fold ($p<0.01$) when compared with control and 1.49 fold ($p<0.05$) when compared with root extract group C. (Fig 1f).

3.2 Experiment II. Comparative effect of melatonin and *B. diffusa* root and stem extracts in peripheral blood and lymphatic tissue (spleen) of mice against antibiotic (ampicillin) induced stress.

3.2.1 TLC

Ampicillin induced significant ($p<0.01$) reduction in circulating leukocyte by 1.43 fold when compared with control. Melatonin, root extract and stem extract when given separately to ampicillin pre-treated animals for 15 days significantly ($p<0.01$) restored leukocyte count in groups C, D and E by 1.4 fold, 1.35 fold, 1.26 fold, respectively when compared with ampicillin pre-treated group only though stem extract plus ampicillin group showed 1.14 fold ($p<0.05$) reduction in TLC when compared with control group A (Fig. 2a).

3.2.2 Apoptotic Index

Ampicillin induced significant ($p<0.01$) increase in apoptotic index of splenocytes (Fig 2b) by 1.9 fold when compared with controls. Melatonin, root extract and stem extract pre-treated animals, upon ampicillin treatment for 15 days, showed significant ($p<0.01$) reduction in apoptotic index in splenocytes by 1.26 fold, 1.45 fold, 1.43 fold of groups C, D and E, respectively when compared with ampicillin treated group only.

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3.2.3 TBARS level

Ampicillin induced significant ($p<0.01$) increase in TBARS level in splenic tissue by 2.84 fold when compared with control. Melatonin, root extract and stem extract pre-treated animals, upon ampicillin treatment showed significantly reduced TBARS level in splenic tissue by 1.54 fold ($p<0.01$), 1.3 fold ($p<0.01$), 1.2 fold ($p<0.01$) of groups C, D and E, respectively when compared with ampicillin treated group only (Fig 2c).

3.2.4 SOD activity

SOD activity got significantly decreased in spleen (Fig 2d) of ampicillin treated group B by 1.72 fold ($p<0.01$) when compared with control group A and got significantly ($p<0.05$) restored to control level in group C by 1.64 fold, group D by 1.56 fold, group E by 1.44 fold when compared with ampicillin treated group B.

5.2.5 TAA

TAA got significantly decreased in ampicillin group B by 1.42 fold ($p<0.01$, Fig 2e) in spleen tissue when compared with control group A. We observed significant ($p<0.01$) increase of 1.44 fold in TAA of spleen with melatonin treatment in group C and non-significant increase in rest groups D and E when compared with ampicillin treated group only.

3.2.6 Blastogenic response

Ampicillin alone (group A) induced 3.1 fold ($p<0.01$) decrease in T-lymphocytes stimulation index of spleen. We observed significant ($p<0.01$) decrease in splenocyte proliferation by 1.26 fold, 1.64 fold, 1.82 folds respectively in melatonin plus ampicillin group C, root extract plus ampicillin group D, stem extract plus ampicillin group E, respectively when compared with control group A. Significant increase in stimulation index of splenocytes by 2.03 fold in melatonin plus ampicillin group C, 1.95 fold in root extract plus ampicillin group D, 1.84 fold in stem extract plus ampicillin group E when compared with ampicillin group only (Fig 2f).

4. Discussions

Boerhaavia diffusa or "Punarnava" is a very important Indigenous plant. From the time unknown the Indian rural people used this medicinal herb for its multifarious functions and actions in human physiology. A number of different flavonoids and retinoids were reported from different vegetative parts of this plant [34]. The most important products of the plants are boeraviene B and boeraviene E which have been reported recently [35], retinoids and alkaloids like "Punarnabins". Reports are available considering the role of this plant in protecting the animals from prostatic hyperplasia [36]. After the qualification, separation and purification of different flavones and alkaloids, they were quantified properly [37]. The therapeutic aspect of this plant product (mostly by ethanolic extract) was tested angiotensin II induced hypertrophy in H9c2 cardiomyoblast cell lines via modulating oxidative stress and down regulating NF-κβ and TGF-β1 [38]. Further, some recent studies have also suggested the anti-inflammatory [37], anti-angiogenic [14], anti-oxidant [39], anti-metastatic [40], anti-estrogenic [16] effects of *B. diffusa* either of root or of stem extracts under differential clinical [41] or under *in vitro* conditions [42, 43].

Neurohormone, melatonin is mostly reported to be pro-inflammatory [9] and anti-stress hormone [10]. Melatonin may exert its function either by receptor dependent or by receptor independent pathways [5]. But, till date there is no single report available stating the comparative effects of *B. diffusa* and melatonin on modulation of immunity and stress management induced due to anti-biotic treatment.

Thus, in the first part of our study the root and stem extracts of *B. diffusa* was studied either alone or in combination with melatonin in immune modulation and stress management of lymphoid organs. Our results suggested, upon BRE + Mel and BSE + Mel treatment that a significant increase in peripheral immune parameters and %SR of splenocytes than control, BRE, BSE and melatonin treatment alone. Our results may suggest that BRE and BSE along with melatonin treatment are having some synergistic immune modulator effects. Further, in BSE + Mel and BRE + Mel treated groups we have found significantly increased expressions of free radical scavenging enzyme expression (i.e. SOD), TAS and significantly decrease in apoptotic parameters and TBARS levels suggesting that melatonin and *B. diffusa* root and stem extracts cumulatively are capable enough to modulate the general body homeostasis than Mel, BRE and BSE alone. However, these results needed further support to demonstrate the roles of BRE, BSE and Mel in induced stress. We used ampicillin, as a molecule for induction of oxidative stress and immune compromised condition of body. The important observation and result was that ampicillin was acting as an anti-inflammatory and oxidative agent regulating the body homeostasis in a negative manner. Thus, this study is of its first kind where any anti-biotic is going to be reported for some detrimental effect apart from its normal clinical significance. Ampicillin caused immune compromised condition of the body by causing oxidative stress and also by provoking apoptosis in immune competent cells. Our results in this context were noteworthy as we studied the effect of melatonin, BRE and BSE in counteracting the stress caused by ampicillin. Our *in vivo* and *in vitro* results in combination with Mel + AMP, BSE + AMP and BRE + AMP were significant in comparison to control and AMP treatment alone as they have ameliorated the immune compromised condition and oxidative damage caused due to AMP treatment. Further, melatonin pre-treatment groups (in AMP + Mel groups) were non-significantly higher than those of BSE + Mel and BRE + Mel groups. Thus, we may suggest that melatonin treatment was sufficient enough to ameliorate the immune compromised condition caused due to ampicillin treatment.

5. Conclusion

The therapeutic efficiencies of *B. diffusa* root extract (BRE) and stem extracts (BSE) was the first aim of the study in terms of immune modulation and counteract the oxidative stress. We found that combined treatment of BSE + Mel and BRE + Mel were more effective than melatonin, BSE and BRE alone. These results were further supported and extrapolated in the ampicillin induced immune compromised condition caused due to oxidative stress. It was found that Mel + AMP treated groups were competent enough to ameliorate the oxidative

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stress caused due to AMP treatment and of AMR + BSE and AMP + BRE groups.

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7. Future Scope of the study

B. diffusa is known for its many biological roles from BRE and BSE. However, for the first time we explained that it may ameliorate the antibiotic induced immune compromised condition caused due to oxidative stress. Hence, pretreatment of *B. diffusa* root and stem extracts during antibiotic treatment may improve oxidative stress and immune compromised condition. However, a combination of *B. diffusa* root and stem extracts with melatonin will provide a better remedy from side effects of antibiotics.

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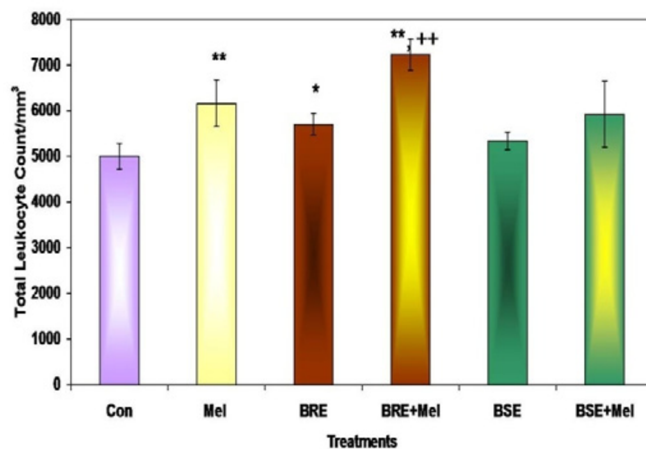

Figure 1 (a)

Effect of Melatonin and *B. diffusa* root and stem extract on TLC of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control, Mel= Melatonin, BRE= *B. diffusa* Root Extract, BRE+Mel= Root Extract+Melatonin, BSE= *B. diffusa* Stem Extract, BSE+Mel= Stem Extract+Melatonin *p<0.05, **p<0.01 *vs* Con; ++p<0.01 *vs* BRE.

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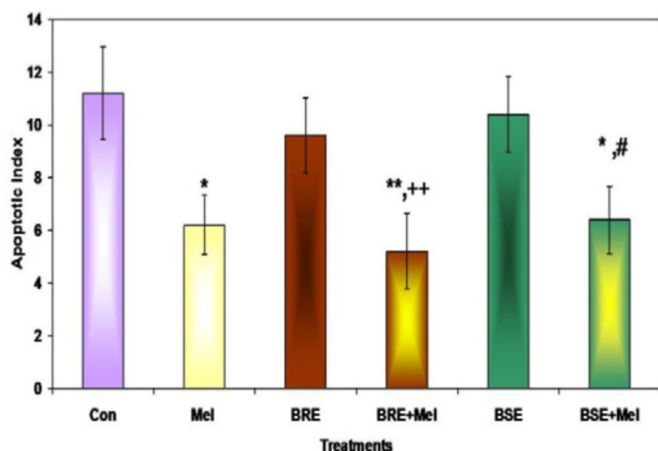

Figure 1 (b)

Effect of Melatonin and *B. diffusa* root and stem extract on apoptotic index of spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control, Mel= Melatonin, BRE= *B. diffusa* Root Extract, BRE+Mel= Root Extract+Melatonin, BSE= *B. diffusa* Stem Extract, BSE+Mel= Stem Extract+Melatonin. *p<0.05, **p<0.01 *vs* Con; ++p<0.01 *vs* BRE; #p<0.05 vs BSE.

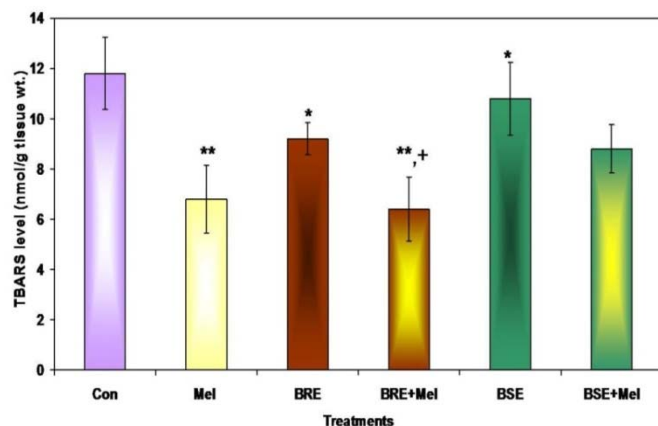

Figure 1 (c)

Effect of Melatonin and *B. diffusa* root and stem extract on TBARS level on spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control, Mel= Melatonin, BRE= *B. diffusa* Root Extract, BRE+Mel= Root Extract+Melatonin, BSE= *B. diffusa* Stem Extract, BSE+Mel= Stem Extract+Melatonin. *p<0.05, **p<0.01 *vs* Con; +p<0.05 *vs* BRE

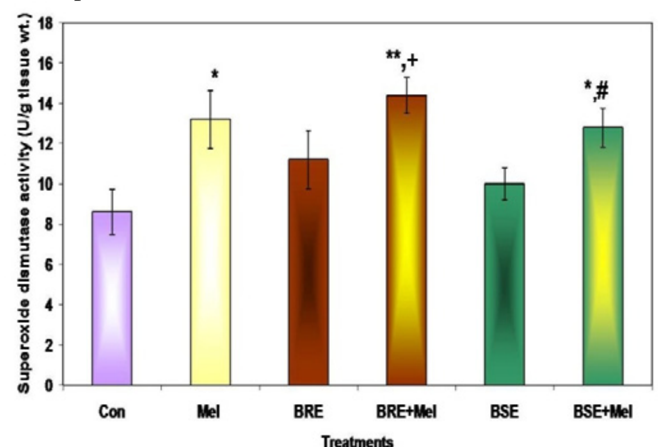

Figure 1 (d)

Effect of Melatonin and *B. diffusa* root and stem extract on Super Oxide Dismutase activity on spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control, Mel= Melatonin, BRE= *B. diffusa* Root Extract, BRE+Mel= Root Extract+Melatonin, BSE= *B. diffusa* Stem Extract, BSE+Mel= Stem Extract+Melatonin. *p<0.05, **p<0.01 *vs* Con; +p<0.05 vs BRE; #p<0.05 *vs* BSE.

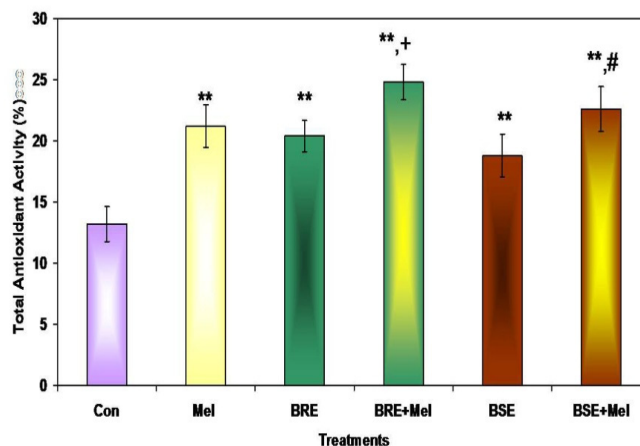

Figure 1 (e)

Effect of Melatonin and *B. diffusa* root and stem extract on Total Antioxidant Activity of spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control, Mel= Melatonin, BRE= *B. diffusa* Root Extract, BRE+Mel= Root Extract+Melatonin, BSE= *B. diffusa* Stem Extract, BSE+Mel= Stem Extract+Melatonin. **p<0.01 *vs* Con; +p<0.05 *vs* BRE; #p<0.05 *vs* BSE.

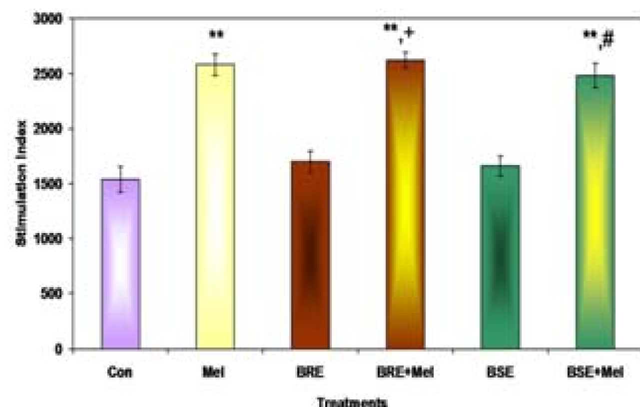

Figure 1 (f)

Effect of Melatonin and *B. diffusa* root and stem extract on stimulation index of splenocytes of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control, Mel= Melatonin, BRE= *B. diffusa* Root Extract, BRE+Mel= Root Extract+Melatonin, BSE= *B. diffusa* Stem Extract, BSE+Mel= Stem Extract+Melatonin. **p<0.01 *vs* Con; +p<0.05 *vs* BRE; #p<0.05 vs BSE.

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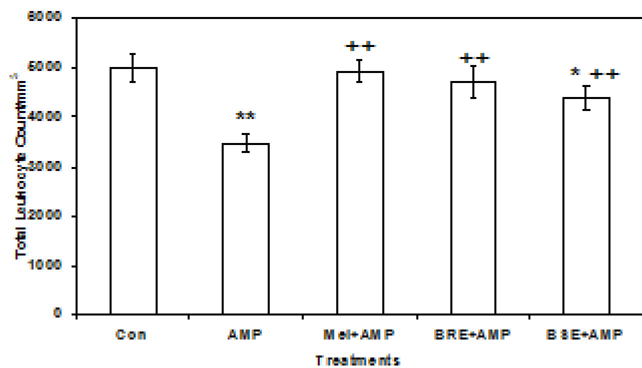

Figure 2 (a)

Comparative effect of melatonin, *B. diffusa* root and stem extracts against ampicillin induced TLC of peripheral blood of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control; AMP= Ampicillin; Mel+AMP= Melatonin to ampicillin pre-treated; BRE+AMP= Root extract to ampicillin pre-treated; BSE+AMP= Stem extract to Ampicillin pre-treated. *p<0.05, **p<0.01 *vs* Con; ++p<0.01 *vs* AMP.

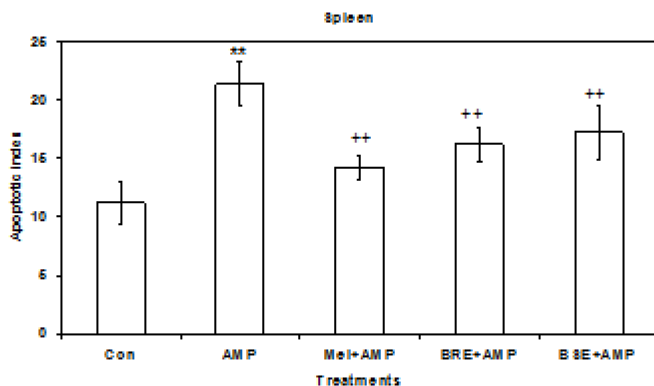

Figure 2 (b)

Comparative effect of melatonin, *B. diffusa* root and stem extracts against ampicillin induced apoptotic index in spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control; AMP= Ampicillin; Mel+AMP= Melatonin to ampicillin pre-treated; BRE+AMP= Root extract to ampicillin pre-treated; BSE+AMP= Stem extract to Ampicillin pre-treated. **p<0.01 *vs* Con; ++p<0.01 *vs* AMP.

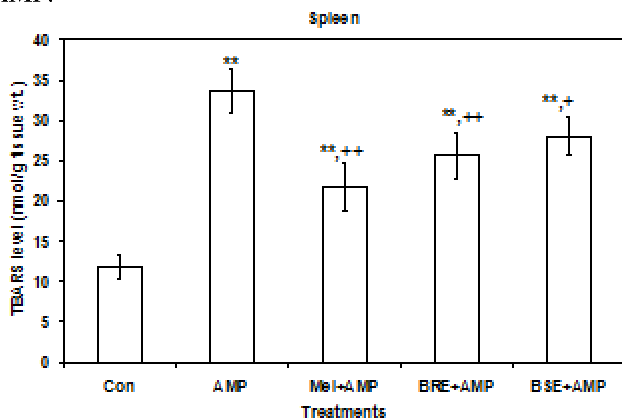

Figure 2 (c)

Comparative effect of melatonin, *B. diffusa* root and stem extracts against ampicillin induced TBARS level production in spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control; AMP= Ampicillin; Mel+AMP= Melatonin to ampicillin pre-treated; BRE+AMP= Root extract to ampicillin pre-treated; BSE+AMP= Stem extract to Ampicillin pre-treated. **p<0.01 *vs* Con; +p<0.05, ++p<0.01 *vs* AMP.

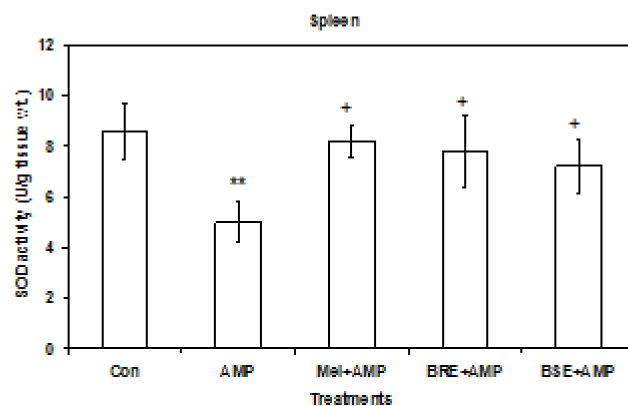

Figure 2 (d)

Comparative effect of melatonin, *B. diffusa* root and stem extracts against ampcillin induced SOD activity in spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control; AMP= Ampicillin; Mel+AMP= Melatonin to ampicillin pre-treated; BRE+AMP= Root extract to ampicillin pre-treated; BSE+AMP= Stem extract to Ampicillin pre-treated. **p<0.01 *vs* Con; +p<0.05 *vs* AMP.

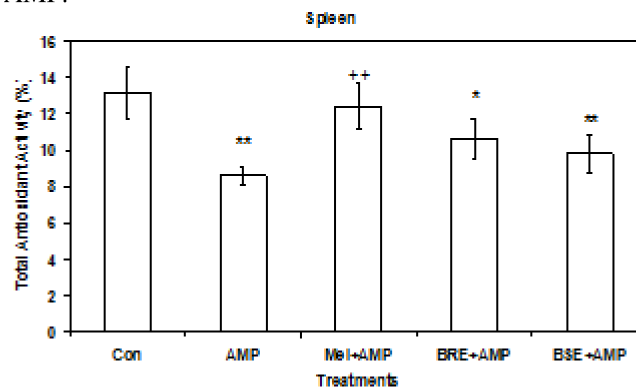

Figure 2 (e)

Comparative effect of melatonin, *B. diffusa* root and stem extracts against ampicillin induced TAA in spleen of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control; AMP= Ampicillin; Mel+AMP= Melatonin to ampicillin pre-treated; BRE+AMP= Root extract to ampicillin pre-treated; BSE+AMP= Stem extract to Ampicillin pre-treated. *p<0.05, **p<0.01 *vs* Con; +p<0.05, ++p<0.01 *vs* AMP.

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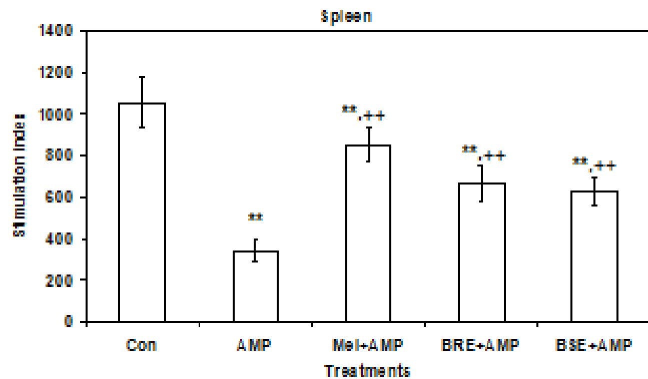

Figure 2 (f)

Comparative effect of melatonin, *B. diffusa* root and stem extracts against ampicillin induced stimulation index in splenocytes of mice. Vertical bars represent mean ± SEM, *n*= 5 for each group. Con= Control; AMP= Ampicillin; Mel+AMP= Melatonin to ampicillin pre-treated; BRE+AMP= Root extract to ampicillin pre-treated; BSE+AMP= Stem extract to Ampicillin pre-treated. **p<0.01 *vs* Con; ++p<0.01 *vs* AMP

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