Journal of Molecular Pathophysiology
www.scopemed.org
DOI: 10.5455/jmp.20151023055630

Short Communication

Linden blossom Freon extract morphological examination based on the model of adjuvant arthritis in rats

Dmitry Demyanenko[1], Natalia Timchenko[2], Galina Belik[3], Maxim Evstigneev[4], Olesya Kudina[3], Yuri Stoletov[3], Maxim Zupanets[3], Dmitry Semeniv[3]

[1]Department of Commodity Science, National University of Pharmacy, Kharkiv, Ukraine
[2]Department of Processing and Food Technologies, Kharkiv Petro Vasylenko National Technical University of Agriculture, Kharkiv, Ukraine
[3]Department of Pharmacology, National University of Pharmacy, Kharkiv, Ukraine
[4]Department of Physics, Sevastopol State University, Sevastopol

Address for correspondence:
Natalia Timchenko,
Department of Processing and Food Technologies, Kharkiv Petro Vasylenko National Technical University of Agriculture, Kharkiv, Ukraine,
timchenko_n@list.ru

Received: September 18, 2015
Accepted: October 15, 2015
Published: October 25, 2015

ABSTRACT

Objective: Treatment of diseases of the rheumatoid arthritis provides use of nonsteroidal anti-inflammatory drugs and corticosteroids which have side effects and some drugs were discontinued. It is necessary to find biologically active substances of natural origin of sufficient anti-inflammatory activity which are safe compared to synthetic non-steroidal anti-inflammatory drugs. Blossom of different types of linden is widely used in medicine due to its diverse therapeutic action which can be sedative, expectorant, diaphoretic, diuretic, anxiolytic and antispasmodic. **Methods:** A powder mixture was formed from Linden blossom extract and inert filler (lactose) (Linden blossom powdered extract – LBPE). Anti-inflammatory properties of LBPE were studied on the model of adjuvant arthritis in white mongrel rats. Diclofenac sodium was selected as a comparison drug. Freund's adjuvant, which was administered to animals subplantarly was used to reproduce adjuvant arthritis model. LBPE was intragastrically administered within the limits of the therapeutic and preventive regimen once a day from the first day after injection of adjuvant for 22 days in arbitrary effective dose of 30 mg / kg. **Results:** LBPE has a normalizing effect on condition of the overlying tissues, reducing the severity of synovitis, whereby no evidence of completely invaded and / or destroyed cartilage surfaces by the synovial membrane was registered. Morphometric parameters are better than in the control pathology, and the density of chondrocytes location reaches intact indices. **Conclusions:** Pronounced anti-inflammatory activity of LBPE are determined due to the model of adjuvant arthritis in rats. Under the influence of LBPE cell density indicator increases to intact values.

KEY WORDS: Adjuvant arthritis, anti-inflammatory effect, articular cartilage, chondrocytes, Linden blossom extract

INTRODUCTION

Treatment of diseases of the locomotor system including rheumatoid arthritis (RA) provides long-term use of drugs of different groups, basic of which are nonsteroidal anti-inflammatory drugs and corticosteroids.

New side effects of new generation (coxibs) nonsteroidal anti-inflammatory drugs which are selective inhibitors of II type cyclooxygenase (COG), were discovered. Some drugs used to treat arthrosis and RA were discontinued.

Given the above, the topical problem lies in the search for biologically active substances (BAS) of natural origin, mainly plant substances of sufficient anti-inflammatory activity which are safe compared to synthetic non-steroidal anti-inflammatory drugs (NSAIDs) and thus suitable for long-term treatment of RA and other inflammatory diseases. From an economic point of view, an important factor is the availability of the resource base of certain plants.

Blossom of different types of linden is widely used in medicine due to its diverse therapeutic action which can be sedative, expectorant, diaphoretic, diuretic, anxiolytic and antispasmodic [1-9].

MATERIALS AND METHODS

Linden blossom Freon extract preparation

The exact botanical species were Tilia cordata L. The exact part of the plant tissue which is used for the herbal drug, according to the official Pharmacopoieas was Inflorescence (Lime flower according to EP7). Herbal drug Lime flower was bought in Lektravy (Ukraine). Six lots of lime flowers X0608, X0609, R0608, R0609, Z0608, Z0609 were the objects of our researches. Lipophilic and lipophilic and hydrophilic complexes were obtained from Tilia cordata blossom by downstream processing of raw materials with different liquefied gases and their mixtures. The authors [10] found the anti-inflammatory effect of difluorochloromethane (Freon-22) linden extract at rectal administration to rats.

Gas liquefied linden blossom extracts were studied, as they were perspectively included into the formula of peroral dosage bands. Difluorochloromethane (Freon-22) was used for the preparation of the extract. The methodology of the preparation of the extract described in details in the patent UA93003 Method of complex processing of linden blossom. We provided comprehensive chemical analysis of all extracts

obtained. The difluorochloromethane extract contained mainly lipophylic components (among them there were essential oil components) and very few phenolics. It was found that main (with content above 1%) components of the extract were the following: saturated hydrocarbons (65.7% in total), octyl-acethylene (4.3%), aldehydes (7.4% in total), hexahydrofarnezyl-acetone (1.5%), ethyl nanoate (1.3%). All the rest compounds were minor ones.

Herbal drug-extract ratio of the obtained extract was 1:15 (approximately 6.5% of extract in the drug). Difluoro chloride methane (Freon-22) lipophilic extract of linden blossoms, active substance A0, was chosen to conduct a study. According to organoleptic attributes A0 object is a mixture of brownish-green resinous mass with yellow-green amorphous particles, at 55-60°C both fractions melt down, forming a homogeneous dense extract; the substance has a strong odor, characteristic of linden during flowering. Object A0 easily dissolves in methylene chloride, acetone, hexane, toluene, partly dissolves in ethanol, propylene glycol (PG) and polyethylene oxide (PEO-400) and is practically insoluble in water.

In view of the above properties, to transform the extract into powder so that it was convenient to dose and administer it to animals, the extract A0 was mixed with inert filler (lactose) at a dilution of 1:15, forming a powder mixture (sample A or LBPE – linden blossom powdered extract).

Treatment with Linden blossom Freon extract

Anti-inflammatory properties of sample A were studied on the model of adjuvant arthritis (AA) in white mongrel rats weighing 180-220 g. Diclofenac sodium [10] which is a classic nonsteroidal anti-inflammatory drug, was selected as a comparison drug. Freund's adjuvant, which was administered (single administration) to animals subplantarly at the rate of 0.1 ml per animal [10] was used to reproduce AA model.

LBPE was intragastrically administered within the limits of the therapeutic and preventive regimen once a day from the first day after injection of adjuvant for 22 days in arbitrary effective dose of 30 mg / kg, which was determined in previous studies [10]. Diclofenac sodium was administered at a dose ED50= 8 mg/kg.

The study of drug influence on the development and course of AA was evaluated due to its ability to reduce swelling of the extremities.

Morphological studies

When conducting morphological studies on the AA model, separated joints were fixed in a 10% neutral formalin solution, after long time rinsing in the running water, they were decalcified in a 6% nitric acid solution. To control the joints exposure time in decalcifying fluid, the bone puncturing with a dissecting needle was performed. After using spirits of increasing concentration tissues were embedded in paraffin-celloidin by standard methods, blocks were used to produce 6-8 micron thick sections, which were stained with hematoxylin and eosin.

The «Bimam P-12» microscope was used to carry out a microscopic examination.

Morphometric measurements were performed to objectivize the data received. Ocular micrometer was used to determine the thickness of the cartilage on micropreparations. It was measured in 3 points: in the central part of the head, upper and lower edge. Thickness of articular cartilage was expressed in arbitrary units. The density of chondrocytes was calculated in the conventional area unit. Each value represents the mean ± standard error of the mean. Statistical processing of the received digital data was performed using INSTAT statistical program. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

When conducting morphological studies, it was found that the articular surface of the ankle joint bones of intact rats is covered with a hyaline cartilage, which has an expressed zonal structure. In the outer zone cells are isolated, small flattened, in the middle zone cells are arranged in columns perpendicular to the surface. Chondrocytes in the area are round large, with a high nuclear-cytoplasmic ratio, isogenic groups of two or four cells are found. Their cytoplasm is slightly basophilic. Deep zone contains small chondrocytes, sometimes with pycnotic nuclei, located in the large lacunae. Deep zone of hyaline cartilage is without clear boundary and it becomes calcified, calcification line is poorly contoured. The average thickness of the articular cartilage of intact rats makes up 11,48 standard units, the density of cells in it equals to 27,15 specimens per unit of area. Intercellular substance staining is homogeneous, its intensity increases towards the subchondral bone (Figure 1a). The bone structure is a typical trabecular bone structure. Plexiform trabecula cells contain active polymorphocellular marrow. Areola type synovial membranes protrude into the joint cavity in the form of cuneated folds. Synoviocytes group rather closely to each other, in some areas they lie in 3-4 rows. Subsinovial tissue is poor in cells, connective-tissue fibers prevail there (Figure 1b). A fat type of synovial layer, which covers the intraarticular fat pads is also found there. Its surface cells are arranged in a single layer.

Periarticular connective tissue, including tendons and subcutaneous fat, are of typical for its localization structure, no signs of cell proliferation are found. A moderate amount of cells of lymphoid and histiocytic series (Figure 2) is found. Muscle fibers are equally stained, its cross-striation is well expressed.

At histological examination of ankle joints of rats, held on the 22nd day after inoculation of adjuvant, morphological signs of inflammatory, hyperplastic and destructive-dystrophic processes were observed in all animals.

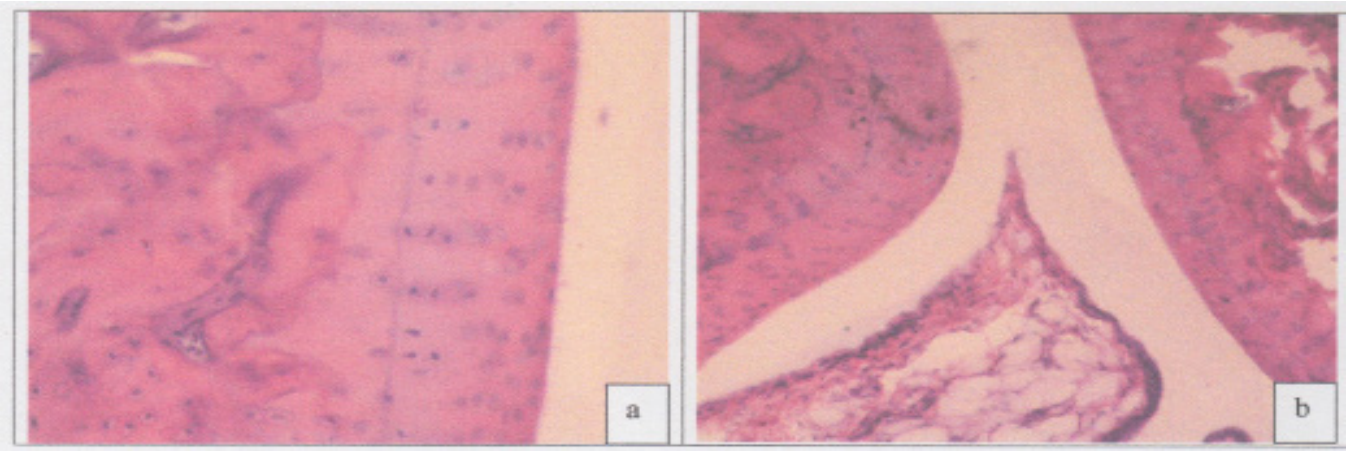

Figure 1. Hyaline cartilage of articular surface of intact rats. (a) Zoning arrangement of cartilage cells is clearly visible. (b) Synovium is of normal structure. Hematoxylin and Eosin x 200

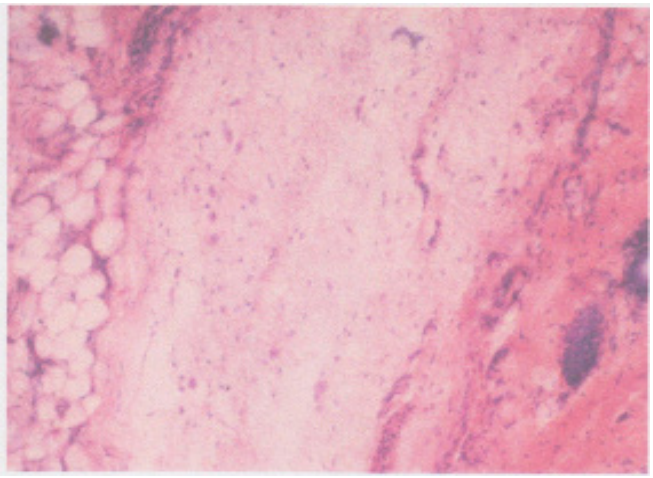

Figure 2. Periarticular tissues of intact rats. Fat, loose connective, muscle tissues with a small content of leukocyte cells are presented here. Hematoxylin and Eosin x 250

Significant tissue changes of synovial membranes were observed in all cases. To such changes belong proliferative and inflammatory processes like: severe proliferation of acentric synoviocytes, which are located in 6-7 and more rows, making the fold thickened and multicellular, the appearance of leukocyte infiltration with an admixture of histiocytes and fibroblasts of different maturity in the thickness of synovium (Figure 3a). Subsinovial layer vessels are dilated, sanguine and contain white blood cells in the lumen.

Hyperplastic coats with pronounced signs of synovitis behave very aggressively towards the cartilage surface. Pannus, a hyperplastic synovial membrane, growing on the sides of the articular surface and tightly adhering to it, has a direct destructive effect. Focally pannus penetrates into the cartilage, replacing it with connective tissue (Figure 3). Cartilaginous surfaces of some joints are completely invaded by synovium, so that it is difficult to distinguish where the cartilage ends and connective tissue begins (Figure 4a). Apparently, mobility of such joints was severely limited. Cartilages, cankered by synovitis from all sides, making cartilage islands which are surrounded by connective tissue (Figure 4b) are found.

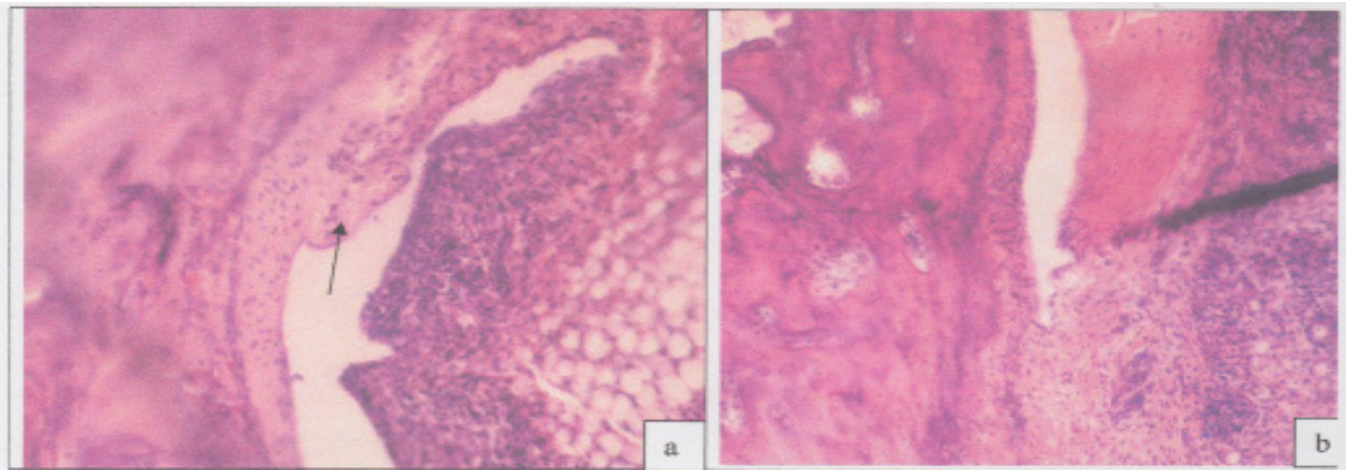

Figure 3. The ankle joint of the rat after Freund's adjuvant inoculation. Hyperplastic synovium (on the right) forms the pannus (arrow) and creeps on the cartilage, invading it. (a) Chondrocytes are randomly arranged. (b) Fusion of the cartilage surface with synovitis, free cartilage is narrowed. Hematoxylin and Eosin x 150

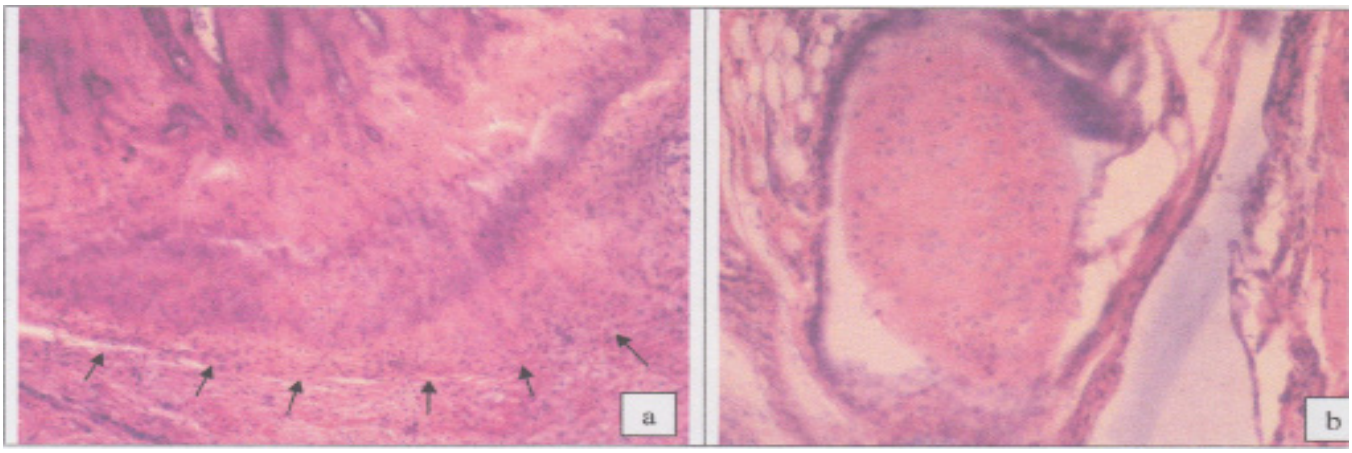

Figure 4. The ankle joint of the rat after Freund's adjuvant inoculation. (a) Complete healing of the cartilage surface with synovitis (arrows) x 250. (b) Cartilage island is in the connective tissue capsule. Hematoxylin and Eosin x 150

On those surfaces where cartilage directly contacts synovitis, it is narrowed (for 26% in comparison with intact) and loses the characteristic chondrocytes zonal location (Figure 3), the density of which is reduced as compared with the intact control.

Diclofenac comparator agent administration in a dose of 8 mg\kg for medicinal purposes does not reduce the severity of inflammatory and proliferative processes in the periarticular tissues. Soft tissue structures are still densely infiltrated by leukocytes, contain granulomas, and muscles are focally lysed (Figure 5). Signs of synovitis are registered, but synovial membranes are somewhat calmer than in the control pathology group. In the case when proliferative processes are localized in subsinovial layer and acentric synoviocytes lie in 1-2 layers, the synovium lies in the joint space, without touching the surface of the cartilage and the cartilage is not changed (Figure 5b).

In the case of pronounced synovitis, the coat forms pannus, which is tightly fused with the articular cartilage (Figure 6). No articular surfaces with fully invaded cartilage were found.

Morphometric parameters do not differ from control pathology, the density of chondrocytes increases in the case of Diclofenac treatment (Table 1).

At a small-leaved linden extract administration in a dose of 30 mg/kg, periarticular tissues morphology does not differ from that in rats of the control pathology group (Figure 7a). Giant cell granular reaction is expressed.

Synovium with proliferating acentric synoviocytes, enlarged and bloodfilled vessels, inflamed subsinovial layers crawls to the articular cartilages. Cells zoning arrangement characteristic of hyaline cartilage, is often violated (Figure 7b). However, no complete fusion of synovitis with the cartilage surface and the destruction of the latter are registered. Morphometric parameters are better than in the control pathology, and the density of chondrocytes location reaches intact indices in the case of linden blossom extract treatment (Table 1).

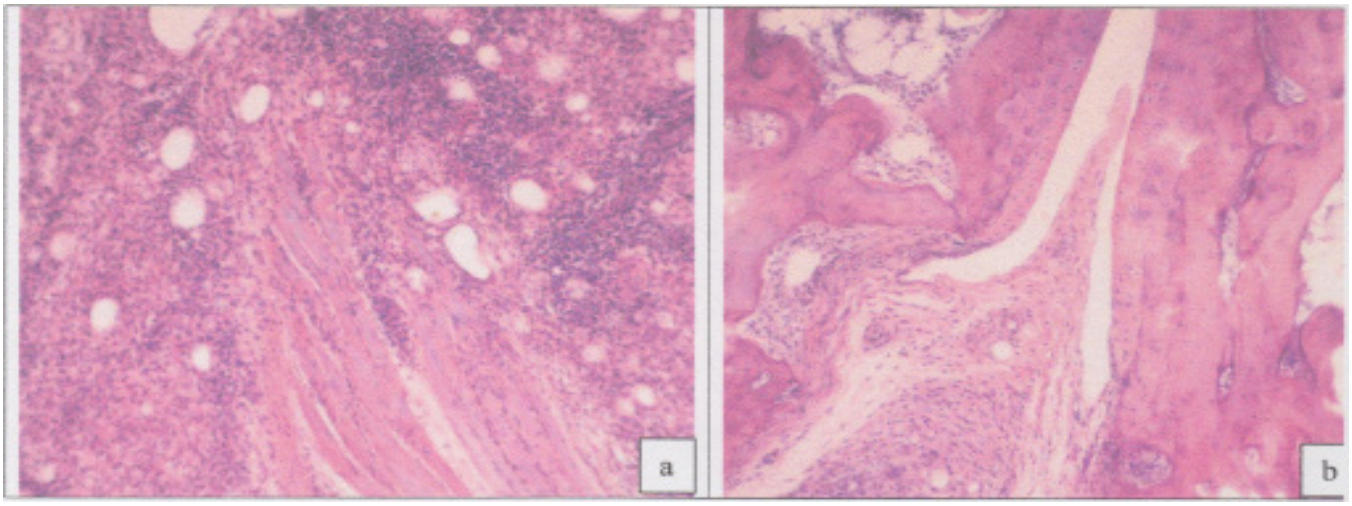

Figure 5. The ankle joint of the rat, Diclofenac, 8 mg / kg. (a) Inflammatory response in the periarticular tissues, myocytolysis. (b) Synovium is without acentric proliferation of synoviocytes, the cartilage is not changed. Hematoxylin and Eosin x 150

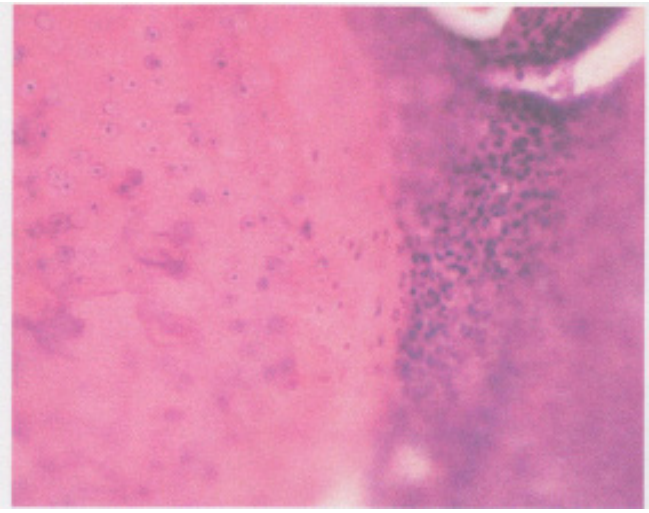

Figure 6. The ankle joint of the rat, Diclofenac, 8 mg / kg. Synovial membrane, which adheres the cartilage surface and invades the segment of the articular cartilage. Hematoxylin and Eosin x 200

DISCUSSION

Some authors have studied the anti-arthritic properties of 2 pharmacological preparations of natural origin: a combination of salt of glucosamine and phenyl-anthranilic acid (BISG-1) and thick extract from the leaves of mountain-ash (TELMA) using the model of collagen-induced arthritis. It was proved that BISG-1 and TELMA show pronounced anti-inflammatory effect on the autoimmune arthritis process [11, 12]. It was shown that BISG-1 regenerates articular cartilage in the pharmacological studies of anti-arthritic activity [12]. The density of chondrocytes location is higher by a 20% than control pathology indices in the case of BISG-1 treatment [12]. Linden blossom extract shows pronounced anti-inflammatory activity also as compared with anti-arthritic pharmacological preparations of natural origin BISG-1 and TELMA. It is necessary to note that the density of chondrocytes location reaches intact indices in the case of linden blossom extract treatment as compared with the density of chondrocytes location in the case of BISG-1 treatment which is higher by a 20% than control pathology indices.

Table 1. Morphometric parameters of articular cartilage in rats

Group	Cartilage thickness	Cell density per unit of area
Intact control	11.48 ± 0.29	27.15 ± 0.94
Control pathology	8.51 ± 0.52*	23.33± 1.09*
Diclofenac	8.14 ± 0.72*	24.66 ± 1.70
Linden blossom	8.94 ± 0.28*	27.15 ± 1.24**

Therefore, pronounced anti-inflammatory activity of LBPE are determined due to the model of adjuvant arthritis in rats. When conducting morphological studies using RA in rats model linden blossom extract has a normalizing effect on condition of the overlying tissues, reducing the severity of synovitis, whereby no evidence of completely invaded and / or destroyed cartilage surfaces by the synovial membrane was registered. In addition, under the influence of the drug cell density indicator increases to intact values.

LIST OF ABBREVIATIONS

LBPE – linden blossom powdered extract; COG – cyclooxygenase; RA – rheumatoid arthritis; BAS – biologically active substances; NSAIDs – nonsteroidal antiinflammatory drugs; PG — propylene glycol; PEO — polyethylene oxide; AA – adjuvant arthritis; BISG-1 – a combination of salt of glucosamine and phenyl-anthranilic acid; TELMA – thick extract from the leaves of mountain-ash.

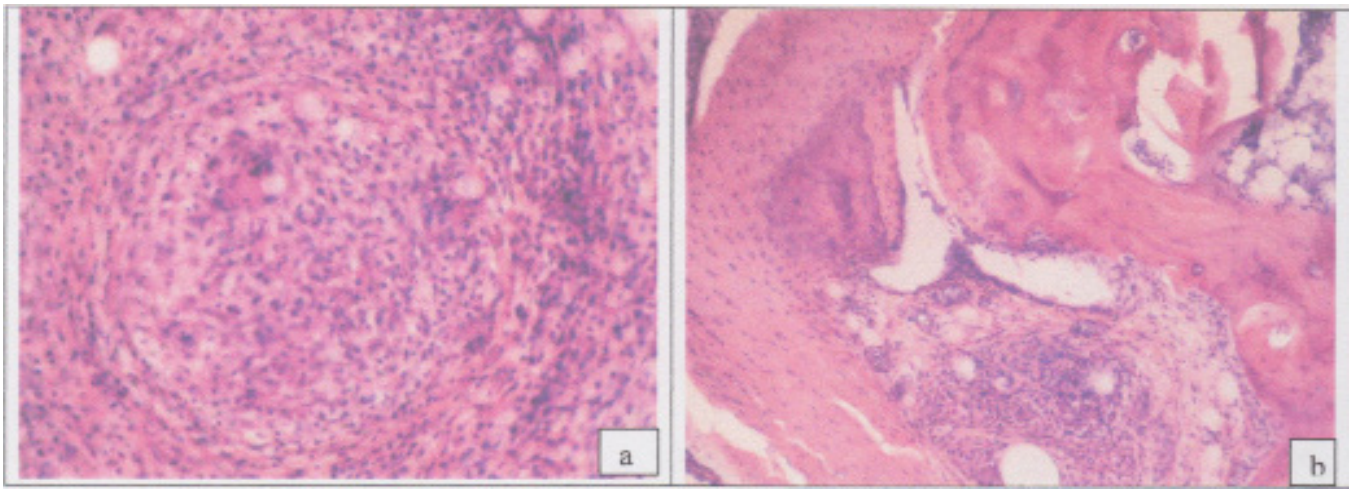

Figure 7. The ankle joint of a rat, linden blossom. (a) Giant cells of foreign bodies and granulomas are in periarticular tissues x 250. (b) Synovitis with signs of synoviocytes creeps on the cartilage, which lost its zoning arrangement of the cells x 150. Hematoxylin and Eosin.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest

REFERENCES

1. Aguirre-Hernandez E. Bioactivity-guided isolation of β-sitosterol and some fatty acids as active compounds in the anxiolytic and sedative effects of tilia americana var. Mexicana. Planta Medica 2007; 73: 1148-5.
2. Coleta M, Campos MG, Cotrim MD, Proença da Cunha A. Comparative evaluation of Melissa officinalis L., Tilia europaea L., Passiflora edulis Sims. and Hypericum perforatum L. in the elevated plus maze anxiety test. Pharmacopsychiatry 2001; 34: 20-1.
3. Cotrim MD, Figueiredo IV, Cavadas C. Effects of Tilia europaea on guinea pig ileum and aorta. Br J Pharmacol 1995; 114: 287.
4. Viola H, Wolfman C, Destein ML. Isolation of pharmacologically active benzodiazepine receptor ligands from Tilia tomentosa (Tiliceae). J Ethnopharmacol 1994; 44: 47-3.
5. Lanza JP, Steinmetz M. Action comparees des extrait aqueux de graines de Tilia platyphylla et de Tilia vulgaris sur l'intestine isole de rat. Fitoterapia 1986; 57: 185-8.
6. Aguirre-Hernández E, Martínez AL, González-Trujano ME. Pharmacological evaluation of the anxiolytic and sedative effects of Tilia americana L. var. mexicana in mice. J Ethnopharmacol 2007; 109: 140-5.
7. Pérez-Ortega G, Guevara-Fefer P, Chávez M. Sedative and anxiolytic efficacy of Tilia americana var. mexicana inflorescences used traditionally by communities of State of Michoacan, Mexico. J Ethnopharmacol 2008; 116: 461-8.
8. Al-Essa MK, Mohammed FI, Shafagoj YA, Afifi FU. Studies on the direct effects of the alcohol extract of tilia cordata on dispersed intestinal smooth muscle cells of guinea pig. Pharm Biol 2007; 45: 246–0.
9. Barreiro Arcos ML, Cremaschi G, Werner S. Tilia cordata Mill. Extracts and scopoletin (isolated compound): differential cell growth effects on lymphocytes. Phytother Res 2006; 20: 34-0.
10. Zhurenko DS, Tsubanova NA. Study of anti-inflammatory activity of suppositories with plant extracts. Current Issues of development of new drugs: Mater. National Scientific Conference for Students and Young Scientists, April 21, 2011. Kharkiv, Ukraine. Kharkiv: National University of Pharmacy, 2011: 284.
11. Shtrygol SY, Drogovoz SM, Zupanets MV, Kononenko AV. Study of the anti-inflammatory activity of pharmacological drugs of natural origin. Herald Pharmacy 2014; 64: 89-3.
12. Drogovoz SM, Zupanets MV. The pharmacological study of anti-arthritic activity of derivatives of glucosamine salts and N-phenylanthranilic acids. Physician-post-graduate 2014; 65: 31-4.

Source of Support: Nil, Conflict of Interest: None declared