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Oxidative stress induced hematological alteration of methotrexate attenuates *Bacopa monnieri* in rats

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Abstract
The ameliorative potential of aqueous leaf extract of *Bacopa monnieri* and silymarin were studied against methotrexate induced heamatological alteration. Total 36 male *Wistar* rats were separated into six groups (n = 6). Group 1 was maintained as normal control. A single dose of Methotrexate (20 mg/kg i.p.) was administered on day 9 to groups 2, 4, 5 and 6. Groups 3 and 5 were administered (p/o) with aqueous leaf extract of *Bacopa monnieri* @ 300 mg/kg bodyweight. Group 4 was administered (p/o) with aqueous leaf extract of *Bacopa monnieri* @ 150 mg/kg bodyweight and while group 6 received *silymarin* @ 200mg/kg body weight. The treatment was given for a period of 14 days. The animals were sacrificed on day 15th of the experimental. The blood was drawn for hematology and serum was collected for assessing total protein, biochemical parameters, and liver tissue was collected for estimation of antioxidant markers and tissue inflammatory markers. This current study revealed significant alterations in Hematology (RBC, WBC, Hb and PCV), antioxidant profile (TBARS, GSH and SOD), in methotrexate control group 2 as compared to treatment groups 4 and 5. There was significant improvement in group 5 and the values were comparable to treatment group 3. The values of groups 3 and 1 were comparable without any significant difference. Groups 4, 5 and 6 showed significant improvement in all the parameters compared to group 2.
In conclusion, *Bacopa monnieri* was found to possess the ameliorating action against methotrexate induced heamatological changes as similar to silymarin and results were more pronounced in the group receiving the higher dose, which was evident in this study by reducing the toxic markers and restoration of antioxidant enzymes. The overall beneficial effects of *Bacopa monnieri* could be attributed to its antioxidant actions.

Keywords: methotrexate, *Bacopa monnieri*, silymarin and heamatological ultrations

Introduction
Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent that effectively inhibits cellular growth. It is being used in many clinical indications, including leukemia, lymphomas, and other malignancies; autoimmune diseases, such as psoriasis and rheumatoid arthritis; and ectopic pregnancy [1, 2]. The therapeutic effect of MTX is by inhibiting the dihydrofolate reductase (DHFR) enzyme which is essential for synthesis of folic acid. Folic acid is converted into metabolically active tetrahydro folic acid by the enzyme dihydrofolate reductase. Tetra hydrate form of folic acid participates in the synthesis of purines, thymidylate and ultimately nucleic acids by transferring methyl groups to nucleotide precursors [3].
The exact mechanism of MTX induced haematological alteration is as yet unclear. Evidence has recently shown that reactive oxygen species (ROS) metabolites play a basic role in their pathogenesis [4, 5, 6].
MTX induced oxidative stress is plays a crucial role in liver damage and the reduction of this negative effect by antioxidant therapies have been demonstrated in experimental studies [7]. Lipid peroxidation (LPO) is known to play an essential role in damage to the cell membrane through reactive oxygen radicals. In the experimental studies, MTX toxicity has been shown to increase malondialdehyde (MDA), an important index of lipid peroxidation, and this increase has been shown to be suppressed by antioxidant therapies [8, 9].
The herbal plants are used for ameliorative agents against various drugs toxicity as they contain several phytochemcals like flavonoids, phenols, steroids and terpenoid which acts as antioxidants in the biological systems. In earlier studies many authors have been shown the protective role of herbal plants like fenugreek in diabetes [10],

Withania somnifera in exercise induced stress [11] spirulina in doxorubicin [12], *Terminalia chebula* against acetaminophen [13], *Withania somnifera* in chlorpyrifos induced renal and cardiac alterations [14] and antituberculosis drug induced hepatotoxicity [15], terminalia arjuna against cisplatin induced hepatotoxicity and nephrotoxicity [16], ascorbic acid in glyphosate induced testicular toxicity [17], *Tinospora cordifolia* and vitamin C in cisplatin induced cardiotoxicity [18].

"*Bacopa monnieri*" is a perennial, creeping herb plant commonly known as 'Brahmi'. It is used for the treatment of epilepsy, asthma, ulcers and tumors [11] skin disorders, specific uses include the treatment of asthma, insanity, cognitive and epilepsy [12], antioxidant, adaptogenic, gastrointestinal effects, endocrine, anti-inflammatory [13] and many experimental studies on different extracts of *Bacopa monnieri* have been shown neuroprotective, antioxidant and hepatoprotective activities [14].

Material and methods

Chemicals

All chemicals were of analytical grade and they are obtained from Qualigens Pvt. Ltd., Mumbai and SRL Pvt. Ltd., Mumbai, India.

Plant material and preparation of leaf extract

The fresh leaves of *B. monnieri* plant were collected from Hyderabad, India. The plant species were authenticated by Scientist, Agricultural College, Hyderabad, India. The fresh leaves of *B. monnieri* were washed twice with distilled water and shade dried at room temperature for 40-45 days. Leaves were powdered using a mechanical blender. Thereafter, 1 g powder was mixed with 100 ml of boiled distilled water and stirred on hot plate for 15 minutes. After the process, extract was filtered through Whatman No. 1 filter paper. The filtrate was kept at low temperature (4 °C) for further use [21].

Animals and Experimental Design

Thirty-six male *Wistar* rats aged about 3 months with an average bodyweight of 180 ± 10 g were obtained from Vyas labs, Hyderabad. They are divided into six equal groups (n=6) with different treatments (Table 1). The animals housed in polypropylene cages, under controlled environmental conditions (20–22 °C) and 12-hour dark and light cycles with sterilized dried clean, autoclaved rice husk was used as bedding material, which was changed on alternate days. The rats were maintained on standard balanced diet with drinking water *ad libitum* throughout the experimental period. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (No.2/22/C.V.Sc., Hyd. IAEC- Rats/29.02.2020) and were in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Table 1: Experimental design with group wise treatment protocol

Groups	Treatments	No. of Animals
1.	Normal saline for 14 days (Control)	6
2.	Methotrexate Control (20 mg/kg B.wt. i.p. single dose on day 9)	6
3.	Aqueous leaf extract of *Bacopa monnieri* Control (300mg/kg B. wt Oral Route for 14 days)	6
4.	Methotrexate (20 mg/kg B. Wt i.p. on 9th day) and Aqueous leaf extract of *Bacopa monnieri* (150 mg/kg B. wt Oral Route for 14 days)	6
5.	Methotrexate (20 mg/kg B. Wt i.p. on 9th day) and Aqueous leaf extract of *Bacopa monnieri* (300 mg/kg B. wt Oral Route for 14 days)	6
6.	Methotrexate (20 mg/kg B. Wt i.p. on 9th day) and Silymarin (200 mg/kg B. wt Oral Route for 14 days)	6

Blood and Serum analysis

Blood collection was carried out at the end of the experiment on day 14th for sero biochemical analysis. Feed was withdrawn 12 h before the blood collection and blood was collected from retroorbital plexus into serum vacutainers and centrifuged at 3000 RPM for 15 min, and serum was separated and stored at -80 ^{0}C till analysis. The sera samples were analyzed for the estimation of albumin, globulin, BUN and creatinine by using serum analyzer. Hematology was carried out at fortnight intervals for TEC, TLC, hemoglobin and PCV.

On the 14th day, after blood collection, rats were euthanized by carbon dioxide exposure and liver tissues were collected, homogenized and stored at -80C for further estimation of GSH, SOD and TBARS,

Analysis

Statistical Analysis

Data were subjected to statistical analysis by applying one-way analysis of variance (ANOVA) using the statistical package for social sciences (SPSS) version 25.0. Differences between means were tested using Duncan's multiple comparison tests and significance was set at $P<0.05$.

Results and Discussion

Body weight changes

Significant reduction ($P<0.05$) in body weight (g) was observed in Methotrexate treated rats (group 2) as compared with non-toxic control (group 1). Rats treated with aqueous leaf extract of *Bacopa monnieri* (group 3, 4 and 5) and silymarin (group 6) showed significant increase ($P<0.05$) in body weight when compared with group 2 (Table 2).

Table 2: Average body weights of different groups

Group	7th DAY	14th DAY
1. Normal Control	210.00±0.68^a	262.33±1.12^a
2. MTX @ 20mg/kg	209.00±0.51^a	222.00±0.57^d
3. *B. monnieri* @ 300mg/kg	208.50±0.76^a	263.60±0.66^a
4. MTX+*B. monnieri* @150mg/kg	207.00±1.03^a	234.50±0.99^c

5. MTX +*B. monnieri* @ 300 mg/kg	209.50±1.33[a]	241.83±1.35[b]
6. MTX+SILYMARIN @200mg/kg	209.16±0.40[a]	231.83±0.74[c]

Values are Mean + SE (n =6); One way ANOVA with Duncan's post hoc test (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05) among the groups (Vertically)

Table 3: Hematology parameters of different groups

Group	Total Erythrocytes (RBC/TEC) count (10^6/µl)	Total leucocytes (WBC/TLC) count (10^3/µl)	Haemoglobin (Hb) concentration (g/dl)	Packed cell volume (%)
1. Normal Control	9.06±0.27[a]	13.82±0.07[a]	15.25±0.22[a]	52.16±1.20[a]
2. MTX @ 20 mg/kg	5.54±0.15[d]	8.76±0.28[d]	11.25±0.08[c]	40.48±1.13[d]
3. *B. monnieri* @ 300 mg/kg	9.30±0.11[a]	13.46±0.21[a]	15.63±0.17[a]	52.23±1.46[a]
4. MTX+*B. monnieri* @150 mg/kg	7.74±0.09[c]	11.30±0.09[c]	12.15±0.17[b]	46.83±1.10[c]
5. MTX +*B. monnieri* @ 300 mg/kg	8.22±0.08[b]	12.76±0.08[b]	12.30±0.34[b]	48.72±1.19[b]
6. MTX+SILYMARIN @200 mg/kg	7.60±0.10[c]	11.08±0.13[c]	12.04±0.16[b]	47.21±1.27[c]

Values are Mean + SE (n =6); One way ANOVA with Duncan's post hoc test (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05) among the groups (Vertically)

Total erythrocytes, total leucocyte count, Hb and PCV levels are found to be significantly decreased (P<0.05) in rats treated with Methotrexate (group 2), whereas treatment with the aqueous leaf extract of *Bacopa monnieri* (group 4 and 5) and silymarin (group 6) was found to protect the rats from such effects of methotrexate (Table 3).

Table 4: Serological parameters of different groups

Group	Serum Blood urea nitrogen (BUN) concentration (mg/dl)	Serum creatinine concentration (mg/dl)	Serum Albumin concentration (g/dl)	Serum Globulin concentration (g/dl)
1. Normal Control	29.93±1.41[c]	0.72±0.07[c]	4.13±0.12[a]	3.24±0.05[a]
2. MTX @ 20 mg/kg	37.83±1.79[a]	1.20±0.98[a]	2.33±0.04[d]	2.40±0.03[d]
3. *B. monnieri* @ 300 mg/kg	30.75±1.40[c]	0.71±0.06[c]	4.22±0.07[a]	3.36±0.06[a]
4. MTX+*B. monnieri* @150 mg/kg	34.36±1.37[b]	0.88±0.04[b]	2.79±0.25[c]	2.60±0.01[c]
5. MTX +*B. monnieri* @ 300 mg/kg	34.51±1.15[b]	0.85±0.05[b]	3.48±0.19[b]	2.89±0.03[b]
6. MTX+SILYMARIN @200 mg/kg	33.84±1.29[b]	0.85±0.11[b]	2.70±0.05[c]	2.69±0.02[c]

Values are Mean + SE (n =6); One way ANOVA with Duncan's post hoc test (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05) among the groups (Vertically)

Serum parameters such as BUN and Creatinine levels were found to be significantly increased (P<0.05) in rats treated with Methotrexate (group 2), whereas treatment with the aqueous leaf extract of *Bacopa monnieri* (group 4 and 5) and silymarin (group 6) was found to protect the rats from such effects of methotrexate. Serum protein parameters such as albumin and globulin levels were found to be significantly decreased (P<0.05) in rats treated with Methotrexate (group 2), whereas treatment with the aqueous leaf extract of *Bacopa monnieri* (group 4 and 5) and silymarin (group 6) was found to protect the rats from such effects of methotrexate (Table 4).

Table 5: Effect on antioxidant markers of different groups

Group	SOD activity (U/mg protein)	GSH concentration (n moles/mg protein)	TBARS concentration (n moles of MDA released/mg protein)
1. Normal Control	8.96±0.29[a]	4.01±0.04[a]	1.18±0.08[d]
2. MTX @ 20 mg/kg	6.00±0.11[d]	2.28±0.06[d]	3.95±0.04[a]
3. *B. monnieri* @ 300 mg/kg	9.09±0.05[a]	3.88±0.04[a]	1.12±0.10[d]
4. MTX+*B. monnieri*@150 mg/kg	8.13±0.05[c]	3.26±0.05[c]	3.48±0.04[b]
5. MTX +*B. monnieri* @ 300 mg/kg	8.42±0.02[b]	3.58±0.02[b]	3.00±0.06[c]
6. MTX+SILYMARIN @200 mg/kg	8.03±0.05[c]	3.30±0.05[c]	3.40±0.02[b]

Values are Mean + SE (n =6); One way ANOVA with Duncan's post hoc test (SPSS)
Means with different alphabets as superscripts differ significantly (P<0.05) among the groups (Vertically)

Anti-oxidant parameters such as SOD and GSH levels were found to be significantly decreased (P<0.05) in rats treated with Methotrexate (group 2), whereas treatment with the aqueous leaf extract of *Bacopa monnieri*(group 4 and 5) and silymarin (group 6) was found to increased and TBARS levels were found to be significantly increased (P<0.05) in rats treated with Methotrexate (group 2), whereas treatment with the aqueous leaf extract of *Bacopa monnieri*(group 4 and 5) and silymarin (group 6) was found to decreased (Table 5).

Discussion

Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent for various cancer treatments. However, this anticancer agent is often exerting severe adverse effects including haematological and serobiocheimcal apart from the hepatoxicity. The mechanism of MTX-induced toxicity is mainly due to reactive oxygen metabolites. Lipid peroxidation is known to play an essential role in damage to the cell membrane through reactive oxygen radicals. MTX toxicity has been shown to increase malondialdehyde (MDA), an important indicator of lipid peroxidation, and this increase has been shown to be suppressed by antioxidant therapies [8, 9]. The present study was carried out with supplementation of *B. monnieri* (BM) at different dose levels to mitigate the adverse effects of MTX and the results are discussed here below.

In the present experiment, the body weights of rats in toxic control i.e., group 2 were significantly reduced when compared to normal control group 1 and per se group 3. The reduction in body weights may be attributed to direct toxicity, anorexia, general body weakness and stress [8, 19]. In the present study, decreased body weights in MTX group is attributed to oxidative stress and oxygen free radicals generated in MTX induced haemato toxicity. Weight gain observed in the animals treated with *B. monnieri* and silymarin may be due to restoration of antioxidant defenses and decreased body weight may be due to direct effect of MTX on feed intake and decreased appetite due to liver injury [20].

The effect of MTX in this study revealed significant lower values of TEC, TLC, Hb and PCV in toxic control group 2 compared to control groups 1 and per se 3. A significant change in hematological parameters may be due to cytotoxic effect of MTX on hemopoietic system [21]. The treatment groups 4, 5 and 6 revealed a significant improvement in TEC, TLC and PCV values. The hemo protective effect of *B. monnieri* extract and silymarin in the treatment groups may be due to the antioxidant and antiinflammatory activity and these results are in agreement with the previous studies [22, 23, 24].

The present study also revealed a significant alteration in the albumin and globulin concentration in toxic control group 2 when compared to the normal control and per se group 3, which might be attributed to the impairment of liver function due to MTX administration resulting in oxidative stress and decreased capacity of liver to synthesize protein [25, 26]. Reduction in serum albumin and globulin induced by methotrexate could be due to several factors like damage to liver, increased intestinal protein loss, protein losing nephropathy and dietary protein deficiency as there was decrease in feed intake [19]. The improvement in the treatment groups may be due to the presence of antioxidant property of the Bacopa as bacoside A3 [24].

Biomarkers of oxidative stress and antioxidant profile were studied to evaluate MTX induced free radical damage in the present experiment. The results revealed a significant decrease in the activity of SOD and concentration of GSH, while significant increase in the concentration of TBARS in the toxic control (group 2) when compared to control group 1and 3. MTX enhances the formation of ROS, which has been implicated in inducing oxidative stress, thus resulting in decreased SOD and GSH. SOD converts superoxide radical to H_2O_2 and GPx prevents oxidative damage to essential intracellular compounds by reduction of H_2O_2 to water, GSH destroys the free radicals formed in the cells. SOD is one of the major enzymes of endogenous antioxidant defense system that catalyses the dismutation of superoxide anions. MTX treatment depletes GSH levels in many tissues that cells could be more sensitive to ROS and leads to a reduction in the effectiveness of the antioxidant enzyme defense system [27]. In our study, the level of MDA in MTX treated rats was significantly higher than the control group. This finding was in agreement with several earlier reports demonstrating that MTX induced oxidative stress in tissues [27]. The increase in MDA levels suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant defense mechanism to prevent the formation of excessive free radicals. Enhancement of antioxidant status in liver by *B. monnieri* [28, 29] under different conditions. The *B. monnieri* extract revealed the presence of several bioactive components [30] in the extract includes triterpenoids, saponins, alkaloids, glycoside, alcohols, triterpenoids and saponins such as bacoside A and bacoside A3 and are reported to be responsible for *B. monnieri*'s hepato protective efficiency. Pretreatment with bacoside A significantly improved the SOD activity to near normal level, restoration of GSH by maintaining the integrity of the membrane and also reduced oxidative stress by modulating the expression of hsp70 and prevent the cells by scavenging the ROS [31]. In our study, a significant improvement was observed in group 5than the other treatment groups (4and 6), *B. monnieri*@300mg dose had improved the activity of SOD and GSH and decreased the concentration of MDA released.

In view of the above results, treatment with *B. monnieri* showed haemato protection as similar to silymarin against MTX induced sero-haematological alterations, but the rats which were treated with high dose of *B. monnieri* showed better results due to the potent anti-oxidant properties of the *B. monnieri*.

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