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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTI-ARTHRITIC ACTIVITY OF *SANGUISORBA MINOR* SUBSP. *BALEARICA* AGAINST FREUND'S COMPLETE ADJUVANT-INDUCED ARTHRITIS IN RATS

SANGUISORBA MINOR SUBSP. BALEARICA'NIN FREUND'S COMPLETE ADJUVAN İLE OLUŞTURULMUŞ ARTRİTLİ RAT MODELİNDE ANTİ-ARTRİTİK AKTİVİTESİ

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ABSTRACT

Objective: *Therapeutic properties of Sanguisorba L. genus plants are supported by numerous in vivo and in vitro studies showing the anti-viral, anti-ulcerogenic, anti-cancer, anti-acetylcholinesterase, radioprotective, anti-allergic, and anti-inflammatory activities. The present study was designed to investigate anti-arthritic activity of the "Sanguisorba minor subsp. balearica (Bourg. ex Nyman) Muñoz Garm. & C.Navarro" (Smb), a subspecies of Sanguisorba L. genus, on Complete Freund's Adjuvant induced (CFA-induced) arthritic rat model.*
Material and Method: *The extract of aerial parts of the Smb was obtained by the consecutive steps of maceration process. The phytochemical content of the extract was analyzed by the High-Performance Liquid Chromatography (HPLC) method. Complete Freund's Adjuvant-induced arthritic rats were used to examine the anti-arthritic activity of the plant extract.*

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Result and Discussion: *HPLC analysis revealed a high amount of phenolic compounds in the Smb extract. The identified phenolics are ellagic acid, gallic acid, quercetin dehydrate and p-coumaric acid with the concentrations of 4.1288; 2.6342; 0.0871 and 0.0633 ppm, respectively. Injection of Smb extract caused a decrease in paw thickness in the CFA-induced arthritic animals. The decrease in paw thicknesses of animals treated by quercetin and Smb 70 mg/kg groups was higher than diclofenac sodium group. Smb 34 mg/kg group also showed* a *decrease in paw thickness, but it was lower compared to quercetin and Smb 70 mg/kg groups and higher than the diclofenac sodium group. According to the histopathological evaluations of the joint tissues, both 34 mg/kg and 70 mg/kg Smb extract treatment were improved the inflammatory deformations related to arthritis on the rats.*
Keywords: *Anti-arthritic activity, arthritis, Complete Freund's Adjuvant (CFA), histopathology, Sanguisorba minor subsp. balearica*

ÖZ

Amaç: *Sanguisorba L. cinsine ait bitkilerin tedavi edici özellikleri pek çok in vivo ve in vitro çalışma ile araştırılmış olup; anti-viral, anti-ülserojenik, anti-kanser, anti-asetilkolinesteraz, radyasyon koruyucu, anti-alerjik ve anti-enflamatuvar etkileri gösterilmiştir. Çalışmamız, Sanguisorba L. cinsine ait bir alt tür olan "Sanguisorba minor subsp. balearica (Bourg. ex Nyman) Muñoz Garm. & C.Navarro" (Smb) bitkisinin artrit modeli oluşturulmuş ratlarda anti-artritik etkisini araştırmayı amaçlamaktadır.*
Gereç ve Yöntem: *Smb'nin toprak üstü kısımlarının ekstresi birbirini takip eden aşamalardan oluşan maserasyon yöntemi ile elde edilmiştir. Ekstrenin fitokimyasal içeriği Yüksek Performanslı Sıvı Kromatografisi (YPSK) metodu kullanılarak tayin edilmiştir. Anti-artritik etkinliğin araştırılması için Freund's Complete Adjuvan (CFA) ile indüklenerek oluşturulmuş artritli ratlar kullanılmıştır.*
Sonuç ve Tartışma: *YPSK analiz sonuçları Smb ekstresinin yüksek miktardaki fenolik içeriğini ortaya koymuştur. Tanımlanan fenolikler şu şekildedir;* elajik asit , gallik asit, kuversetin dehidrat *ve p-kumarik asittir ve tespit edilen miktarları sırasıyla 4.1288;2.6342; 0.0871 and 0.0633 ppm'dir. Bitki ekstresi uygulanması CFA ile indüklenen artritli ratların ayak bölgelerindeki enflamasyonda bir azalmaya sebep olmuştur. Enflamasyon miktarındaki en yüksek baskılanma kuversetin ve Smb 70 mg/kg uygulanan grupta tespit edilmiştir ve bu etki diklofenak uygulanan gruptan daha yüksektir. Smb 34 mg/kg uygulanan grupta enflamasyon yine diklofenak uygulanan gruptan daha yüksek oranda baskılanmıştır. Ayak eklemlerinden alınan mikro kesitlere yapılan histopatolojik analizlere göre hem 34 mg. hem de 70 mg. Smb ekstresi artrite bağlı enflamatuvar deformasyonu iyileştirmiştir.*
Anahtar Kelimeler: *Anti-artritik aktivite, artrit, Complete Freund's Adjuvant (CFA), histopatoloji, Sanguisorba minor subsp. balearica*

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by symmetrical synovitis in large and small joints, leading a progressive functional destruction of synovial, cartilage and bone. The disease affects about 1-2% of the population and its incidence increases with age and gender, women being affected three times more than men [1,2]. Final outcome of uncontrolled rheumatoid arthritis is the impairment of articular cartilage, bone deformity, disability of joint function and pain. It has a negative impact on life quality of the patients and increase their morbidity rate [3,4]. The ultimate goal of RA treatment is to heal or minimize joint damage, relieve pain and maintain normal joint functions. A panel of drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease modifying anti-rheumatoid arthritic drugs (DMARDs) have been used to treat the symptoms of RA. However, all these drugs have several undesired effects. Recent researches aim to discover new therapeutical agents which have high safety profile and low adverse effects for the treatment of arthritis [5-7]. In this context, natural products have an increasing interest for therapeutical purposes. A well known medicinal plant from Turkey, *Sanguisorba minor* subsp. *balearica* was chosen for the current study with its therapeutic properties and biologically active phytoconstituents potential.

"Sanguisorba minor subsp. *balearica* (Bourg. ex Nyman) Muñoz Garm. & C.Navarro" is a subspecies of *Sanguisorba minor* genus and belonging to the Rosaceae family. It is an edible, perennial herb with pinnate leaves and it is famous with their reddish-green flowers. It is widely distributed throughout West Asia, North America and Europe [9,10]. In Turkey, it is known as a medicinal plant and intensively used for ethnobotanical purposes. Based on its location, it is known with many different names [11,12]. Ethnobotanical use and traditional names of the members of *Sanguisorba minor* genus plants are given in Table 1.

Table 1. Ethnobotanical use of *Sanguisorba minor* plants

Botanical name	Local name	Plant part used	Traditional use	Reference
Sanguisorba minor scop. subsp.muricata (spach)Briq.	Otukesme otu	Aerial parts	Skin diseases/eczema	[13]
Sanguisorba minor subsp. magnolii	Bostan güzeli	Aerial parts	Urinary system diseases	[14]
Sanguisorba minor subsp. minor	Küçük çayır düğmesi	Aerial parts	Constipation/Gastrointestinal disorders	[15]
Sanguisorba minor scop. muricata	Kelek ayağı	Leaves	Hypothyroidism/ Hyperthyroidism	[16]

Apart from its traditional use, *Sanguisorba* species were reported with their anti-viral [17,18], anti-ulcerogenic [19], anticancer [20,21], anti-acetylcholinesterase [22], radioprotective [23], immunomodulatory [24,25], anti-allergic [26], and anti-arthritic [10,27] properties. Among these therapeutic properties, ability to relieve inflammation is important. It was known that inflammation plays a central role in the pathogenesis of many diseases such as cancer, diabetes, obesity, cardiovascular diseases and rheumatoid arthritis. For this reason, inflammation and inflammation-based disorders are areas of interest and studied extensively. Limited number of studies were carried out on the anti-inflammatory effect of the *Sanguisorba* genus. In a case study, it was revealed that the ethanolic extract of *Sanguisorba officinalis* exerts inhibitory effects on Prostaglandin E (PGE) production and suggests a potent anti-inflammatory activity mediated by Nuclear factor kappa-B (NF-κB) and Activator protein 1 (AP-1) inhibitory properties [28]. The anti-inflammatory effect of ethanolic extract of *Sanguisorba officinalis* L. on skin disorders was tested in human keratinocyte HaCaT cells, and it was reported that ethanolic extract of *Sanguisorba officinalis* L. exerts anti-inflammatory effect by suppressing the expression of Tumour necrosis factor-alpha/ Interferon- γ (TNF-α/IFN-γ) stimulated chemokines and pro-inflammatory molecules in human keratinocyte HaCaT cell lines [29]. In view of its potent anti-inflammatory activity, the present study was designed to evaluate the anti-arthritic activity of "*Sanguisorba minor* subsp. *balearica" (Smb)*. Therefore, the present study was aimed to evaluate the therapeutic effect of *Smb* in rats with experimental arthritis model induced by Complete Freund's adjuvant (CFA). CFA-induced arthritis (AA) in rats has been used as an animal model for rheumatoid arthritis in the development of new therapeutic approaches. Because, rats exhibit a systemic inflammatory disease with similar bone and cartilage alterations to those observed in rheumatoid arthritis in human.

MATERIAL AND METHOD

Plant Material

Plant samples were collected from the locality of Kayseri, Pınarbaşı (Turkey), between "Eğrisöğüt village and Aşağı Beyçayır village Kumuk Ali Çeşmesi" at an altitude of 1750 meter in July 2017.

Voucher specimens are kept in AEF (Herbarium of Ankara University Faculty of Pharmacy) with the herbarium number of AEF 26985.

Preparation of The Plant Extracts

Aerial parts of the air-dried plant material (50 g) were powdered and subjected to maceration process in sterile distilled water for 24 hours by using a mechanical shaker (Heidolph Instruments) at 300 rpm at room temperature. Then, extract was filtered through Whatman filter paper, lyophilized (Christ Gamma 2-16 LSC), and weighed. Final yield of extraction was calculated as 15% for *Smb* weight/weight and plant extract was stored at -20ºC in the absence of oxygen.

HPLC Analysis

HPLC analysis were performed with Agilent 1200 LC series under the standardized conditions. Plant extract was filtered through 0.22 µm membrane filters and pass through a C18 HPLC column (4.6 mm x 25 cm and 5µm particle size). Flow rate was arranged as 0.3 ml/min with the 13 minutes gradual mobile phase flow, and the sample injection volume was 5 µl. Spectra were monitored between 200 – 500 nm.

Animal Model

Adult "Sprague- Dawley" male rats weighing 200-250 gram were used in this study. Animals were housed under standard conditions (12 hours light, 12-hour dark cycle; 27 ± 3℃ and 35-50 % humidity) and fed with a standard pellet diet. All animal experiments were carried out in accordance with the "National Institutes of Health Guide for Care and Use of Laboratory Animals" procedures. Complete Freund's Adjuvant (CFA) is applied as an immunization stimulant reagent and commonly used in many experimental models to mimic chronic inflammatory diseases. CFA-induced arthritis is a scientifically justified experimental model for rodents [30-34].

Induction of Arthritis and Treatment Protocol for Animals

Experimental animal groups were randomly assorted into six groups before the onset of "Complete Freund's Adjuvant" (CFA) injection. Groups were named according to their treated reagents as Group 1 (healthy control), Group 2 (negative control), Group 3 (diclofenac sodium treated), Group 4 (quercetin treated), Group 5 (*Smb* 34 mg/kg treated) and Group 6 (*Smb* 70 mg/kg treated) and each group comprised of six animals.

Except healthy control group, CFA reagent was injected on the left hind paw of the rats intraplantarly. After CFA injection, when the paw thicknesses were reached at the stable level in all experimental groups (on day 7) each group was treated with its specific reagent.

Dosages of the therapeutical reagents were adjusted by the reference to similar studies performed under the same experimental conditions. Diclofenac sodium is used as a member of non-selective COX inhibitor in this study for reducing the signs of the arthritis (positive control) [10,35-38]. The details of the animal treatments were given in Table 2.

Table 2. Experimental treatment protocol of animal groups

Group	Treatment
Group 1	Healthy control
Group 2	Negative control (CFA)
Group 3	CFA+Diclofenac* (5mg/kg)
Group 4	CFA+Quercetin* (25mg/kg)
Group 5	CFA+*Smb** (34mg/kg)
Group 6	CFA+*Smb** (70mg/kg)

*Reagents were administered daily orally from day 8 to 28.

Arthritic Score and Assessment of Paw Thickness

Basal paw volumes of all the animals were measured by plethysmometer just before CFA injection on day 0. After CFA injection, hind paw measurements were taken at different intervals till day 28. Morphological features of the arthritis were monitored by a set a visual scoring on a scale of 0-4 which refers to the clinical signs and symptoms of the arthritis, where 0: no change, 1: slight swelling and edema of the paw, 2: mild swelling and edema of the paw and/or limb, 3: severe swelling and edema of the paw and/or limb, 4: deformity and inability of paw and/or limb [35-37]. Inhibition degree of paw thickness as percentage was calculated as follows: "(1-T0/Tt) ×100, where T0 is the mean of paw thickness at day 0 and Tt is the mean of paw thickness at a particular time".

Histopathological Examinations of Joint Tissues

Lesioned hind paw tissues were cut at the metacarpal joint and fixed in 10% buffered formalin for 48-72 hours. Fixed tissues were decalcified for 72 hours in the decalcification solution. Then tissue samples were dehydrated through graded alcohol series. Finally, processed tissues were embedded in paraffin at 56°C- 58°C. Three sections of 4-5 µm thickness were taken from the prepared paraffin blocks, each of them was stained with haematoxylin-eosin (HE) and then evaluated histopathologically under the light microscope. Microphotographs were taken with focusing on the synovium, cartilage, and joint space.

Mean Histopathological Scores of Experimental Groups

Histopathologically examined and stained tissues were evaluated by a visual scoring system on a scale of 0-5 in terms of the severity of pathological damage of tissues [38,39]. Scoring were done according to four parameters which are; edema, inflammation, bleeding and necrosis.

Statistical Analysis

All analyzes were performed in triplicate and the average of measurements were calculated. Statistical analyses were calculated with GraphPad Prism version 9.1 program and One-Way ANOVA and Tukey post-hoc tests were used. The results obtained were averaged with "Standard Error of Means (SEM)" and the probability between $p<0.05$ and $p<0.005$ was considered statistically significant.

RESULT AND DISCUSSION

Total phenolic content of the plant extract were analyzed by HPLC method using a total of 7 reference standarts including coumarin, p-coumaric acid, gallic acid, kaempferol, catechin hydrate, ellagic acid and quercetin dihydrate. A standard curve was drawn based on the serial dilutions of reference standarts. Standart mixture includes 1 ppm of each phenolics. The HPLC chromatogram profile of the *Smb* extract were shown in Figure 1.

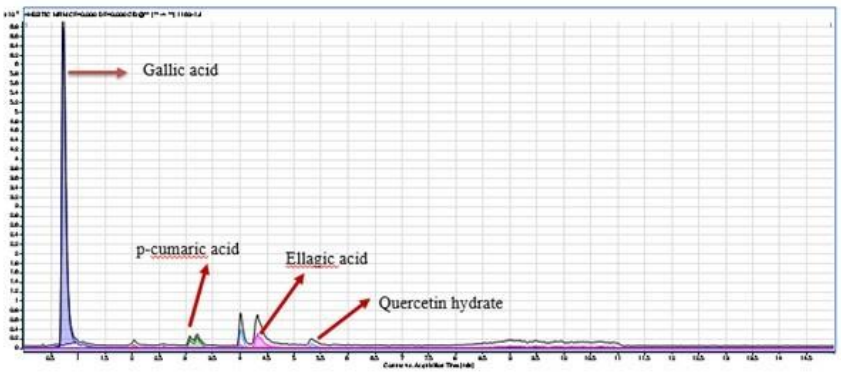

Figure 1. HPLC chromatogram profile of Smb extract. Solid line represents standard mixture. Purple area represents gallic acid, green area represents p-cumaric acid, pink area represents ellagic acid and blue area represents quercetin hydrate

The HPLC analysis of the plant extract showed that phenolic compounds were included 4.1288 ppm of ellagic acid, 2.6342 ppm of gallic acid, 0.0871 ppm of quercetin hydrate and 0.0633 ppm of p-coumaric acid. In addition to these phenolic compounds, low amount of catechin hydrate, coumarin and kaempferol (less than ≤ 0.005 ppm.). Quantitative results of the phenolic compounds of the *Smb* extract were given in Table 3.

Table 3. Quantitative phenolic compounds of *Smb* extract determined by HPLC

Plant Extract	Ellagic acid (ppm)	Gallic acid (ppm)	Quercetin hydrate (ppm)	p-coumaric acid (ppm)	Coumarin, catechin hydrate and kaempferol (ppm)
Sanguisorba minor subsp.balearica	4.1288	2.6342	0.0871	0.0633	≤ 0.005

Animal paw thicknesses were measured regularly by plethysmometer after CFA injection to obtain similar inflammation degree on the injection side. Measurements of the characteristic signs of rheumatoid arthritis on animal's paws is more accurate and well accepted method. It enables to express the effects of the treatments quantitatively on paw inflammation of the animals [41]. According to the measurements, paw thicknesses were reached at the stable level on day 7 in all experimental groups as shown in Figure 2.

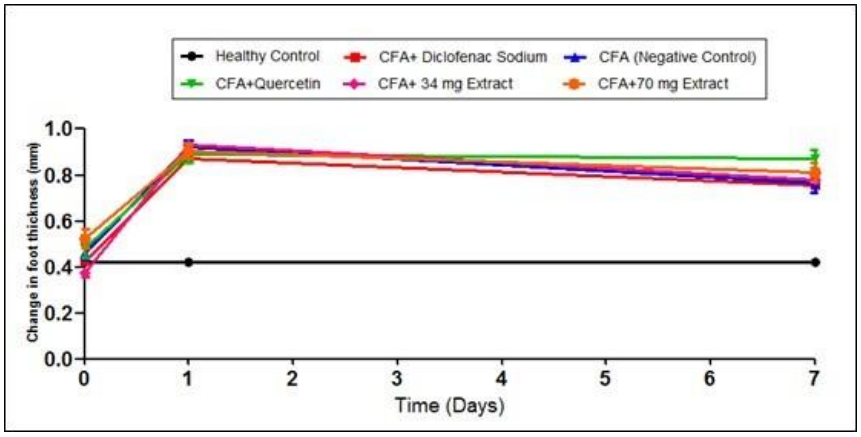

Figure 2. Animal paw thicknesses over a time period after CFA injection

Moreover, animal paw thicknesses were measured at regular intervals during the subacute and chronic phases between day 7 and day 28 of the arthritis. Treatment of animals with the plant extract and quercetin as well as diclofenac sodium caused a decrease in paw thicknesses. On day 10, 18, 24 and 28, the decrease in paw thicknesses of all the experimental groups diclofenac sodium, quercetin, "34 mg/kg *Smb"* and "70 mg/kg *Smb"* were significant when compared to negative control (CFA) group. In addition, "*Smb* 70 mg/kg" and quercetin groups had the highest decrease in paw thickness compared to diclofenac sodium and "*Smb* 34 mg/kg" groups as shown in Figure 3 and Table 4.

In order to express the differences between the groups more clearly, change in the paw thicknesses were calculated as " % decrease in paw thickness = (1-T0/Tt) ×100 ", where T0 is the mean of paw thickness at day 0 and Tt is the mean of paw thickness at a particular time. The highest decrease of paw thickness was calculated in quercetin and "*Smb* 70 mg/kg" groups as 70.08% and 61.23%, respectively, which showed higher decrease in paw thickness compared to the diclofenac sodium group having 49% of decrease. Moreover, "*Smb* 34 mg/kg" group had more decrease in paw thickness with 56% compared to the diclofenac sodium group. The inhibition degree of paw thickness in all experimental groups were given in Figure 4.

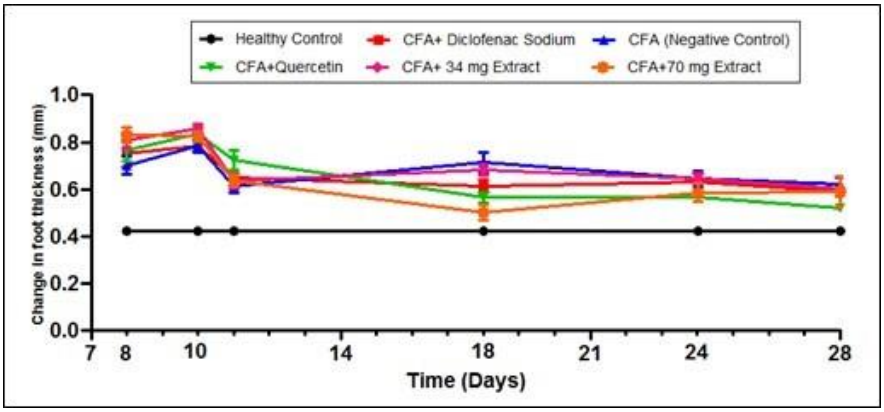

Figure 3. Change in paw thicknesses in subacute and chronic phases after the treatments

Table 4. Assessment of paw thicknesses after the treatment of animals

Groups	Treatment	Paw thickness (mm)				
		Day 7	Day 10	Day 18	Day 24	Day 28
1	Healthy Control	0.42±0.006	0.42±0.006	0.42±0.06	0.42±0.006	0.42±0.006
2	CFA Control	0.70±0.040	0.78±0.028	0.71±0.043	0.64±0.032	0.62±0.026
3	Diclofenac (5mg/kg)	0.75±0.055	0.78±0.026	0.61±0.026	0.63±0.033	0.59±0.019
4	Quercetin (25mg/kg)	0.76±0.049	0.83±0.033	0.56±0.021	0.56±0.018	0.52±0.017
5	*Smb* 34mg/kg	0.808±0.033	0.85±0.020	0.68±0.024	0.64±0.024	0.60±0.011
6	*Smb* 70mg/kg	0.832±0.033	0.82±0.019	0.50±0.034	0.58±0.039	0.58±0.066

*Values expressed as Mean ± SEM, n=6

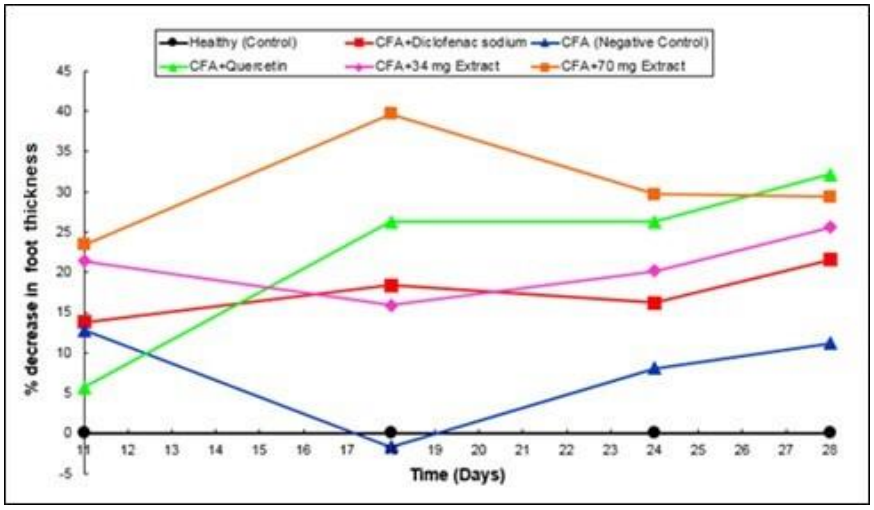

Figure 4. Change in paw thickness between the groups as percent compared to CFA control from day 11 till day 28.

Histopathological examination of joint tissues of animals enables the detection of prominent morphological disorders due to rheumatoid arthritis. Histopathological changes were evaluated basically on three parameters, including cartilage and/or bone destruction, inflammation, and appearance of the joint space. Microsections of joint tissues of the healthy control group animals had shown intact articular cartilage and normal joint space without inflammation. In CFA group (negative control), damage of the articular cartilage and narrowing of joint space with the severe inflammation were seen. Treatment of rats with diclofenac sodium has a slight recovery effect on arthritic signs. Multifocal bordered inflammatory areas were observed. There were varying degrees of decrease in inflammation and recovery between the joint spaces in animal groups treated with *Smb* at both "34 mg/kg and 70 mg/kg" doses. The improvement of histological appearance produced in *Smb* groups was better compared to the diclofenac group. Quercetin treatment of rats produced the most significant improvement in all arthritic parameters compared to all other groups (Figure 5 and Figure 6).

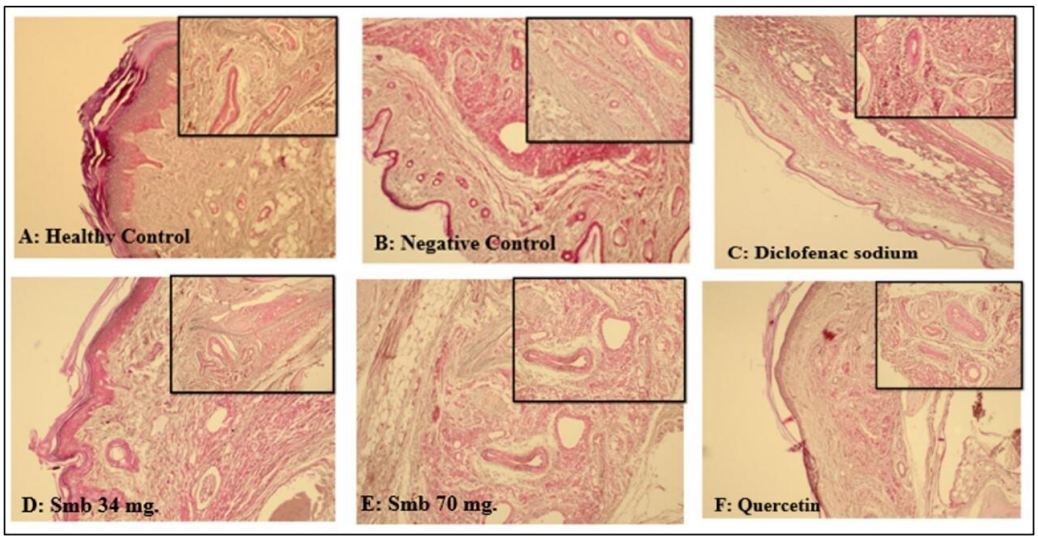

Figure 5. Microphotographs showing the histopathology in adjuvant-induced arthritic rats for six treatment groups (light microscopy 200X)

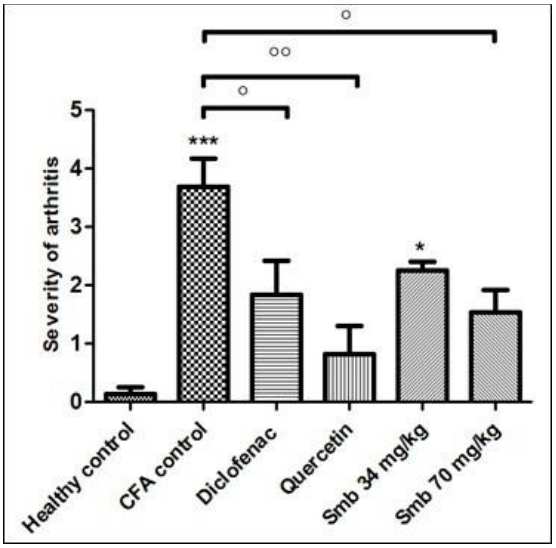

Figure 6. Mean histopathological scores of experimental groups. Values expressed as Mean ± SEM, n = 6, * p <0.05 34 mg *Smb* and *** p< 0.005 negative control compared to healthy control. ° p <0.05 positive control and 70 mg *Smb*, °° p <0.01 quercetin compared to the negative control

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects the joints and characterized by inflammation, swelling, deformity, pain and malfunction of the symmetrical joints. In the present study, CFA-induced arthritic rats were used as an experimental model which have close similarities to human RA disease for clinical and histopathological features. "*Sanguisorba minor* subsp. *balearica (Smb)"* belongs to the "*Sanguisorba"* genus in the family "Rosaceae", known as a medicinal plant and a number of studies have been carried out in the literature belonging to this genus. These studies demonstrated their high quantity of phytochemical content and also their therapeutical properties. *Sanguisorba minor* plant is mentioned as a "Promising Medicinal Plant" due to its high amount of bioactive compound [40]. More than 120 phytochemical compounds belonging to *Sanguisorba* genus plants, especially *S. officinalis* and *S. minor*, have been identified and major phytochemical compounds of plant extract have been described. These identified phytochemicals grouped as phenolics, flavonoids, neolignans and terpenoids [21,41-43]. It is emphasized that the therapeutical properties of the *Sanguisorba* species, were largely due to their polyphenolic and flavonoid content [26,42,44]. Ranfa et al., reported that *Sanguisorba minor* exhibited the highest total polyphenolic content among the other *Sanguisorba* genus [17]. It has also been shown that there is a relationship between flavonoid content of the plant and its anti-inflammatory effects [27,28,45,46]. Our HPLC results obtained from *Smb* water extract were in a correlation with the results from the literature. HPLC data revealed that, *Smb* extract composed of high amount of phenolic content (ellagic acid, gallic acid and coumaric acid) as well as flavonoid (quercetin) content. Hence, in this study we hypothesized that *Sanguisorba minor subsp. balearica*, which have high phenolic and flavonoid content, more likely to have anti-inflammatory effect on CFA- induced arthritic rats and we tested this possible effects both local and systemic aspects. Measurements of the characteristic signs of rheumatoid arthritis on animal's paws is more accurate and well accepted method. It enables to express the effects of the treatments quantitatively on paw inflammation of the animals [47]. Therefore, in the present study, paw thickness measurement of rats was used as an index that refers the anti-arthritic activity of *Smb* at the doses of "34 mg/kg and 70 mg/kg". The decrease in paw thicknesses in all experimental groups between day 10 to 28 were found to be significant when compared to negative control (CFA). "*Smb* 70 mg/kg" and quercetin groups have similar and the highest decrease in paw thickness compared to diclofenac and *Smb* 34 mg/kg groups. These results point out that, treatment with the *Smb* extract (both 34 mg/kg and 70 mg/kg) have an ameliorating effect on the paw inflammation in CFA-induced arthritic rats and this effect is better than the diclofenac sodium (member of non-selective COX inhibitor) treatment. All these results revealed that *Smb* extract with both the doses have a strong therapeutic effect compared to diclofenac sodium. Histopathological analysis provides important informations about the morphological changes and pathological signs of rheumatoid arthritis (RA) on the joint tissues. In the current study, histopathological analysis showed that *Smb* extract with both doses "34 mg/kg and 70 mg/kg" had a reducing effects on the severity of histopathological parameters including cartilage and/or bone destruction, inflammation, and appearance of the joint space as compared to negative control (CFA) group. This effect is also comparable with diclofenac and quercetin treated groups. Moreover, quercetin has the best-reducing effect in histopathological parameters among all treatment groups. All these results were found to be statistically significant.

In conclusion, supressing effect of *Sanguisorba minor subsp. balearica* extract on joint inflammation and destruction in CFA-induced arthritic rats were verified in the current study for the first time. Our data in paw thickness of animals and histopathological parameters provide important information for the anti-arthritic activity of *Smb* extract. The results that we have obtained are compatible with other studies [30-35,48]. RA have been treated with many different therapeutic approaches such as use of "prostaglandin inhibitors", "glucocorticoids", and "COX-2 inhibitors". Although these agents having good therapeutical effects, but they also have numerous side effects [49,50]. Current study showed at the first time therapeutic potential of *Smb* extract on arthritis. Therefore, *Smb* extract may serve as a source of natural anti-inflammatory plant but this needs further evaluation for use as an alternative treatment. Underlying mechanisms of this therapeutical effect of *Smb* extract on arthritis should be further evaluated by advanced biochemical and molecular studies.

AUTHOR CONTRIBUTIONS

Concept: A.İ.G., A.M.G.Ö., O.A.; Design: A.İ.G., A.M.G.Ö., O.A.; Control: A.İ.G., A.M.G.Ö., O.A.; Sources: A.İ.G., A.M.G.Ö., O.A.; Materials: A.İ.G., A.M.G.Ö., O.A.; Data Collection and/or Processing: A.İ.G., A.M.G.Ö., O.A.; Analysis and/or Interpretation: A.İ.G., A.M.G.Ö., O.A.; Literature Review: A.İ.G., A.M.G.Ö., O.A.; Manuscript Writing: A.İ.G., A.M.G.Ö., O.A.; Critical Review: A.İ.G., A.M.G.Ö., O.A.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

Animal studies were performed at "Gülhane Experimental Animal Production and Research Center" with the Ethical Committee Permission number of 17/13 on 28.03.2017. All animal experiments were carried out in accordance with the "National Institutes of Health Guide for Care and Use of Laboratory Animals."

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