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Protective Effect of *Bacopa monnieri* against Methotrexate Induced Hepatotoxicity in *Wistar* Rats

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ABSTRACT

Background: Methotrexate (MTX) is an anticancer drug and its clinical efficacy is limited to the toxicity associated with the liver. In this study, we examine the potential role of aqueous leaf extracts of *Bacopa monnieri* to counter methotrexate-induced hepatotoxicity.

Methods: This study was undertaken by considering 36 numbers. Wistar male rats were randomly segregated into six groups, with six rats per group. Groups 1 and 2 were kept as normal controls and methotrexate was treated on the 9th day. While groups 3 and 4 received an aqueous extract of the leaf of *B. monnieri* at two varied doses of 150 and 300 mg/kg, respectively, as a treatment, Whereas groups 5 and 6 kept *B. monnieri* perse and the standard hepatoprotective drug silymarin @ 200 mg/kg and experiment was conducted for 14 days period. After the experimental procedure, AST, ALT, TNF-α and IL-10 were measured and histopathology of the liver was done.

Result: The present study revealed significant alterations in AST, ALT, TNF-alfa and IL-10 in methotrexate-treated rats (group 2) when compared to group 1 and treatment groups 4 and 5 revealed significant improvement and these results were validated through imaging in the histopathology of the liver. We conclude that *Bacopa monnieri* can efficiently reduce methotrexate-induced liver inflammation and can be used in the management of hepatotoxicity in the liver.

Key words: *Bacopa monnieri*, Hepatotoxicity, IL-10, Methotrexate, TNF-α.

INTRODUCTION

Methotrexate (MTX) is a folate antimetabolite and derivative of aminopterin used as a cytotoxic anticancer agent in malignancies discovered during 1940, it acts by interfering with DNA biosynthesis, repair and cellular replication (Pawankalyan *et al*., 2022). Methotrexate was first employed as a treatment for acute leukemia in children back in 1948 and later on, it was also introduced as a therapy for psoriasis and rheumatoid arthritis (RA). The drug received FDA (Food and Drug Administration) approval for RA treatment in 1988 and for psoriasis treatment in 1972, becoming one of the most commonly used disease-modifying antirheumatic drugs globally (Tenti *et al*., 2023). The precise mechanism of MTX-induced hepatotoxicity is unknown at this time. Reactive oxygen species (ROS) metabolites have recently been discovered to play a key role in the hepatotoxicity of a variety of xenobiotics and medications. There is evidence that MTX can reduce oxygen uptake in isolated mitochondria and inhibit oxidative phosphorylation in the mitochondria, it can also inhibit the enzymes 2-oxoglutarate, isocitrate, malate and pyruvate dehydrogenases in mitochondria as well (Ghoneum and El-Gerbed, 2021). The consequences of ROS formation lead to stimulating transcription factors such as nuclear factor-kappa B (NF-κB) which can trigger an increase in the expression of genes related to the synthesis of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-6. Subsequently, these agents contribute to tissue harm and the initiation of apoptosis (Sayed *et al*., 2022). For the treatment of liver disorders plants have been used as excellent sources for the management of liver toxicity due to the presence of phytochemicals like flavonoids, phenols, terpenoids and steroids (Gopi *et al*., 2010; Priyanka *et al*., 2020).

For centuries, *Bacopa monnieri* has been utilized as a traditional ayurvedic medicine in India to treat various illnesses and conditions, particularly as a nerve tonic and cardiotonic (Adams *et al*., 2007; Jain, *et al*., 2016). The plant

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is known to contain triterpenoid saponins, alkaloids and flavonoids, which have been found to have antioxidant properties (Hou *et al*., 2002; Allawadhi *et al*., 2021). In fact, studies have shown that alcohol extracts of *Bacopa monnieri* are effective in acting as antioxidants, free radical scavengers and anti-lipid peroxidative agents (Bhattacharya, 2011). Moreover, previous research has indicated that *B. monnieri* can inhibit the formation of superoxide anions in a dose-dependent manner and can also mitigate nitric oxide toxicity in rat astrocytes (Chougle *et al*., 2021).

B. monnieri's phytochemicals, such as the alkaloid brahmine, nicotine, herpestine, bacosides A and B, saponins A, B and C, triterpenoid saponins, stigmastanol, -sitosterol, betulinic acid, D-mannitol, stigmasterol, -alanine, aspartic acid, glutamic acid and serine and pseudojujubogenin, are believed to have hepatoprotective effects (Manvitha *et al*., 2019: Jeyasri *et al*., 2020).

MATERIALS 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

To obtain the plant material, fresh leaves of *B. monnieri* were gathered near to Hyderabad, India and authenticated by a Scientist at Agricultural College, Hyderabad, India. Approximately 40-45 days were required for air-drying the leaves after being washed twice with distilled water. The dried leaves were then ground into a fine powder using a mechanical blender. Aproximately measured 100 gram of powder taken and was boiled by blending in a 100 ml of distilled water and stirred for 15 minutes on a hot plate to obtain extract. Subsequently the extract was filtered by susing Whatman No.1 filter paper and stored at low temperature (4°C) until further use.

Animals and experimental design

In this experimental study, 36 Wistar male rats weighing in between 180±10 g were acquired from Vyas labs located in Hyderabad and were assigned to 6 groups (n=6) for administering varied treatments scheduled. The rats were placed in polycarbonated cages by maintaining ambient temperature, including a temperature of 20-22°C and a 12-hour light and dark cycle. The bedding material consisted of sterilized, dried, clean, autoclaved rice husk, replaced every other day. The rats were provided a standard balanced diet and access to drinking water *ad libitum* for the entire experimental period. Throughout the experimental period, the rats were given a nutritionally balanced diet.

Additionally, they were allowed to Reverse osmosis drink water freely. This ensured that the rats remained healthy and hydrated throughout the experiment. The Institutional Animal Ethical Committee reviewed and approved all the procedures and protocols used in the study, ensuring that the research complied with ethical standards. (No.2/22/C.V.Sc., Hyd. IAEC- Rats/29.02.2020) and the experiment was conducted in the Lab Animal House, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Rajendranagar, Hyderabad, in August 2021 in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Experimental design with group-wise treatment protocol

Group 1 received normal saline (PO) as a sham, while group 2 rats were administered Methotrexate @ 20 mg BW via the IP route as a single dose on the 9th day of the experiment. Similarly, groups 4 and 5 were given low dose (150 mg/kg BW) and high dose (300 mg/kg BW) via the PO route and MTX administration on the 9th day. However, groups 3 and 6 received *Bacopa* (300 mg/kg, PO) and silymarin as a standard (200 mg/kg BW, PO), respectively. The treatment schedule was instituted for 14 days.

Blood and serum analysis

On the 14th day of the experiment, blood was withdrawn from the retro orbital flexus of the rats using serum vacutainers, then centrifuged at 3000 RPM for 15 minutes and the serum was separated and stored at -80°C until further analysis of liver biomarkers such as AST and ALT (Erba kit procedure by following IFCG Method, Kinetic). Following the blood collection, the rats were euthanized using carbon dioxide exposure and their liver tissues were collected, homogenized and stored at -80°C for TNF-á and IL10 (As per the Elisa kit procedure procured from Thermo Fisher Scientific, Bangalore) liver homogenate estimation and some peace of liver collected in the formalin for the histopathology (Singh and Sulochana, 1997) to draw possible conclusions.

Statistical analysis

The collected data was analyzed using the Statistical Package for Social Sciences (SPSS) version 25.0 and one-way analysis of variance (ANOVA) was applied. Duncan's multiple comparison tests were used to test for differences between means and significance was considered at $P<0.05$.

RESULTS AND DISCUSSION

Liver serum biomarkers

Rats treated with Methotrexate (Group 2) showed a significant increase ($P<0.05$) in serum parameters such as AST and ALT levels. However, the rats treated with the extract of *Bacopa monnieri* in groups 4 and 5 exhibited protection from the detrimental effects of methotrexate on these parameters. These values are comparable to the silymarin treated group (6) (Table 1).

Pro-inflammatory and anti-inflammatory activity

Group 2 showed considerable ($P<0.05$) elevated levels in the TNF-α and IL-10 (pg/mg) in liver homogenate compared to group 1. On the other hand, groups 4 and 5, which were treated with the *B. monnieri,* showed a magnificent ($P<0.05$) decrease in TNF-α and IL-10 concentration when compared

to group 2. Notably, these values in groups 4 and 5 revealed similar to those of group 6, which was treated with the standard silymarin. The results are depicted in Table 1.

Histopathology of liver

The tissue sections of the liver of group 2 showed moderate to severe congestion of the central vein, degenerated hepatocytes with the presence of Kupffer cells and mild fibrous tissue proliferation in the perivascular area with disruption of the portal triad and dilated sinusoids (Fig 2 and 2a). In contrast, treated groups 4, 5 and 6 showed mild to moderate congestion of the central vein, dilated sinusoidal space. Mild degeneration of the hepatocytes in group 4 (Fig 4 and 4a), mild degeneration, mild proliferation of Kupffer cells and mild dilation of the periportal area in group 5 (Fig 5 and 5a), Normal radiating appearance of hepatic cords with mild dilation of central vein in group 6 (Fig 6 and6a), however, groups 1 and 3 revealed normal architecture (Fig 1, 1a, and 3,3a).

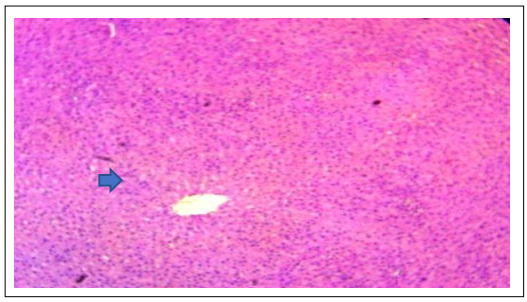

Fig 1: Group 1: Photomicrograph of liver demonstrating the liver's normal architecture with the appearance of radiating pattern hepatic cords (arrow). H and E×10.

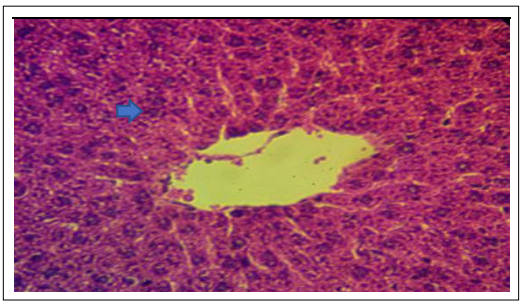

Fig 1a: In Group 1, a photomicrograph of the liver was obtained, which revealed the normal architecture of the liver with hepatic cords appearing in a radiating pattern (Indicated by an arrow). H and E×40.

MTX is a chemotherapeutic agent with cytotoxic properties that finds broad application in the treatment of various forms of cancer. However, MTX often exerts severe adverse effects and causes hepatotoxicity, including progressive development of fibrosis and cirrhosis. The primary cause of MTX-related liver damage is toxic metabolites produced by the drug. The toxicity of MTX was demonstrated in our study, where the toxic control group (Group 2) showed a significant increase in AST and ALT levels compared to the normal control groups (Groups 1 and 3). This finding is consistent with a previous report (Helal and Said, 2020; Namratha *et al*., 2021) that showed marked liver injury in rats treated with MTX. Similarly other authors also deomonstrated that protective effect of resveratrol and vitamin-e in 5-flourouracil induced hepatotoxicity (Harish *et al*., 2021), Terminalia arjuna against Experimental Hepatotoxicity due to Cisplatin in Rats (Sneha *et al*., 2021) and postulated protective effects due to antioxidant properties of these plants. However, our study also found that the administration of *B. monnieri*, which has antioxidant properties, significantly reversed the changes in serum AST and ALT activities in the toxic control group. This indicates that *B. monnieri* has the potential to alleviate MTX-induced liver damage. Furthermore, the groups treated with different doses of *B. monnieri* showed significant improvement in hepatic biomarkers and the results were comparable to the group treated with the standard silymarin extract containing silybinin, silydianine and silychristine, which also attenuated the increased AST and ALT levels.

Concurrently with oxidative stress, inflammation is pivotal in MTX-induced hepatotoxicity's pathogenesis (Fouad *et al*., 2020). The current data indicate that a single i.p. injection of MTX was able to increase level of TNF-á which might be due to excess generation of ROS in MTX-treated rats increase neutrophil infiltration and enhance transcription of proinflammatory cytokines including TNF-α (Kumar and Reddy *et al*., 2012; Taskin *et al*., 2021). In the present investigation, MTX administration significantly elevated the tissue TNF-α levels compared to normal control rats. Administration of *B. monnieri* and silymarin for 14 days

Table 1: The activity of AST, ALT, TNF α and IL-10 in varied treatment groups of rats.

Group	AST activity (IU/L)	ALT activity (IU/L)	TNF α (pg/mg tissue)	IL-10 (pg/mg tissue)
1. Normal control	92.10±2.73^{d}	33.66±1.23^{d}	8.45±0.14^{d}	77.33±0.42^{a}
2. MTX @ 20 mg/kg	140.95±2.74^{a}	49.90±1.44^{a}	19.33±0.25^{a}	55.16±0.30^{d}
3. *B. monnieri* @ 300 mg/kg	93.40±2.57^{d}	33.78±1.24^{d}	8.36±0.13^{d}	74.33±0.33^{a}
4. MTX+*B. monnieri* @150 mg/kg	132.01±2.19^{b}	46.41±1.33^{b}	14.13±0.20^{b}	65.66±0.33^{c}
5. MTX+*B. monnieri* @ 300 mg/kg	125.25±2.52^{c}	44.83±1.28^{c}	12.35±0.16^{c}	71.00±0.36^{b}
6. MTX+Silymarin @200 mg/kg	129.90±2.22 ^{b}	47.25±1.20^{b}	14.26±0.14^{b}	65.16±0.30^{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).

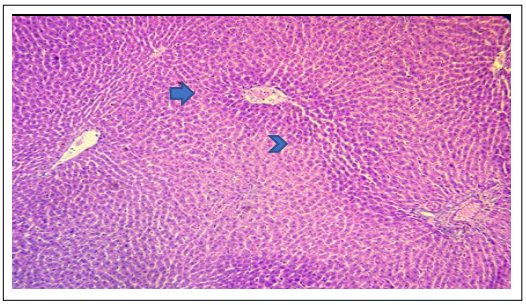

Fig 2: Group 2: A photomicrograph of the liver displayed moderate congestion in the central vein (Indicated by an arrow), as well as mild dilation of sinusoidal spaces (Indicated by a chevron) H and E×10.

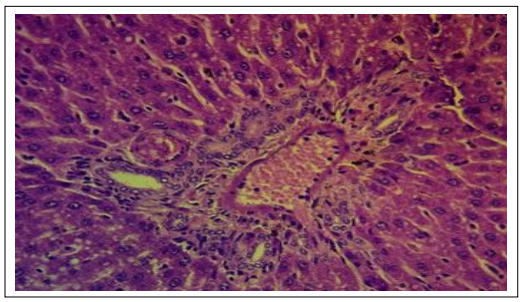

Fig 2a: In Group 2, a photomicrograph of the liver revealed moderate congestion in the central vein (Indicated by an arrow), mild dilation of sinusoidal spaces (Indicated by a chevron) and mild proliferation of Kupffer cells (Indicated by a down arrow head). H and E×40.

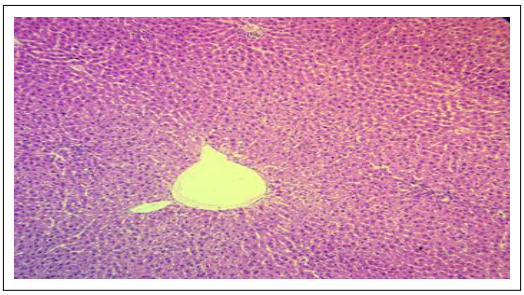

Fig 3: Group 3: A photomicrograph of the liver demonstrated the normal architecture of the liver with the appearance of hepatic cords in a radiating pattern: H and E×10.

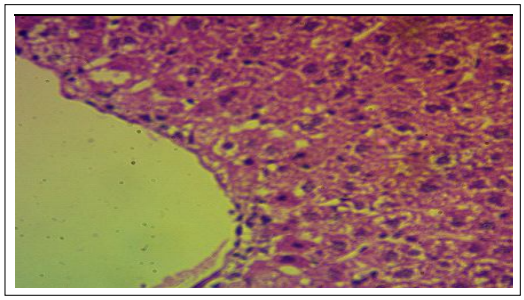

Fig 3a: In Group 3, a photomicrograph of the liver was obtained, which exhibited the normal architecture of the liver with hepatic cords appearing in a radiating pattern. H and E×40.

before MTX decreased inflammatory cytokine levels. The anti-inflammatory effects of *B. monnieri* and silymarin could be mediated by suppressing NF-kB regulation including COX-2, LOX and TNF α (Sharma *et al*., 2020). The reactive metabolite 7-hydroxy methotrexate is formed as a result of methotrexate metabolism. This metabolite has been found to modify cellular macromolecules, generate intracellular oxidative stress and mediate cytokine responses. These events are considered critical components in the pathophysiology of hepatotoxicity. Furthermore, the intracellular stress pathways lead to the sensitization of

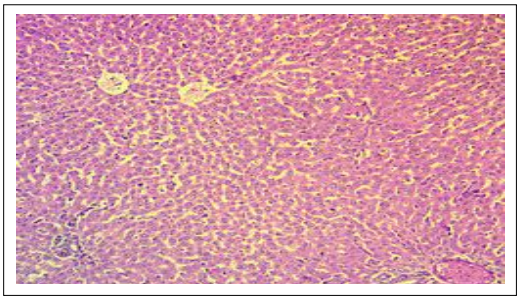

Fig 4: In Group 4, a photomicrograph of the liver revealed the dilation and mild congestion of the central vein (Indicated by an arrow) and irregular hepatic cords with dilatation of sinusoidal space (Indicated by a chevron). H and E×10.

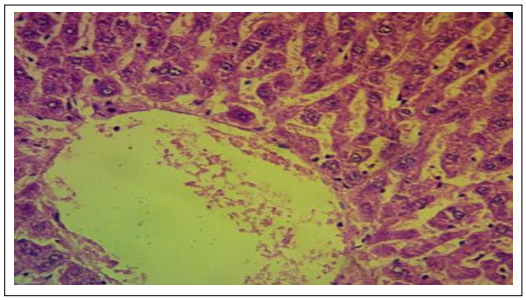

Fig 4a: Group 4: A photomicrograph of the liver displayed the dilation and mild congestion of the central vein (Indicated by an arrow) along with irregular hepatic cords exhibiting dilatation of sinusoidal spaces (Indicated by a chevron). H and E×40.

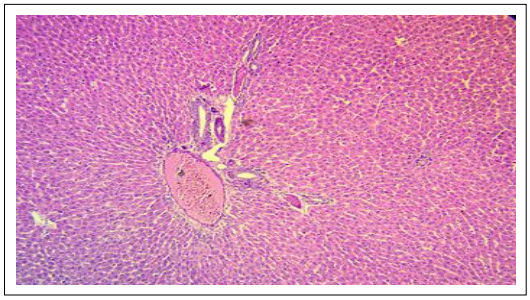

Fig 5: In Group 5, a photomicrograph of the liver demonstrated congestion of the central vein (Indicated by an arrow) and mild dilation of sinusoidal spaces (Indicated by a down arrow). H and Ex10.

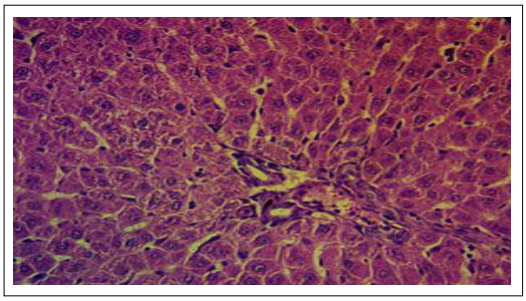

Fig 5a: Group 5: A photomicrograph of the liver displayed congestion of the central vein (Indicated by an arrow), mild proliferation of Kupffer cells (Indicated by a chevron) and mild dilation of sinusoidal spaces (Indicated by a down arrow): H and E×40.

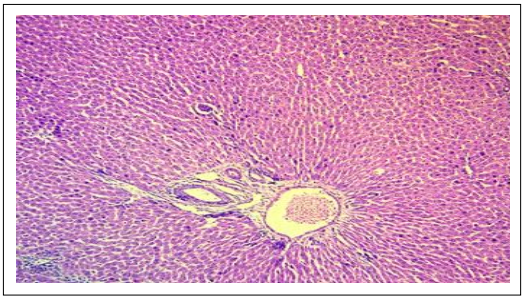

Fig 6: In Group 6, a photomicrograph of the liver depicted congestion of the central vein (Indicated by a down arrow) and mild dilation of sinusoidal spaces (Indicated by an arrow). H and E×10.

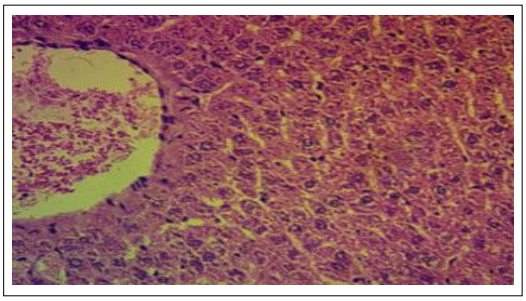

Fig 6a: In Group 6, the photomicrograph of the liver shows a central vein with congestion (Indicated by a down arrow) and mild dilation of sinusoidal spaces (Indicated by an arrow): H and E×40.

proinflammatory cytokines and the inhibition of anti-inflammatory cytokines. In this work, animals treated with *B. monnieri* and silymarin significantly showed the elevated level of anti-inflammatory interleukin IL-10 in groups 4, 5 and 6 compared to MTX group, suggesting that *B. monnieri* and silymarin possessed immune modulating activity in liver and immune system. Further these results were substantiated by the change in histopathology of liver in the MTX treated group and appreciable improvement in *Bacopa* treated groups.

Based on the findings, *B. monnieri* treatment provided hepatoprotection similar to silymarin against MTX-induced hepatotoxicity, although the rats given a high dose of *B. monnieri* had better results due to the presence of phytoconstituents in the *B. monnieri.* Based on the findings and histological analysis, it can be determined that *B. monnieri* extract can prevent MTX-induced hepatotoxicity and that *B. monnieri* extract can be administered as a hepatoprotective agent to combat the drug's harmful effects.

CONCLUSION

The study found that MTX resulted in liver injury by increasing inflammatory markers, liver biomarkers and causing structural changes under microscopic examination. However, administering aqueous leaf extract of *B. monnieri* to rats injected with MTX helped reduce inflammation and liver damage. This suggests that the protective action of *B. monnieri* against Methotrexate toxicity is due to the presence of phytochemicals. Overall, the study confirms the beneficial role of *B. monnieri* in protecting against MTX-induced liver toxicity.

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Conflict of interest: None.

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