Original Article

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The effect of hydro-alcoholic extract of Kelussia odoratissma Mozaffarian (KOM) seed on histopathology & joints diameter in experimental model of Incomplete Freund,s adjuvant induced arthritis in rat

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ABSTRACT

BACKGROUND AND OBJECTIVE: Arthritis is one of the inflammatory diseases that can cause debilitating problems. Existing drugs have numerous side effects that limit their use. KOM has anti-inflammatory properties due to its flavonoid compounds. Therefore, this study was performed to investigate the anti-inflammatory effects of hydroalcoholic extract of KOM in an experimental model of arthritis.

METHODS: In this experimental study, 46 male Wistar rats were randomly divided into 7 groups: healthy and patient control groups, normal saline recipient , hydrocortisone recipient and 3 groups treated with hydroalcoholic extract at doses of 300, 500 and 700 mg/kg. First, KOM was extracted then arthritis induced by injection of 0.1 cc of Incomplete Freund's adjuvant, and from 15th day KOM extract was injected intraperitoneally. On the last day (31st), their ankle joint was prepared for histological examination and the groups were compared.

FINDINGS: The results showed that doses of 300, 500 and 700 mg/kg of hydroalcoholic extract did not significantly reduce joint diameter. Histologic studies of positive control group showed subcutaneous inflammation, destruction and fibrosis of cartilage and panus formation which subcutaneous inflammation was reduced in the low and high doses of the extract ($p<0.05$).

CONCLUSION: The results of this study showed that hydroalcoholic extract of KOM reduced subcutaneous inflammation but had no significant effect on joint diameter and other histopathologic and immunological changes in IFA induced arthritis.

KEY WORDS: *Kelussia odoratissma Mozaffarian, Arthritis, Inflammation, Incomplete Freund's adjuvant.*

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Introduction

Inflammation is an immunological defense mechanism in response to mechanical damage, burns, microbial infections, allergens and other fatal stimuli and enables the immune system to effectively avoid harmful stimuli and begin the healing process (1, 2). Complex processes and different mediators are involved in inducing, maintaining or exacerbating inflammatory reactions. Factors that affect macrophage inflammation are mediated by the release of factors such as nitric oxide, prostaglandin mediators and cytokines. The effect of anti-inflammatory agents is due to their ability to inhibit prostaglandin formation by cyclooxygenases. Cytokines also play a critical role in the inflammatory process, including the most important pro-inflammatory cytokines TNF-a, 1IL-β and IL-6 (3, 4).

Among the inflammatory diseases, arthritis can be a transient effect of bacterial and viral infection or a chronic debilitating condition and as a chronic disease can lead to poor quality of life, disability and premature death. There are various forms of arthritis including osteoarthritis, rheumatoid arthritis and spondyloarthritis (5). Rheumatoid arthritis is a chronic autoimmune disease and one of the debilitating problems of current societies and medical science.

Pro-inflammatory cytokines are thought to play an important role in the progression of the disease, especially the factors listed above. In patients with rheumatoid arthritis, the levels of these pro-inflammatory cytokines in synovial fluid and articular tissues are significantly increased. The major pathogenesis of this disease is generally characterized by inflammation of the synovial joints and subsequently deformities and destruction of cartilage and bone. Over time, the inflamed synovial tissue grows irregularly, forming an invasive tumor-like tissue called panus (6). Osteoarthritis is the most common joint disorder and the most common cause of long-term disability in most societies, occurring in 60 to 90% of people older than 65 years (7, 8).

The major histologic change of osteoarthritis is assosiated with complete or partial destruction of hyaline cartilage, new bone formation (osteophytes), softening, and cartilage erosion. In addition, a process of correction is seen on the articular surface of the joints with osteoarthritis called pannus-like tissue. Microscopic manifestations have shown that the pannus is composed of fibroblast-like cells and invading macrophages (9, 10). Since current arthritis treatments, including non-steroidal anti-inflammatory drugs, have limited and short-term effects, including nausea, peptic ulcer, gastrointestinal bleeding, exacerbation of heart failure, hypertension, hepatic, renal, respiratory, neurological disorders and inhibiting the synthesis of cartilage matrix in humans, the use of herbs for minimal side effects can be a good alternative treatment. Kelussia odoratissma Mozaffarian is a fragrant plant from the Chatterian family that is native to specific areas of Iranian cities including Chahar-mahal Bakhtiari, Isfahan, Kohglouye-Va boyer-Ahmad, and has not yet been reported in other parts of the world (11). In traditional medicine, the plant's organs have anti-pain, anti-inflammatory, sedative and anti-cough effects (12). Also in scientific studies their effects such as anti-inflammatory, analgesic, anxiolytic and hypnotic effects, antioxidant, anti-allergic, vasodilatory, anti-hypertensive, high-density lipoprotein enhancer, fibrinolytic, gastric acid lowering agent, antibacterial, anti-diabetic, anti-lipid peroxidation and anti-tumor have been identified (13-18).

Studies on the fractions of whole Kelussia odoratissima Mozaffarian extract showed that rotin 3, 4, and 7-trihydroxyflavonol caffeic acid and phthalide exist, which inhibit arachidonic metabolism.The presence of the 5-hydroxy group in the flavonoid structure enhances the inhibition of lipoxygenase enzyme and consequently reduces inflammation (17). Also, phytochemical evaluation of Kelussia odoratissima Mozaffarian seed showed the presence of epigenin as a major component of anti-inflammatory flavonoids that induced dose-dependent phosphorylation of MAP kinase and modulated MAP kinase message cascade as an increasing regulation mediator of interleukin 6 and 8 (15).

According to other studies, epigenin also reduces serum levels of IL-6 and TNF-α in the acute inflammation model (18). Based on previous studies on the chemical composition and anti-inflammatory effects of Kelussia odoratissima Mozaffarian, this study was conducted to investigate the anti-inflammatory effects of hydroalcoholic extract of Kelussia odoratissima Mozaffarian seed in an experimental model of arthritis in rats.

Methods

This experimental-in-vitro study was approved by the Ethics Committee of the Babol University of Medical Sciences with code, on 42 Wistar male rats in

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the age range of 8 to10 weeks with approximate weight of 180-200 gr at the Laboratory of Animal Breeding of Babol University of Medical Sciences research and technology. Kleussia Odoratissma Mozaffarian from Shahrekord University of Medical Sciences was prepared, after removing the excess parts and washing and drying in the shade, it was powdered by electric grinder.

The dried Kleussia Odoratissma Mozaffarian powder was mixed in Erlen with 70% ethanol and then shaken for 72 hours. The solution was then passed through the filter paper and the resulting hydroalcoholic extract was transferred to a rotary evaporator and the solvent was removed. The extract was then weighed and dissolved in normal saline to obtain doses of 300,500 and 700 mg/kg.

Rheumatoid Arthritis Model: To induce rheumatoid arthritis, rats were anesthetized by chloroform then 0.1ml Incomplete Freund, s Adjuvant (IFA) injected to the tail of all rats except control and healthy controls on day 1 and 0.1ml IFA was injected subcutaneously into the left paw of the rats on the eighth day of the study.Rats were randomly divided into 7 groups, as follows:

Group1 (healthy control): 6 rats with no arthritis were received 1 ml of oral saline for 156 days from day 15 study.

Group2 (Patient Control): consisted of 6 rats with arthritis in which they received no medication and received only water and food.

Group 3 (Arthritis and Normal Saline): 6 rats in which arthritis was induced and received 10 ml of saline daily for 16 days by gavage f from day 15 study.

Group 4 (Arthritis and Hydrocortisone): 6 rats in which arthritis was induced and received 10 mg / kg hydrocortisone daily for 16 days intraperitoneally from day 15 study.

Group 5 (Arthritis and Kleussia Odoratissma Mozaffarian): consisted of 6 rats in which arthritis was induced and received Kleussia Odoratissma Mozaffarian at a dose of 300 mg/kg daily by gavage for 16 days, from day 15 study.

Group 6 (Arthritis and Kleussia Odoratissma Mozaffarian): 6 rats in which arthritis was induced and received Kleussia Odoratissma Mozaffarian at a dose of 500 mg / kg daily by gavage for 16 days from day 15 study.

Group 7(Arthritis and Kleussia Odoratissma Mozaffarian): 6 rats in which arthritis was induced and received Kleussia Odoratissma Mozaffarian at a dose of 700 mg/kg daily by gavage for 16 days from day 15 study.

Observations and weight measurements of rats and the diameter of left knee were performed on day 0 and 1and then every four days by a caliper. From the eighth day, left and right ankle joint diameters were added to the measurements. On the 14th and 31st day of the study, the rat ankle joint was sampled for histopathological examination. Joints were fixed in 10% formalin and molded in paraffin for staining after tissue processing. Then, 5 µm serial slices were prepared from the ankle joint using a rotary microtome machine (Leitz, Germany). Finally, the slides stained with hematoxylin-eosin (H&E).

After staining, the specimens were mounted by entellan. The variables of subcutaneous inflammation, cartilage destruction, cartilage fibrosis, chondrocyte hyperplasia, and reduction of articular space and formation of pannus state were examined under light microscopy. Descriptive statistics were used to describe the histological features and the Kruskal Wallis test was used for quantitative data and Mann-Whitney test for pairwise comparisons. Scoring was performed to semi-quantify the qualitative data. Results were described as zero, one, two, and three, respectively, with no, low, moderate, or severe scores, and group scores were compared in pairs and $p<0.05$ was considered significant.

Results

Comparison between groups using Mann-Whitney test showed that the difference between the saline and the patient group in subcutaneous inflammation and cartilage destruction were $p<0.001$ and $p=0.002$, respectively. Also, the difference between the patient and Kelussia odoratissima Mozaffarian group of 300 mg/kg in subcutaneous inflammation was ($p=0.037$), in the Kelussia odoratissima Mozaffarian group of 500 mg/kg was ($p=0.02$) and in the Kelussia odoratissima Mozaffarian group of 700 mg/kg was $p=0.027$(Table 1). There was a significant difference in histologic examination for subcutaneous inflammation and cartilage destruction between healthy control group and other groups that induced the disease ($p<0.005$). The patient group receiving 300 and 700 mg Kelussia odoratissima Mozaffarian showed a significant decrease in subcutaneous inflammation compared to the control group ($p<0.005$).

The effects of Kelussia odoratissima Mozaffarian on the other variables were not significantly different (Table 1). Figures 1 and 2 show severe subcutaneous inflammation and reduction of articular space and

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severe cartilage destruction in the patient control group and compared to the patient group receiving Kelussia odoratissima Mozaffarian at dose of 700. From the 21st day until the end of the study, all study groups had a statistically significant increase in the diameter of the left ankle joint compared to the healthy control group ($p<0.005$). The groups receiving different doses of Kelussia odoratissima Mozaffarian had no significant decrease in the diameter of the left ankle joint compared to the control group and the patient group receiving hydrocortisone.

Comparison and analysis of the right ankle and left knee measurements also showed no significant differences between groups (Fig 3).

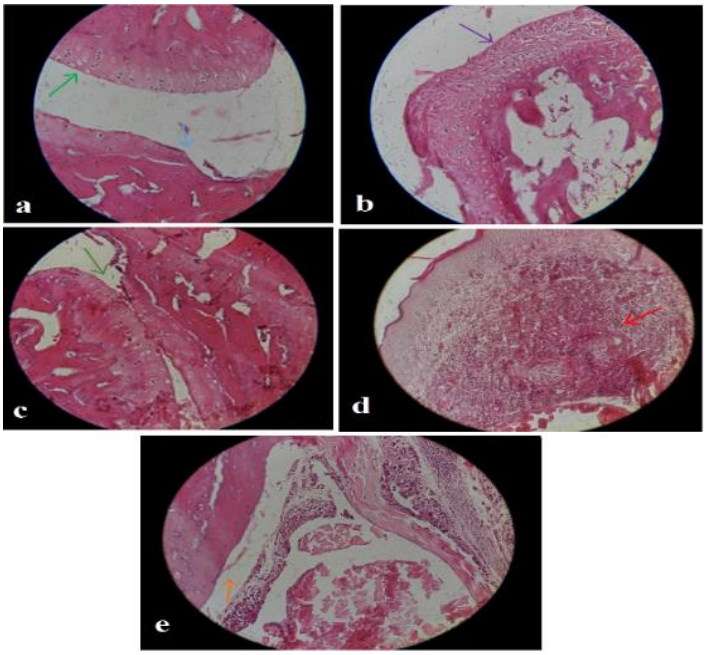

Figure 1. Histological image of the left ankle joint of the patient control group. (a) Severe cartilage destruction that has reached the bone surface, cartilage hypertrophy. b) Disruption of the cartilage structure. c) Reduction of articular space. d) Severe arthritis. (e) Formation of the pannus (presence of connective tissue, infiltration of inflammatory cells). Zooming 40 X, H&E staining

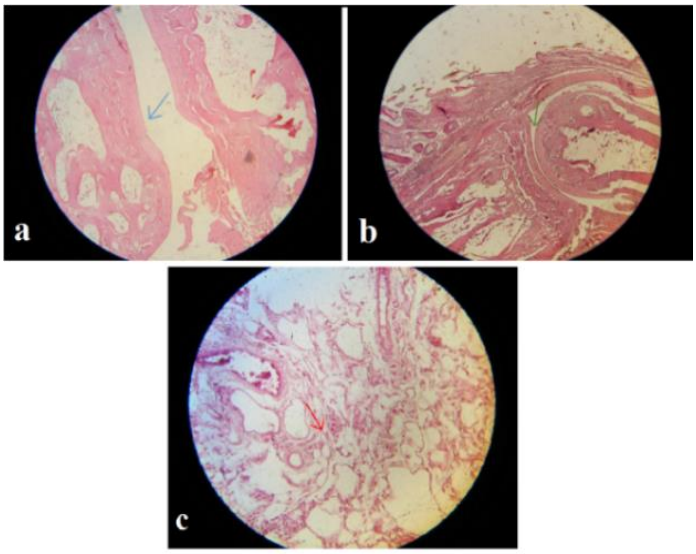

Figure 2. Histological image of the left ankle joint of the patient group receiving KOM at dose of 700. a) Mild cartilage destruction, magnification 40X (b. reduction of joint space; magnification 10X c.) Mild inflammation, magnification 40X, H&E staining

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Table1. Comparison of severity score of histopathologic complications in control, patient and recipient groups of KOM extract at doses of 300, 500 and 700 mg / kg in rat model of Fraund Adjuvant-induced arthritis

Groups / Variabels	Healthy control	Patient control	Patient+ hydrocortisone	Patient+KOM at dose of 300	Patient+KOM at dose of 500	Patient+KOM at dose of 700	P-value
Subcutaneous inflammation	0^a	14^b	11	5^c	10^c	7^c	<0.05
Cartilage destruction	0^a	8^b	8	6	7	4	>0.05
Cartilage fibrosis	0	3	0	0	2	0	>0.05
Chondrocyte hyperplasia	0	2	4	6	3	2	>0.05

In the treatment groups a with b were significant with p<0.001. C with b are also significant with p <0.05. Inflammation rate up to = 6 means mild, between 6 to 12 means moderate, greater than 12 to 18 means severe

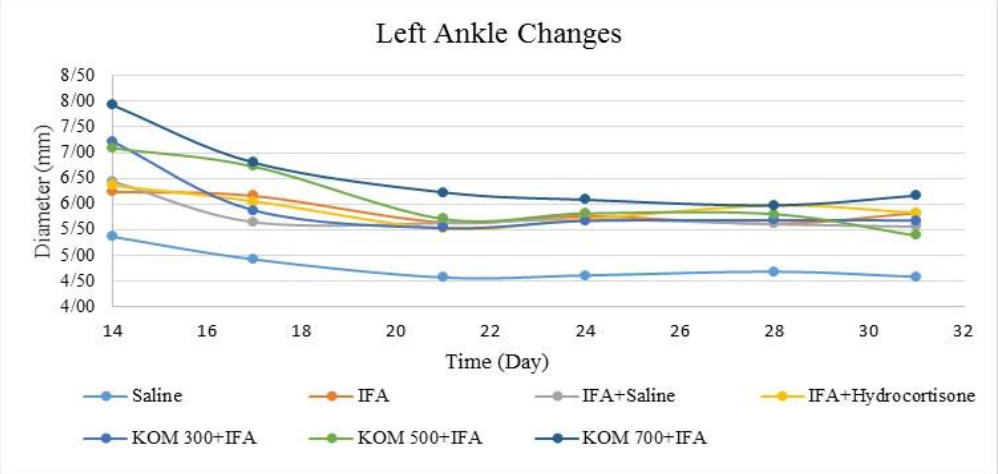

Figure 3. Left ankle diameter mean (mm) Changes diagram of rats versus time (day) in the seven experimental groups. IFA: Incomplete Freund, adjuvant, KOM: Kleussia odoratissim Mozaffarian

Discussion

The results showed that there was no significant difference in the diameter of the left leg between the control group and the group receiving KOM, while the difference between the healthy control group and the other groups was significant. Since there was no significant difference between the patient group receiving hydrocortisone and the control group and the anti-inflammatory effect of hydrocortisone was also significant; and cannot easily deny the anti-inflammatory effect of KOM.

Cartilage destruction is a multistep process that is triggered by matrix-degrading enzymes such as metalloproteinases, which are a large group of zinc-dependent proteinases that can destroy the extracellular synovium composition such as gelatin, collagen, and casein (19). The most prominent histopathological symptoms in rheumatoid arthritis include synovial layer hyperplasia, infiltration of inflammatory cells mainly lymphocyte and plasmacells, an increase in the density of synovial tissue, such as fibrocytes and fibroblasts, endothelial cells that leads to the formation of a pannus and damages cartilage and bone (20). In a study of the effect of Kleussia odoratissma Mozaffarian extract on rheumatoid arthritis induction model with Complete Freund's adjuvant in rats, it was shown that the mean clinical condition and serum CRP level in the group of patients receiving KOM decreased that it was observed at doses of 200 mg/kg and 300 of KOM and had a significant difference with the negative control group (11). As in our study in a study on hydroalcoholic extract of Angus plant, it was shown that different doses of this extract showed a good anti-inflammatory effect on rheumatoid arthritis, which was dose-dependent (6).Another study found synovial and panus tissue in patients with rheumatoid arthritis, vascular panus osteoarthritis or fibrosis and inflammatory infiltration and decreased extracellular matrix proteins in all tissue samples (10). Consistent with this study, our study also found a pannus formation in IFA recipient groups

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during histologic studies of the joint tissue. Also, IFA-treated groups showed histological evidence of joint inflammation, cartilage hyperplasia, bone and cartilage destruction, while the healthy control group did not. There was a significant difference in subcutaneous inflammation and cartilage destruction between healthy control and IFA receiving groups. Hydrocortisone recipient group the severity of cartilage destruction was lower but not significantly different from the control group, whereas there was a significant difference in subcutaneous inflammation between the control group and the dose of 300 mg/kg and 700 mg/kg of KOM. Based on this finding and previous studies, it is predicted that this plant has an anti-inflammatory effect on arthritis, but further studies are needed on cartilage destruction and other evidence of arthritis (21, 22, 4). It is recommended that further studies use a plethysmometer to measure rat paw volume and to measure inflammatory cytokines in the articular fluid. Measurement of the levels of other factors involved in arthritis, including collagenases, elastases, proteases, IL-17, Rank, and OPG can also be useful. The results of this study showed that the hydroalcoholic extract of the KOM has an anti-inflammatory effect in a dose-dependent manner and the 700 mg/kg of the extract showed more inhibitory effects on cartilage destruction and inflammatory processes of the disease.

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