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## Hepatoprotective Effect of Silymarin on Arthritic Rats Treated with Leflunomide

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### ABSTRACT

The disease-modifying antirheumatic drug leflunomide is associated with hepatic toxicity as a side effect. This study aimed to lesser hepatic toxicity of leflunomide by using silymarin in combination with leflunomide to avoid the hepatic toxicity resulting from using leflunomide in treatment of rheumatoid arthritis. Drugs were orally administered after arthritis was induced by subplantar injection of 0.1 mL Freund's complete adjuvant. Oral administration of leflunomide and silymarin inhibited the dermal hypersensitivity of adjuvant-induced arthritis and significantly reduced the paw oedema in comparison to the control group. This combination normalized serum nitric oxide concentration. Leflunomide caused a significant elevation of the serum liver enzymes activity Glutamate-Oxalate Transaminase and Glutamate-pyruvate Transaminase which were improved by silymarin co-treatment. These results revealed that silymarin has positive effect on adjuvant-induced arthritis and marked antioxidant activity which minimized the toxic effect of leflunomide on the liver. In conclusion silymarin could be used in combination with leflunomide to minimize its toxic effect on liver.

**Key words:** Rheumatoid arthritis, leflunamide, silymarin, malondialdehyde, reduced glutathione

### INTRODUCTION

Leflunomide inhibits the mitochondrial dihydroorotate dehydrogenase, as fourth enzyme step of pyrimidine synthesis pathway (Breedveld and Dayer, 2000). It has immunomodulatory effect and anti-inflammatory activity which was suitable to treat rheumatoid arthritis in 1998 (Alcorn *et al.*, 2009). Drugs metabolized by CYPs to reactive metabolites consequently react with cellular macromolecules and subsequently disable hepatocellular homeostasis (Lammert *et al.*, 2010). Although, leflunomide is metabolized *in vivo* in both humans and experimental animals, its metabolic pathways and the relationship to hepatotoxicity is indistinct (Rozman, 2002).

Silymarin purithoughfied extract is widely used to treat toxic effects in the liver. Further purification into individual compounds is difficult, expensive and therefore not commercially available (Sonnenbichler *et al.*, 1996). The aim of the present study was for identifying the risk factors and understanding the mechanisms of leflunomide-induced liver injury are of critical

importance. Therefore to minimize hepatic toxicity of leflunomide by using silymarin, to provide more detailed evaluation of the anti-inflammatory and anti-arthritic effects produced by silymarin in the rat.

## **MATERIALS AND METHODS**

**Animals:** Adult albino wistar rats, weighting 120-130 grams, were obtained from the animal house colony in National Research Center (Giza, Egypt). Animals were housed in a conditioned room at  $25\pm 2^{\circ}\text{C}$ , standard diet and tap water source were supplied *ad libitum*.

**Drugs:** Leflunomide, N-[4-trifluoro-methylphenyl]-5-methylisoxazol-4-carboxamide; was obtained from Aventis Pharma, (Cairo, Egypt). Silybum marianum was a provided from Sedico Pharmaceutical Company (Cairo, Egypt) as pure powder. The dose was calculated according to Paget and Barnes (1964).

### **Methods**

**Adjuvant-induced arthritis:** It was done according to Newbould (1963) method animals were divided into six groups, each consisting of 8 rats. One group acts as control normal and in five groups, adjuvant arthritis was induced in the right hind paw by subplantar injection of 0.1 mL Freund's Complete Adjuvant (FCA) on zero day to induce inflammation. Drugs were orally administration according to the following scheme starting daily from 10th day till 39th day. Group (1): Received saline and served as arthritic control, group (2): Received leflunomide in dose of ( $10\text{ mg kg}^{-1}$ ), group (3): Received silymarin in dose of ( $100\text{ mg kg}^{-1}$ ), group (4): Received combination of leflunomide and silymarin, finally group at the same time and group (5): Received silymarin for two weeks followed by leflunomide for another two weeks, at the end of experiment.

**Estimation of stable oxidation products of nitric oxide (NO):** Reduction of nitrate by vanadium (III) combined with detection by the acidic Griess reaction according to Miranda *et al.* (2001). method, using an plate reader (Bio-Tek Instruments, Inc.).

**Assessment of liver function:** The activity of serum Glutamate-Oxalate Transaminase (sGOT) and Glutamate-pyruvate-Transaminase (sGPT) were measured according to the method described by Reitman and Frankel (1957).

**Assessment of antioxidant properties:** Lipid peroxides were determined according to the method described by Ruiz-Larrea *et al.* (1994) and expressed as  $\text{nmol g}^{-1}$  wet tissue and expressed as  $\text{nmol g}^{-1}$  wet tissue. Lipid peroxidation products were estimated by the determination of the level of TBARS that were measured as malondialdehyde (MDA). The latter is the decomposition product of the process of lipid peroxidation and is used as an indicator of this process. The principle of the assay depends on the colorimetric determination of a pink pigment product, resulting from the reaction of TBARS with thiobarbituric acid (TBA) in an acidic medium, at high temperature. Reduced glutathione (GSH) content was determined in stomach homogenates according to the method of Ellman (1959). The data were expressed as  $\mu\text{mol g}^{-1}$  tissue. The method depends on the fact that both protein and non-protein thiol (SH-) groups (mainly GSH) react with Ellman's reagent [5,5'-dithiobis (2-nitrobenzoic acid)] to form a stable yellow color of 5-mercapto-2-nitrobenzoic acid, which can be measured colorimetrically at 412 nm.

**Statistical analysis:** Values were expressed as Means±SE. Results experiments were analyzed using one way ANOVA followed by Least Significant Difference (LSD) multiple comparisons test.  $p < 0.05$  was accepted as being significant in all types of statistical tests. Statistical analysis of results, were done using software SPSS 14.

## RESULTS

**Effect on paw edema induced by FCA:** Induction of arthritis by 0.1 mL FCA was accompanied with significant increased in paw edema reaching about 106% of the normal value. Oral treatment of leflunomide and silymarin starting from day 10th of induction succeeded to cause decrease in paw edema. Moreover, the combination of them decreased paw edema significantly compared to arthritic group (Fig. 1).

Adjuvant arthritis was induced in the right hind paw by subplantar injection of 0.1 mL FCA. The magnitude of swelling of the injected hind paw was measured and paw volume % change was calculated. Saline was administrated in one group of animals orally and the group served as control. LEF ( $10 \text{ mg kg}^{-1}$ ) and SIL ( $100 \text{ mg kg}^{-1}$ ) and their combination were administrated orally starting from day 10th of induction. Paw volume was duplicated measured just prior to adjuvant injection and every three days for 39 days after adjuvant injection using water displacement plethysmometer and the mean values were recorded. Results are expressed as Means±SEM ( $n = 8$ ). Significantly different from arthritic group at  $p < 0.05$ . Statistical analysis was done using one way ANOVA followed by LSD for multiple comparisons, respectively.

**Effect on serum nitric oxide:** There was a significant increase in NO level was observed in arthritic groups by 92% compared to normal group. Conversely, leflunomide, silymarin and their compination are normalized serum nitric oxide (Fig. 2).

Adjuvant arthritis was induced in the right hind paw by subplantar injection of 0.1 mL FCA. The magnitude of swelling of the injected hind paw was measured and paw volume % change was

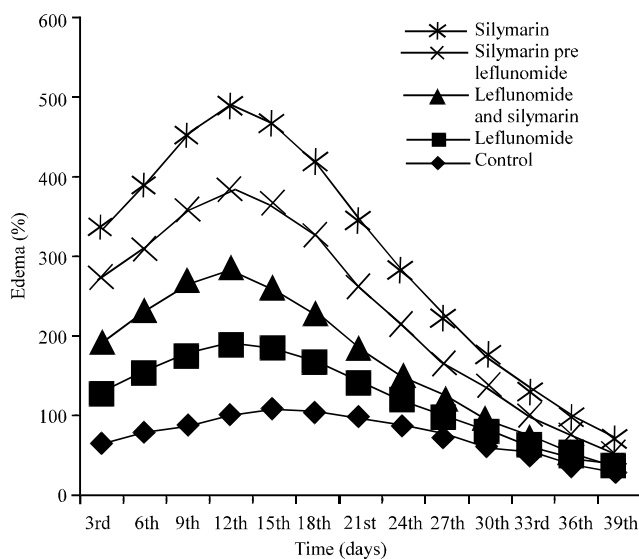


Fig. 1: Effects of leflunomide, silymarin and their combination on Freund's complete adjuvant-induced paw edema in rats

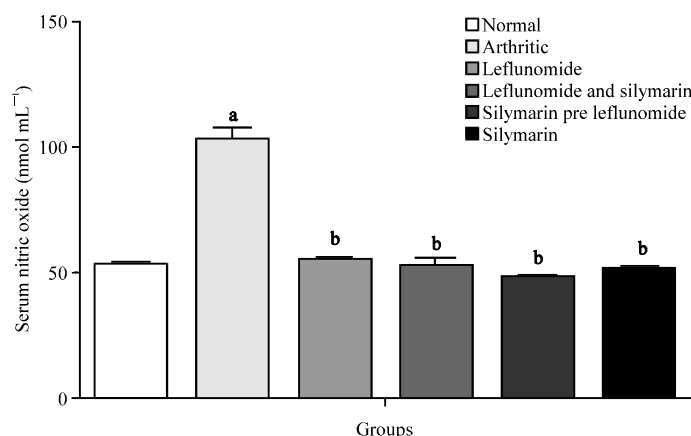


Fig. 2: Effects of leflunomide, silymarin and their combination on serum nitric oxide level of adjuvant-induced arthritic rats

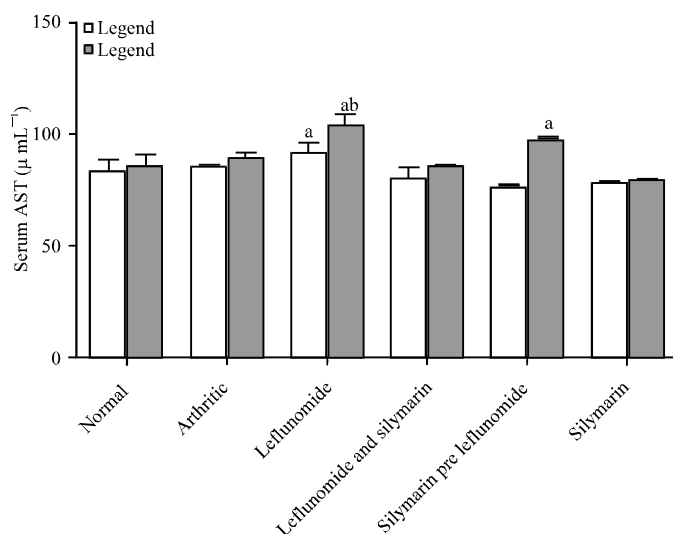


Fig. 3: Effects of leflunomide, silymarin and their combination on serum AST level of adjuvant-induced arthritic rats

calculated. Saline was administrated in one group of animals orally and the group served as control. LEF (10 mg kg<sup>-1</sup>) and SIL (100 mg kg<sup>-1</sup>) and their combination were administrated orally starting from day 10th of induction. At the end of experiment period, blood was collected from the retro-orbital venus plexus. Then serum nitric oxide level was measure. Results are expressed as Means±SEM (n = 8). An (a)-Significant difference from normal rats p<0.05 and (b) Significant difference from hyperglycaemic rats p<0.05.

### Effect on liver function

**Effect on serum AST:** Induction of arthritis by 0.1 mL FCA did not show any change in the serum AST level. However, treatment with leflunomide (10 mg kg<sup>-1</sup> day<sup>-1</sup> o.p.) showed an increase in the serum AST level reaching about 22% of the normal value. On the other hand, combination of leflunomide and silymarin did not change in the serum AST level (Fig. 3).

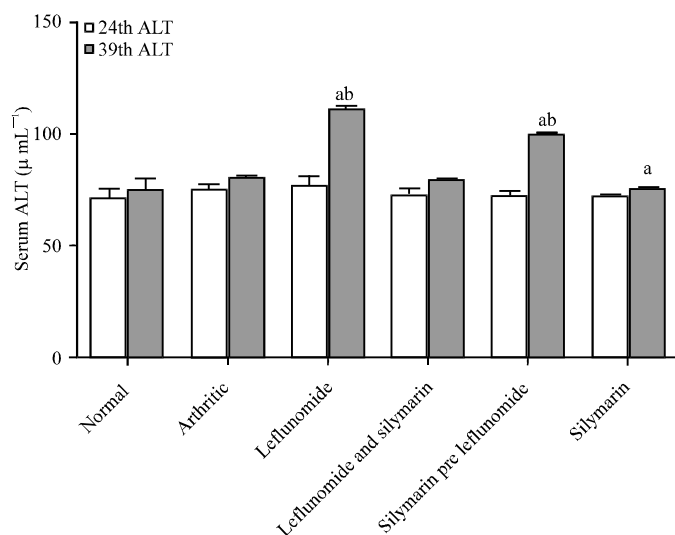


Fig. 4: Effects of leflunomide, silymarin and their combination on serum ALT level of adjuvant-induced arthritic rats

Adjuvant arthritis was induced in the right hind paw by subplantar injection of 0.1 mL FCA. The magnitude of swelling of the injected hind paw was measured and paw volume % change was calculated. Saline was administrated in one group of animals orally and the group served as control. LEF (10 mg kg<sup>-1</sup>) and SIL (100 mg kg<sup>-1</sup>) and their combination were administrated orally starting from day 10 till 39th of induction. Blood samples from animals were withdrawn using heparinized capillary tubes and serum was used for AST determination after 24 and 39 consecutive days of FCA injection. Results are expressed as Means±SEM (n = 8). An (a) Significant difference from normal rats p<0.05 and (b) Significant difference from hyperglycaemic rats p<0.05.

**Effect on serum ALT:** Induction of arthritis by 0.1 mL FCA did not show any change in the serum ALT level. However, treatment with leflunomide (10 mg kg<sup>-1</sup> day<sup>-1</sup> o.p.) showed an increase in the serum ALT level reaching about 48% of the normal value. Whereas silymarin in combination with leflunomide was normalized the elevated serum ALT level (Fig. 4).

Adjuvant arthritis was induced in the right hind paw by subplantar injection of 0.1 mL FCA. The magnitude of swelling of the injected hind paw was measured and paw volume % change was calculated. Saline was administrated in one group of animals orally and the group served as control. LEF (10 mg kg<sup>-1</sup>) and SIL (100 mg kg<sup>-1</sup>) and their combination were administrated orally starting from day 10th till 39th of induction. Blood samples from animals were withdrawn using heparinized capillary tubes and serum was used for ALT determination after 24 and 39 consecutive days of FCA injection. Results are expressed as Means±SEM (n = 8). An (a) Significant difference from normal rats p<0.05 and (b) Significant difference from hyperglycaemic rats p<0.05.

**Effect on liver homogenate MDA:** Induction of arthritis by 0.1 mL FCA showed by 24% increase in the liver MDA level. Furthermore, treatment with leflunomide (10 mg kg<sup>-1</sup> day<sup>-1</sup> o.p.) showed an increase in the liver MDA level reaching about 35% of the normal value. Conversely, treatment with silymarin leflunomide decreased the liver MDA level of the normal value (Fig. 5).

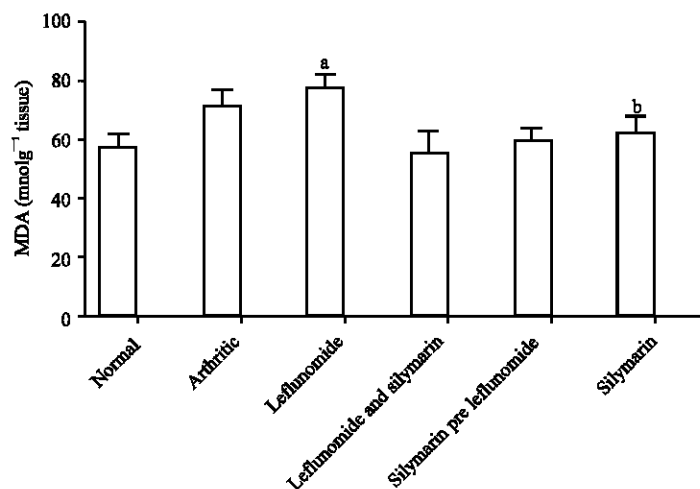


Fig. 5: Effects of leflunomide, silymarin and their combination on liver thiobarbituric acid reactive substances (MDA) content of adjuvant-induced arthritic rats

Adjuvant arthritis was induced in the right hind paw by subplantar injection of 0.1 mL FCA. The magnitude of swelling of the injected hind paw was measured and paw volume % change was calculated. Saline was administrated in one group of animals orally and the group served as control. LEF (10 mg kg<sup>-1</sup>) and SIL (100 mg kg<sup>-1</sup>) and their combination were administrated orally starting from day 10th of induction. At the end of experiment period, animals were sacrificed by cervical dislocation under ether anesthesia. Liver were dissected and part of each liver was homogenized and the homogenate was used for the determination of liver thiobarbituric acid reactive substances (MDA) content. Each value represents the mean of 8 rats±SE. Statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) multiple comparisons test:

- Significantly different from normal group at p<0.05
- Significantly different from control group at p<0.05

**Effect on liver homogenate GSH:** Induction of arthritis by 0.1 mL FCA showed 17% in the liver GSH level. Furthermore, treatment with leflunomide (10 mg kg<sup>-1</sup> day<sup>-1</sup> o.p.) showed decrease in the liver GSH level reaching about 28% of the normal value. Conversely, treatment with silymarin reversed the decrease in the liver GSH content elicited by FCA or leflunomide when compared to the control group (Fig. 6).

Adjuvant arthritis was induced in the right hind paw by subplantar injection of 0.1 mL FCA. The magnitude of swelling of the injected hind paw was measured and paw volume % change was calculated. Saline was administrated in one group of animals orally and the group served as control. LEF (10 mg kg<sup>-1</sup>) and SIL (100 mg kg<sup>-1</sup>) and their combination were administrated orally starting from day 10th of induction. At the end of experiment period, animals were sacrificed by cervical dislocation under ether anesthesia. Liver were dissected and part of each liver was homogenized and the homogenate was used for the determination of liver reduced glutathione content (GSH) content. Each value represents the mean of 8 rats±SE. Statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) multiple comparisons test:

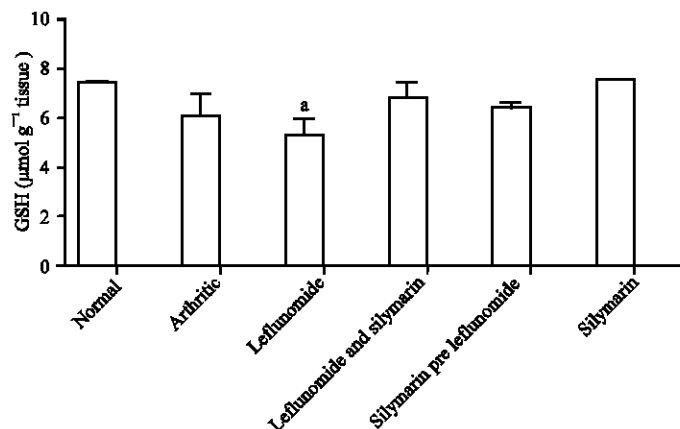


Fig. 6: Effects of leflunomide, silymarin and their combination on serum GSH level of adjuvant induced arthritic rats

- Significantly different from normal group at  $p < 0.05$
- Significantly different from control group at  $p < 0.05$

## DISCUSSION

Leflunomide converted to active metabolite (malononitrilamide) (Schuna and Megeff, 2000), that excreted in the bile, but it is efficiently reabsorbed by enterohepatic recirculation (Bartlett *et al.*, 1996).

In rheumatic diseases, oxidative stress resulted from migration of active phagocytes and leukocytes into synovial and periarticular tissues (Aaseth *et al.*, 1998). Therefore, supplementation of antioxidants resulted in benefit effect in rheumatoid arthritis.

Findings of the present study indicate that leflunomide in a dose of  $10 \text{ mg kg}^{-1}$  showed significant decreased in rat paw volume in comparison to control group; silymarin had less effect than leflunomide. Additionally, their co-administration showed same manner like leflunomide on FCA model.

The anti-arthritic activity of leflunomide may be attributed to inhibition of de novo pyrimidine synthesis in activated T cells by selective inhibition of DHODH (Fox *et al.*, 1999). It has also other activities as inhibition of tyrosine kinase is one of the most important mechanisms of action of leflunomide (Xu *et al.*, 1997). Leflunomide also inhibits the expression of cell adhesion molecules, which facilitate cellular interactions involved in antigen presentation, secretion of cytokines and production of matrix metalloproteinases that degrade articular cartilage and bone (Kraan *et al.*, 2000).

Leflunomide significantly decreased serum nitric oxide level in arthritic rats, may be due to inhibition of iNOS activation (Jankovic *et al.*, 2000; Miljkovic *et al.*, 2001). Furthermore, treatment with silymarin showed a significant decrease on serum nitric oxide level in comparison to arthritic group.

Silymarin was less active and only at the highest concentration in modifying iNOS expression their inhibitory activity on  $\text{NO}^{-2}$  production. Effect of co-administration of both leflunomide and silymarin on the same parameters showed improvement in liver enzymes. While pre-treatment of silymarin to leflunomide did not show significant improvement in liver enzymes.

According to the results, leflunomide significantly increased liver lipid peroxides content, while it was significantly decreased GSH represented in liver homogenate of arthritic rats. Peroxidation



of lipids is a common consequence of tissue damage by free radicals and the presence of the reaction product malondialdehyde (MDA) is an indication that such process has taken place (Furuno *et al.*, 1998).

On the other hand, silymarin normalized lipid peroxides contents and GSH content in liver homogenate of arthritic rats as silymarin was used pretreatment with leflunomide or even together.

## CONCLUSION

Finding of the present study suggest that silymarin has anti arthritic activity which may be related to its anti oxidant effect and decreasing of NO as one of the most important mediators of inflammation. The present study indicates that it is better to use leflunomide with silymarin that control the liver toxicity resulted from leflunomide treatment in arthritic rats.

## ACKNOWLEDGMENT

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