

Antiinflammatory effect of herbal preparations on adjuvant arthritis in rats

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Abstract: The present study aimed to investigate the antiinflammatory effects of 2 herbal extracts (CDS and CDSM) in rats with experimental adjuvant arthritis (AA). Joint swelling, development of polyarthritis, blood indices, pro/antioxidant activity, and proinflammatory cytokine IL-17 in blood serum, as well as the liver and paw histopathology and histochemistry, were assessed. Oral injections of extracts showed an antiinflammatory effect analogous to that of diclofenac (DF), improved blood indices and suppressed level of IL-17, significantly diminished joint swelling, and histological changes in the joints. Increase in CAT activity and AOA and reduction in lipid peroxidation was noted. The preparations did not show toxic effects on the liver. The obtained results suggest that the prophylactic use of CDS and CDSM have a significant effect on slowing AA progression, similar to the effect of DF. Both preparations could be potential preventive or therapeutic candidates for the treatment of autoimmune processes in combination with other drugs.

Key words: Adjuvant arthritis, herbal extracts, antioxidant activity

1. Introduction

With the aging of the global population, such diseases as rheumatoid arthritis (RA), osteoarthritis, and other bone metabolic disorders are increasingly impacting public health as well as the economy (1,2). Because current treatments for arthritis are inefficient, produce substantial side effects, and tend to be expensive, natural products, which are devoid of such disadvantages, offer novel treatment opportunities (3). In recent years, there has been a vast increase in the number of complementary drugs purported to have joint-protecting and free-radical scavenging properties (4,5). A number of natural substances that exhibit antiinflammatory activity and minimal side effects are also being increasingly tested in experimental models of arthritis (6,7).

The preparation CDS is a compound consisting of turmeric (*Curcuma longa*) dry extract (curcumoids 98%), *Harpagophytum procumbens* (Hp) dry extract (harpagosides 8%), and milk thistle (*Silybum marianum*) dry extract (silymarin 60%). The compound CDSM is a composition of these extracts plus methylsulfonylmethane (MSM), which is widely used as a dietary supplement for its beneficial effects against arthritis, reducing arthritic and rheumatic pain. It is used to support the function of joints,

often in combination with glucosamine and chondroitin or other compounds like *Boswellia serrata* extract and hyaluronic acid (8). Previous studies have demonstrated that MSM possesses antioxidant and antiinflammatory properties (9,10), increasing antioxidant enzyme levels and reducing malondialdehyde (MDA), myeloperoxidase (MPO), and TNF- α (9). Its administration protects against the development of type II collagen-induced arthritis in mice by modifying immune responses (11).

Among the substances in the preparations CDS and CDSM, curcumin, derived from the root of the plant *Curcuma longa* (turmeric), has been one of the most promising natural active ingredients (12). Biomedical research on curcumin is ever-increasing because of its antiinflammatory and antioxidant effects (13,14). It is used for the treatment of inflammation, rheumatism, and other chronic and inflammatory diseases (14). The antiinflammatory activity of curcumin seems to be comparable to that of steroidal and nonsteroidal drugs such as indomethacin and phenylbutazone (13). Curcumin has been shown to suppress the production of cytokines such as interferon- γ (IFN- γ), interleukins, and TNF- α (15).

Another component, *Harpagophytum procumbens* (Hp), commonly known as devil's claw, is a perennial plant

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that thrives in arid conditions in southern Africa. Hp is widely recommended as a popular antiinflammatory and analgesic preparation for musculoskeletal disorders. The efficacy of Hp in the relief of arthritic symptoms has been investigated in numerous animal, clinical, and in vitro studies (16). Hp acts by way of interleukin and leukocyte migration to the painful and inflamed joint area (17). It is an effective and well-tolerated serious treatment option for mild to moderate degenerative rheumatic disorders, providing improved quality of life (16).

Silymarin in the preparations CDS and CDSM is an extract from the seeds of the milk thistle plant, *Silybum marianum*. There exists a widely held view that silymarin promotes liver health through antioxidant, antiinflammatory, antiproliferative, and immunomodulatory effects (18).

The purpose of the present study was to examine the effect of CDS and CDSM on clinical and histological scores by using Freund's adjuvant-induced arthritis (AA), because there is a strong correlation between the efficacy of therapeutic agents in this model and in RA in humans (17). Pro/antioxidant activity, antiinflammatory, and immunomodulatory effects of the preparations were investigated and compared with the effects of diclofenac (DF).

2. Materials and methods

2.1. Materials

Complete Freund's adjuvant (CFA) was purchased from Sigma Aldrich (Calbiochem, USA). Acetic acid, trichloroacetic acid, orthophosphoric acid, thiobarbituric acid, nitric acid, ascorbic acid, ferrous sulfate, ammonium molybdate, hydrogen peroxide, 10% neutral buffered formalin, hematoxylin, eosin, picrofuxin, and toluidine blue were obtained from Sigma-Aldrich Chemie and Fluka Chemie GmbH (Germany); ketamidol from Richer Pharma AG (Wels, Austria); sedaxylan from Eurovet Animal Health B.V. (Holland); tetrachloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) and tannic acid from Carl Roth GmbH & Co (Germany); sodium citrate from Penta (Czech Republic). Diclofenac sodium was purchased from Ciba-Geigy Ltd. (Switzerland).

Herbal preparations with the code names CDS and CDSM used in this experiment were prepared at the pharmaceutical company Aksada and kindly proposed for investigation. Each dose from the CDS composition consisted of turmeric (*Curcuma longa*) dry extract (curcumoids 98%), devil's claw (*Harpagophytum procumbens*) dry extract (harpagosides 8%), and milk thistle (*Silybum marianum*) dry extract (silymarin 60%). Each dose from the CDSM composition consisted of the abovementioned extracts and MSM (99.9%). Extracts contained in CDS and CDSM were standardized by the HPLC method and the quality was based on Certificates

of Analysis, which corresponds to the European Pharmacopeia requirements.

2.2. Animals

A total of 46 adult male Wistar rats (age, 8–10 weeks; weight, 175–200 g) were used for the study. The animals were housed in plastic cages and maintained under standard laboratory conditions in the vivarium of the Department of Biomodels, State Research Institute Centre for Innovative Medicine. They were fed a standard pellet diet and received water ad libitum. Throughout the study, the animals were cared for in accordance with the European Convention Guide for the Care and Use of Laboratory Animals, and Lithuanian laws. All experiments on animals were performed with prior approval of the Lithuanian Laboratory Use Ethics Committee under the State Food and Veterinary Service (Protocol No. G2-31).

2.3. Adjuvant-induced arthritis, its evaluation, and treatment

The animals were randomly divided into 5 groups: 4 experimental (10 animals in each) and a healthy rat group (6 animals). Adjuvant arthritis (AA) was induced in 40 rats by the subplantar injection of 0.1 mL of complete Freund's adjuvant (CFA). The doses of preparations used in the animals corresponded to the doses used for humans, and were estimated by the pharmaceutical company Aksada. The preparations were suspended in 1% starch gel and injected orally by gastric intubation 5 times a week in 0.5 mL volume per rat following AA-inducing day. The first group received 39.73 mg/kg CDS per day; the second, 117 mg/kg CDSM per day; the third, 1 mg/kg DF per day; the fourth group was the control AA group without treatment; the fifth, healthy rats. The control AA group and healthy rat group received 0.5 mL of starch gel as vehicle. The experiment lasted 17 days. The antiarthritic effect of the preparations was evaluated by measuring the paw volume plethysmometrically 3 times a week by using a plethysmometer (PVP1001; Kent Scientific Corporation). On day 17, the rats were euthanized by decapitation under ketamidol–sedaxylan anesthesia. Blood, liver, and injected paws were collected for further investigation. The leukocyte count (performed by using a hematological analyzer from Picoscale, Hungary) and erythrocyte sedimentation rate (ESR) were determined in the blood. Blood samples were centrifuged at $800 \times g$ for 10 min to obtain serum samples which were stored frozen at 20 °C until testing.

2.4. Pro/antioxidant activity of blood serum

Lipid peroxidation, assessed as malondialdehyde (MDA) levels in the blood serum and expressed as nmol/mL, was determined by thiobarbituric acid reaction at a wavelength of 532 nm by the method of Gavrilov et al. (19). Catalase (CAT) activity, expressed in nmol/L per min, was measured at the wavelength 410 nm as described by Koroliuk et al. (20). Total antioxidant activity (AOA) was determined

in the reaction with thiobarbituric acid, as described by Galaktionova et al. (21). The precise description of the methods used has been published previously (22). The optical density of investigated indices was determined with a spectrophotometer (Marcell Pro 300; Poland).

2.5. Histology

The liver and injected paws from AA and healthy rats were excised followed by routine neutral formalin fixation, decalcification, and paraffin embedding. Paraffin beeswax tissue blocks were prepared for sectioning at 5- μ m thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained with hematoxylin-eosin, picrofuxin, and toluidine blue for histopathological and histochemical examination with the light microscope. Histological assessment of changes in the liver and joint tissues were estimated by using a 4-point score (0–3), where 0 indicated the absence of changes and 3 was the most severe expression of a particular symptom. During the processing and analysis of tissues, the pathologist was blinded to the animal groups and the treatments.

2.6. Measurement of cytokine IL-17 level

Cytokine IL-17 level was measured in the serum of the control and experimental animals by enzyme-linked immunosorbent assay (ELISA) kit specific for rats (ab119536-IL-17 Rat ELISA Kit) according to the procedure recommended by manufacturer's instructions (abcam, UK). Each sample was assayed in duplicate. The concentration of cytokine was determined with the help of a standard curve.

2.7. Statistical analysis

Statistical analysis was done by one-way analysis of variance ANOVA using PRISM Software (GraphPad Software, San Diego, CA, USA) followed by Student's t-test. The nonparametric Mann-Whitney U test was used to evaluate the histological changes. All data were expressed as the mean \pm SEM and considered to be statistically significant at P values smaller than or equal to 0.05.

3. Results

3.1. Clinical and hematological indices

The CDS preparation significantly suppressed joint swelling until day 13 ($P < 0.01$ – 0.001); CDSM significantly suppressed joint swelling throughout the experiment ($P < 0.02$ – 0.001) (Figure 1). Polyarthrititis did not develop in the treated rats, whereas in the control AA group it was observed in 30% of the animals.

There was a significant increase in ESR and the leukocyte count of the arthritic rats when compared with the healthy-animal group (Figure 2A). The CDS and CDSM preparations significantly reduced ESR by 60.4% ($P < 0.001$) and 45.4% ($P < 0.01$) respectively, and leukocyte count by 45.6% ($P < 0.001$) and 44.5% ($P < 0.001$) compared with the control AA group.

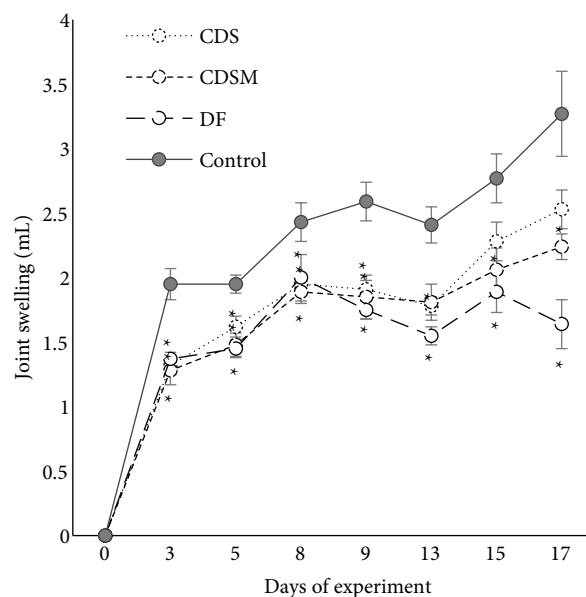


Figure 1. Joint swelling of rats with adjuvant arthritis (AA) treated with CDS, CDSM, and diclofenac (DF). *The differences are statistically significant in comparison with the control AA group.

3.2. Effect of CDS and CDSM on IL-17 level in the blood serum

The level of cytokine IL-17 in the serum was detected on day 17 after immunization with CFA (Figure 2B). It significantly increased in the control AA group compared with the healthy animals ($P < 0.0001$). CDS and CDSM suppressed IL-17 level by 45.4% ($P < 0.05$) and 30.23%, respectively.

3.3. Effect of CDS and CDSM on lipid peroxidation and antioxidant activity

The levels of lipid peroxidation (MDA) were found to be significantly ($P < 0.0001$) increased in the control AA rats compared to the normal (healthy) control group (Figure 3). This was accompanied by a marked ($P < 0.001$) 43% and 39.9% decrease in AOA and CAT activity. In contrast, treatment with CDS (Group 1) and CDSM (Group 2) showed a significant lowering effect on MDA level (suppression by 50% and 46% respectively; $P < 0.0001$), similar to DF. An elevation of CAT activity by 17.3% and 20.4%, and AOA by 54.2% and 41.2% ($P < 0.001$), was also observed in comparison with the control AA group (Figure 3). It should be noted that the effect of both preparations and DF on MDA, AOA, and CAT activity was similar.

3.4. Histological changes in the liver

There was no histopathological alteration of the normal structure in hepatic parenchyma of healthy animals (data not shown).

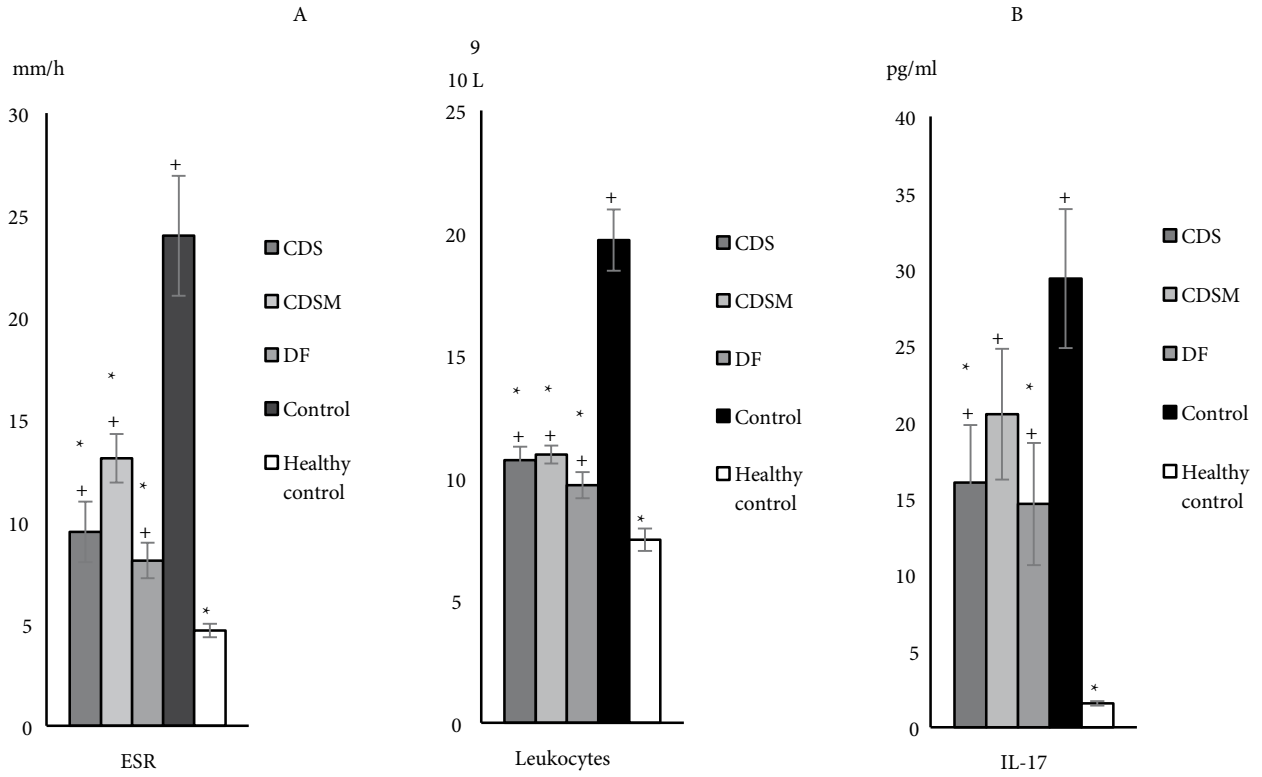


Figure 2. Blood indices (A) and IL-17 level (B) in rats with adjuvant arthritis (AA) treated with CDS, CDSM, and diclofenac (DF). * The differences are statistically significant in comparison with the control AA group. + The differences are statistically significant in comparison with the healthy animals group.

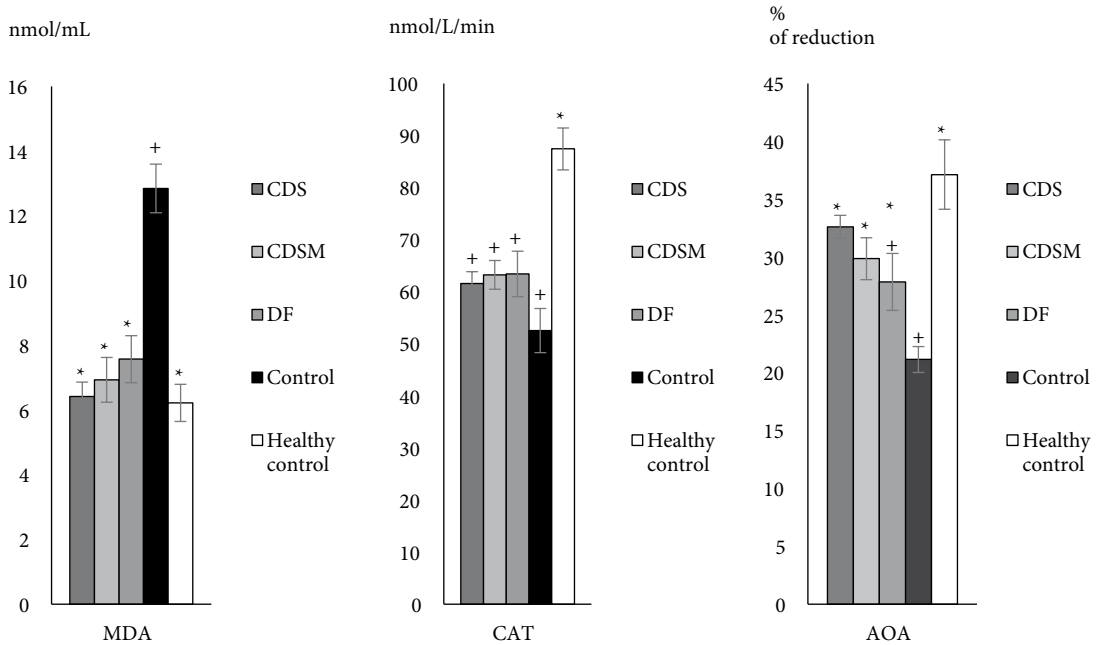


Figure 3. Pro/antioxidant activity in rats with adjuvant arthritis (AA) treated with CDS, CDSM, and diclofenac (DF). * The differences are statistically significant in comparison with the control AA group. + The differences are statistically significant in comparison with the healthy animals group.

The effect of CDS and CDSM on the liver of rats with AA was similar to the effect of DF (Table 1). Preparations significantly diminished inflammatory infiltration of hepatic stroma with lymphocytes by 63% ($P < 0.01$) and 56.8% ($P < 0.01$). General inflammatory reaction decreased by 43.4% ($P < 0.05$) in the CDS-treated group and by 48.1% ($P < 0.05$) in the DF group in comparison with the control AA group. CDS also markedly diminished alteration of parenchyma by 60.2% ($P < 0.02$). No toxic effects on the liver were observed after the treatment.

3.5. Effect of CDS and CDSM treatment on histological and histochemical findings in the joints

There were no abnormalities observed in the joints of the healthy rats (data not shown). The control CFA-administered group showed joint cartilage abrasions and erosion, pannus formation, extensive infiltration of inflammatory cells in the soft periarticular tissues and synovium, edema, and angiomas (Table 2; Figure 4).

In the soft periarticular tissues, the preparations of CDS and CDSM significantly reduced the general inflammatory

Table 1. Histological changes in the liver of rats with adjuvant arthritis treated with preparations CDS and CDSM.

Index		1 gr. AA + CDS	2 gr. AA + CDSM	3 gr. AA + DF	4 gr. AA control
Alteration of parenchyma		0.35 ± 0.11*	0.70 ± 0.17	0.65 ± 0.11	0.88 ± 0.16
Inflammatory infiltration of hepatic stroma	Lymphocytes	0.30 ± 0.11*	0.30 ± 0.17*	0.35 ± 0.11*	0.81 ± 0.09
	Macrophages	0.65 ± 0.15	0.60 ± 0.22	0.70 ± 0.18	0.88 ± 0.16
	General	0.60 ± 0.14*	0.60 ± 0.22	0.55 ± 0.17*	1.06 ± 0.11

Note: Hepatic tissue was fixed in neutral formalin solution, embedded in paraffin, and 5- μ m-thick histological sections of the tissue were stained with hematoxylin–eosin (for visualization of inflammation and inflammatory cell infiltration and necrosis of hepatocytes). The histological assessment of changes in the liver was performed in a blinded manner by a pathologist. Each parameter was scored on a 0- to 3-point scale, where 0 means the absence of changes, 0.5 – traces of changes, 1 – minimal changes, 2 – moderate changes, 3 – heavy changes. * The differences are significant in comparison with the control AA group.

Table 2. Histological changes in the joints after the treatment of adjuvant arthritis in rats with CDS and CDSM preparations.

Tissue	Index		1 gr. AA + CDS	2 gr. AA + CDSM	3 gr. AA + DF	4 gr. AA control
Soft periarticular tissues	Inflammatory Infiltration	Leukocytes	1.25 ± 0.29	1.00 ± 0.23	0.60 ± 0.18*	1.63 ± 0.29
		General	1.65 ± 0.17*	1.40 ± 0.16*	1.35 ± 0.08*	2.13 ± 0.08
	Edema		1.15 ± 0.08*	0.90 ± 0.14*	0.65 ± 0.11*	2.06 ± 0.11
	Angiomatosis		0.85 ± 0.13*	0.65 ± 0.11*	0.65 ± 0.08*	1.31 ± 0.13
Synovium	Inflammatory Infiltration	Leukocytes	0.30 ± 0.13*	0.35 ± 0.11*	0.25 ± 0.11	1.13 ± 0.23
		General	0.80 ± 0.08*	0.55 ± 0.14*	0.55 ± 0.12*	1.44 ± 0.15
	Synovium villi proliferation		0.90 ± 0.12*	0.75 ± 0.08*	0.80 ± 0.08*	1.63 ± 0.12
	Edema		0.50 ± 0.15*	0.55 ± 0.14*	0.25 ± 0.11*	1.31 ± 0.21
	Angiomatosis		0.60 ± 0.10*	0.60 ± 0.10*	0.40 ± 0.10*	1.25 ± 0.23
Cartilage	Alteration	Abrasion	0.75 ± 0.24	0.70 ± 0.13*	0.65 ± 0.15*	2.00 ± 0.00
		Fissures	0.15 ± 0.11*	0.10 ± 0.07*	0.20 ± 0.11*	1.06 ± 0.27
	Pannus		0.15 ± 0.10*	0.25 ± 0.08*	0.05 ± 0.05*	1.00 ± 0.27

* The differences are significant in comparison with the control AA group.

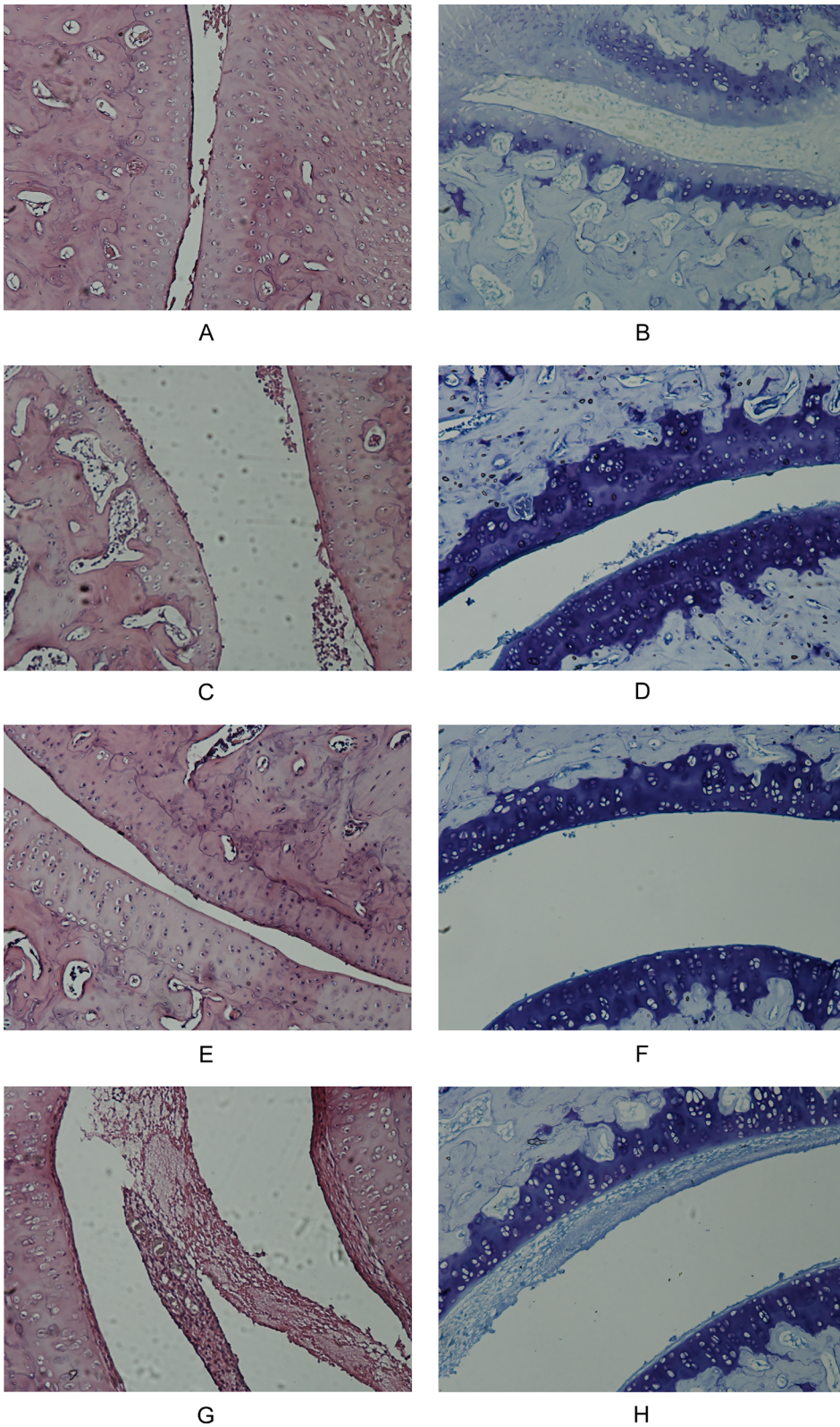


Figure 4. Histologic and histochemical appearance of the joints, original magnification 100×, stained with H & E (A, C, E, G), toluidine blue (B, D, F, H) after treatment with CDS (A, B), CDSM (C, D), DF (E, F), and from untreated control rats (G, H).

infiltration by 22.5% ($P < 0.05$) and 34.3% ($P < 0.002$) respectively, edema by 44.2% ($P < 0.001$) and 56.3% ($P < 0.001$), and angiomatosis by 35.1% ($P < 0.05$) and 50.4% ($P < 0.002$) as compared with the control AA group. Significantly lower periarticular tissue γ -metachromasia was observed after the treatment with CDS (0.25 ± 0.08 , CDS; 0.94 ± 0.26 , control; $P < 0.05$).

The highest pathological changes in the synovium were found in the control untreated AA group. CDS markedly suppressed synovium villi proliferation by 44.8% ($P < 0.001$) and CDSM by 54% ($P < 0.001$). Both CDS and CDSM administration diminished edema (by 61.8% and 58%; $P < 0.01$), inflammatory infiltration with leukocytes (by 73.5% and 69%; $P < 0.01$), general inflammatory reaction (by 44.4%, $P < 0.002$ and 61.8%, $P < 0.001$), and angiomatosis (by 52%; $P < 0.02$ – 0.001) compared with the control AA group (Table 2). Synovium inflammatory infiltration with macrophages was markedly lower (by 66.7%) in the CDSM-treated group (0.25 ± 0.08 , CDSM; 0.75 ± 0.09 , control; $P < 0.001$).

Both preparations significantly diminished fibrillations (abrasions) ($P < 0.001$ – 0.0001) and fissures ($P < 0.01$) in the joint cartilage, as well as pannus formation ($P < 0.05$), compared with the control AA group (Figure 4).

4. Discussion

Because current treatments for arthritis result in unwanted side effects and tend to be expensive, natural products devoid of such disadvantages offer a novel opportunity. The main task of this study was to confirm the idea that better pharmacological antiinflammatory response can be achieved by using a mixture of herbal extracts, the mechanism of action of which is different from that of diclofenac, an ordinary NSAID and a powerful inhibitor of COX-2 and prostaglandin synthase activities. It is known that the use of NSAIDs is constrained by the presence of gastrointestinal side effects, which can be prevented by the use of herbal preparations, such as curcumin, which is able to prevent and treat gastric ulcers (23).

We have demonstrated the antiarthritic ability of the preparations CDS and CDSM in AA. It was found that they may have a significant effect on slowing AA progression and this effect was similar to the therapeutic effect of diclofenac (DF). Inhibition of joint swelling was associated with marked decrease in histological changes in the joint tissues. The main pathological changes of RA are that leukocytes infiltrate into articular cavities and cause recurrent synovitis (24), and that invasive pannus causes damage to cartilage, bone, and surrounding tissue. As observed in our study, CDS and CDSM compounds significantly decreased synovium infiltration by leukocytes, the general inflammatory reaction in periarticular tissues and synovium, pannus formation, and fibrillations and fissures in the cartilage.

This is not surprising, because the ingredients of the analyzed preparations are dry extracts from turmeric (*Curcuma longa*), *Harpagophytum procumbens* (Hp), milk thistle (*Silybum marianum*), and MSM. These extracts are widely used for their beneficial effects against inflammation and arthritis. The protection of joints after the use of these extracts with various antioxidant, antiinflammatory, immunomodulatory, antiproliferative effects are widely described (12–15,17,18).

Multiple therapeutic activities of curcumin are associated with its antiinflammatory and antioxidant effects (12,13). Hp is widely recommended as a popular antiinflammatory and analgesic preparation for musculoskeletal disorders (16,25), and silymarin for its antioxidant, antiinflammatory, antiproliferative, and immunomodulatory effects (18). MSM also possesses antioxidant, antiinflammatory, and antiapoptotic effects (9,10,26).

Hence, the antiarthritic potential of the investigated CDS and CDSM preparations is a result of their ingredients, which show antiinflammatory activity mediated through inhibition of different molecules involved in inflammation including NF- κ B, COX-2, 5-LOX, TNF- α , IL-1b, IL-6, matrix metalloproteinases (MMPs), and nitric oxide (12–15,23,27).

No particular behavior or clinical or physiological signs were observed in the animals treated with either compound, suggesting that the doses used of the preparations are probably not toxic in vivo. It was confirmed by histological examination of the liver, where lower alterations in hepatic parenchyma and stroma were found in the treated groups.

Cytokine IL-17, a major product of T cells, which plays an important role as an upstream mediator of RA pathogenesis, and promotes inflammation via enhancing the production of IL-1 β , TNF- α , and IL-6 (28,29), significantly increased in rats with AA compared to the healthy controls in our study. In response to treatment with CDS, its level significantly decreased. The CDSM preparation showed a tendency to reduce this cytokine level compared with the control AA group.

Increase in leukocyte count has been suggested to be one of the characteristic diagnoses of arthritis. In the present study, arthritic animals showed raised leukocyte level. The preparations CDS and CDSM significantly decreased leukocytes, revealing their beneficial role against arthritis. ESR, which markedly increased in the arthritic control group, was significantly lower after treatment with both preparations and the standard drug DF, demonstrating the significant benefit of the preparations in arthritic conditions.

Antioxidant enzymes that are present in biological systems can protect the tissue from oxidative injury. Our results demonstrate that the activity of CAT and AOA

was diminished in AA animals, which may have been due to increased production of free radicals. The decrease in antioxidant enzyme activity correlated with increased lipid peroxidation quantified by measurement of the MDA. It is well known that MDA is a terminal product of lipid peroxidation, and so the content of MDA can be used to estimate the extent of lipid peroxidation (30).

Our data of biochemical assessments showed significantly lower levels of MDA and higher AOA and CAT activity in the treated groups compared with the control AA group.

Data on therapeutic effects of CDS and CDSM led us to assay the ability of these preparations to prevent development of AA in rats. These findings are consistent with those reported by other authors, who have demonstrated the ability of the extracts that composed our preparations to reduce inflammation and to enhance

antioxidant activity, as well as induction of direct scavenging of free radicals (9,10,12,16,18,25,26).

In conclusion, the data of this study demonstrated that the compounds CDS and CDSM could play a role in protecting against clinical, biochemical, immunological, and histological alterations in rats with AA. The therapeutic benefit was comparable with the effect achieved by treating with the clinically available drug DF. We suggest that the antiarthritic effect of the investigated compounds occurs through the inhibition of oxidative stress in AA, and increased antioxidant activity. These findings suggest that the investigated compounds may be promising agents for prevention and treatment of autoimmune diseases, in combination with other drugs.

The obtained results can be used in further research of safer arthritis treatments to avoid side effects caused by standard therapy.

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