

Rice bran phytic acid protects against methotrexate -induced oxidative stress and acute liver injury in rats

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Abstract

Methotrexate is an effective and extensively used chemotherapeutic agent to treat range of malignancies, but its therapeutic use is limited because of dose dependent hepatotoxicity. Several published reports advocate that supplementation with antioxidant can influence methotrexate induced acute liver injury. Twenty four adult male rats (aged 56 days and weighted $138 \pm 8.8g$) were randomly assigned into four equal groups (control and three treated groups), first group as control group given normal saline, a second single injection 20mg /kg B.W. i.p. of methotrexate, third group given 15mg/kg B.W. of phytic acid, then given single injection 20mg /kg B.W. i.p. of methotrexate and fourth group given 15mg/kg B.W. of phytic acid for 10 days, and male rats were sacrificed. Blood and liver, subcellular fluid was obtained to assess subcellular activity of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), reduced glutathione (GSH), glutathione reductase, glutathione-S-transferase (GST), glutathione peroxidase (GPx), catalase, quinone reductase (QR), superoxide dismutase (SOD), γ -glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), lipid peroxidation, glucose-6-phosphate dehydrogenase (G6PD) and total protein. The results of liver injury rats (T) showed significant increased activity of ALT, AST, GGT, LDH, decreased GSH, GR, GST, GPx, CAT, QR, SOD, ($p < 0.05$) when compared with the control, and histological findings further supported the protective effects of phytic acid against methotrexate induced acute liver injury. phytic acid (D) therapy moderated liver damage and normalized the activities of all antioxidant enzymes. In conclusion, present study demonstrate that oxidative stress and liver injury are closely associated with methotrexate induced toxicity and phytic acid shows the protective efficacy against methotrexate induced acute liver injury possibly via attenuating the oxidative stress and inflammatory response.

Key words: Methotrexate, Phytic acid, Oxidative stress, Liver injury, Rats.

دراسة التأثير الكابح لحامض الفايترك ضد الاجهاد التأكسدي في ضرر الكبد الحاد المستحدث بلميثوتكسيت

في الجرذان

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الخلاصة:

الميثوتركسيت من العقارات التي تستخدم في نطاق واسع في علاج الاورام السرطانية, الاستخدام العلاجي محدد بضرر الكبد الحاد الذي يرتبط مع الاجهاد التأكسدي, هناك عدة ابحاث تشير الى ان استخدام مضادات الاكسده ممكن يوثبط التسمم الكبدي الناتج عن ميثوتركسيت في الدراسة الحالية تم التركيز على التأثير الوقائي لحمض الفايترك في الضرر الكبدي الحاد المستحدث تجريباً في ذكور الجرذان باستخدام عقار الميثوتركسيت.

استحدث ضرر الكبد الحاد في 18 ± أجريت الدراسة الحالية باستخدام 24 جرذاً ذكراً ناضجاً بعمر 65 يوماً و وزن 138 الاولى المجموعة ضمت 12أجرذ بواسطة حقنة منفردة عقار من الميثوتركسيت ملغمبتركيز 15/كغم وزن الجسم البريتونفي المجموعة الثالثة السيطره جرعت ملحل فسيولوجيملحلول, المجموعة الثانية حقنت بعقار ميثوتركسيت لمدة 10يوم حقنت بعقار ميثوتركسيت بعدو ذلك جرعت حامض الفايترك بتركيز 150ملغم/كغم لمدة 10يوم المجموعة الرابعة جرعت حامض الفايترك بتركيز 150ملغم/كغم يومخراً من التجربة تم التضحة بلحيوانات و جمعت عينات الدم الكبدو وتم تحضر السائل الخلوي بعد لغرض قياس تراكيز أنزيمات المضادة للاكسدة و الانزيمات الاخرى. أضهرت جميع ذكور الجرذان المعاملة بلميثوتركسيت انخفاض معنوي في أنزيمات المضادة للاكسدة و ارتفاع معنوي في أنزيمات التسمم الكبدي مقارنة مع مجموعة السيطرة. أظهرت المجموعة الثالثة ارتفاع معنوي في الانزيمات المضادة للاكسدة و انخفاض معنوي في انزيمات التسمم الكبدي مع المقارنة في المجموعة الثانية.

لوحظ لا يوجد فرق معنوي بين المجموعة الرابعة بلمقارنة مع مجموعة السيطرة.

نستنتج من الدراسة الحالية ان هناك علاقة وثيقة بين تكوين الاجهد التاكسدي و التسمم الكبدي الناتج من الميثوتركسيت, حامض الفايترك له القابلية على تثبيط الضرر الكبدي عن طريق مثبطات الاكسدة و الاستجابة الاتهابية. الكلمات الافتتاحية: الميثوتركسيت, حامض الفايترك, الاجهاد التأكسدي, الجرذان.

Introduction

Methotrexate (MXT) (2S)-24-[(2,4-diaminopteridin-6-yl) methyl methylamino] benzoyl-amino] pentanedioic acid [1], it is a potent antineoplastic agent commonly used in many cancers including headneck, lung, kidney, bladder, ovary and testis tumors [2]. It is an organic platinum derivative in contrast to other antitumoric agents. Despite its potent anti-tumoral effect, it has which restricted its clinical use because toxic side effects such as neurotoxicity, nephrotoxicity and ototoxicity [3], which restricted its clinical use therefore, liver injury also promote as a result of disturbance of metabolites [4]. The available evidence seems to suggest that higher doses of methotrexate causes hepatotoxicity development in spite of hepatic damage [5].

However, liver injury is also progressed which noted during low-dose methotrexate therapy [6]. Oxidative stress

resulting from ROS might be a main acting among these factors [7]. The authors showed the oxidative stress, hepatoprotective also plays a significant effect in methotrexate induced liver injury [8]. as shown previously that the investigate several antioxidant substances in methotrexate induced liver injury [9]. Because it is assumed that methotrexate-related adverse effects can occur through various ways, the idea that different chemical agents that may decrease the potential adverse effects can be used as combination which be reasonable to minimize effect [10].

This study is an attempt to investigated the positive impact of rice bran phytic acid, a potent free radical scavenger and antioxidant, against methotrexate-induced hepatotoxicity [11]. Rice bran phytic acid (PA) is phytate, inositol polyphosphate and inositol

hexakisphosphate (IP6) is a highly phosphorylated molecule abundant in plants and legumes, including corn, soy beans, nuts, wheat bran, rice bran and nuts and many plants bran and seeds [12]. Nutritional or dietary factors are attracting the main deal of concern since long time due to their property a highly effective antioxidant agents [13]. Phytic acid has gained attention because it has notable antioxidant, inflammatory and anticarcinogenic properties [14]. In this study we investigated protective effect of phytic acid, on the liver injury induced by methotrexate in vivo

Materials & Methods

Experimental design

Mature male rats (150-200 g), 6-8 weeks old, were obtained from animal house facility of veterinary faculty/ kufa university. Rats were housed in an animal care facility under room temperature (25 °C) with 12 h light/dark cycles and were given free access to standard pellet diet and tap water. Before the treatment, rats were left for 7 days to acclimatize. To study the effect of prophylactic treatment with phytic acid on methotrexate-induced oxidative stress and hepatotoxicity responses in the liver, four groups each of six male rats were kept in different cages as per the different dose and modulator combinations requirement. Rats were randomly divided in to four groups, six animals in each.

Group I (control group) received only normal saline injection intraperitoneally at a dose of 10 ml/kg body weight from days once for 10 days.

Group II were given with a single ip injection of methotrexate (20 mg/kg body weight, intraperitoneally) at seventh day according to Tugba et al [15].

Groups IV received phytic acid (PA) at the dose of 150 ml/kg orally body weight (b.wt.) from days once for 10 days according to El-Sheikh et al [16].

Group III received both methotrexate and phytic acid treatments as previously indicated.

Preparation of subcellular fluid

Liver tissues were perfused with distilled water until a pink color

appeared. Tissues were homogenized by about 20 up and down strokes in a ground-glass tissue grinder. Sucrose (0.88 M) was used for homogenization, washing and re-suspension of the particulate fractions. Using cooled ultracentrifuge, homogenates were fractionated for obtaining subcellular fluid according to Ayako and Fridovich, 2002 [17].

Activity of reduced glutathione (GSH)

Reduced glutathione was determined by the method method of Habig et al. [18].

Assay for glutathione reductase activity

The GR activity was measured by the method of Carlberg and Mannervik [19].

Assay for glutathione-S-transferase (GST) activity

Glutathione- S- transferase activity was experimentally measured by the method of Habig et al. [20].

Activity of glutathione peroxidase (GPx)

Glutathione peroxidase activity was recorded by the method of Mohandas et al. [21].

Assay for catalase activity

Catalase activity was assayed by the method of Claiborne [22].

Measurement of quinone reductase (QR) activity

The QR activity was estimated by the method of Benson et al. [23].

Measurement of superoxide dismutase (SOD) activity

The SOD activity was determined by the method of Marklund and his colleagues [24].

Assay for γ -glutamyl transpeptidase
 γ -GGT Activity was done by the method of Orłowski et al. [25].

Assay for lactate dehydrogenase (LDH) activity

Lactate dehydrogenase (LDH) activity was determined in serum by the method of Kornberg et al. [26].

Estimation of lipid peroxidation

The assay for microsomal lipid peroxidation was done following the method of Wright et al. [27].

Measurement of glucose-6-phosphate dehydrogenase (G6PD) activity

The activity of glucose-6-phosphate dehydrogenase was determined by the method of Zaheer et al. [28].

Assay for serum aspartate aminotransferase and alanine aminotransferase activity

Alanine aminotransferase (AST) and aspartate aminotransferase (ALT) activity were measured through comparisons [32].

the method of Reitman and Frankel [29].

Measurement of protein

The protein concentration in all samples was measured through the method of Lowry et al. [30]. using bovine serum albumin as the standard.

Histological investigation

For histopathology study, the liver was removed and immediately fixed in freshly prepared 10% neutral buffered formalin at 48°C processed as described [31].

Statistical analysis

The data from individual groups are presented as the mean \pm standard error of the mean (SEM). Differences between groups were analyzed by using one way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test and minimum criterion for statistical significance was set at $p \leq 0.05$ for all

Results

Table 1

Results of pretreatment of phytic acid on glutathione and related enzymes like GSH, GR, GST and GPX on methotrexate induced redox imbalance, values are expressed as mean \pm SEM.

| Treatment groups | GSH (nmol GSH/g tissue) | GR (nmol NADPH oxidized/min/ mg protein) | GST (nmol CDNB conjugate formed/ min/mg protein) | GPX (nmol NADPH oxidized/min/mg protein) |
|--------------------------------------|-------------------------|--|--|--|
| Group I vehicle only | 0.46 \pm 0.004 | 384.72 \pm 11.1 | 81.23 \pm 14.31 | 272.18 \pm 31.12 |
| Group II methotrexate only | 0.31 \pm 0.006 | 189.1 \pm 15.3 | 39.21 \pm 7.36 | 105.71 \pm 12.54 |
| Group IV Phytic acid D+ methotrexate | 0.43 \pm 0.02 | 298.7 \pm 14.6 | 69.23 \pm 3.25 | 187.51 \pm 13.42 |
| Group V Phytic acid D only | 0.47 \pm 0.05 | 393.4 \pm 13.8 | 83.15 \pm 6.71 | 268.71 \pm 11.64 |

Results obtained are significantly different from control group ($p < 0.001$). Results obtained are significantly different from methotrexate treated group ($p < 0.02$, $p < 0.04$ and $p < 0.001$) phytic acid.

Table 2

Results of pretreatment of phytic acid on the level of Catalase, QR and SOD on methotrexate, values are expressed as mean±SEM.

| Treatment groups | Catalase (nmol H ₂ O ₂ consumed/min/mg protein) | QR (nmol NADPH oxidized/min/mg protein) | SOD (units/mg protein) |
|--------------------------------------|---|---|------------------------|
| Group I vehicle only | 123.17 ± 36.4 | 195.6 ± 12.4 | 145.6 ± 2.5 |
| Group II methotrexate only | 85.31 ± 15.6 | 97.61 ± 13.8 | 107.4 ± 3.2 |
| Group IV Phytic acid D+ methotrexate | 101.43 ± 41.38 | 162.21 ± 31.1 | 129.7 ± 4.3 |
| Group V Phytic acid D only | 125.11 ± 31.52 | 197.21 ± 31.5 | 147.3 ± 2.7 |

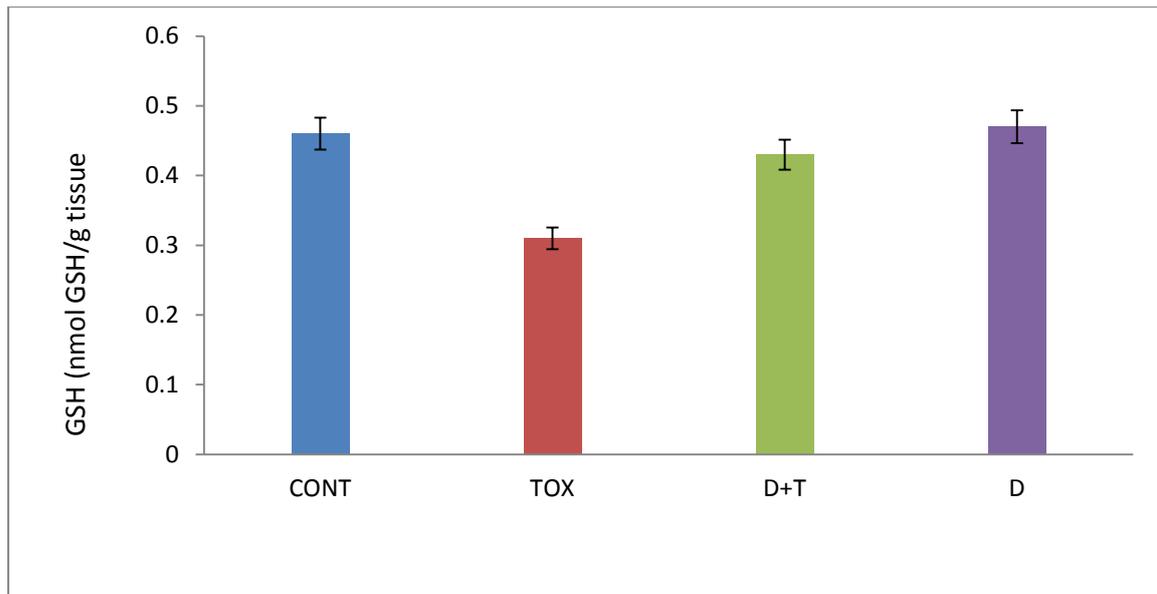
Results obtained are significantly different from control group ($p < 0.003$). Results obtained are significantly different from methotrexate treated group ($p < 0.01$, $p < 0.02$ and $p < 0.005$) phytic acid.

Table 3

Results of pretreatment of phytic acid on serum liver toxicity markers like AST, ALT, LDH and g-GGT on methotrexate induced enhancement, values are expressed as mean±SEM.

| Treatment groups | ALT (IU/L) | AST (IU/L) | g-GGT (nmol p-nitroaniline formed/min/mg protein) | LDH (nmol NADH oxidized/min/mg protein) |
|--------------------------------------|-------------|-------------|---|---|
| Group I vehicle only | 11.34 ± 1.5 | 27.14 ± 2.1 | 491.3 ± 11.8 | 202.5 ± 20.11 |
| Group II methotrexate only | 28.12 ± 1.3 | 55.81 ± 1.7 | 791.4 ± 21.1 | 338.3 ± 8.92 |
| Group IV Phytic acid D+ methotrexate | 17.21 ± 1.9 | 38.53 ± 1.3 | 541.8 ± 14.9 | 220.7 ± 23.41 |
| Group V Phytic acid D only | 10.32 ± 1.1 | 30.17 ± 1.1 | 484.5 ± 13.7 | 198.9 ± 7.38 |

Results obtained are significantly different from control group ($p < 0.002$). Results obtained are significantly different from methotrexate treated group ($p < 0.04$, $p < 0.02$ and $p < 0.0005$) phytic acid.



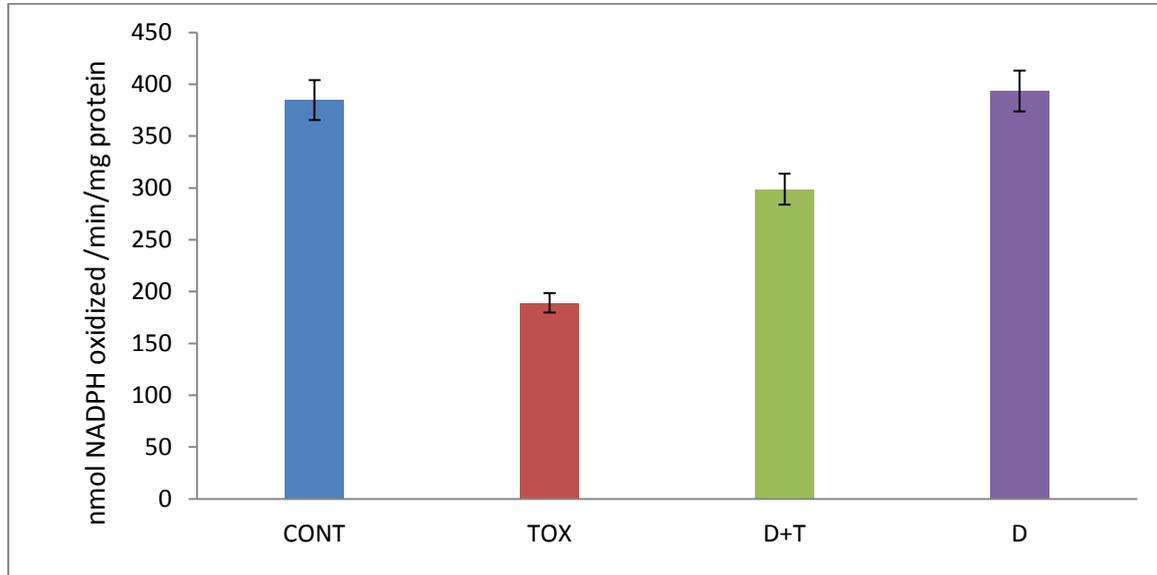
CONT = Salin Only

TOX = MTX Only

D + T = PA + MTX

D = PA Only

Fig. 1. Effect of PA pre-treatment on hepatic GSH induced by MTX. Values are expressed as mean \pm SEM. MDA content was significantly ($p \leq 0.05$) as compared of treated with control group. PA= Phytic acid, MTX = Methotrexate.



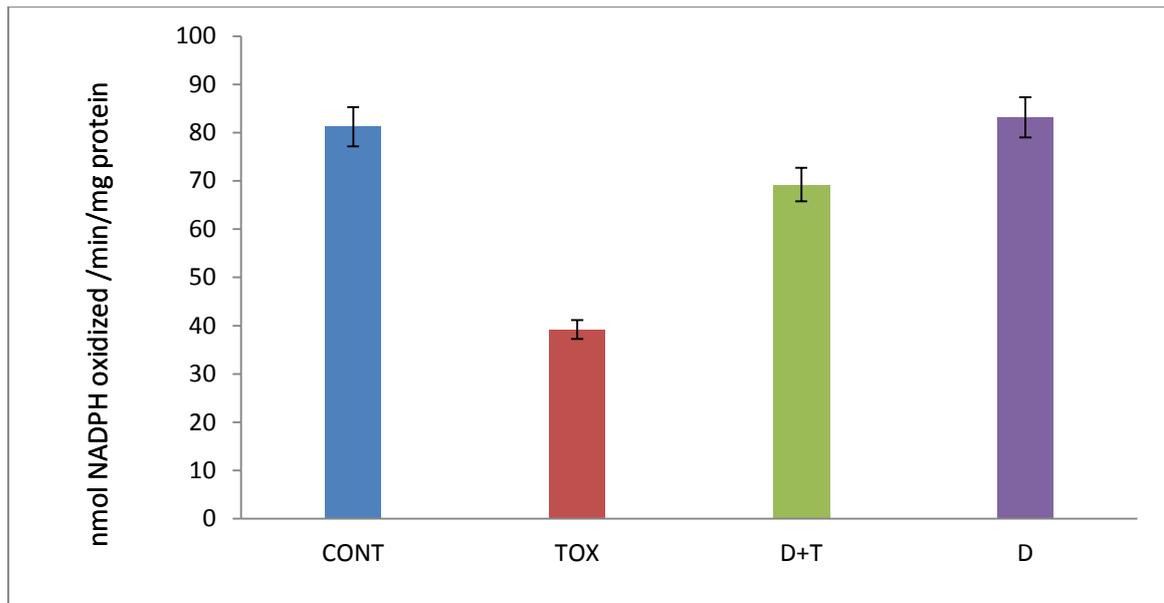
CONT = Salin Only

TOX = MTX Only

D + T = PA + MTX

D = PA Only

Fig. 2. Effect of PA pre-treatment on hepatic GR induced by MTX. Values are expressed as mean \pm SEM. MDA content was significantly ($p \leq 0.05$) as compared of treated with control group. PA= Phytic acid, MTX = Methotrexate.



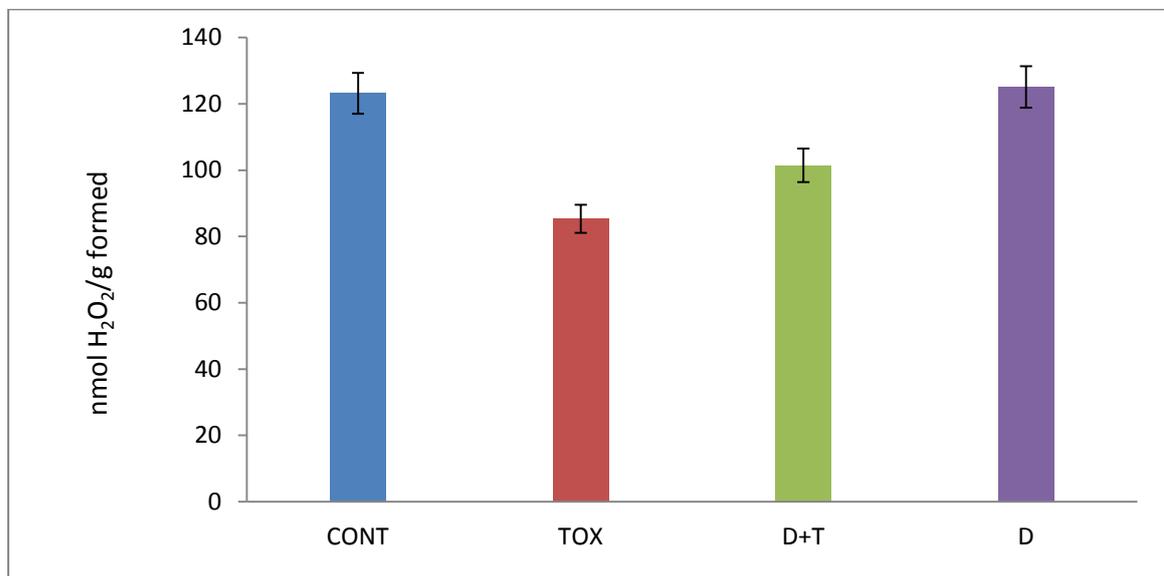
CONT = Salin Only

TOX = MTX Only

D + T = PA + MTX

D = PA Only

Fig. 3. Effect of PA pre-treatment on hepatic GST induced by MTX. Values are expressed as mean \pm SEM. MDA content was significantly ($p \leq 0.05$) as compared of treated with control group. PA= Phytic acid, MTX = Methotrexate.



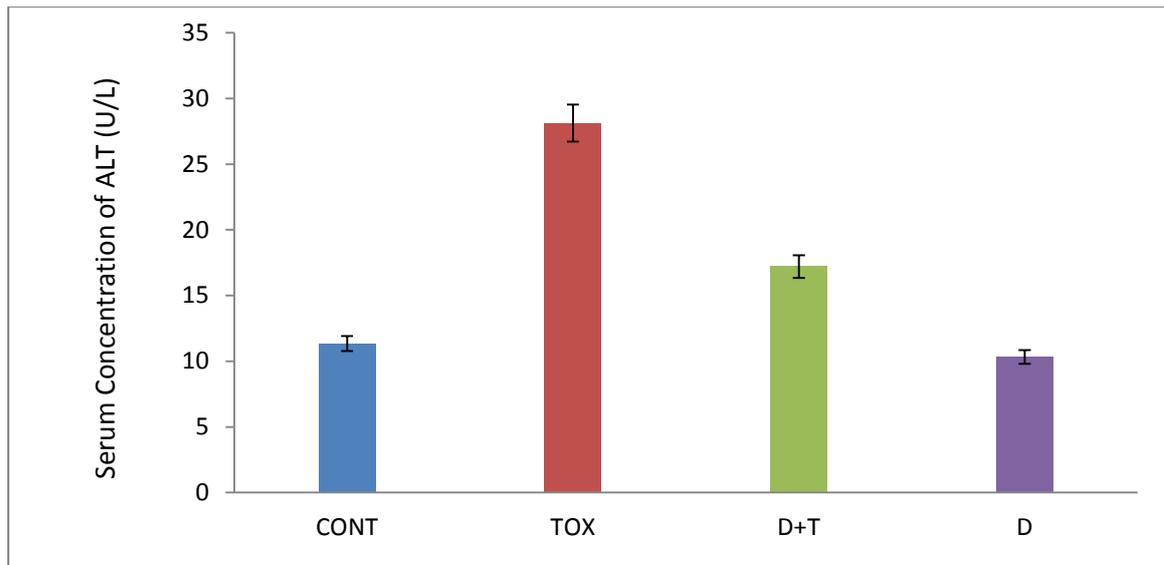
CONT = Salin Only

TOX = MTX Only

D + T = PA + MTX

D = PA Only

Fig. 4. Effect of PA pre-treatment on hepatic Catalase induced by MTX. Values are expressed as mean \pm SEM. MDA content was significantly ($p \leq 0.05$) as compared of treated with control group. PA= Phytic acid, MTX = Methotrexate.



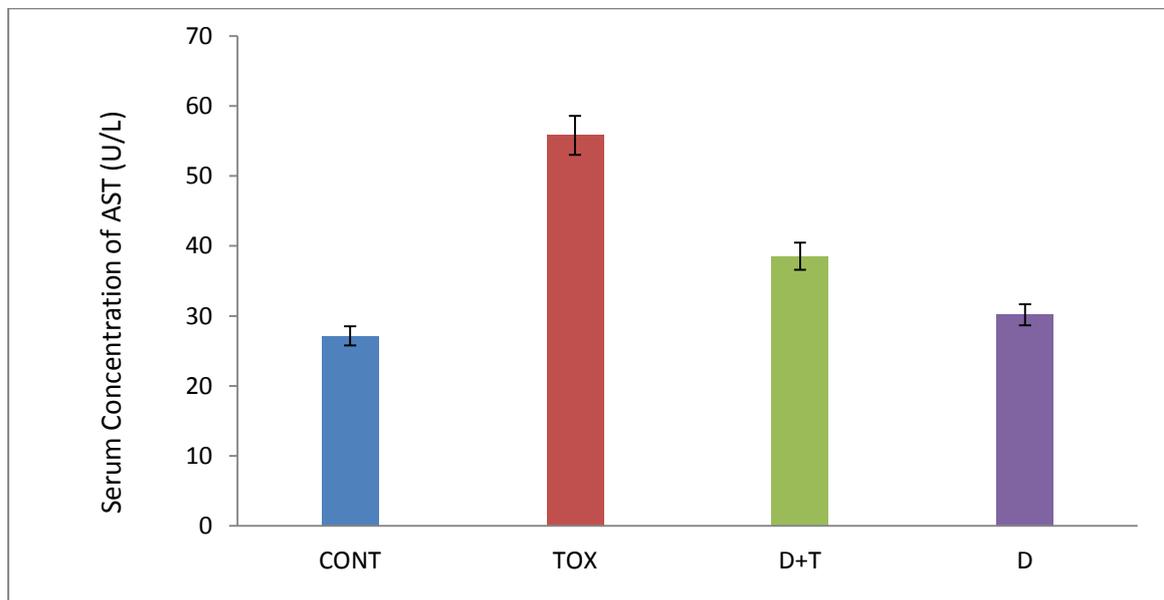
CONT = Salin Only

TOX = MTX Only

D + T = PA + MTX

D = PA Only

Fig. 5. Effect of PA pre-treatment on hepatic ALT induced by MTX. Values are expressed as mean \pm SEM. MDA content was significantly ($p \leq 0.05$) as compared of treated with control group. PA= Phytic acid, MTX = Methotrexate.



CONT = Salin Only

TOX = MTX Only

D + T = PA + MTX

D = PA Only

Fig. 6. Effect of PA pre-treatment on hepatic AST induced by MTX. Values are expressed as mean \pm SEM. MDA content was significantly ($p \leq 0.05$) as compared of treated with control group. PA= Phytic acid, MTX = Methotrexate

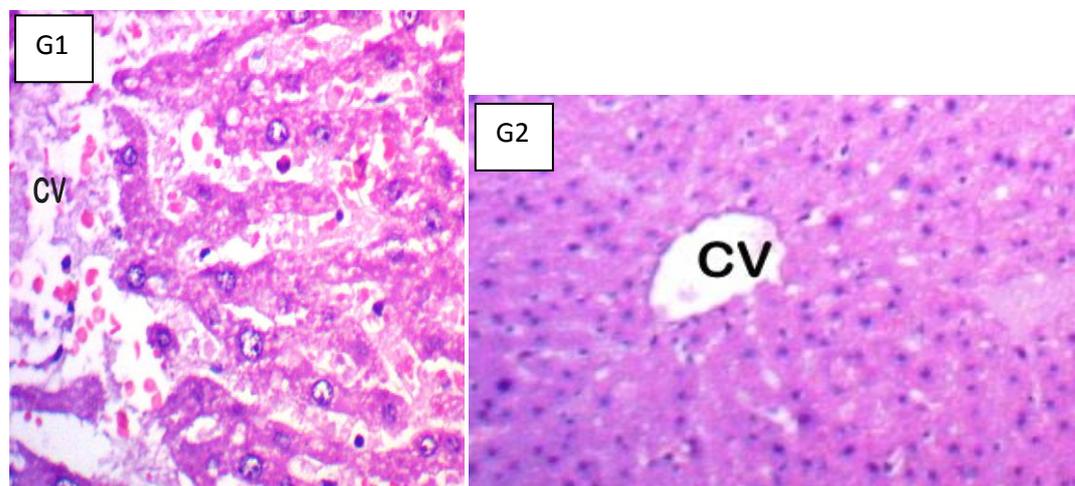


Fig. 7. Effect of phytic acid pretreatment on methotrexate induced hepatic histological alterations (CV) central vein. Representative photomicrographs (magnification $\times 40$), There was marked infiltration of neutrophils and activated sinusoids in methotrexate treated animals (Group II) as compared with only phytic acid treated animals (Group I, IV). However there was no significant difference in the liver histology in the animals only treated with phytic acid (Group IV) as compared to only vehicle treated animals (Group I).

Phytic acid pre-treatment decreased MDA formation MDA formation was measured to demonstrate the oxidative damage on LPO of methotrexate induced liver injury in rats. A significant ($p < 0.002$) increase of the MDA formation was found in the methotrexate treated group when compared with control. We have observed that pre-treatment with phytic acid at leads to the significant ($p < 0.04$ and $p < 0.002$ respectively) prevention of membrane damage when compared to methotrexate treated group. No significant difference was found in the MDA level between control and only D group.

Phytic acid attenuates the serum LDH, ALT and AST activity Protective effect of phytic acid on serum LDH, AST and ALT level observed. Significance change in these parameters was found in the methotrexate treated groups as compared to control ($p < 0.003$). Pre-treatment with phytic acid was found significantly ($p < 0.02$, $p <$

0.003) effective in the normalization of these markers when compared to methotrexate treated group (Figs. 5-6). Phytic acid alone did not show any significant difference as compared to control.

Phytic acid pre-treatment restored the hepatic GSH level Protective effect of phytic acid on hepatic GSH level was marked. The level of GSH was depleted significantly ($p < 0.003$) in methotrexate treated group as compared to control group. Phytic acid pre-treatment increased its level significantly ($p < 0.02$, $p < 0.001$) in III and IV groups as compared to methotrexate treated group. Phytic acid alone pre-treated group exhibited no significant changes in GSH level as compared to control group (Fig. 1).

Phytic acid pre-treatment ameliorates the activities of hepatic antioxidant enzymes Intra-gastric methotrexate ingestion was found to deplete hepatic antioxidant enzymes

(GST, G6PD, GPx, GR and catalase) significantly as compared to control ($p < 0.003$). Pre-treatment with phytic acid before methotrexate ingestion was found significantly effective in restoring these enzymes at both, dose one and dose two ($p < 0.004$). We have observed that there is no significant difference in the activity of these antioxidant enzymes between control and only phytic acid treated group (Tables 1 and 2).

Discussion

This study advances our understanding of attention has been given to natural compounds for the prevention of different human disease [57]. natural compound, rice bran phytic acid comes under the class of flavonoid, is nontoxic ubiquitous and anticarcinogenic [34]. The results thus obtained are compatible with supplementation of rice bran phytic acid before methotrexate administration leads to prevention of hepatic injury. There is a number of findings associated with the methotrexate induce toxicity [58], the correlation between oxidative stress and hepatotoxicity which representative thought multi etiological factors [59]. the imbalance between reactive nitrogen species (RNS) and generation of reactive oxygen species, as resulting from oxidative stress and their clearance by free radicals scavenger defense system like impairment of balance between antioxidants and pro-oxidants[60]. Hepatic LPO by methotrexate ingestion has been largely known to serve as bio-indicator of oxidative stress induced injury in animal experimental module as well as in human studies gone through clinical trials [61]. It was the main purpose of the paper to draw attention to observed a noticeable increase of the LPO in methotrexate ingested group and further it was found that there is a significant impact in LPO by phytic acid pre-treatment.

In spite of lipid peroxidation methotrexate induced hepatic toxicity

mediated through accumulation of protein carbonyl formation, formation of the 1-hydroxyl ethyl radical, formation of lipid peroxidation due to developing of oxidative, and decreases in hepatic antioxidant defense, especially GSH. GSH is the major antioxidative tripeptide in the cell [62], which have essential effect in the detoxification of toxicants, metabolism of nutrients and regulation of various pathways to human body homeostasis. Different nonenzymatic and enzymatic reflex could be effect in GSH mediated scavenging of free radicals and other oxygen species [63]. It has been observed that chronic and acute intake of methotrexate causes GSH depletion both in time as well as dose dependent fashion. Previous studies indicate that chronic alcoholics have shown a variety of approaches to deplete level of GSH [64]. Various mechanisms are considered associated with the hepatic GSH depletion by methotrexate intake probably by oxidative stress, increased lipid peroxidation, binding to acetaldehyde and by affixing GSH discharge from the hepatic tissue and/or by depletion of the synthesis, could be lead for the decrease level of hepatic. Homeostatic balance of GSH activity could limit the oxidative stress and improve methotrexate mediated hepatic toxicity.

Conclusion

In the present study, we have shown that methotrexate ingestion causes significant depletion in the hepatic GSH which is in agreement with the previous observations. Pre-treatment with phytic acid improves the level of liver GSH significantly and also shows to enhance the activity of GSH dependent enzymes like GPx, GR, and G6PD. Catalase plays an important effect in the development of hepatotoxicity by methotrexate.

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