# Evaluation of The Cinical Use of Silymarin in Knee Osteoarthritis: Application of the Dual Inhibitory Concept of Cyclooxygenase and 5-Lipoxygenase

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## ABSTRACT:

**BACKGROUND:**

Many compounds from natural sources, including silymarin, proved to have effective inhibitory effects on cyclooxygenase (COX) and 5-lipoxygenase (5-LO) *in vitro* qnd experimental animals. The exellent pharmacological pre-clinical profile of these compounds indicates a broad range anti-inflammatory effectiveness devoid of the most troublesome side effects, which have at least impart impaired the clinical use of the classical COX inhibitors, including the newer selective COX-2 inhibitors.

**OBJECTIVES**:

This project designed to evaluate the clinical utility of silymarin, as a dual inhibitor of COX and 5-LO, as a single agent or in combination with non-steroidal anti-inflammatory drugs (NSAIDs) of both types, selective and non-selective COX inhibitors, in the treatment of knee osteoarthritis (OA).

**PATIENTS AND METHODS:**

Randomized, double blinded clinical study was performed on 220 patients who have symptomatic and radiologic evidence of painful OA of the knee. Patients were allocated into five groups, treated with either meloxicam (15mg/day), piroxicam; (20mg/day), silymarin (300mg/day) + piroxicam (20mg/day), silymarin (300mg/day) + meloxicam (15mg/day) or silymarin (300mg/day) alone. The treatment was followed for 8 weeks through measurement of the clinical effects of drugs each 7 days, using the Knee Injury and Osteoarthritis Outcome Score (KOOS) system.

**RESULTS:**

The results showed that silymarin, when used alone or in combination with NSAIDs resulted in significant improvement in the components of KOOS, higher than that produced by meloxicam or piroxicam when each used alone.

**CONCLUSION:**

In conclusion, oral administration of 300mg/day silymarin in OA patients produced very well characterized analgesic and anti-inflammatory activities, and when co-administered with piroxicam or miloxicam improves their therapeutic profile.


***KEY WORDS:*** Silymarin, Osteoarthritis, Knee injury, KOOS.

## INTRODUCTION:

Osteoarthritis (OA) is a common disorder of synovial joints. It is characterized pathologically by focal areas of damage to the articular cartilage, centered on load-bearing areas, associated with new bone formation at the joint margins (osteophytosis), change in the subchondral bone, variable degrees of mild synovitis, and thickening of the joint capsule [(1)]. When this disease is advanced, it is visible on plain radiographs, which show narrowing of joint space (due to cartilage lose) , osteophytes, and some times changes in the subchondral bone [(2)]. Gauging the severity of OA involves assessment of both joints and patients. This assessment may be done in the clinical setting in support of diagnosis, treatment decision, or evaluation of response to treatment. Clinical examination of the osteoarthritic joints can be helpful in assessing the extent of joint damage, such as deformity and instability, but the reproducibility of findings is low [(3)]. Many types of drugs, exemplified by non-steroidal anti-inflammatory agents NSAIDS0, currently being used to treat OA. However, NSAIDs elicit adverse effects particularly gastrointestinal ulcerations [(4)]. Moreover, some of these agents have been reported to disrupt extracellular matrix metabolism, particularly proteoglycans synthesis [(5)]. The need

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for more effective treatment of arthritis with fewer side effects has encouraged the search for complementary or alternative approaches, and has been attracted the interest of clinicians as well as patients [6,7]. Many investigators have proven that varieties of flavonoid molecules possess anti-inflammatory activity in various models of inflammation [8,9] and some of them were found to inhibit chronic inflammation in several experimental animal models [10].

Silymarin is a mixture of flavolignans isolated from the ripe seeds of the medicinal plant *Silybum marianum* (Milk thistle); contained mainly silybinin A and B, isosilybinin, silychristin, silydianin and taxifolin. The dihydroflavonol taxifolin and the flavolignans (silybinin, isosilybinin and taxifolin) are usually encompassed by the term silymarin [11]. Of the isomers that contribute silymarin, silybinin is the most active and milk thistle extracts are standardized to contain 70-80% silybinin [12]. In addition to many diverse pharmacological and anti oxidant activities demonstrated by silymarin, several studies reported variety of anti-inflammatory effects, including mast cells stabilization [13], inhibition of neutrophils migration [14], inhibition of leukotriene synthesis and prostaglandin formation [15,16]. The molecular bases of the anti-inflammatory effects of silymarin are not yet completely known; they might be related to the inhibition of the transcription factor NF-$_K$B which regulates the expression of various genes involved in the inflammatory processes [17,18].

This study designed top evaluate the clinical utility of using silymarin, as a dual inhibitor of cyclooxygenase and 5-lipoxygenase, as a single agent or in combination with NSAIDs of both types, selective and non-selective COX-inhibitors, in the treatment of knee osteoarthritis.

## PATIENTS AND METHODS:

Randomized, double blind clinical study was performed on (220) randomly selected patients (79 males and 141 females) with painful osteoarthritis (OA) of the knee, at the Out Patients Clinic in Baghdad Teaching Hospital with age range 38-75 years (53.07 ± 8.18). All patients have symptomatic and radiological evidence of OA in one or both knee joints; their clinical features were in accordance with the description of OA in UK and North American Clinical Guidelines. They also show no significant differences in their initial pain, morning stiffness or global assessment; all patients were informed about the nature and the aim of the study. During patient selection certain exclusion criteria were followed, based on the following: 1. patient with positive history of gastric ulcer, 2. patients with end-stage radiological events of joint destruction, 3. patients with positive history of allergic reactions to any one of the known NSAIDs, 4. any patient who miss one week of treatment assessment indicated in the present study and/or his medication for any reason, 5. pregnant or lactating patients, 6. patients with renal or hepatic damage and those who are on treatment with drugs that interfere with the assessment method.

The selected patients were randomly allocated into five groups as follow: Group A, includes 50 (21 males and 29 females) patients with negative GIT risk factors, treated with meloxicam tablets (15mg/day) taken at night for eight weeks (32 patients only completed the study). Group B, includes 50 (10 males and 40 females) patients with negative GIT risk factors, treated with piroxicam capsules (20mg/day) taken at night for eight weeks (35 patients only completed the study). Group C, includes 50 (17 males and 33 females) patients with negative GIT risk factors, treated with silymarin capsules (150mg) taken twice daily with piroxicam capsules (20mg/day) for eight weeks (40 patients only completed the study). Group D, includes 50 (19 males and 31 females) patients with negative GIT risk factors, treated with silymarin capsules (150mg) taken twice daily with meloxicam tablets (15mg/day) for eight weeks (40 patients only completed the study). Group E, includes 20 (8 males and 12 females) patients with negative GIT risk factors, treated with silymarin capsules (150mg) taken twice daily for eight weeks (all patients completed the study). Effects of drug treatment were assessed each seven days by clinical evaluation and direct interview with patients through a questionnaire method known as Knee Injury and Osteoarthritis Outcome Score (KOOS) [19]. The results were expressed as mean ± SEM; paired *t*-test and ANOVA were used to examine the degree of significance; *P* values less than 0.05 were considered significantly different.

## RESULTS:

### *Effect on Pain Score*

Before enrolment in the study (zero time), OA patients demonstrated poor pain control with their previous therapy, manifested by low pain score in all groups, which indicate severe or extreme symptoms of pain. Treatment with silymarin alone resulted in significant increase in pain score started from the first week (117%) reaching maximum level after 4 weeks (193%) and remain at this level until the last week of the study (eight weeks) (table 1). Significant improvement in pain score also

produced in patients treated with piroxicam or meloxicam alone, but the level of improvement was significantly lower than that observed in silymarin alone treated group. Combination of silymarin with piroxicam or meloxicam resulted in significantly higher levels of improvement in the pain score, started from the first week of treatment and remains elevated until the end of the study. The level of pain score in those groups seems comparable with those produced by treatment with silymarin alone.

**Effects on Symptom Score**

At zero time (before starting treatment), all selected OA patients showed poor management of OA symptoms, manifested by low score of symptoms according to the outcome of KOOS (table 2). Treatment with silymarin alone resulted in time-dependent increase in this score, reaching maximum level 9198%) compared to base line level ($P$<0.05), and remain at this value for the remaining three weeks of the study. Treatment with piroxicam or meloxicam resulted in significant elevation in symptom score (27% and 12% respectively), values which are significantly lower than those produced by silymarin at the same period. Meanwhile, the increase in symptom score in those two groups remains significantly lower than that observed in silymarin-treated group. Combination of NSAIDs with silymarin improves symptom score, which is comparable to that observed during silymarin alone treatment, and significantly higher than those reported during treatment with NSAIDs alone (table 2).

**Effects on Daily Living Activity (ADL) Score**

In table 3, ADL score was found relatively low before starting treatment in all patients (zero time) enrolled in study. During treatment with silymarin, ADL score showed time –dependent increase started after 1 week and reaching maximum after 4 weeks (117% and 199% respectively, $P$<0.05 with respect to baseline value). No further increase in ADL score was reported with increasing the period of treatment up to 8 weeks. Table 3 also showed that treatment with piroxicam or meloxicam resulted in significant elevation in ADL score, started after 1 week of treatment (8% and 12% respectively), values which are significantly lower than those produced by silymarin treatment alone at the same period. Combination of piroxicam or meloxicam with silymarin resulted in significantly higher improvement in ADL score compared to their use alone, and comparable to those reported when silymarin was used alone (table 3).

**Effects on Sport/Recreation Score**

Table 4 revealed low sport/recreation score at zero time levels before starting drug treatment.

Treatment with silymarin alone resulted in significant increase in sport/recreation score started after the first week (170%), reaching maximum level after 5 weeks (198%) and remain at this level until the end of the study (8weeks). Table 4 also demonstrated significant improvement in sport/recreation score produced by piroxicam and meloxicam, but started after the second week of treatment (51% and 46% respectively). However, the level of improvement that produced by the NSAIDs alone was significantly lower than that observed due to treatment with silymarin alone. Combination of silymarin with piroxicam or meloxicam resulted in significantly higher levels of improvement in the sport/recreation score after the first week of treatment, and found to be comparable to those produced by silymarin alone.

**Effects on Quality of Life Score (QOL)**

At zero time (before treatment), all patients showed relatively low QOL score, indicating worse consequences of OA on the quality of patients' life (table 5). Treatment with silymarin alone resulted in time-dependent improvement in QOL score, started after 1 wee; of treatment (117%, $P$<0.05 compared to baseline value), reaching maximum level after 5 weeks (198%) compared to baseline value ($P$<0.05), and remain at this level until the end of the study. Treatment with piroxicam or meloxicam resulted in significant improvement in the QOL score (26% and 41% respectively) after 2 weeks of treatment, and found significantly lower than those produced by silymarin alone. Combination of piroxicam or meloxicam with silymarin resulted in a pattern of improvement in the QOL score similar to that observed during treatment with silymarin alone, but comparable to it only after 3 weeks of treatment. This effect was found significantly different compared to those reported due to the use of NSAIDs alone (table 5).

## DISCUSSION:

Despite the significant advances in understanding mechanisms of pain, many people with arthritis experience different levels of acute and chronic pain that impair their daytime function [(20)]. Additionally, unrelieved pain leads to serious negative consequences, like those observed in pain score belongs to OA patients before treatment (table 1), with many other physiological effects associated with increased catabolic demands [(21)]. Pain with movement is the principle symptom of OA patients; although cartilage tissue contains no pain receptors, sensation of pain likely results from inflammatory mediators, bone edema and mechanoreceptors in the surrounding joints.

Patients with OA of the knee often complain of instability or buckling, especially when they are describing stairs or stepping off crumbs, a situation that was clearly revealed by poor score according to KOOS results (table 1). Most patients with OA seek medical attention because of pain, and the safest initial approach is to use simple oral analgesics such as acetaminophen.

In the present study, the reported effect for silymarin in improving pain score can be explained according to its nature of biological activity, which attributed to many factors. Silymarin has anti-inflammatory activity and inhibits the production of tumor necrosis factor alpha (TNF-α) both *in vitro* and *in vivo* [22, 23]. Because of its anti-inflammatory properties, it may be useful in the treatment of many inflammatory disorders. It produces anti-arthritic activity in animal models, and in dose-dependent manner inhibits adjuvant-induced arthritis [24], probably mediated through inhibition of the enzyme 5-lipoxygenase. Many investigators also observed the inhibitory effect of silymarin on COX-2 and production of interleukin-1α [25].

The lack of satisfaction of patients and doctors with NSAIDs treatment reflected by that fewer than 20% of patients with hip or knee OA, in whom NSAIDs treatment initiated, are still taking the same drug 12 months later [26]. In the present study, most of selected patients were currently maintained on one or more than one of the commonly used NSAIDs, but clinical improvement in the degree, type and incidence of pain and other parameters seems to be at lower levels when evaluated by KOOS.

Osteoarthritis is the single most common cause of disability in older adults, and many studies revealed 10% prevalence of painful disabling knee OA in people over 55 years, of whom quarter are severely disabled [27]. Taking into account the assumption that, if the pathological processes which give rise to x-ray changes could be slowed down, this would be an important mean of preventing pain and disability, and improve symptom score in OA patients, especially those who present with knee pain, morning stiffness and joint crepitus, according to the reported guidelines of the American college of Rheumatology about classification of OA as a clinical symptom in older adults [28]. In this respect, treatment with silymarin alone or in combination wit NSAIDs may give us promising indication about the possible role of silymarin or other flavonoids in reversing or retarding the progression of degenerative processes that predispose to pain and other consequent disabling symptoms in OA. In spite of having various agents offering possibilities for pharmacological treatment of OA, among them are NSAIDs (selective and non-selective inhibitors of COX), analgesics and steroids [29], therapeutic outcome, toxicity profile and significant interference with the pathogenesis of the disease are not so much hopeful, and necessitate the search for new therapeutic models in this respect.

Polyphenolic flavonoids, the active constituent in silymarin, have different biological activities including antioxidant, free radical scavenging, anti-inflammatory and cytoprotective activities. They interfere with the inflammatory processes through blocking both COX and 5-LO pathways [24], inhibiting LTB4 formation [30], suppress TNF-α – induced activation of NF-κB (18), in addition to the powerful inhibitory effect on NO production within the immune system through interfering with iNOS gene expression [31].

Osteoarthritis is though to be the leading cause to chronic loss of work and severely reduced quality of life. In most instances, patients with OA have such severe symptoms that they are unable to function independently [32], a situation clearly revealed in the low value for this parameter in KOOS score reported in the present study (table 3). Moreover, OA is considered as a significant worldwide health problem owing to the progressive and debilitating nature of the disease, which results in high morbidity and marked decrease in quality of life (QOL) [33]. In the same respect, physical disability arising from pain and loss of functional capacity reduces quality of life and interferes with daily activities [34], and consequently the significant improvement in pain score, observed due to the use of silymarin compared to NSAIDs might correspond to the improvement in quality of life and daily activity score. The consequences of pain are widely spread and when chronically sustained may lead to depression, restricted social life, sleep problems and impaired mental function [35]. Therefore, the major goal of OA treatment is to improve mentally related consequences of pain to optimize algo-functional features and improves patient's QOL. The data presented in table 5 confirm the later idea, where silymarin significantly improves all parameters of KOOS system, when used alone or in combination with piroxicam or meloxicam compared to using NSAIDs alone. This activity of silymarin may be attributed to high compliance and satisfaction in the silymarin-treated patients, which can be explained according to the quality of analgesia and improved recovery of joint function (range of

motion) attributed to the cytoprotective and tissue regenerative power of silymarin. The eight weeks of the study is a short duration, being in line with acute OA flare episode, and elongation of treatment period may show more accurately the impact on the chronic consequences of the disease, patient compliance and side effects profile of the given medication. However, the present study was long enough to document how rapid the onset of silymarin action and its sustained efficacy, both when used alone or in combination with piroxicam or meloxicam.

From clinical perspective, current medical treatments of OA did not provide cure or elimination of arthritis-related pain and disability. Therefore, management of patients with OA should not aim only at decreasing pain, but remodeling the pathological processes to improve functions of the affected part and enhancing quality of life [36,37,38]. In this respect, the extract of ginger rhizome has been used as an anti-inflammatory agent for musculoskeletal pain [39,40], and several species of this plant have been reported to reduce inflammation and relieve arthritic joint pain. Additionally, some flavonoids such as luteolin, galangin and moria are good inhibitors of both COX and 5-LO, and the ratio of selectivity to each of the two enzymes depend on the structure activity relationship in each compound [41,42]. In conclusion, silymarin can be used clinically, alone or in combination with NSAIDs, for the management of patients with knee OA.

Acknowledgment

We gratefully acknowledge University of Baghdad and Department of Rheumatology in Baghdad Teaching Hospital for supporting the present work.

**CONCLUSION:**

Oral administration of 300mg/day silymarin in OA patients produced very well characterized analgesic and anti-inflammatory activities, and when co-administered with piroxicam or miloxicam improves their therapeutic profile.

**Table 1. Effect of treatment of OA patients with silymarin, piroxicam, meloxicam or combination of NSAIDs with silymarin on pain score.**

| Treatment groups | Pain score during treatment periods (week) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 |
| silymarin (300mg/day) n=20 | 32.7 ± 4.0 | 71.0 ± 3.9*[a] | 83.4 ± 2.7*[a] | 96.0 ± 1.1*[a] | 98.0 ± 1.0*[a] |
| piroxicam (20mg/day) n=35 | 32.2 ± 1.8 | 35.9 ± 1.9[b] | 46.6 ± 2.0*[b] | 54.0 ± 1.9*[b] | 57.2 ± 2.0*[b] |
| piroxicam (20mg/day) + silymarin (300mg/day) n=40 | 34.7 ± 2.1 | 69.6 ± 2.3*[a] | 84.3 ± 1.8*[a] | 97.0 ± 0.8*[a] | 97.7 ± 0.8*[a] |
| meloxicam (15mg/day) n= 32 | 30.4 ± 2.6 | 35.9 ± 2.5*[b] | 42.8 ± 2.5*[b] | 47.6 ± 2.7*[c] | 47.4 ± 2.8*[c] |
| meloxicam (15mg/day) + silymarin (300mg/day) n=40 | 39.5 ± 2.4 | 67.5 ± 2.4*[a] | 82.6 ± 1.7*[a] | 93.4 ± 1.1*[a] | 96.3 ± 1.0*[a] |

Data were expressed as mean ± SEM, * significantly different compared to baseline value, values with non-identical superscripts (a,b,c) among different groups at the same period are considered significantly different ($P<0.05$).

**Table 2. Effect of treatment of OA patients with silymarin, piroxicam, meloxicam or combination of NSAIDs with silymarin on symptoms score**.

| Treatment groups | symptoms score during treatment periods (week) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 |
| silymarin (300mg/day) n=20 | 32.7 ± 4.0 | 71.0 ± 3.9*[a] | 83.4 ± 2.7*[a] | 96.0 ± 1.1*[a] | 98.0 ± 1.0*[a] |
| piroxicam (20mg/day) n=35 | 39.0 ± 2.0 | 49.6 ± 1.9*[b] | 55.5 ± 1.6*[b] | 60.5 ± 1.8*[b] | 68.2 ± 1.0*[b] |
| piroxicam (20mg/day) + silymarin (300mg/day) n=40 | 41.0 ± 2.6 | 76.3 ± 2.3*[a] | 89.6 ± 1.4*[a] | 96.0 ± 0.5*[a] | 96.9 ± 0.5*[a] |
| meloxicam (15mg/day) n= 32 | 38.1 ± 3.3 | 42.6 ± 3.5*[b] | 48.8 ± 3.1*[b] | 53.1 ± 3.3*[c] | 53.2 ± 3.1*[c] |
| meloxicam (15mg/day) + silymarin (300mg/day) n=40 | 44.7 ± 2.7 | 72.4 ± 2.0*[a] | 86.3 ± 1.2*[a] | 94.8 ± 0.5*[a] | 95.7 ± 0.4*[a] |

Data were expressed as mean ± SEM, * significantly different compared to baseline value, values with non-identical superscripts (a,b,c) among different groups at the same period are considered significantly different ($P$<0.05).

**Table 3. Effect of treatment of OA patients with silymarin, piroxicam, meloxicam or combination of NSAIDs with silymarin on activity of daily life (ADL) score.**

| Treatment groups | ADL score during treatment periods (week) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 |
| silymarin (300mg/day) n=20 | 32.7 ± 4.0 | 71.0 ± 3.9*[a] | 83.4 ± 2.7*[a] | 96.0 ± 1.1*[a] | 98.0 ± 1.0*[a] |
| piroxicam (20mg/day) n=35 | 43.9 ± 1.6 | 47.2 ± 1.9*[b] | 55.5 ± 2.7*[b] | 63.7 ± 2.7*[b] | 63.0 ± 2.6*[b] |
| piroxicam (20mg/day) + silymarin (300mg/day) n=40 | 38.9 ± 2.3 | 76.4 ± 2.7*[a] | 88.6 ± 1.5*[c] | 96.4 ± 0.5*[a] | 97.5 ± 0.5*[a] |
| meloxicam (15mg/day) n= 32 | 36.7 ± 3.2 | 40.6 ± 3.0*[b] | 46.6 ± 2.7*[b] | 52.0 ± 2.8*[c] | 52.0 ± 2.7*[c] |
| meloxicam (15mg/day) + silymarin (300mg/day) n=40 | 43.9 ± 2.4 | 72.7 ± 2.4*[a] | 87.2 ± 1.5*[c] | 95.4 ± 0.6*[a] | 97.3 ± 0.6*[a] |

Data were expressed as mean ± SEM, * significantly different compared to baseline value, values with non-identical superscripts (a,b,c) among different groups at the same period are considered significantly different ($P$<0.05).

**Table 4. Effect of treatment of OA patients with silymarin, piroxicam, meloxicam or combination of NSAIDs with silymarin on sport/recreation score**.

| Treatment groups | sport/recreation score during treatment periods (week) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 |
| silymarin (300mg/day) n=20 | 32.7 ± 4.0 | 71.0 ± 3.9*[a] | 83.4 ± 2.7*[a] | 96.0 ± 1.1*[a] | 98.0 ± 1.0*[a] |
| piroxicam (20mg/day) n=35 | 20.7 ± 1.4 | 23.0 ± 1.7 [b] | 31.3 ± 2.1*[b] | 37.0 ± 2.1*[b] | 38.8 ± 2.0*[b] |
| piroxicam (20mg/day) + silymarin (300mg/day) n=40 | 25.0 ± 2.4 | 58.1 ± 2.3*[c] | 77.1 ± 2.1*[c] | 86.4 ± 1.3*[c] | 89.6 ± 1.1*[c] |
| meloxicam (15mg/day) n= 32 | 20.5 ± 2.4 | 22.7 ± 2.4[b] | 29.9 ± 2.8*[b] | 33.8 ± 2.9*[b] | 34.1 ± 2.9*[b] |
| meloxicam (15mg/day) + silymarin (300mg/day) n=40 | 27.3 ± 2.2 | 49.1 ± 2.5*[d] | 67.3 ± 2.1*[d] | 82.3 ± 1.3*[c] | 84.9 ± 1.2*[d] |

Data were expressed as mean ± SEM, * significantly different compared to baseline value, values with non-identical superscripts (a,b,c,d) among different groups at the same period are considered significantly different ($P$<0.05).

**Table 5. Effect of treatment of OA patients with silymarin, piroxicam, meloxicam or combination of NSAIDs with silymarin on quality of life (QOL) score.**

| Treatment groups | QOL score during treatment periods (week) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 |
| silymarin (300mg/day) n=20 | 32.7 ± 4.0 | 71.0 ± 3.9*[a] | 83.4 ± 2.7*[a] | 96.0 ± 1.1*[a] | 98.0 ± 1.0*[a] |
| piroxicam (20mg/day) n=35 | 27.0 ± 1.9 | 22.5 ± 2.0*[b] | 34.0 ± 2.6*[b] | 41.2 ± 2.5*[b] | 42.8 ± 2.3*[b] |
| piroxicam (20mg/day) + silymarin (300mg/day) n=40 | 21.1 ± 1.8 | 52.5 ± 2.7*[c] | 78.6 ± 2.0*[c] | 92.8 ± 0.9*[a] | 93.6 ± 0.9*[c] |
| meloxicam (15mg/day) n= 32 | 21.9 ± 2.8 | 22.8 ± 2.8[b] | 30.8 ± 2.6*[b] | 34.2 ± 2.5*[c] | 34.8 ± 2.6*[d] |
| meloxicam (15mg/day) + silymarin (300mg/day) n=40 | 24.4 ± 1.9 | 46.3 ± 2.9*[d] | 65.2 ± 2.3*[d] | 83.6 ± 1.5*[d] | 88.4 ± 1.7*[c] |

Data were expressed as mean ± SEM, * significantly different compared to baseline value, values with non-identical superscripts (a,b,c,d) among different groups at the same period are considered significantly different ($P$<0.05).

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