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The effect of complex herbal extract and methotrexate on suppressing adjuvant arthritis in rats

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ABSTRACT

The present study evaluated the therapeutic benefits of complex herbal preparation named CBMDS, consisting of turmeric (*Curcuma longa*), Boswellia (*Boswellia serrata*), Methylsulphonylmethane, Devil's Claw (*Harpagophytum procumbens*) and Silymarin, using it in combination with methotrexate, in order to suppress adjuvant arthritis in rats, and to attenuate methotrexate-induced liver damage. Adjuvant arthritis was induced in 28 rats by a single subplantar injection of complete Freund's adjuvant (0.1 mL) into the left hind paw. The animals were divided into four groups (with seven animals in each). Group I received CBMDS, Group II - CBMDS in combination with methotrexate, and Group III just methotrexate. The treatment lasted from day 0 to day 17 (CBMDS was given daily except weekends in a dose of 160 mg/kg, methotrexate - 2 mg/kg once a week). Group IV was the control group. Clinical (body weight, hind paw volume, erythrocyte sedimentation rate, leukocyte count), biochemical (serum pro-/antioxidant activity markers), immunological (serum interleukin levels) and histological changes in joint and liver tissues were evaluated. CBMDS significantly alleviated arthritis and reduced hepatic damage, which was more evident in the methotrexate group. The combined treatment also markedly reduced arthritic symptoms and levels of malondialdehyde. Antioxidant activity was significantly higher in treated Groups I and II. CBMDS and its combination with methotrexate promoted anti-arthritic action, reduced histological changes in the joint tissues, and minimized methotrexate-induced liver toxicity.

Key words: adjuvant arthritis; complex of herbal extracts; methotrexate; antioxidant activity

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease, characterized by inflammation of joints, destruction of articular tissues, and deformation of fingers and palms. This disease requires sustained treatment, so medications used in management of RA should be well tolerated. Methotrexate (MTX), an antiproliferative and immunosuppressive agent, is the disease modifying anti-rheumatic drug (DMARD) used for the treatment of RA (FRIEDMAN and CRONSTEIN, 2019; GREMESE et al., 2019). It is a folic acid antagonist, used alone or in combination with other therapeutic agents. However, despite the efficacy of MTX in reducing inflammation, its benefits are impaired by its toxicity resulting in poor compliance (BANJI et al., 2011; FRIEDMAN and CRONSTEIN, 2019). Due to adverse effects, combined therapy of MTX with a number of natural substances is being explored to eliminate or minimize MTX toxicity (ISMAIL et al., 2018; ZUO et al., 2018; HE et al., 2019).

Among these substances, curcumin, derived from the root of *Curcuma longa* (turmeric), which is rich in diferuloylmethane, has been investigated as one of the most promising natural active ingredients (TATY ANNA et al., 2011; MESHKIBAF et al., 2019) for its anti-inflammatory (SHEHZAD et al., 2013) and antioxidant effects (MESHKIBAF et al., 2019).

Boswellic extract (BSE) is used for the treatment of chronic inflammatory diseases (AMMON et al., 2016; MAJEED et al., 2019). Due to its anti-inflammatory effect, BSE can be used as an anti-arthritic drug (KUMAR et al., 2019).

Methylsulphonylmethane (MSM) has been widely used as a dietary supplement for its beneficial effects against various diseases, especially arthritis, in order to reduce arthritic and rheumatic pain (DEBBI et al., 2011; EZAKI et al., 2013; BUTAWAN et al., 2017) and (often in combination with other supplements, such as glucosamine and chondroitin) to treat or prevent osteoarthritis (LUBIS et al., 2017). It also used in sport horses following jumping exercise, to reduce oxidative stress (MARAÑÓN et al., 2008).

Devil's Claw is widely recommended as a popular anti-inflammatory and analgesic agent for the treatment of musculoskeletal disorders (ANDERSEN et al., 2004; ZHANG et al., 2011), and the antioxidant properties of Devil's Claw extract have been highlighted in experimental studies (SCHAFFER et al., 2013; GRABS et al., 2014). It has been used for the treatment of inflammatory symptoms and degenerative disorders in horses for many years (AXMAN et al., 2018).

The complex of flavonoids (silibinin, silydianin, silychristin and others) known as silymarin is a biologically active preparation (WIANOWSKA and WIŚNIEWSKI, 2015). It exhibits anti-inflammatory, antioxidant, anti-apoptotic, and immunosuppressive properties (SHAVANDI et al., 2017; DUPUIS et al., 2018; KANDEMIR et al., 2017). Silymarin has the potential to alleviate the adverse effects of oxidative stress on poultry farms (BARADARAN et al., 2019), and shows hepatoprotective effects on CCl_4-induced hepatic damage in a broiler chicken model, and on acetaminophen-induced hepatotoxicity in mice (BARADARAN et al., 2019; ELSAYED ELGARAWANY et al., 2019).

Numerous plant extracts, including curcumin, Boswellia extract, and MSM, have shown effect, by reducing pain and functional disability, stronger than that observed with analgesics and glucosamine/chondroitin products (HENROTIN and MOBASHERI, 2018). Devil's claw extract (3% harpagoside), in addition to bromelain extract, glucosamine hydrochloride, chondroitin sulfate and MSM, is able to prevent cartilage destruction, and decrease oxidative stress, by reducing malondialdehyde (MDA), and inflammation (UCUNCU et al., 2015).

Our experiments of recent years have revealed that some substances derived from herbs also show an anti-arthritic effect using an adjuvant arthritis model (AKRAMAS et al., 2017; AKRAMAS et al., 2019).

In this study, we explored the anti-arthritic effect of a preparation named CBMDS, alone or in combination with MTX, by using adjuvant arthritis (AA) as the experimental model of RA, which is extensively used for testing natural (AKRAMAS et al., 2019) or therapeutic (BRADŪNAITĖ et al., 2001) products. We also evaluated the potential properties of CBMDS to reduce hepatotoxicity of MTX when both preparations are used in combination.

Materials and methods

Preparations and chemicals. Complete Freund's adjuvant (CFA - Sigma Aldrich, USA), acetic acid, trichloracetic acid, orthophosphoric acid, thiobarbituric acid, nitric acid, ascorbic acid, ferrous sulphate, ammonium molybdate, hydrogen peroxide, 10% neutral buffered formalin, hematoxylin, eosin, toluidine blue, was obtained from Sigma-Aldrich Chemie and Fluka Chemie GmbH (Germany), ketamidor - from Richter Pharma AG (Wels, Austria) and sedaxylan - from Eurovet Animal Health B.V (Holland); tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$) and tannic acid - from Carl Roth GmbH&Co (Germany), sodium citrate - from Penta (Czech Republic), MTX - from Ebewe (Austria). The Commercial ELISA abcam® Kit for IL-17 and Thermo Scientific Rat IL-1β ELISA Kit for IL-1β were used for detection of IL-levels in blood serum. The preparation with the code name CBMDS consisting of turmeric dry extract (curcuminoids 98%), Boswellia dry extract (boswellic acid 30%), MSM, Devil's Claw extract and Silymarin used in experiment was prepared by the Pharmaceutical company "Aksada" (Kaunas, Lithuania), and provided for investigation in rats with experimental AA.

Animals. Twenty-eight male albino Wistar rats weighing 180-230 g, were obtained from the breeding center of the State Research Institute, Centre for Innovative Medicine, Department of Biomodels (Vilnius, Lithuania), and used for the study. The animals were housed in suitable cages and acclimatized to laboratory conditions for a period of one week before the commencement of the experiment. The rats were fed with standard rodent food pellets (JCS; Litagra Company Group, Lithuania), and received water *ad libitum*. All animals were humanely treated, in accordance with the Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. The study design was approved by the Lithuanian Laboratory Animal Use Ethics Committee, under the State Food and Veterinary Service (Protocol No. G2-31).

Induction of arthritis and treatment schedule. Arthritis was induced by subplantar injection of 0.1 mL CFA into the left hind paw of anaesthetized animals (ketamidor and sedaxylan - 0.1 mL i.m.). The animals were divided into four groups (with seven animals in each). Group I received CBMDS, Group II - CBMDS in combination with MTX, and Group III - MTX alone. Group IV was the arthritis control group. The treatment lasted from day 0 to day 17 (CBMDS was given daily, except weekends in a dose of 160 mg/kg, MTX - 2 mg/kg, once a week). Preparations were suspended in 1% starch gel and injected orally by gastric intubation at a volume of 1 mL per rat. The control group received the same volume orally of starch gel as the vehicle on the treatment days.

Measurements. Arthritis development was evaluated clinically and histologically. Body weight and joint swelling were evaluated 7 times during the whole experiment, which lasted for 17 days. Paw volumes were measured using a Plethysmometer PVP1001 (Kent Scientific Corporation, USA).

At the end of experiment (on day 18) the animals were humanely sacrificed by decapitation under ketamidor and sedaxylan anesthesia (0.1 mL i.m.). The general condition of the animals, their weight, macroscopic changes to internal organs, and indices of inflammatory processes were evaluated. Erythrocyte sedimentation rate (ESR) and leukocyte count were estimated using a Picoscale hematological analyzer (Hungary). Blood samples were collected in graded tubes during decapitation, and centrifuged at 800 x *g* for 10 min. to obtain serum samples, which were stored frozen at -20 °C until testing. The concentration of MDA, catalase (CAT) activity and the total antioxidant activity (AOA) in the blood serum were investigated according to the methods described by GAVRILOV et al. (1987), KOROLIUK et al. (1988) and GALAKTIONOVA et al. (1998). A precise description of these methods has been published previously (AKRAMAS et al., 2015).

Estimation of IL-17 and IL-1β in blood serum. Cytokine IL-17 levels in the blood serum of the control and test animals were measured by an enzyme-linked immunosorbent assay (ELISA) kit specific for rats (ab119536-IL-17 Rat ELISA Kit) according to the procedure recommended by the manufacturer's instructions (abcam®, UK). IL-1β levels in blood serum were measured using a Thermo Scientific Rat IL-1β ELISA Kit. Each sample was assayed in duplicate.

Histology. The livers and damaged joints of the rats were taken, fixed in formalin, decalcified,

and embedded in paraffin. Deparaffinized 5 μm histological sections of joints and liver were stained with hematoxylin-eosin (H&E) and toluidine blue for histopathological examination with a light microscope. All histological material was evaluated by a blind method by two independent pathologists. 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 - severe changes.

Statistics. ANOVA statistical analysis was performed using PRISM Software (GraphPad Software, San Diego, CA, USA) and the differences between the groups were determined 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 value ± SEM and were considered to be statistically significant at $P<0.05$.

Results

Clinical and hematological indices. Although an increase in animal body weight was observed in all treated groups during the experiment, no significant differences were found between the test and the control AA groups (data not shown). Only on the last day of the study was a marked increase in body weight noticed in animals that received CBMDS (257.43 ± 9.22 g - test group; 223.12 ± 11.71 g - the control AA group; $P<0.05$).

Visual changes were not observed in the macroscopic examination of the internal organs. The relative weight of kidneys was significantly lower ($P<0.05$) in Groups II and III in comparison with Group IV (control). The spleen weight was markedly lower in all treated groups (Table 1): in Groups I and II spleen weight decreased by 21% ($P<0.01$ and $P<0.02$ respectively), and in the Group III - by 34.2% ($P<0.001$) showing the positive effect of the preparations. The thymus was found to have a significantly lower relative weight ($P<0.001$) after the treatment with CBMDS.

During the study, significant suppression of joint swelling was observed in all treated groups (Fig. 1). The suppressing effect of the investigated compounds on the joints was similar, and exceeded by more than 50% the control group at the end of experiment: 57.9% ($P<0.0001$), 51.5% ($P<0.0001$), and 54.5% ($P<0.0001$) in groups receiving CBMDS, its combination with MTX, and MTX alone, respectively.

The analysis of blood parameters at the end of experiment (Fig. 2) showed a statistically significant decrease in ESR in groups treated with CBMDS ($P<0.0001$), its combination with MTX ($P<0.0001$) and MTX alone ($P<0.001$) in comparison with the control AA group. All investigated compounds also significantly diminished the leukocyte count: CBMDS - by 48.6% ($P<0.001$), CBMDS+MTX - by 48% ($P<0.001$), MTX - by 39.2% ($P<0.002$).

Changes in pro-inflammatory cytokines IL-17 and IL-1β in blood serum. Treatment with the preparation CBMDS, its combination with MTX and

Table 1. Body and relative organ weight in rats with adjuvant arthritis treated with CBMDS preparation, its combination with MTX and MTX alone

Groups	Body weight (g)	Liver (g/kg^{-1})	Kidneys (g/kg^{-1})	Spleen (g/kg^{-1})	Thymus (g/kg^{-1})
I CBMDS	257.43 ± 9.22*	3.45 ± 0.10	0.82 ± 0.02	0.30 ± 0.01 *	0.14 ± 0.01 *
II CBMDS+MTX	242.29 ± 11.02	3.30 ± 0.14	0.79 ± 0.01 *	0.30 ± 0.02 *	0.21 ± 0.02
III MTX	243.86 ± 8.85	3.45 ± 0.13	0.80 ± 0.01 *	0.25 ± 0.01 *	0.24 ± 0.01
IV Control	223.12 ± 11.71	3.42 ± 0.15	0.89 ± 0.04	0.38 ± 0.02	0.24 ± 0.02

Adjuvant arthritis (AA) was induced by injection 0.1 mL of complete Freund's adjuvant (CFA) into the left hind paw. The test groups with AA were treated from AA induction day: Group I with CBMDS preparation, Group II - with a combination of CBMDS and methotrexate (MTX), Group III - with MTX. Group IV - AA control without treatment. * The differences are significant in comparison with the control AA group.

Table 2. Pathomorphological changes in the liver of rats with adjuvant arthritis treated with CBMDS preparation, its combination with MTX and MTX alone

Index		Groups			
		I CBMDS	II CBMDS+MTX	III MTX	IV AA control
Alterations of parenchyma		0.57 ± 0.07	0.21 ± 0.10 *	0.86 ± 0.14	0.64 ± 0.09
V. centralis hypervolemia		0.28 ± 0.10 *	0.28 ± 0.10 *	0.64 ± 0.09	0.57 ± 0.07
Inflammatory infiltration of hepatic stroma	Lymphocytes	0.50 ± 0.00	0.21 ± 0.10 *	0.50 ± 0.10	0.71 ± 0.10
	Macrophages	0	0	0	0.07 ± 0.07
	General	0.50 ± 0.00	0.21 ± 0.10 *	0.43 ± 0.07	0.71 ± 0.10
	Penetration into the lobule	0.21 ± 0.10	0.14 ± 0.09	0.43 ± 0.17	0.43 ± 0.17

The test groups with adjuvant arthritis (AA) were treated from AA induction day: Group I with CBMDS preparation, Group II - with a combination of CBMDS and methotrexate (MTX), Group III - with MTX. Group IV - AA control without treatment. 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 - severe changes. * The differences are significant in comparison with the control AA group.

MTX alone significantly diminished IL-17 level by 70% (P<0.02), 59.5% (P<0.05) and 71.3% (P<0.01) respectively in comparison with the control AA group (Fig. 3). Although a lower level of IL-1β was observed after the treatment with CBMDS and its combination with MTX, no significant differences in comparison with the control AA group were found. The herbal preparation diminished IL-1β concentration by 17.7% and CBMDS+MTX - by 14.5%.

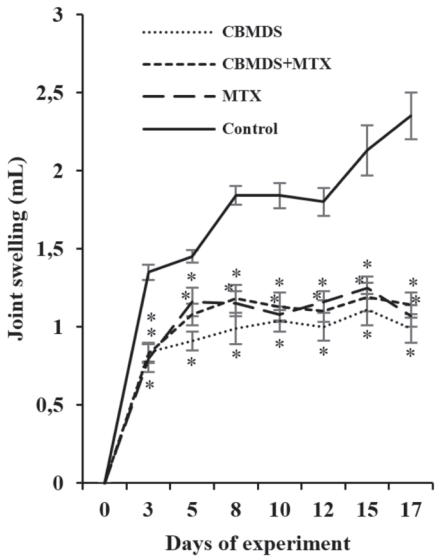


Fig. 1. Joint swelling in rats with adjuvant arthritis (AA) treated with CBMDS, its combination with methotrexate (MTX) and MTX alone. * The differences are significant in comparison with the control AA

Effect of preparations on lipid peroxidation and antioxidant activity. AA induced a rise in MDA level, reflecting serum lipid peroxidation. MDA decreased significantly, by 26.1% (P<0.01) after the treatment of AA with CBMDS (Group I) and by 36% (P<0.001) after combined treatment (Group II). CBMDS also enhanced CAT activity by 17.7% and CBMDS+MTX - by 14.5% (Fig. 4).

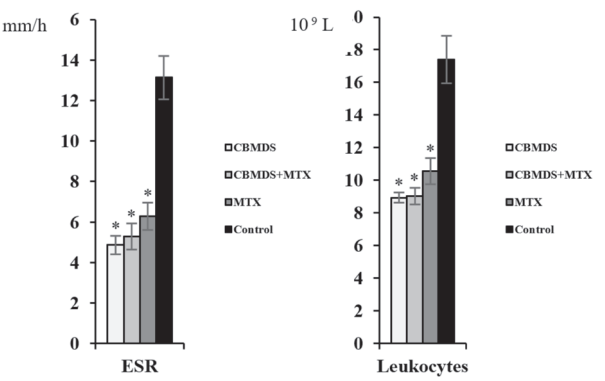


Fig. 2. Blood indices (erythrocyte sedimentation rate - ESR, leukocyte count) in rats with adjuvant arthritis (AA) treated with CBMDS, its combination with methotrexate (MTX) and MTX alone. * The differences are significant in comparison with the control AA group.

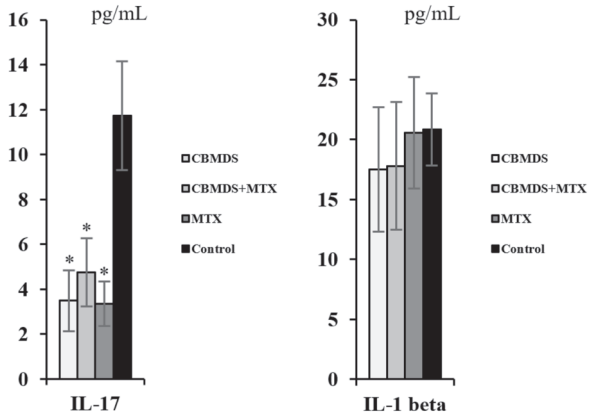


Fig. 3. Interleukin (IL-17 and IL-1β) levels in rats with adjuvant arthritis (AA) treated with CBMDS, its combination with methotrexate (MTX) and MTX alone. * The differences are significant in comparison with the control AA group.

AOA increased by 74.3% (P<0.001) and 53.6% (P<0.05) in both Groups I and II, treated with CBMDS and CBMDS+MTX combination, respectively. MTX alone only insignificantly diminished the MDA level by 16.6%, and enhanced CAT activity and AOA by 7.9% and 2.5%, respectively.

Histological changes in the liver. Treatment of AA with CBMDS and its combination with MTX significantly reduced *V. centralis* hypervolemia (P<0.05) compared to the control AA group (Table 2). The CBMDS+MTX also markedly decreased alteration of parenchymal hepatocytes (hyperchromatic and atypical nuclei, eventual small lipid droplets in cytoplasm, apoptotic fragments, and eventual tiny pericellular fibrosis; P<0.01). CBMDS+MTX diminished inflammatory infiltration of hepatic stroma with lymphocytes, and the general inflammatory reaction (P<0.01). So, the most beneficial effect on the liver was observed using the combined preparation CBMDS+MTX. The addition of the herbal preparation to MTX improved hepatic tissue changes caused by MTX toxicity.

Histological changes in the joint tissues. As shown in Table 3 and Fig. 5, the combined preparation CBMDS+MTX most strongly reduced changes in the soft periarticular tissues. It significantly decreased inflammatory infiltration with lymphocytes (P<0.02), leukocytes (P<0.002), and general inflammatory reaction (P<0.001).

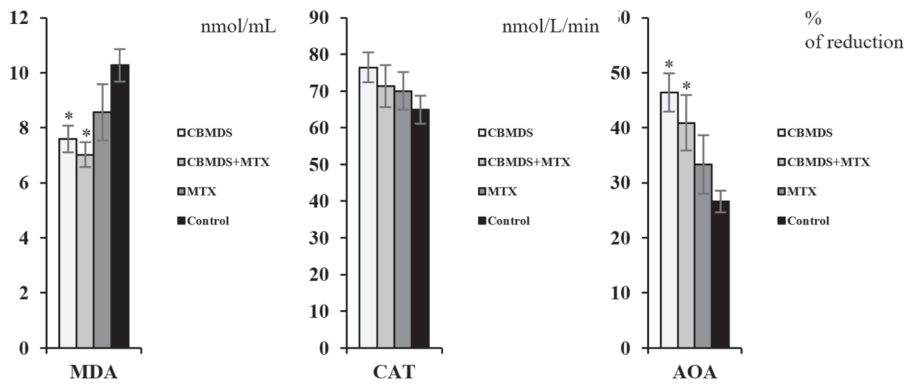
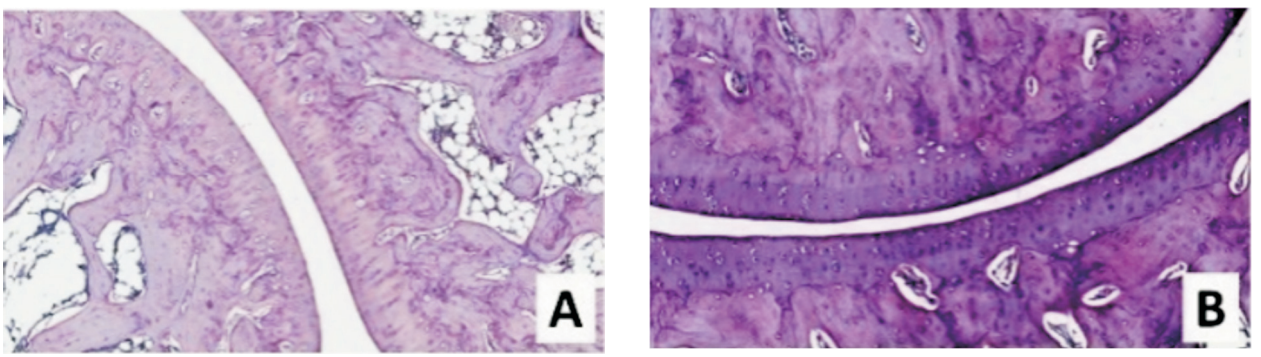


Fig. 4. Pro-/antioxidant indices (malondialdehyde - MDA, catalase - CAT, antioxidant activity - AOA) in blood serum of rats with adjuvant arthritis (AA) treated with preparation CBMDS, its combination with methotrexate (MTX) and MTX alone. * The differences are significant in comparison with the control AA group

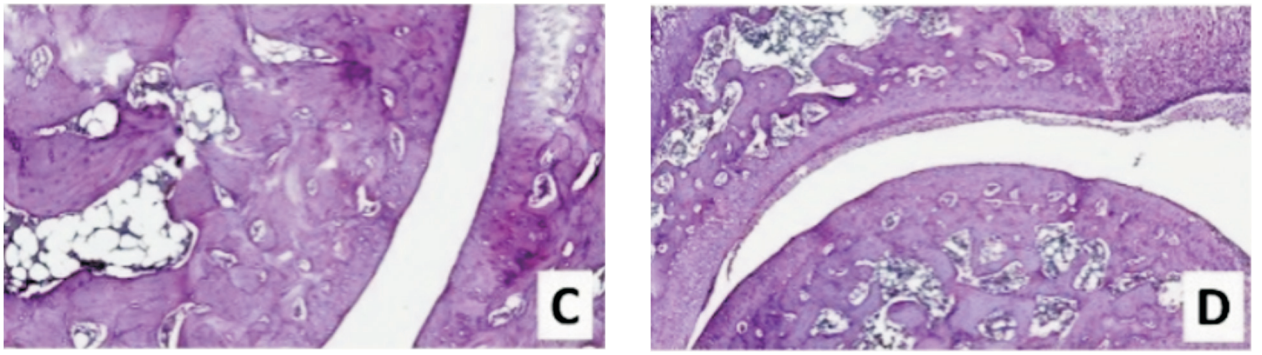
Table 3. Histological changes in joint tissues of rats with adjuvant arthritis treated with CBMDS preparation, its combination with MTX and MTX alone

Tissue	Index		Groups			
			I CBMDS	II CBMDS+MTX	III MTX	IV AA control
Soft periarticular tissues	Inflammatory infiltration	Lymphocytes	1.75 ± 0.21	1.28 ± 0.15 *	1.92 ± 0.20	1.93 ± 0.17
		Leukocytes	0.17 ± 0.10 *	0.07 ± 0.07 *	0.25 ± 0.11 *	1.71 ± 0.39
		Macrophages	1.33 ± 0.16	0.93 ± 0.17	1.67 ± 0.25	1.36 ± 0.14
		General	1.83 ± 0.16 *	1.36 ± 0.14 *	2.08 ± 0.20	2.50 ± 0.15
	Edema		0.91 ± 0.08 *	0.50 ± 0.00 *	1.17 ± 0.10 *	2.29 ± 0.10
	Angiomatosis		0.83 ± 0.10 *	0.64 ± 0.09 *	1.25 ± 0.11 *	2.00 ± 0.15
	γ-metachromasia		0.33 ± 0.16 *	0 *	0 *	1.36 ± 0.18
Synovium	Proliferation		1.41 ± 0.20 *	0.93 ± 0.17 *	1.17 ± 0.17 *	2.21 ± 0.15
	Edema		0.83 ± 0.16	0.36 ± 0.09 *	0.58 ± 0.08 *	1.29 ± 0.18
	γ-metachromasia		0.17 ± 0.10	0 *	0.08 ± 0.08	0.36 ± 0.14
	Inflammatory infiltration	Lymphocytes	1.25 ± 0.17 *	0.43 ± 0.20 *	0.67 ± 0.17 *	1.79 ± 0.15
		Leukocytes	0.17 ± 0.10 *	0.14 ± 0.09 *	0.25 ± 0.17 *	1.43 ± 0.33
		Macrophages	0.91 ± 0.27	0.21 ± 0.15 *	0.33 ± 0.21	0.79 ± 0.15
		General	1.33 ± 0.21 *	0.50 ± 0.19 *	0.75 ± 0.17 *	2.00 ± 0.15
	Angiomatosis		0.75 ± 0.11 *	0.50 ± 0.10 *	0.67 ± 0.10 *	1.57 ± 0.31
Cartilage	Alteration	Erosion	1.33 ± 0.42	0.64 ± 0.23	0.83 ± 0.38	1.14 ± 0.40
		Usura	0.50 ± 0.18 *	0.14 ± 0.14 *	0.17 ± 0.10 *	1.36 ± 0.28
	Pannus		0.25 ± 0.17 *	0.14 ± 0.14 *	0.17 ± 0.10 *	1.43 ± 0.27
	Thinning of cartilage		0.08 ± 0.08 *	0.07 ± 0.07 *	0.08 ± 0.08 *	0.71 ± 0.18

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



Figs. 5 A and 5 B. Histological image of the joints in rats with adjuvant arthritis (AA) after treatment with preparation CBMDS, its combination with methotrexate (MTX) and MTX alone. A - CBMDS (160 mg/kg daily except weekends): smooth articular surface with mild hypocellularity, mild bone resorption, irregular tide mark. B - CBMDS+MTX (2 mg/kg once a week): normal joint cartilage.



Figs. 5 C and 5 D. Histological image of the joints in rats with adjuvant arthritis (AA) after treatment with preparation CBMDS, its combination with methotrexate (MTX) and MTX alone. C - MTX (2 mg/kg once a week): cartilage hypocellularity, superficial clefts, destroyed tide mark, mild bone resorption. D - AA control without treatment: pannus, clefts to transitional zone, destroyed tide mark, moderate bone resorption. Original magnification 100 x, stained with hematoxylin and eosin (H&E).

Tissue infiltration with macrophages was near to significant (t = 1.95) compared to the changes observed in the control AA group. MTX alone suppressed only infiltration with leukocytes (P<0.01), CBMDS - infiltration with leukocytes (P<0.01) and general inflammatory reaction (P<0.01). Notable suppression of edema (P<0.0001), angiomatosis (P<0.001; P<0.002) and γ-metachromasia (P<0.002-0.001) were found in all treated groups.

Synovium proliferation was significantly (P<0.01-0.001) reduced in all treated groups, however, significant suppression of edema was only observed in Groups II and III treated with CBMDS+MTX (P<0.001) and MTX (P<0.01), respectively. Suppression of edema was only found to be close to significant (t = 1.91) by applying a single CBMDS mixture for the treatment of AA. No signs of γ-metachromasia were observed in Group II (P<0.05 compared to the control AA group). CBMDS+MTX also significantly suppressed inflammatory infiltration with macrophages (P<0.02). A significant decrease in infiltration with lymphocytes (P<0.05-0.001), and leukocytes (P<0.01), a diminished general inflammatory reaction (P<0.05-0.001), and less angiomatosis (P<0.05-0.01) in the synovium were observed after treatment with the all preparations.

Studies of cartilage erosions showed no significant differences between the control AA group and the treated groups. Meanwhile, the *usurae* were found markedly suppressed in all the treated groups (P<0.05-0.002) (Table 3).

All preparations significantly inhibited the formation of pannus (P<0.01-0.001). Decreased thinning of cartilage was observed using all protocols of treatment (P<0.01, Table 3).

Discussion

On the basis of studies by many researchers who have intensively investigated the biologically active substances of various plants and tried to apply them in the treatment of inflammatory processes and RA (JIANG et al., 2010; GANESAN et al., 2016), and also from our experience over the past years (AKRAMAS et al., 2017; AKRAMAS et al., 2019), we investigated for the first time a combined preparation of CBMDS alone or in combination with MTX on rats with AA, and compared their anti-inflammatory and antioxidant effects with MTX alone, which is widely used in the treatment of RA.

MTX alone or in combination with other medications is one of the most effective disease-modifying antirheumatic drugs (DMARDs). Despite the well-known inflammation-reducing benefits of MTX, the use of this drug is associated with toxic effects (CHATZIDIONYSIOU and SFIKAKIS, 2019; FRIEDMAN and CRONSTEIN, 2019). 8-19% of patients discontinue MTX treatment due to adverse effects, such as gastrointestinal, hepatic, renal, pulmonary, and hematological disturbances,

or even damage to the central nervous system (VARATHARAJAN et al., 2009; MAHMOUD et al., 2017). Other authors state that 26% of patients refuse MTX treatment due to poor response, high toxicity, or both (CHATZIDIONYSIOU and SFIKAKIS, 2019). MTX hepatotoxicity was demonstrated in our study, and these findings are in line with reports by other authors, showing damage of liver tissue following MTX therapy (BANJI et al., 2011; HE et al., 2019). An alternative solution is to combine MTX with other medications or natural products with anti-inflammatory and antioxidant properties, in order to eliminate or reduce MTX toxicity (BANJI et al., 2011; ZUO et al., 2018; ISMAIL et al., 2018; HE et al., 2019). On the basis of histological liver findings, we confirmed that there was less alteration of the hepatic parenchyma and fewer changes to the stroma in Group I (CBMDS) and particularly Group II (CBMDS+MTX) as compared to Group III (MTX) and the control AA group. The lowest scores of changes to the liver tissues were observed in the group of animals treated with CBMDS+MTX, which showed that the CBMDS preparation can inhibit MTX-induced hepatotoxicity. This effect might be mostly associated with the silymarin in the investigated preparation. Silymarin can neutralize free radicals, suppress lipid peroxidation and lymphocyte proliferation, and improve liver function (HUSSAIN et al., 2009; POLYAK et al., 2010; ASHKAVAND et al., 2012; GHARAGOZLOO et al., 2013; FEDERICO et al., 2017; BARADARAN et al., 2019). The beneficial effect on the liver of other compounds in CBMDS also cannot be ruled out (BANJI et al., 2011).

In our trial, the combination of CBMDS with MTX for AA management significantly reduced joint swelling by 51.5% ($P<0.0001$). Although the reduction of joint swelling in the MTX group (54.6%, $P<0.0001$) appeared to be slightly higher in comparison with the control group than in the CBMDS+MTX group, it should be noted that difference between the CBMDS+MTX and MTX groups is small and insignificant. Changes in joint tissues, confirmed by the histological examination, showed that the combination of CBMDS with MTX showed the best effect on the joints, with the smallest changes observed in soft joint tissue, synovium and cartilage. Also, there were no significant differences in terms of decreased leukocyte count between the treated groups, although CBMDS and CBMDS+MTX diminished leukocytosis to a higher degree. The greater reduction of leukocyte count in the CBMDS and CBMDS+MTX groups shows the positive anti-inflammatory effect of the test preparation. In addition, the course of arthritis in individual animals may not be completely identical. Apparently, this is what caused these small and insignificant differences between the treated groups.

The results obtained are not surprising, because the CBMDS preparation consists of dry extracts from turmeric, Boswellia, MSM, Devil's Claw, and silymarin. All the extracts in this mixture exert anti-inflammatory properties, and are suggested for the additional treatment of inflammatory arthritic processes (SHEHZAD et al., 2013; ROY et al., 2019; KARABAY et al., 2014; MENGHINI et al., 2019; AVATO and ARGENTIERI, 2019; POLYAK et al., 2013). In addition, the anti-inflammatory activity of some of them, such as curcumin, seems to be comparable to steroid and non-steroid medications, such as indomethacin and phenylbutazone (MENON and SUDHEER, 2007).

The positive effects on the joints are apparently related to MSM and Devil's claw, which are included in the CBMDS. Preparation MSM has a beneficial effect on OA chondrocyte metabolism, probably due to the modulation of the NF-kB pathway (CHELESCHI et al., 2018), and is considered to be a source of sulfur for the production of sulfur-containing amino acids, because sulfur is needed for the formation of connective tissue and in maintaining its function (PARCELL, 2002). Devil's claw extract not only inhibits the inflammatory process and cartilage degeneration in the joints, but also greatly improves blood circulation in the synovium, hence ensuring joint nutrition and toxin elimination (VLACHOJANNIS et al., 2008; WACHSMUTH et al., 2011).

The data from our research confirm and supplement the work of other authors on individual components of CBMDS, or some combinations, in which anti-arthritic, antioxidant and immunosuppressive effects were observed in animal models of arthritis (SCHAFFER et al., 2013;

HENROTIN and MOBASHERI, 2018; UCUNCU et al., 2015; KHAYYAL et al., 2018; MESHKIBAF et al., 2019; WANG et al., 2019; ESCOBEDO-MARTÍNEZ et al., 2019).

The important mediators in the pathogenesis of RA are pro-inflammatory cytokines, such as IL-1β, IL-17, TNF-α, and IL-6 (MATEEN et al., 2016; ABOREHAB et al., 2017; WU et al., 2018). Elevated levels of TNF-α and IL-1β in damaged tissues, and IL-1β in serum were also observed in animals with arthritis (KUNCHA et al., 2013; ISMAIL et al., 2018). The T-cell product IL-17 plays a marked role as a mediator in RA pathogenesis. It stimulates inflammation by enhancing the production of IL-1β, TNF-α and IL-6 (ZHANG et al., 2013; SHI et al., 2015). Its levels significantly increase in rats with AA compared with healthy animals, as has been demonstrated in our studies (AKRAMAS et al., 2017). A marked decrease in IL-17 by 59.5% and IL-1β by 14.5% in blood serum was found using CBMDS+MTX in our study. The fact that the levels of cytokines, such as IL-1β and IL-17, decrease after treatment with CBMDS and its combination with MTX, indicates the positive effect of these agents on the course of the pathological process.

No adverse animal behavior, clinical or physiological symptoms were observed during the treatment with the investigated products, which indicates their non-toxicity *in vivo*. In our study, the control AA group showed marked leukocytosis (which is a sign of AA). The treatment improved blood parameters. The significant decrease in ESR and leukocyte count in the treated groups, as compared with the control AA group, indicates the positive effect of tested agents.

Thus, the treatment with CBMDS and CBMDS+MTX did not show liver toxicity, but improved blood indices, and exhibited immunomodulatory effects by reducing the levels of pro-inflammatory cytokines, such as IL-17.

Excessive production of free radicals in arthritic animals and RA patients is associated with a stimulated pro-oxidant system and a deficient antioxidative defense system, leading to oxidative stress and lipid peroxidation. It causes not only synovium damage, but also injures other internal organs (HARUNA et al., 2007; COMAR et al., 2013). Antioxidant enzymes present in biological systems can protect the tissues from oxidative damage. The antioxidant, anti-inflammatory, anti-proliferative and immunomodulatory effects of separate compounds in the CBMDS preparation have been described by other authors (POLYAK et al., 2013; ZANG et al., 2013; VAN DER MERWE and BLOOMER, 2016; CHELESCHI et al., 2018). Our trials have shown that the activity of AOA and CAT is lower in animals with AA, which is apparently associated with increased free radical production. The reduced activity of antioxidant enzymes correlates with the increased peroxidation of lipids, measured as the amount of MDA. It is well known that MDA is the end product of lipid peroxidation, and therefore its content can be used to evaluate lipid peroxidation (HUANG et al., 2012). Effective scavengers of reactive oxygen species are curcumin (MENON and SUDHEER, 2007; ESCOBEDO-MARTÍNEZ et al., 2019), Boswellia (UMAR et al., 2014), MSM (EZAKI et al., 2013; LUBIS et al., 2017), and Devil's claw (SCHAFFER et al., 2013). Our data from biochemical evaluation showed that MDA levels were significantly lower in the treated groups and the activity of AOA and CAT was higher than in the AA group without treatment. The increase in AOA in the CBMDS group was most pronounced, which is not surprising, as the compounds included in CBMDS have antioxidant activity. MTX, although effective in ameliorating the progress of AA, induces hepatic changes. As opposed to MTX, CBMDS and its combination with MTX protected the livers of AA rats, and downregulated oxidative stress by decreasing the MDA level and increasing AOA.

CBMDS+MTX showed the most beneficial effect on the liver in our study. This indicates that the preparation has a hepatoprotective effect, as was also observed by some other authors using curcumin, silymarin, and flavonoids together with MTX (BANJI et al., 2011; BARADARAN et al., 2019; HE et al., 2019).

In summary, it can be concluded that CBMDS and its combination with MTX could be useful as an alternative for reducing the side-effects of MTX in the treatment of adjuvant arthritis. Since the tested

CBMDS preparation showed anti-inflammatory, antioxidant and immunosuppressive activity in the treatment of experimental arthritis, it could also probably serve as a preventive or therapeutic agent for other autoimmune diseases. Experiments with larger numbers of animals, and other autoimmune disease models are needed, in order to prove its efficacy. The combination of natural substances such as CBMDS with conventional DMARDs such as MTX could be an alternative or complementary medicine therapy for RA in the future.

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SAŽETAK

U ovom je radu procijenjena terapijska korist mješavine biljnih preparata pod imenom CBMDS, koja sadržava kurkumu (*Curcuma longa*), tamjanovo drvo (*Boswellia serrata*), metilsulfonilmetan, vražju kandžu (*Harpagophytum procumbens*) i silimarin. Biljni preparati su upotrijebljeni u kombinaciji s metotrekstom s ciljem supresije adjuvantnog artritisa u štakora i smanjenja oštećenja jetre uzrokovanog metotreksatom. Adjuvantni artritis izazvan je u 28 štakora pojedinačnom subplantarnom injekcijom mineralne uljne emulzije Freund's Complete Adjuvant (0,1 mL) u stražnju lijevu šapu. Životinje su podijeljene u četiri skupine (u svakoj po sedam jedinki). Skupina I primila je CBMDS, skupina II – CBMDS u kombinaciji s metotreksatom, a skupina III – metotreksat. Pokus je trajao od nultog do 17. dana (CBMDS je davan svaki dan, osim vikendom, u dozi od 160 mg/kg, metotreksat u dozi od 2 mg/kg jedanput tjedno). Skupina IV bila je kontrolna skupina. Procijenjene su kliničke (tjelesna masa, opseg stražnje šape, sedimetacija eritrocita, broj leukocita), biokemijske (marker serumske aktivnosti prooksidansa i antioksidansa), imunološke (razine serumskog interleukina) i histološke promjene u zglobovima i jetri. CBMDS je znakovito ublažio artritis i smanjio oštećenje jetre, što je bilo očitije u skupini s metotreksatom. Kombinirana terapija također je znatno smanjila simptome artritisa i razine malondialdehida. Antioksidacijska aktivnost bila je znakovito veća u skupinama I i II. CBMDS u kombinaciji s metotreksatom imao je antiartritično djelovanje, smanjio je histološke promjene u zglobovima i minimizirao toksičnost metotreksata za jetru.

Ključne riječi: adjuvantni artritis; kompleks biljnih ekstrakata; metotreksat; antioksidacijska aktivnost