Proceedings S.Z.P.G.M.I. Vol: 32(1): pp. 7-12, 2018. PSZMC-662-32-1-2018

Protective Role of *Beta vulgaris* and *Moringa oleifera* Leaves in Methotrexate Induced Hepatotoxicity in the Rat Model

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ABSTRACT

Introduction: Methotrexate (MTX) is an antimetabolite used in the treatment of a variety of cancers and autoimmune disorders with significantly higher hepatotoxic potential ranging from fibrosis to cirrhosis in Western versus Asian populations with no known treatment. Focus on phytotherapy has emerged in this regard. **Aims and Objectives:** The aim of this work was to test the possible protective effect of *Beta vulgaris* and *Moringa oleifera* leaves against MTX induced hepatotoxicity in rats. **Place and Duration of Study:** The study was conducted in experimental research laboratory, PGMI, Lahore and was completed in 1 year. **Material and Methods:** 45 rats were divided into 5 groups of 9 animals each. Group 1 served as healthy controls and Group 2 as diseased control. All extracts were administered in single oral doses daily for 24 days. Groups 3 and 4 were administered 200mg /kg of ethanolic extract of Beta vulgaris (EEBV) and 800 mg/kg ethanolic extract of Moringa oleifera (EEMO) respectively, while Group 5 was given their combined extracts (100mg/kg EEBV+400mg/kg EEMO). On 21st day hepatotoxicity was produced in groups 2-5 by giving single intraperitoneal injection of 20mg/kg MTX. Blood samples were drawn through cardiac puncture at baseline, days 21 and 24 and LFTs were determined. Qualitative data was analyzed using chi-square and p values <0.05 were labeled significant. **Results:** Ethanolic extracts of Beta vulgaris and Moringa oleifera individually and in combination exhibited significant hepatoprotective effect when administered prior to methotrexate in rats, by maintaining normal murine LFTs (ALP.AST,ALT) with P value <0.05. **Conclusion:** EEBV and EEMO leaves and their combined extracts possess significant hepatoprotective activity.

Key words: Methotrexate, Ethanolic extract of *Beta vulgaris*, Ethanolic extract of *Moringa Oleifera,* Liver function tests, alkaline phosphatase, aspartate aminotransferase, alanine transaminase.

INTRODUCTION

The liver play a vital role in maintenance of body's homeostasis which includes synthesis and metabolism of proteins, carbohydrates, lipids, vitamins, plasma proteins, maintenance of acid balance, secretion of hormones, transport of oxygen and carbon dioxide, detoxification and excretion of exogenous and endogenous metabolites[1]. It is therefore often vulnerable to insult caused by toxins, microbes, various metabolites and xenobiotics such as prescribed and over the counter drugs (OTC)[2]. Drug induced hepatotoxicity is an important condition. It has become the leading cause of fulminant liver failure accounting for 20-40 % of such cases referred for liver transplantation in US [3]. These drugs affect liver cells by different mechanisms like oxidative stress, fatty acid peroxidation, fat accumulation, antibody mediated cytotoxicity and apoptosis[4].

Methotrexate (MTX) is an antimetabolite and folic acid antagonist[5]. It has been used for treatment of many diseases such as acute lymphocytic leukemias, choriocarcinoma, breast cancer, head and neck carcinomas, rheumatoid arthritis, psoriasis and Crohn's disease[6]. High dose and chronic administration of this drug can cause hepatotoxicity

and nephrotoxicity as well[7]. Until now leucovorin has been used for the management of toxicities induced by methotrexate. It provides normal tissues with a mechanism to bypass blocked enzyme and replenish the folate pool. It can rescue only rapidly proliferating cells i.e. gastrointestinal cells and hematopoietic cells. Therefore it lacks the ability to manage other toxicities induced by MTX[8].

7-hydroxymethotrexate is a major metabolite of MTX. Its formation occurs in liver. MTX is stored as polyglutamated form within cells. This accumulation of polyglutamated form of MTX is responsible for MTX induced hepatotoxicity. Oxidative stress is thought to be main reason of MTX induced toxicities within liver and kidney[9]. The basic mechanism underlying this toxicity is decrease in the level of NADPH in cells, which is required for synthesis of glutathione, an important antioxidant[10]. Therefore decreased glutathione levels lead to decrease in antioxidant enzymatic defense system, making cells more vulnerable to Reactive Oxygen Species (ROS)[11].

Many plants have been evaluated for their protective effect against drug induced toxicities so far. For this purpose different extracts of different herbs have been used.

Keeping in mind the protective effect of different plants we selected two herbs *Beta vulgaris* and *Moringa oleifera*. Beet root (*Beta vulgaris*) is a herb, which belongs to family Amaranthaceae. Its leaves are tonic, diuretic, anti-inflammatory and are useful in diseases of spleen and liver[12].

Moringa oleifera is another herb, which belongs to family Moringacea. Preliminary phytochemical studies on leaf extracts of both *Beta vulgaris* and *Moringa oleifera* have revealed them to contain various phytochemicals like sterols, triterpenoids, tannins, flavonoids, alkaloids, glycosides and saponins[12,13]. These active principles are known antioxidants, so these active principles might prove helpful in playing their protective role against adverse effects of MTX.

Based on research stated previously, no study had been done regarding protective effect of *Beta vulgaris* and *Moringa oleifera* leaves against MTX induced hepatotoxicity. This dimension remained under investigated, so the main objective of our research project was to evaluate their protective role.

MATERIAL AND METHODS

Animals Required:

The study was approved by Ethical Review Board, Shaikh Zayed Medical Complex. Male rats obtained from animal house of PGMI (Post Graduate Medical Institute) Lahore, weighing 150-200 g were used. Total 45 animals were randomly selected. 9 animals were included in each group. They were housed in standard Propylene cages and kept under controlled room temperature (25 ± 10^0C), and fed with pelletsas standard laboratory diet and tap water ad libitum.

Chemicals and Drugs:

Methotrexate (50mg/2ml in injectable form) was obtained from Signoir Chemicals. Other chemicals included chloroform, Ethanol (95%), Formalin (10%) solution. Kits for ALP, AST, ALT were purchased from Randox. Soxhlet Apparatus was provided by PCSIR.

Selection of Plant Materials:

Beta vulgaris and *Moringa oleifera* leaves were purchased from Hamdard Dawakhana Lahore. The leaves were identified and authenticated by Department of Botany, Punjab University Lahore.

Experimental Settings:

This one year study was carried out in experimental research laboratory in PGMI Lahore. Biochemical tests were performed at the Biochemistry lab in Shaikh Zayed Hospital Lahore.

Ethanolic extracts of *Beta vulgaris* and *Moringa oleifera*:

These were prepared at PCSIR (Pakistan Council of Scientific and Industrial Research) Lahore.

After washing with distilled water leaves were air dried under shade at room temperature and stored in a tightly closed and properly labeled plastic container. Dried leaves were grounded with the help of mortar and pestle to obtain 100 gram powder.

Beta vulgaris Leaf Extraction:

100 grams of powdered leaf material of *Beta vulgaris* was extracted gradually in multiple cycles with 90% ethanol using soxhlet apparatus. The process involved a slow dissolution of the non-volatile compound in solvent during each cycle and concentration in the distillation flask followed by maceration with distilled water for 24 hours.

Solvent removal was performed using rotary evaporator under reduced pressure below 45˚C to get EEBV (Ethanolic extract of Beta vulgaris) a 11.5% w/w greenish paste [12].

Moringa oleifera Leaf Extraction:
Dried leaves *of Moringa oliefera* were collected and pounded into 100 gram powder before extraction. The powder was macerated into absolute alcohol at room temperature, the filtrate was concentrated under reduced pressure and later evaporated in water bath using evaporating dish at 60˚C to a greenish paste carrying a percentage yield of 12.4%[14].

Animals Grouping: The animals were randomly divided into 5 groups of 9 animals each as follows

Group 1 (negative control): Healthy control group was given distilled water only.

Group 2 (positive control): Experimental control group were given only distilled water followed by a single injection of MTX 20mg/kg i/p on the 21st day to induce toxicity.

Group 3 (Test): EEBV (Ethanolic Extract of *Beta vulgaris*) leaves 200mg/kg p/o once daily for 24days. Methotrexate 20mg/kg i/p was given on 21st day.

Group 4 (Test): EEMO (Ethanolic Extract of *Moringa oleifera*) leaves 800mg/kg p/o for 24 days. Methotrexate 20 mg/kg i/p was given on 21st day.

Group 5 (Test): EEBV (Ethanolic extract of *Moringa oleifera*) 400 mg/kg and EEBV (Ethanolic Extract of *Beta vulgaris*) leaves 100 mg/kg p/o for 24 days. Methotrexate 20 mg/kg i/p was given on 21stday.[12]

Blood Sampling and Determination of LFTS:
1.5-3ml blood was collected in 5ml syringes by intracardiac puncture on Days 0, 21 and 24. Serum was separated after centrifugation and evaluated for ALP, AST, ALT levels in all groups using standard referred protocols.

Statistical analysis:
Data was analyzed by using SPSS version 20.0. Quantitative parameters were described by using mean±S.D/median (IQR) for each group. The comparison amongst groups was made using ANOVA followed by Tukey's test. A p-value of < 0.05 was considered statistically significant.

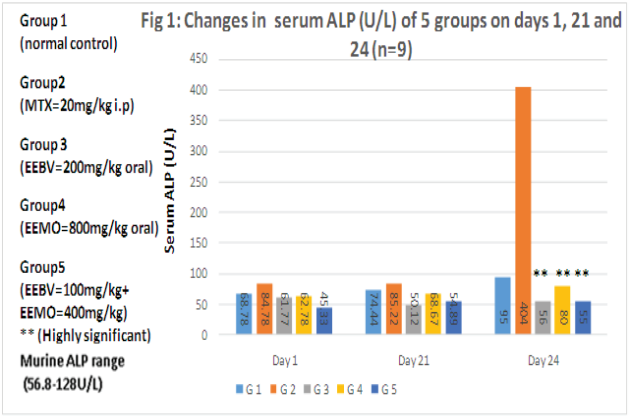
RESULTS

At baseline and on the 21st day LFT values of all 5 groups lay within range reflecting safety of these extracts.

On the 24th day, highest mean ALP levels of 404.2±64.3 U/L were recorded for the diseased control group 2 while other groups 3&4 had significantly lower levels of ALP as compared to it with p-values <0.001. However the lowest levels of 55.0 ±7.0U/L were found in group 5 which was administered combined EEBV and EEMO extracts.(Fig-1)

The serum AST levels also followed a similar trend. In group 2 AST values were the highest recorded as 422.3±43.4 U/L, while those of healthy control group 1 and treatment groups 3, 4 and 5 were recorded as 94.6±12.6, 142.8± 18.3, 157.2 ±51.5, and 140.3 ±34.6 U/L respectively. The difference amongst groups was significant with p-value <0.001. (Fig-2)

At the end of the study on the 24th day mean ALT levels were also found significantly raised for group 2 at 78.6±9.4 U/L as compared to groups 1, 3, 4 and 5 which were recorded as 27.6±7.4, 39.3±8.2, 41.0±5.1 and 40.3±8.7 U/L respectively. (Fig-3)



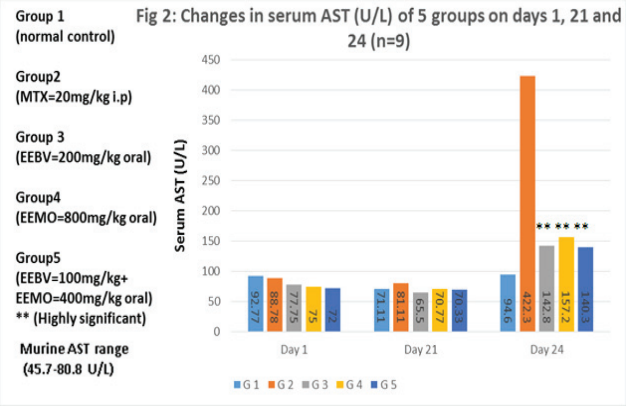


Fig 2: Changes in serum AST (U/L) of 5 groups on days 1, 21 and 24 (n=9)

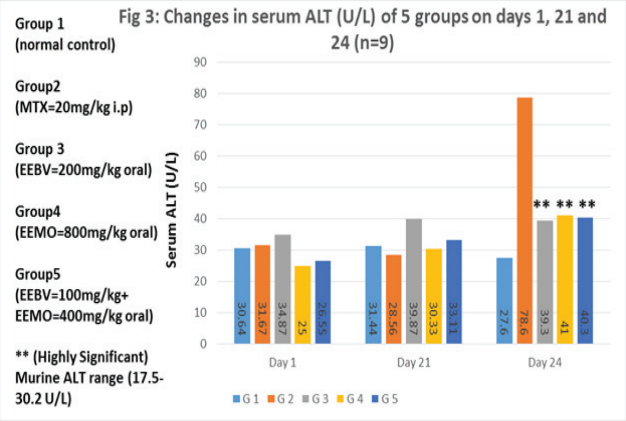


Fig 3: Changes in serum ALT (U/L) of 5 groups on days 1, 21 and 24 (n=9)

DISCUSSION

Beta vulgaris commonly called 'chikandar' and *Moringa oleifera* commonly termed 'sohanjana' have extensive medicinal properties.[12,13] MTX a folic acid antagonist[5] used for the treatment of many diseases like Rheumatoid arthritis, psoriasis, leukemias but multiorgan toxicity remains a major drawback of this drug[6].

This research project was designed to evaluate hepatoprotective effect of MO and BV leaves prior to and after MTX administration in rats for 24 days as these aspects had not been investigated before. Evaluations of biochemical parameters (ALP, AST, ALT) were done on day 0, 21 and 24. Quantitative data was analyzed by Mean ± S.D (IQR), and comparison amongst groups was made using ANOVA. P values <0.05 were labeled significant.

At baseline and on day 21, LFTS in all groups were within normal murine range. Hepatotoxicity was initiated on the 21st day in groups 2-5 by giving single intraperitoneal injection of 20mg/kg MTX resulting in elevation of ALP levels of 14.48% in diseased control group 2 as compared to healthy controls indicative of liver damage which worsened by 24th day to 325.47% as no therapeutic intervention was made. All these results were consistent with results of previous literature [15].

However this depreciation in liver function was not seen in other groups where continuous administration of daily single oral doses of EEMO & EEBV extracts for 24 days afforded variable degrees of hepatoprotection. Here in comparison to disease control group an improvement in ALP levels of 86.14%, 80.20 and 86.39% was seen in Groups 3 and 4 administered 200mg /kg of ethanolic extract of *Beta vulgaris* (EEBV) and 800 mg/kg ethanolic extract of *Moringa oleifera* (EEMO) and Group 5 where combined extracts of (100mg/kgEEBV +400mg/kg EEMO) were used respectively. Thus maximum improvement was seen in the combination group where half doses of both EEMO & EEBV were used. No comparable study could be found to support or refute our findings. The difference amongst groups was also significant with p-value <0.001.

A similar profile emerged with AST levels which lay within normal range in diseased and experimental groups at baseline and immediately following methotrexate injection on day 21, gradually increasing to highest values of 77.59% by 24th day in group 2. Following continuous administration of EEBV, EEMO, and their combined extracts AST values of groups 3, 4 and 5 showed a 66.1%, 62.77% and 66.77% improvement as compared to the diseased control group. The difference among groups was also significant with p-value <0.001.

The pattern was retained for serum ALT values as well where Groups 3,4 and 5 showed a significant (50%,47.8% & 48.72%) improvement in ALT by the end of the study by day 24 as compared to group 2(MTX). The overall difference was significant with p-value <0.001 supporting hepatoprotective nature of plants used in our research project.

The overall consistent improvement in LFTS noted in the *Moringa oleifera* and *Beta vulgaris* treated groups could be attributed to the presence of antioxidants like tannins, saponins, glycosides, flavonoids and alkaloids which are widely distributed in plants. These antioxidants have

medical functions such as anti-inflammatory actions[16]. The active principles contained within exert antioxidant effect and augment the defense system by inhibiting the production of reactive oxygen species (ROS)[17]. Hence the presence of these antioxidants may be contributory to hepatoprotective effect of these plants.

Different studies have reported these active constitutes to be the main reason behind protective role of herbs where hepatoprotective effect of EEBV and EEMO leaves was observed against ethanol and acetaminophen drugs mediated hepatotoxicity respectively[12,18]. Beta vulgaris root has also shown hepatoprotective action against CCL4 induced hepatic injury in rats[19], while similar protection has been afforded by *Moringa oleifera* against antituberculous drugs induced hepatotoxicity[20].

The significant hepatoprotective activities of these plants justify their traditional use in the prevention of toxicities induced by MTX. Pharmacodynamic studies should be undertaken to establish the mechanism of action of these plant extracts.

CONCLUSION

On the basis of results of our research project, it can be concluded that EEBV and EEMO and their combined extracts possess hepatoprotective properties, which may be of potential benefit for the management of hepatotoxicity induced by MTX. Moreover, in our study we did not observe any adverse effect of the plants which indicates safety of this plant if used as potential medicine.

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