



Anti-inflammatory effect of egg white-chalcanthite and purple bamboo salts mixture on arthritis induced by monosodium iodoacetate in Sprague-Dawley rats

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The aim of this study is to investigate the potential of anti-osteoarthritis effects on egg white-chalcanthite (EC), purple bamboo salts (PBS), and a mixture of EC and PBS (EC+PBS). EC is a mixture of egg white and pulverized chalcanthite. PBS has been widely used as one of functional foods in Korea and shows unique features compared with common salt. Osteoarthritis was induced by intra-articular injection of monosodium iodoacetate (MIA, 4 mg/kg bw) in Sprague-Dawley (SD) rats. Test substances were administered once daily for 6 weeks at doses of 10 mg EC, EC+100 mg PBS, EC+200 mg PBS before and after MIA injection. Each substance was assessed by blood chemistry parameters, and by serum cytokines including IL-1 β and IL-6, and nitric oxide (NO) and prostaglandin-E₂ (PGE₂). Structural changes of articular cartilage were also evaluated by histopathological examination. As a result, body weight and blood chemistry parameter were not different in all experimental groups. EC+PBS mixture reduced the production of PGE₂, NO, IL-1 β , and IL-6. In histological grade of osteoarthritis, EC+PBS mixture had a tendency to ameliorate damage of articular cartilage induced by MIA in a dose-dependent manner. In conclusion, EC+PBS mixture was demonstrated to have a potential for anti-inflammatory effect against osteoarthritis induced by MIA in a dose-dependent manner.

Keywords: arthritis, purple bamboo salts (PBS), egg white-chalcanthite (EC), monosodium iodoacetate (MIA)

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Osteoarthritis (OA) known as degenerative arthritis is the most common type of joint disease that results from breakdown of articular cartilage and subchondral bone, ligaments, and synovial membrane [1]. The most common symptoms are significant pain and functional disability [2]. The prevalence of OA in South Korea is continuously increased due to the increase of aging population and obesity. In 2008, 10.7 % of Korean adults are suffering from OA and it is estimated to be up to 34.5% in persons over 80 years old [3,4]. Pathogenesis of OA is not clear. However, mechanical stress, cytokines, nitric oxide, and reactive oxygen species (ROS) have

been known to be major factors of OA [5-7]. Monosodium iodoacetate (MIA), an inhibitor of glyceraldehyde-3-phosphate dehydrogenase activity, is the most common material used to produce animal models of osteoarthritis. Intra-articular injection of MIA caused destruction of the articular cartilage by inhibiting glycolysis and eventually by inducing cell death [8]. OA induced by MIA is very similar to human OA in view of histopathological changes of articular cartilage and chondrocytes [9,10]. In addition, the model has been used to evaluate the effectiveness of the pharmacologic agents related with arthritis in many previous studies [11-13].

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Egg white-chalcanthite (EC) is a mixture of egg white and pulverized chalcanthite. In the egg white-chalcanthite prepared as noted above, the toxicity of the chalcanthite is neutralized by egg white, so that the toxicity is reduced or removed and the pharmaceutical properties are increased [14]. Recently, it has been reported that EC inhibited inflammation induced by lipopolysaccharide in BV2 cell and also inhibited the growth of NCI-H460 cells by induction of apoptotic cell death [14,15]. Purple bamboo salts (PBS) is widely used functional foods in Korea and shows unique features compared with common salt or sun-dried salt. PBS was produced according to the traditional procedure using a sun-dried salt and bamboo. In this procedure, toxicity of sun-dried salt was decreased and acidity is converting to strong alkalinity [16,17]. Also, when compared with sun-dried salt, PBS contained high concentration of iron, potassium, and phosphate, but the concentration of sulfate is low [16]. It has been reported that PBS have protective effects on inflammatory disease [18], oxidative stress [17,19], cancer [21] and gastric ulcers [12].

Based on these properties of the EC+PBS mixture, we investigated anti-osteoarthritis effects of EC, PBS and EC+PBS mixture against MIA-induced osteoarthritis in male Sprague-Dawley (SD) rats.

Materials and Methods

Reagent

Monosodium iodoacetate (MIA) was purchased from Sigma Chem. Co. (St. Louis, USA) and purple bamboo salts and egg white-chalcanthite were provided by Insan Bamboo Salt Inc. (Hamyang, Korea). IL-1 β , IL-6, NO and PGE2, assay kit were purchased from R&D Systems, Inc. or Cayman Chemical Co. Inc. (Ann Arbor, USA).

Animals

Thirty four male Sprague Dawley (SD) rats weighing 210-240 g were purchased from Daehan-biolink (Eumsung, Korea). The animals were housed two or three per polycarbonate cage in a temperature and humidity controlled room (temperature 20~24°C, humidity 40~70%). Dark/light cycle was controlled for 12 hours. The sterile feed and R/O water were *ad libitum*. All the experimental procedures were examined and approved by the Animal Research Ethics Committee at the Hoseo Toxicological Research Center of Hoseo University.

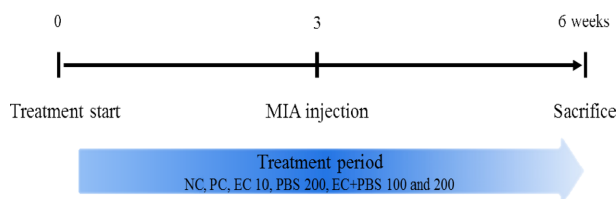


Figure 1. Line diagrams showing experimental design for NC, PC, EC 10, PBS 200, EC+PBS 100 and 200 treatment group in MIA treated rats. MIA: monosodium iodoacetate 4 mg/kg b.w. intra-articular injection, NC: normal control, PC: MIA alone, EC 10: egg white-chalcanthite 10 mg/kg b.w, PBS 200: purple bamboo salt 200 mg/kg b.w, EC+PBS 100: EC+PBS 100 mg/kg b.w, EC+PBS 200: EC+PBS 200 mg/kg b.w..

Development of osteoarthritis and test substance treatment

For induction of osteoarthritis, rats were anesthetized with ketamine (Yuhan Co. Ltd., Seoul, Korea) and xylazine (Bayerkorea Co. Ltd., Seoul, Korea) and then given single intra-articular injection at dose of 4 mg/kg b.w. monosodium iodoacetate (MIA; Sigma, St. Louis, USA) using 27G needle. MIA was dissolved in physiological saline. Control rats were injected with an equivalent volume of saline. Test substance dissolved in saline was given orally once per day for 6 weeks. MIA was injected in all group except normal control group at the 3 weeks of the experiment (Figure 1).

Measurements of blood chemistry parameter

At the 6 weeks after the experiment, animals were anesthetized by CO₂ inhalation. Blood was collected from abdominal aorta and centrifuged at 3,000 rpm for 10 min (2236HR high-speed centrifuge, GYROZEN Co., Ltd, Daejeon, Korea) to obtain serum. The serum were used to measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CRE) and high-sensitivity C-reactive protein (hs-CRP) using the Automatic Chemistry Analyzer (Hitachi Co. Ltd., Tokyo, Japan).

Measurements of NO, PGE2, IL-1 β and IL-6 production

Concentrations of NO, PGE2, IL-1 β and IL-6 in the serum were measured by commercial assay kit following the manufacturer's instructions. NO was determined by measuring nitrite, which is a major stable product of NO, using the Nitrite Colorimetric Assay Kit (Cayman, Ann Arbor, USA). PGE2 was determined using the ELISA kit (Cayman, Ann Arbor, USA). IL-1 β and IL-6 were

determined using the ELISA kit (R&D Systems, Inc. Minneapolis, USA).

Tissue preparation and histological examination

Both knee joints from each animal were dissected and fixed in 10% neutral buffered formalin. Tissues were decalcified in 5% formic acid for 48 hours. After decalcification, the tissues were processed dehydration, clearing, paraffin infiltration step and embedded in paraffin blocks. 5 µm sections were prepared and stained with safranin O-fast green or hematoxylin-eosin. The sections were examined under the light microscope (Olympus BX51, Olympus Co., Ltd., Tokyo, Japan) and evaluated histologically for the severity of lesions according to a modified Mankin grading system [22].

Statistical analysis

Group differences were assessed with a one way ANOVA followed by a Tukey's multiple comparison test. All analyses were conducted using the Statistical Package for Social Sciences for Windows software, version 12.0 (SPSS Inc., Chicago, USA). Statistical significance was assessed at $P < 0.05$. All data were expressed as the mean \pm SD.

Results

Effects of EC, PBS and EC-PBS on body weight and serum biochemical parameter

To investigate the effects of EC, PBS and EC+PBS, blood biochemical parameters were measured in the rat serum of MIA-induced OA model. Compared to normal group, all test groups were no significant difference in the levels of blood parameters including aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen

(BUN), creatinine (CRE). Although high-sensitivity C-reactive protein (hs-CRP), indicator of inflammation, was increased in normal control group, other groups except normal control group did no significant difference (Table 1). Body weight was not different in all experimental groups (Figure 2).

Effects of EC, PBS and EC+PBS on Nitrite and Prostaglandin E2

Nitrite, which was major product of nitric oxide assay, was analyzed to investigate the effect of EC, PBS and EC+PBS mixture in MIA-induced osteoarthritis rat. MIA showed significant increase of nitrite concentration when compared to normal control group. EC+PBS mixture induced significant decrease of nitrite at doses of 100 and 200 mg PBS, whereas EC alone and PBS alone did not show a decrease of nitrite (Figure 3A). MIA also showed significant increase of prostaglandin-E2 concentration when compared to normal control group. EC+PBS mixture induced significant decrease of prostaglandin E2 at dose of 200 mg PBS when compared to MIA alone (Figure 3B).

Effects of EC, PBS and EC+PBS on IL-1 β and IL-6 production in serum

IL-1 β and IL-6 assay were performed to investigate the anti-inflammatory effect of EC, PBS and EC+PBS mixture in the MIA-induced osteoarthritis rat. MIA showed significant increase of IL-1 β and IL-6 levels when compared to normal control group. EC+PBS induced significant decrease of IL-1 β and IL-6 levels at dose of 200 mg PBS when compared to MIA alone. Other groups did no significant difference (Figure 4A, 4B).

Table 1. Changes in blood biochemical parameter of EC, PBS and EC+PBS in MIA-induced rats

Group	AST	ALT	BUN	CRE	Hs-CRP
	IU/L	IU/L	mg/dL	mg/dL	mg/dL
NC	112.4 \pm 13.5	53.4 \pm 7.4	17.7 \pm 2.1	0.4 \pm 0.1	14.5 \pm 4.3
PC	123.6 \pm 23.4	63.4 \pm 13.6	18.1 \pm 2.5	0.4 \pm 0.1	38.4 [#] \pm 8.9
EC 10	129.4 \pm 30.5	65.3 \pm 16.5	17.8 \pm 2.1	0.4 \pm 0.0	40.2 \pm 11.5
PBS 200	115.3 \pm 20.4	63.9 \pm 10.3	18.2 \pm 2.0	0.4 \pm 0.1	36.4 \pm 13.1
EC+PBS 100	118.2 \pm 20.5	58.8 \pm 9.3	18.1 \pm 2.5	0.4 \pm 0.1	42.6 \pm 13.4
EC+PBS 200	110.6 \pm 18.4	57.3 \pm 11.5	17.9 \pm 2.0	0.4 \pm 0.0	38.4 \pm 9.4

AST: aminotransferase, ALT: alanine aminotransferase, BUN: blood urea nitrogen, CRE: creatinine Hs-CRP: high-sensitivity C-reactive protein, NC: normal control, PC: MIA alone, EC 10: egg white-chalcanthite 10 mg/kg b.w, PBS 200: purple bamboo salt 200 mg/kg b.w, EC+PBS 100: EC+PBS 100 mg/kg b.w, EC+PBS 200: EC+PBS 200 mg/kg b.w.

[#], Significantly different from the normal control at $P < 0.05$.

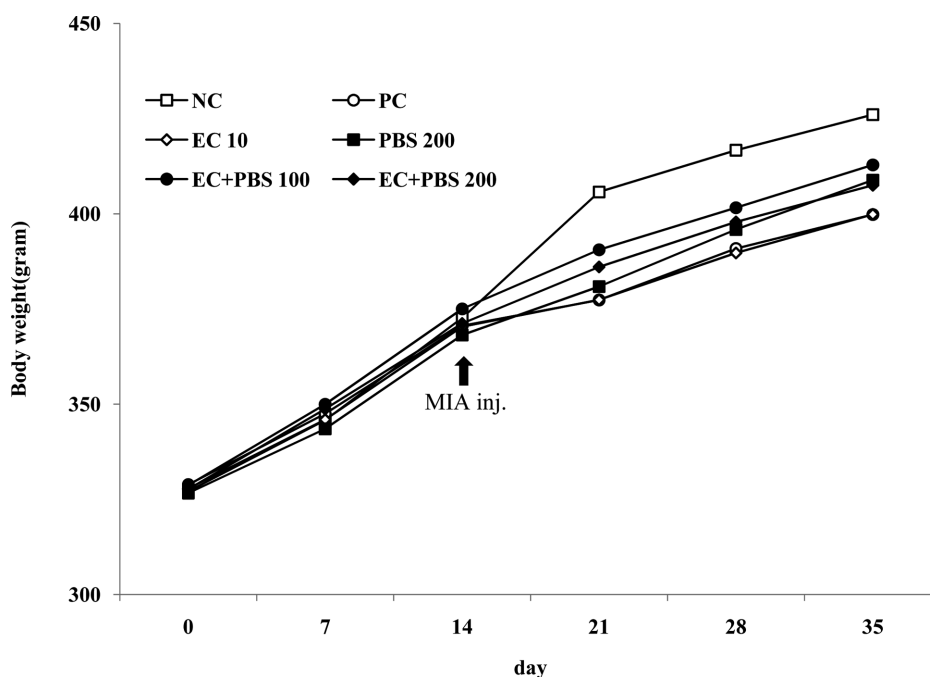


Figure 2. Effect of EC, PBS and EC+PBS on the body weight in rats. The data were expressed as mean \pm SD. NC: normal control, PC: MIA alone, EC 10: egg white-chalcanthite 10 mg/kg b.w, PBS 200: purple bamboo salt 200 mg/kg b.w, EC+PBS 100: EC+PBS 100 mg/kg b.w, EC+PBS 200: EC+PBS 200 mg/kg b.w.

Effects of EC, PBS and EC+PBS on histopathological changes of the articular damage

Histopathological examinations were performed to evaluate morphological changes and severity of the articular damage in the MIA-induced osteoarthritis rat. MIA induced surface irregularities and surface cleft, death of chondrocyte and fibrosis in the cartilage. We also measured histopathological findings which indicate severity of surface irregularity and surface cleft by H&E staining and matrix loss and cell death of articular cartilage by Safranin O staining. Histopathological lesions of articular cartilage are summarized in Table 2 and Fig. 5. PBS and EC+PBS mixture ameliorated severity of articular morphological changes such as surface irregularity and surface cleft induced by MIA (Table 2 and Figure 5A). EC+PBS mixture also decreased severity of matrix loss in articular cartilage (Table 2 and Figure 5B).

Discussion

Osteoarthritis (OA) is a degenerative arthritis characterized by defect of articular cartilage and arthralgia. Although the pathogenesis of OA is not fully understood, death of chondrocyte, only cell in articular cartilage, is one of major factor of osteoarthritis [23]. Death of chondrocytes

cause disturbance of synthesis and re-absorption in cartilage that result in the loss of cartilage matrix components and defect in the structural and functional properties of the cartilage [24].

Although the results of experimental OA model cannot be correct extrapolation to human OA, a recent study showed that there was a similar correlation between OA in animal model induced by MIA and human OA [39]. The experimental models have often been used for confirm to efficacy in OA [40]. MIA is known to disturb chondrocyte metabolism and to induce cytokine, resulting in increase of NO and PGE2 level that induce chondrocyte death and loss of cartilage matrix. It is not clear whether chondrocyte death cause or consequence of arthritis [25], however it is becoming increasingly apparent that IL-1 β and IL-6 is involved in pathogenesis of OA [26-28]. IL-1 β has an important role in the osteoarthritis. IL-1 β induces matrix metalloproteinases, inhibits extracellular matrix synthesis [29] and inhibits chondrocyte proliferation [30]. IL-6 may inhibit the differentiation of mesenchymal cells into chondroblasts [31] and can induce loss of cartilage [32]. In the present study, EC+PBS mixture decreased cytokine level such as IL-1 β , IL-6, the MIA increased these parameters. These results suggest that EC+PBS mixture have a potential of prevention against

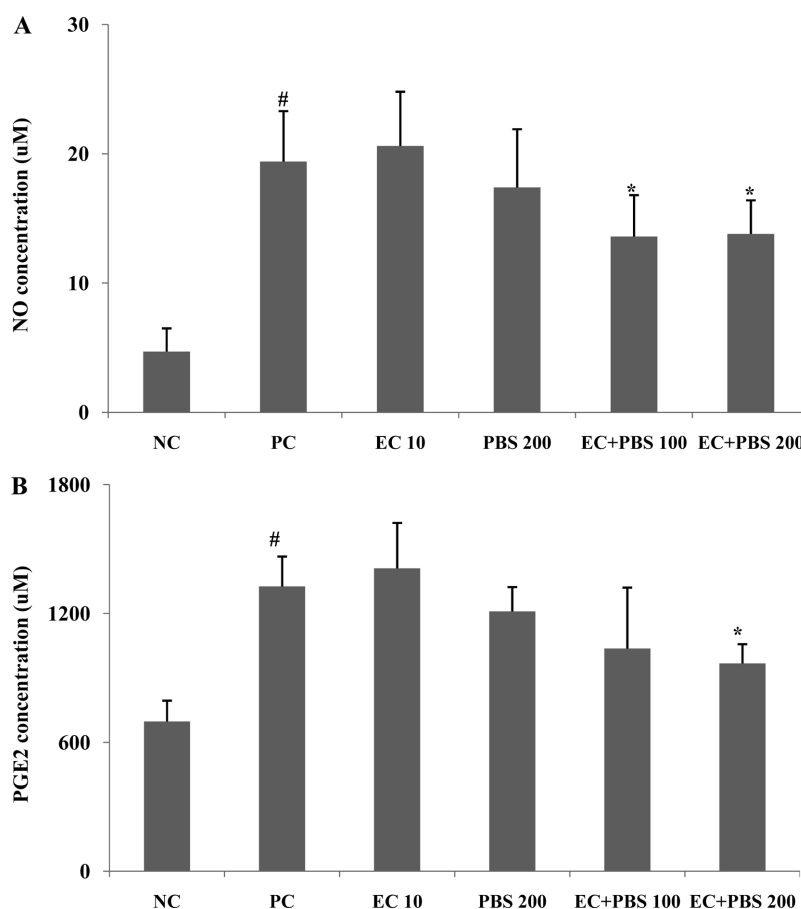


Figure 3. Effects of EC, PBS and EC+PBS on concentration of nitric oxide (A) and prostaglandin E2 (B) The data were expressed as mean \pm SD. The data were expressed as mean \pm SD. NC: Normal control, PC: MIA alone, EC 10: Egg white-chalcanthite 10 mg/kg b.w, PBS 200: Purple bamboo salt 200 mg/kg b.w, EC-PBS 100: EC+PBS 100 mg/kg b.w, EC+PBS 200: EC+PBS 200 mg/kg b.w. #, significantly different from NC at $P < 0.05$. *, significantly different from PC at $P < 0.05$.

inflammation, and subsequently reduce damage of articular cartilage induced by MIA. These results are consistent with a previous report that PBS reduced production of pro-inflammatory cytokine such as IL-1 β , IL-6. [33].

These cytokines can stimulate inflammatory mediators such as NO and PEG2 production [34]. The chondrocytes stimulated with cytokine, especially IL-1 β produce NO and PEG2. NO and PEG2 plays a crucial role in the pathogenesis of OA [35,36]. High level of NO and PEG2 have been found in cartilage and synovial tissues from OA patients and several models of cartilage degradation [36-38].

Rahmati *et al.* [37] reported that these cytokines are thought to be generated by the synovial membrane and subsequently dispersed by the synovial fluid into cartilage. And these cytokines activate chondrocyte, inducing them to nitric oxide and prostaglandins. Notoya *et al.* [38] reported that PGE2 enhanced NO mediated cell

death in analysis of interactions between PGE2 and the cell death. It means that PGE2 may stimulate human OA chondrocytes to the cell death induced by NO. In the present study, the MIA increased NO and PEG2 production, EC+PBS mixture decreased both of these parameters. These results are considered as decrease of cytokine by EC+PBS mixture. These results are consistent with a previous report that PBS reduced NO and PEG2 production [18].

In this study, MIA induced degenerative joint disease, which showed irregularities of articular surface and microfissures perpendicular to the articular surface. Chondrocytes responded to the loss of matrix by undergoing hypertrophy and proliferated to form clusters or clones. Death of chondrocyte were shown in the damaged cartilage induced by MIA. Sometimes, the epiphyseal marrow lose its hematopoietic elements, which were replaced by fibrous connective tissue.

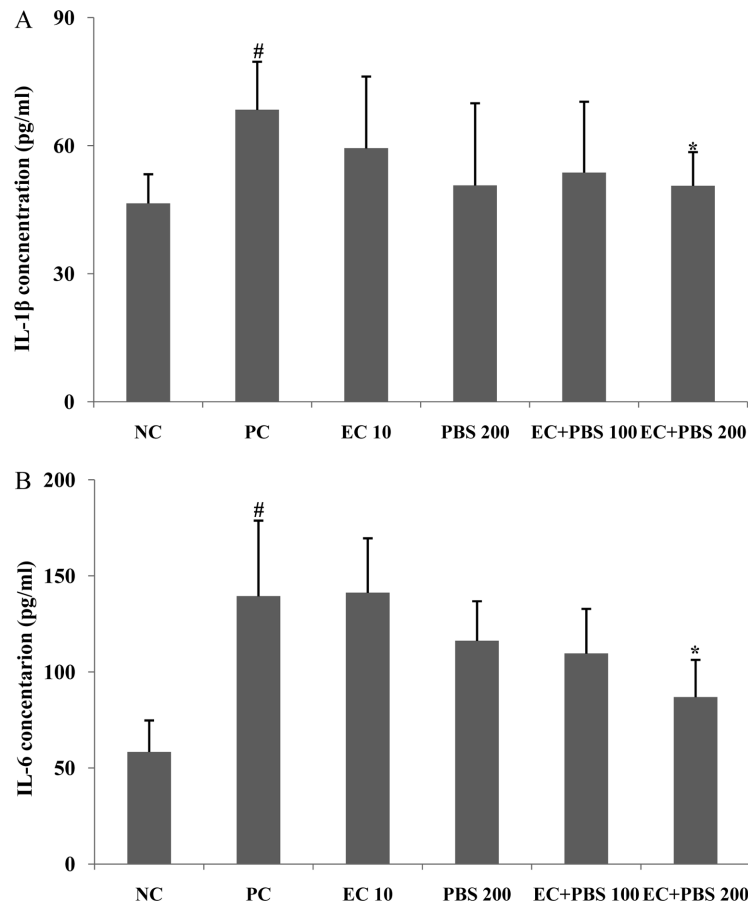


Figure 4. Effects of EC, PBS and EC+PBS on concentration of IL-1 β (A) and IL-6 (B). The results were expressed as mean \pm SD. The data were expressed as mean \pm SD. NC: normal control, PC: MIA alone, EC 10: egg white-chalcanthite 10 mg/kg b.w, PBS 200: purple bamboo salt 200 mg/kg b.w, EC+PBS 100: EC+PBS 100 mg/kg b.w, EC+PBS 200: EC+PBS 200 mg/kg b.w. #, significantly different from NC at $P < 0.05$; *, significantly different from PC at $P < 0.05$.

In summary, EC+PBS mixtures decreased levels of cytokines such as IL-1 β , IL-6, and inflammatory mediator

Table 2. Cartilage destruction assessment of EC, PBS and EC+PBS mixture in the MIA-induced rats

Group	Number of Animal	Structure ^a	Safranin O stain ^b
NC	4	0 \pm 0	0 \pm 0
PC	6	2.27 \pm 0.99	4.49 \pm 0.27
EC 10	6	2.15 \pm 0.96	4.59 \pm 0.04
PBS 200	6	1.05 \pm 0.05	4.12 \pm 0.32
EC+PBS 100	6	1.25 \pm 0.00	3.83 \pm 0.30
EC+PBS 200	6	1.06 \pm 0.05	3.58 \pm 0.25

NC: normal control, PC: MIA alone, EC 10: egg white-chalcanthite 10 mg/kg b.w, PBS 200: purple bamboo salt 200 mg/kg b.w, EC+PBS 100: EC+PBS 100 mg/kg b.w, EC+PBS 200: EC+PBS 200 mg/kg b.w. a, structure represents mean \pm SD of severity of surface irregularity and surface cleft. b, safranin O stain represents mean \pm SD of severity of matrix loss in the cartilage.

such as NO and PGE2 in the OA animal model induced by MIA. EC+PBS mixture protected the articular cartilage damage by reducing IL-1 β and IL-6 level, and prevented the loss of cartilage matrix, which is major symptoms of osteoarthritis. Taken together, these findings indicate that EC+PBS mixtures protect the MIA induced osteoarthritis based on anti-inflammatory actions.

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Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

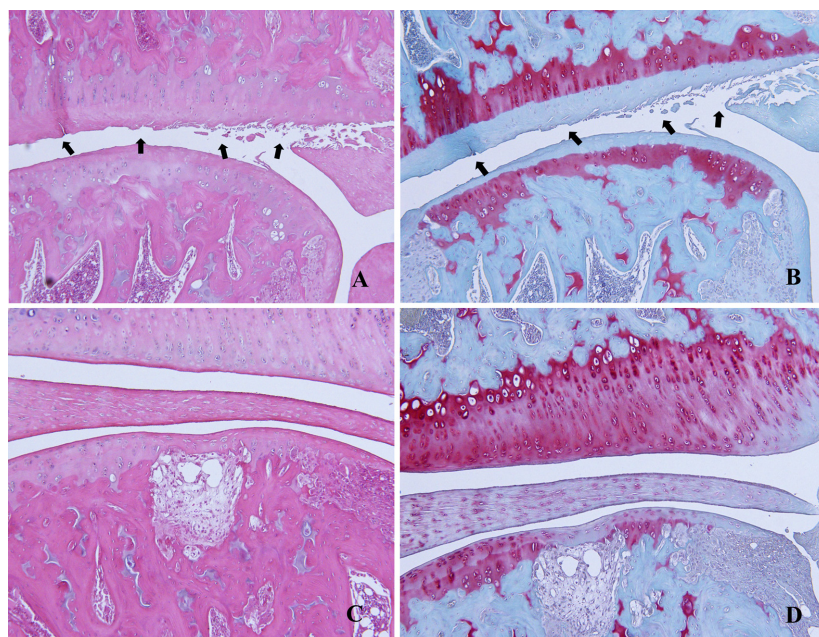


Figure 5. Histopathological findings of articular structure (A) damaging articular structure (surface irregularity and surface cleft, arrows) of rat treated with MIA alone, H&E stain (B) damaging articular structure (matrix loss of cartilage, arrows) of rat treated with MIA alone, Safranin O stain (C) repairing articular structure of rat treated with MIA+EC+200 mg PBS, H&E stain (D) recovering articular structure of rat treated with MIA+EC+200 mg PBS, Safranin O stain.

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