

INVESTIGATION OF NEPHROPROTECTIVE EFFECT OF SILYMARIN AGAINST METHOTREXATE AND IFOSFAMIDE INDUCED TOXICITY IN RATS.

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ABSTRACT

The present study was undertaken to investigate the Nephroprotective effect of silymarin against Methotrexate (MTX) induced nephrotoxicity in rats.

In MTX model rats of either sex (n=6) were pre-treated with silymarin (50mg/kg, *p.o.*) for 6 days in MTX model, MTX toxicity was induced by administering MTX (20mg/kg) on third day by intra-peritoneal route. The influence of prophylactic treatment was analyzed by quantification of Serum/Urinary biomarkers and antioxidants and histopathological observations. Silymarin treatment in presence of MTX was responsible for significant reduction in Serum; Urea, Creatinine, Aspartate transaminase (AST), Alanine transaminase (ALT) and Urinary; Total Protein, Sodium and Potassium compared to MTX control group. Silymarin treatment was also responsible for significant increase in Serum Albumin and antioxidants such as superoxide dismutase (SOD), Glutathione (GSH) and Catalase activities in kidney tissue homogenate compared to MTX control group. Similarly, there was an increase in the urine volume and decrease in the kidney weight in the silymarin treated groups compared to the MTX control group. Results were further supported by histopathological studies. Investigation witnessed the administration of silymarin 50mg/kg dose was effective in normalizing the abnormal conditions of kidney induced by MTX.

Thus investigational finding conclude that silymarin possess potential benefits in treating animals with nephrotoxicity.

Key words: Silymarin, Nephroprotective, MTX.

MATERIALS AND METHODS:

Experimental animals:

Wister rats of either sex weighing 175-250 g were housed in standard polypropylene cages and maintained under controlled room temperature ($25^{\circ} \pm 5^{\circ}\text{C}$) and humidity ($55 \pm 5\%$) in a well-ventilated animal house under 12:12 h light and dark cycle. All the rats were provided with commercially available standard pellet diet, water *ad libitum*. Prior to each study, they were made to fast for 12-14 h but had free access to water. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPSCEA), Govt. of India were followed and prior permission was sought from the institutional animal ethics committee for conducting the study.

Drug and dosage:

Pure sample of silymarin was procured from Sigma Chemicals, USA. Silymarin was dissolved in distilled water then administered orally to the animals by gastric intubation using a force feeding needle. Based on earlier literature review therapeutic dose of silymarin in rat was found to be 50mg/kg the same dose was selected for the present study.¹¹

Treatment:

Albino Wister rats were grouped into 6 groups of 6 rats each. Group I and II were treated with saline and served as normal control and MTX control respectively. For Group III and IV Silymarin treatment was given with a dose of 50mg/kg.¹¹ All treatments were given for six days and by oral route. On third day apart from normal control group all other treatment groups were treated with MTX (20 mg/kg)¹² by intra peritoneal route to induce the nephro-toxicity.

Methods:

Methotrexate(MTX) induced nephrotoxicity in rat:¹²

The Wister rats weighing between 140-160gm were randomly divided into 6 groups of 6 rats each. Twenty four hour after the last treatment different biochemical analysis were performed on collected serum and urine. Then after sacrificing, the rat's kidney were removed and weighed. For half of the kidney samples kidney tissue homogenate (KTH) were prepared with sucrose solution (0.25M) and subjected for antioxidant studies. For remaining kidney samples were subjected for histological examination.

Group-I: Animal kept as normal control (without pre treatment)

Group-II: Animals kept as toxic control (MTX only)

Group-III: Animals treated with Silymarin (50mg/kg)

Group-IV: Animals treated with Silymarin (50mg/kg) for 6 days and subjected to MTX toxicity.

The different parameters estimated were:

1. Serum: Albumin, Creatinine, Urea, Alanine aminotransferase (ALT) & Aspartate aminotransferase (AST).
2. Urine: Urine volume, Na⁺, K⁺ ions & Total Protein.
3. Kidney homogenate: Glutathione Peroxidase, Catalase & Super Oxide Dismutase.
4. Physical parameters: Kidney weight
5. Histological analysis

Kidney Weight

Before the preparation of kidney homogenate, kidneys were placed on butter paper and weighed on an analytical balance to obtain the wet weight. Kidney weight was expressed as percentage body weight.

Histopathological observation:¹³

Representative kidney samples were fixed in 10% neutral buffered formalin for approximately 48 hours, processed, embedded in paraffin, sectioned at 4- μ m, mounted on glass slides, de-paraffinized, and stained with hematoxylin and eosin for subsequent microscopic evaluation

Statistical analysis

Results are expressed as mean \pm SE. Statistical significance was assessed using one-way Analysis of variance (ANOVA) followed by Tukey-karmer multiple comparison tests. P<0.05 was considered significant.

RESULTS:

1) Effect on urine biomarkers:

a) Effect on urine volume:

MTX control demonstrated extremely significant decrease (p<0.001) in urine volume compared to normal control. Silymarin+MTX group showed extremely significant increase (p<0.001) in urine volume compared to MTX control (Table 1).

b) Effect on total protein:

MTX control showed extremely significant increase (p<0.001) in total protein compared to normal control. Silymarin+MTX group showed moderately significant decrease (p < 0.01) in total protein compared to MTX control (Table 1).

c) Effect on sodium:

MTX control documented extremely significant increase (p<0.001) in sodium compared to normal control. Silymarin+MTX group showed extremely significant decrease (p<0.001) in sodium compared to MTX control (Table 1).

d) Effect on potassium

MTX control documented extremely significant increase (p<0.001) in potassium compared to normal control. Silymarin+MTX group showed extremely significant decrease (p<0.001) in potassium compared to MTX control (Table 1).

2) Effect on Serum Biomarkers

Effect on serum albumin

MTX control demonstrated extremely significant decrease (p<0.001) in albumin compared to normal control. Silymarin+MTX group showed moderately significant increase (p < 0.01) in albumin compared to MTX control (Table no 2).

Effect on serum creatinine

MTX control showed extremely significant increase ($p < 0.001$) in creatinine compared to normal control. Silymarin + MTX group showed extremely significant decrease ($p < 0.001$) in creatinine compared to MTX control (Table no 2).

Effect on serum urea

MTX control reported extremely significant increase ($p < 0.001$) in urea compared to normal control. Silymarin+MTX group showed extremely significant decrease ($p < 0.001$) in urea compared to MTX control (Table no 2).

Effect on serum ALT

MTX control showed extremely significant increase ($p < 0.001$) in ALT compared to normal control. Silymarin+MTX group showed extremely significant decrease ($p < 0.001$) in ALT compared to MTX control (Table no 2).

Effect on serum AST

MTX control documented extremely significant increase ($p < 0.001$) in AST compared to normal control. Silymarin+MTX group showed extremely significant decrease ($p < 0.001$) in AST compared to MTX control (Table no 2).

3) Effect on Antioxidants**Effect on GSH in kidney tissue homogenate (KTH)**

MTX control demonstrated extremely significant decrease ($p < 0.001$) in GSH compared to normal control. Silymarin + MTX group showed extremely significant increase ($p < 0.001$) in GSH compared to MTX control (Table 3).

Effect on catalase (KTH)

MTX control reported extremely significant decrease ($p < 0.001$) in catalase compared to normal control. Silymarin+MTX group showed moderately significant increase ($p < 0.01$) in catalase compared to MTX control (Table no 3).

Effect on SOD (KTH)

MTX control documented extremely significant decrease ($p < 0.001$) in SOD compared to normal control. Silymarin+MTX group showed moderately significant increase ($p < 0.01$) in SOD compared to MTX control (Table no 3).

Effect on kidney weight

MTX control demonstrated extremely significant increase ($p < 0.001$) in kidney weight compared to normal control. Silymarin + MTX group showed extremely significant decrease ($p < 0.001$) in kidney weight compared to MTX control. (Table 3)

4) Histopathological analysis: The section of kidney of rats of different groups were stained by haematoxyline and eosin and photographed at magnification 100X.

The kidney of Normal control (figure 1) and only silymarin treated (figure 3) groups showed the normal texture of kidney did not reveal any pathological alterations. The kidney of MTX intoxicated rats (figure 2) showed severe tissue degranulation. Silymarin + MTX treated rats (figure 4) showed recovery compared to toxic group characterised by mild tissue de-granulation.

Table 1: Effect on urine biomarkers against MTX induced nephrotoxicity

Treatment	Urine volume (ml)	Total protein (mg/dl)	Sodium (meq/l)	Potassium (meq/l)
Normal control	14.30±0.5	22.64±1.20	152.50±1.20	4.60±0.04
MTX control	4.50±0.8 ^{***}	40.34±1.78 ^{***}	246.40±1.78 ^{***}	6.40±0.02 ^{***}
Silymarin	10.50±0.6 ^{###}	25.65±1.00 ^{###}	156.90±1.00 ^{###}	2.50±0.05 ^{###}
Silymarin + MTX	8.60±0.80 ^{**###}	31.45±1.40 ^{**###}	208.04±1.40 ^{**###}	3.10±0.03 ^{**###}

Values are expressed as MEAN±SEM. n=6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to normal. ## $p < 0.01$, ### $p < 0.001$ when compared with MTX control.

Table 2: Effect on serum biomarkers against MTX induced nephrotoxicity

Treatment	ALBUMIN (mg/dl)	CREATININE (mg/dl)	UREA (mg/dl)	ALT (U/L)	AST (U/L)
Normal control	28.54±1.98	0.68±0.04	21.54±1.11	47.77±1.23	67.42±1.87
MTX control	9.99±1.87 ^{***}	2.22±0.05 ^{***}	64.87±1.21 ^{**} *	134.00±1.54 ^{**} *	143.00±1.54 ^{***}
Silymarin	22.43±1.65 ^{###}	0.98±0.08 ^{###}	32.11±0.23 ^{##} #	75.76±2.76 ^{###}	79.88±2.11 ^{##} #
Silymarin+MTX	19.22±1.34 ^{**###}	1.32±0.02 ^{**###}	54.99±0.23 [*] **###	85.22±2.77 ^{**} *###	90.33±1.55 [*] **###

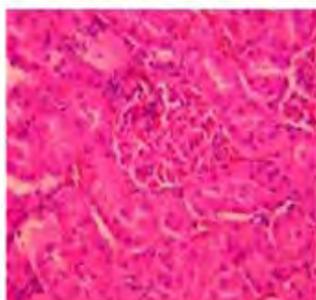
Values are expressed as MEAN±SEM. n=6. **p < 0.01, ***p<0.001 when compared to normal. ##p < 0.01, ###p<0.001 when compared with MTX induced.

Table 3: Effect on antioxidants in MTX induced nephrotoxicity

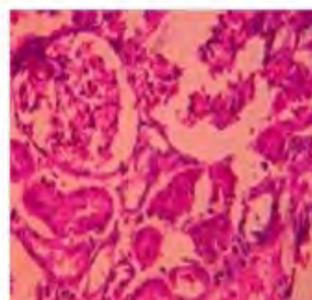
Treatment	GSH U/Mg	CATALASE U/Mg	SOD U/Mg	KIDNEY WEIGHT Gms
Normal control	4.65±0.22	40.33±1.97	8.45±0.21	1.20±0.02
MTX control	2.01±0.11 ^{***}	20.21±1.65 ^{***}	4.11±0.32 ^{***}	1.60±0.01 ^{***}
Silymarin	4.05±0.43 ^{###}	35.11±1.78 ^{###}	7.03±0.43 ^{###}	1.30±0.03 ^{###}
Silymarin+MTX	3.90±0.11 ^{**###}	30.76±1.67 ^{**###}	6.32±0.63 ^{**###}	1.40±0.04 ^{**###}

Values are expressed as MEAN±SEM. n=6. *p < 0.05, **p < 0.01, ***p<0.001 when compared to normal. ##p < 0.01, ###p<0.001 when compared with MTX induced.

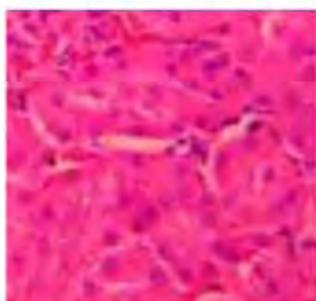
Histopathological studies:



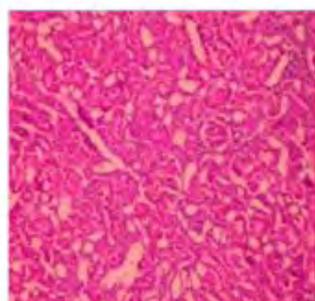
(1) Normal Control
Normal Texture Of Tissue



(2) MTX Control
Severe Tissue Degranulation



(3) Silymarin
Normal texture of tissue



(4) Silymarin+MTX
Mild tissue degranulation

DISCUSSION:

The aim of the present study was to investigate the nephroprotective activity of silymarin against methotrexate (MTX) induced toxicity in rats.

Observed results suggested that silymarin (50mg/kg, p.o.) showed beneficial results. silymarin indicated better results against MTX induced nephro toxicity in rats.

Silymarin (*Silybum marianum*) a polyphenolic flavanoid is having a rich source of flavonoids. Silymarin was proven to be antioxidants which elevate the levels of antioxidant biomarkers, and possess anti carcinogen. It reduces the chances of development of certain cancer. It also has the hepato protective activity. It is used for the treatment of numerous liver disorders and also able to neutralize the hepato toxicity of several agents like ethanol, paracetamol and carbon tetrachloride.¹⁰

Methotrexate (MTX) belongs to the group of antimetabolites is a drug of choice against many types of cancer including osteosarcoma, certain non-Hodgkin lymphomas (NHL), acute lymphocytic leukaemia (ALL) and malignant mesothelioma.

In MTX induced nephrotoxicity model, toxicity was induced by treating the experimental animals with dose of 20 mg/kg by intra-peritoneal route. MTX is responsible for development of nephrotoxicity by the following mechanism. MTX and its active metabolites compete for the folate binding site of the enzyme dihydrofolate reductase. Folic acid must be reduced to tetrahydrofolic acid by this enzyme for DNA synthesis and cellular replication to occur. Competitive inhibition of the enzyme leads to blockage of tetrahydrofolate synthesis, depletion of nucleotide precursors, and inhibition of DNA, RNA, and protein synthesis. MTX also inhibits thymidylate synthase and the transport of reduced folates into the cell.⁵

In this present study, it has been demonstrated that significant increase in urine biomarker level such as total protein, sodium, potassium level. It was also revealed significant decrease urine volume was seen in the MTX intoxicated rat.

Treatment with silymarin reversed the elevated levels of all the urine biomarkers such as total protein, sodium, potassium decreased urine volume to the near normal levels in the model.

It has also shown that significant increase in serum biomarkers level such as creatinine, urea, AST, ALT and significant decrease in albumin level. It was also revealed significant decrease in antioxidants like GSH, catalase, SOD.

Treatment with silymarin reversed the elevated levels of all the serum markers such as creatinine, urea, ALT, AST and decreased level of albumin, decreased antioxidant enzyme to the near normal levels in this model. The histopathological parameters of MTX induced nephrotoxicity were normalized by the treatment with silymarin.

In this this model it has been reported that significant increase in serum biomarkers level such as creatinine, urea, AST, ALT and significant decrease in albumin level was seen.

Treatment with silymarin reversed the elevated levels of all the serum markers such as creatinine, urea, ALT, AST and decreased level of albumin, decreased antioxidant enzyme to the near normal levels in this model.

Silymarin is a potent anti-oxidant. It neutralizes free radicals and restores SOD, GSH and catalase levels in tissue. This adaptogenic property helps to prevent kidney from free radical stress.

silymarin maybe recommended as a nephro protective agent to attenuate toxicity of some valuable drugs such as MTX that currently have a high likelihood of inducing nephrotoxicity.

CONCLUSION:

With the findings of the present study it can be concluded that the Silymarin has demonstrated significant nephroprotective effect against methotrexate (MTX) induced nephrotoxicity in rats.

Treatment with silymarin reversed the MTX induced elevated levels of all the urine biomarkers such as total protein, sodium, and potassium, decreased urine volume near to the normal levels.

It is worth mentioning that silymarin efficiently trims down the elevated levels of serum biomarkers such as AST, ALT, creatinine and urea. It has also restored antioxidant parameters without producing any adverse effect. The results from the present study and histological analysis indicate the administration of silymarin has protective effects against MTX induced renal necrosis state.

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