Anti-artritic effects of Anchusa strigosa extracts on complete Freund’s adjuvant-induced arthritis in rats

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Abstract
Background: Anchusa strigosa, widely distributed in the Middle East region, has been used in traditional medicine to alleviate inflammation and arthritis.
Objective: In this study the aqueous (AqE) and methanol (ME) whole plant-extracts were evaluated for their anti-arthritis effects on Complete Freund’s adjuvant (CFA) -induced arthritis in rat model, and compared to untreated healthy control group (CTRL) as well as to arthritis control and betamethasone (BTM)-treated groups.
Materials and Methods: Dried plant material of Anchusa strigosa was subjected to hot continuous extraction using different solvents to obtain methanol and water extracts. Arthritis was induced by intradermal injection of Complete Freund's adjuvant into the right hind paw of rats. Extracts, at doses of 250 and 500 mg/kg, were administered orally after CFA injection up to the completion of study (for 21 days). Betamethasone (3 mg/kg) was used as a positive control. The effect of the extracts on paw edema, arthritis index, body weight and on hematological parameters was carried out.
Results: Methanol and aqueous extracts significantly decreased paw edema, arthritis index, and attenuated body weight loss. Furthermore, plant extracts reduced the elevated serum WBC level in CFA-induced rats.
Conclusion: The results of this study suggest that A. strigosa has the potential effect for the treatment of inflammatory disorders and rheumatoid arthritis (RA).

Keywords: Anchusa strigosa, betamethasone, Complete Freund's adjuvant (CFA), induced arthritis, rheumatoid arthritis (RA)

Introduction
Rheumatoid arthritis (RA) is a progressive chronic, inflammatory, autoimmune systemic disease [1]. The risk of morbidity and mortality by this disease remains high in the last decade [2], RA is characterized by aggressive synovial hyperplasia and synovitis [3]. There are many valuable strategies for the treatment of RA including traditional nonsteroidal anti-inflammatory drugs (NSAIDs) combined with steroids or disease-modifying anti-rheumatic drugs (DMARDs) and also the novel biologics, such as tumor necrosis factor-α (TNF-α) antibody and the decoy TNF-α receptor [4]. However, the high cost as well as the incidence of serious related-drug side effects still associated with these treatments [5]. The optimal treatment procedure to prevent long-term joint damage and functional decline is still unclear [6]. For this reason, alternative therapies, using medicinal plants, to provide an effective but safer treatment of arthritis are receiving increasing public interest.
Animal disease models, that faithfully reproduce key aspects of human RA, are of great importance. These models play a crucial role in assessing various aspects of many new potential RA treatments. RA-like disease, created in rodent populations by complete Freund's adjuvant (CFA), shares a number of characteristics with RA [7], CFA behaves similar to the pathology of RA, by creating hyperplasia of the synovial tissues, inflammatory infiltration of the joints, and the destruction of bone and cartilage [8].
Anchusa strigosa Banks et Sol (Boraginaceae), which called Lisan-el-thor in Arabic, is a perennial herb that is very common and widely distributed in the Middle Eastern region. This plant exhibited a broad spectrum of biological activities. It is used locally as antiulcer [9] for wound healing [10], diuretic and for abdominal pain [11], arthritis, rheumatism, gout[12, 13]. The antimicrobial activity of both volatile and fixed oil extracted from Anchusa strigosa was studied [14] while Nader et al. [15] showed that the whole plant extract has anti-bacterial and anti-fungal activities. Antidiabetic activity of the aqueous extract of A. strigosa was also studied [16].
Later six new pyrrolizidine alkaloids and glycoside compounds have been isolated from *Anchusa strigosa* roots, and its antifeedant activity was investigated [17]. In Syria, people use this plant in the form of hot aqueous extracts in the treatment of inflammatory diseases (folk medicine). However, no previous studies have investigated the effect of *A. strigosa* extracts in the treatment of arthritis. Thus, the present study aims to evaluate the anti-arthritic activity of *A. strigosa* extracts against complete Freund’s adjuvant-induced arthritis in experimental rat model.

**Materials and Methods**

**Plant material**
The whole plant of *Anchusa strigosa* (Boraginaceae) was collected from Al-Qmaitera governorate in Syria. The plant was identified and authenticated taxonomically by Dr. A. Al-Khatib (botanist), Department of Botany at the Faculty of sciences, University of Damascus.

**Drugs and chemicals**
CFA and betamethasone were purchased from Sigma Aldrich (USA). All other solvents and chemicals used for the study were of analytical grade and purchased from authentic vendors.

**Extraction procedure**
Plant material of *A. strigosa* was shade dried and powdered. Dried powder (50 g) was subjected to hot continuous extraction using soxhlet apparatus with different solvents (methanol or water) at a temp. 70 °C up to 72h. The total methanol extract (ME) of *A. strigosa* was filtered and then dried under vacuum which gave a yield of 33.12% (w/w). The aqueous one (AqE) was also filtered and concentrated over a water bath and further dried under vacuum till constant weight and a yield of 32.92% (w/w).

**Preliminary phytochemical screening**
Both aqueous and methanol extracts were subjected to qualitative phytochemical screening to assess the presence of the main phytochemical constituents such as alkaloids, phenols, flavonoids, and saponins using standard methods [18, 19].

**Pharmacological assay**

**Animals**
42 male wistar albino rats (150-200 g) were selected for the present study and received from the animal house (college of Pharmacy, Damascus). They were conditioned in a small cages (6 rats per cage), and maintained under controlled conditions of humidity 45-55%, temperature 25±1°C and 12 h of light/dark cycle. All the animals were fed with standard diet and free access to water. Animals were sacrificed at the end of the study using an overdose of chloral hydrate, as per the guidelines of institutional animal ethics committee.

**Induction of arthritis and treatment regimen**
On day 0, all rats were subjected to assessment of body weight, behavioral tests, and measurements of the right paw. Subsequently, animals were anesthetized by light ether inhalation and then 0.1 mL of complete freund’s adjuvant (complete fraction of *Mycobacterium butyricum* suspended in mineral oil; Sigma Chemical Co., USA) was injected into the sub-plantar tissue of the right hind paw of male wistar rats. Rats were randomly divided into 7 groups (n=6): Group CTRL (normal control), Group CFA (arthritis control) received vehicle with CFA, Group BTM (positive control) received betamethasone (3 mg/kg) with CFA, Groups ME-250 and ME-500 received methanol extract of *A. strigosa* (250 and 500 mg/kg, respectively) with CFA, Groups AqE-250 and AqE-500 received aqueous extract extracts of *A. strigosa* (250 and 500 mg/kg, respectively) with CFA. All treatments were administered orally thirty minutes before CFA induction (day 0), then the animals were treated daily for up to 21st day.

**Evaluation of arthritis**
The diameter of right hind paw was measured using a Vernier caliper (Mitutoyo, Japan) before and after induction of arthritis on subsequent testing days of 3 days of interval up to day 21. The change of hind paw edema was calculated using the following formula:

\[
\text{The paw edema degree} = \frac{(\Delta V \text{ treated} - \Delta V \text{ untreated})}{\Delta V \text{ untreated}}
\]

Where \(\Delta V\) treated is paw-diameter mean changes of treated animal on respective day. \(\Delta V\) untreated refers to mean changes in initial paw diameter before CFA induction (day 0).

The body weight of rats was recorded using digital weighing balance, and the body weight gain, after 21 days of CFA induction, was calculated for all groups and compared to control group (CTRL).

**Arthritis index**
The severity of arthritis was evaluated using the visual arthritis index as described previously [20, 21]. In this scoring blind test, where observer was blind to the study, rats were observed visually and scored depending on redness and swelling in the right hind paw and the appearance of arthritis in other parts of the animal body as like as ears and tails. An arthritis score (ranged from 0 to 3) was given for every rat, where 0 indicates no visual change, and 1, 2, and 3 indicates mild, moderate, and severe significant inflammation, respectively.

**Biochemical assays**
On day 21 after CFA induction, samples of blood were drawn from the retro-orbital vein puncture, 1 h after the last administration. These samples were used to evaluate some hematological parameters like hemoglobin (Hb), red blood cell (RBC), white blood cell (WBC) and erythrocyte sedimentation rate (ESR) as an indicative of inflammation. An automated hematology analyzer (Sysmax KX-21) was used [22].

**Statistical analysis**
The data expressed as mean ± standard error of the mean (n = 6). Differences between experimental groups were assessed using two independent samples t-test to analyze data by using SPSS program (Statistical Package for the Social Sciences); it showed differences between treated groups and each of normal and CFA-induced control groups.

**Results**

**Chemical constituents**
Phytochemical investigation of the aqueous and methanol extracts of the whole plant of *Anchusa strigosa* showed the presence of hydrocarbons particularly in the methanol extract. The aqueous extract was rich in free amino acids. Saponins...
and steroids were found in both extracts which is in agreement with the results showed by Abbas and co-workers [23] where four triterpenes were isolated from A. strigosa roots. Tests for alkaloids were positive which is in accordance with the presence of Pyrrolizidine alkaloids already isolated from A. strigosa [17, 24]. Previous studies have identified the fat composition in A. strigosa [14, 25]. Lipids and fats were also shown to be present in the extracts used in our study. Alali and coworkers have studied the total phenolic contents of Anchusa strigosa aqueous extract and methanol extracts [26].

In the present investigation of phytochemicals, tests revealed also the presence of flavonoids in both kinds of extracts [Table 1].

Table 1: Preliminary phytochemical screening on the whole plant aqueous and methanol extracts of Anchusa strigosa.

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Test name</th>
<th>Presence / absence ( + / - )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous extract</td>
<td>Methanol extract</td>
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<tr>
<td>Carbohydrates</td>
<td>Molisch’s test</td>
<td>+</td>
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<tr>
<td></td>
<td>Fehling’s test</td>
<td>+</td>
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<td></td>
<td>Borloed’s test</td>
<td>+</td>
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<tr>
<td></td>
<td>Borntrager’s test</td>
<td>+</td>
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<tr>
<td></td>
<td>Modified borntrager’s test</td>
<td>+</td>
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<tr>
<td></td>
<td>Legal’s test</td>
<td>+</td>
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<tr>
<td></td>
<td>Killer kellani test</td>
<td>+</td>
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<tr>
<td></td>
<td>Shinoda test</td>
<td>+</td>
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<td></td>
<td>Alkaline reagent test</td>
<td>+</td>
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<tr>
<td>Glycosides</td>
<td>Foam test</td>
<td>+</td>
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<tr>
<td></td>
<td>Liberman – buchard test</td>
<td>+</td>
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<td></td>
<td>Salkowski test</td>
<td>+</td>
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<td></td>
<td>Sulphur test</td>
<td>+</td>
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<tr>
<td></td>
<td>Hager’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Wager’s test</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Spot test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Saponification test</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Ninhydrin test</td>
<td>+</td>
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<tr>
<td></td>
<td>Biuret test</td>
<td>+</td>
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<tr>
<td></td>
<td>Xanthoproteic test</td>
<td>+</td>
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<tr>
<td>Fixed oils and fats</td>
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<tr>
<td>Amino acids</td>
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</table>

Treatment effects on paw swelling and arthritis index in CFA-induced rats

All CFA-induced rats developed arthritis. They showed statistically significant (P<0.05) increase in the right hind paws edema degree and inflammation was maintained for 21 days compared with normal control group [Figure 1]. However, treatment with A. strigosa aqueous and methanol extracts, at doses of 250 and 500 mg/kg, significantly reduced the paw edema degree in a dose-dependent manner from day 9 to day 21 compared with arthritis control group. Furthermore, paw swelling was also significantly inhibited by Betamethasone.

![Fig 1: Effect of betamethasone, aqueous and methanol extracts of A. strigosa (at doses of 250 and 500 mg/kg) on paw edema degree, evaluated for 21 days at 3-days intervals, in CFA-induced rats as described in “Materials and methods” Section.](image)
Besides, a significant increase of arthritis index was shown in the animals [Figure 2], after CFA induction, compared with normal control group. However, treatment with aqueous and methanol extracts of *A. strigosa* at 500 mg/kg, and betamethasone significantly decreased the arthritis index induced by CFA from day 9 to day 21 compared with CFA arthritis group. Both extracts at 250 mg/kg significantly decreased the arthritis index from day 12 compared with CFA arthritis group.

**Fig 2:** Effect of betamethasone, aqueous and methanol extracts of *A. strigosa* (at doses of 250 and 500 mg/kg) on arthritis index, evaluated at 3-days intervals, in CFA-induced rats as described in “Materials and methods” Section.

**Changes in Body Weight of the Rats over Time**

In this study, the relationship between the weight gain and the CFA-induced arthritis was investigated. The body weight changes of rats were observed up to 21 days. The results showed [Figure 3] a significant decrease (*p*<0.05) in body weight gain in CFA arthritis control group compared with the normal control group (CTRL) at the end of experimentations. Treatment with Betamethasone at 3 mg/kg as well as with *A. strigosa* aqueous and methanol extracts, at 250 and 500 mg/kg, significantly attenuated the difference in body weight gain observed between the CFA arthritis control group and the normal control group (CTRL). Thus, the effect of CFA-induced arthritis on body weight could be reversed by administration of betamethasone or plant extracts. Although the weight gain in rats of all diseased groups was lower than it had been initially, it was still much higher than that of CFA-arthritis control group [Figure 3].

**Fig 3:** Effect of induced arthritis and treatment by betamethasone and *A. strigosa* extracts on body weight gain at the completion of the experiment (21 days).
Rats with CFA-induced arthritis had statistically significant (P<0.05) decreased levels of % hemoglobin (Hb) in CFA-arthritis control group as compared with normal control group. Treatment with betamethasone as well as with plant extracts showed statistically significant (P<0.05) increase in levels of % Hb as compared with CFA-arthritis control group. The aqueous and methanol extracts produced comparable effect to that of betamethasone [Table 2].

### Discussion

It is well known that Adjuvant-induced paw edema in rats has close similarities to human arthritis. Thus it is frequently used for preclinical studies, as experimental model, to screen antiarthritic agents [27].

In the present study, CFA-induced arthritis was used for the first time as a model to assess the effect of *Anchusa strigosa* extracts on arthritis in rats. The obtained results demonstrate that these extracts have the potential protective effect against adjuvant-induced arthritis in rats. Paw edema is an index of assessing therapeutic effects of different treatment. The severity of arthritis could be evaluated using arthritis index which is a visual tool [28]. Determining the degree of swelling of the injected paw and arthritis index is the most objective measurement commonly used to evaluate the anti-arthritis activity in rat model [29]. The data, presented in this study, showed that treatment with both aqueous and methanol extracts of *A. strigosa*, at doses of 250 and 500 mg/kg, significantly and dose dependently, attenuated paw edema and arthritis index induced by CFA compared with CFA arthritis control group. Thus, the principal signs related to the inflammatory reactions like swelling, redness, and inactivity of the affected joints were significantly reduced in the treated groups compared to the arthritis control one.

Furthermore, body weight loss has been previously reported in several studies following the injection of the Freund’s adjuvant [30] and was used to evaluate the anti-arthritis activity of the treatment in arthritic rats. These studies demonstrated a close relationship between the arthritis severity and the body weight loss, attributed to the alterations in the metabolic activities of arthritic animals. In the present study, arthritic rats showed a decrease in body weight gain compared to the healthy control rats. This result agrees with the fact that rheumatoid arthritis is associated with loss of lean tissues containing most of the body's protein. The decrease of body weight gain may also be due to muscle wasting in Freund’s adjuvant arthritis, resulting of the enhanced protein breakdown by the ubiquitin-proteasome proteolytic pathway [31, 32]. It was shown that reduction of the body weight during arthritic conditions may be caused by a deficient absorption of nutrients through the intestine [33]. The treatment of diseased rats with aqueous and methanol extracts of *A. strigosa* attenuated the body weight loss induced by CFA compared with CFA control group. This effect may be explained by the inhibitory effect of the treatment on either the loss of lean tissues containing body's protein or muscle wasting and also on the inflammatory conditions by increasing the nutrients absorption through the intestine of diseased rats. Besides, arthritic control rats presented a reduction in RBC count and in Hb levels, and also an increasing in WBC and ESR. This indicates symptoms of anemic condition. Anemia is the most common hematological deformity seen in patients with rheumatoid arthritis [34]. The extracts of *Anchusa strigosa* exhibited a significant anti-arthritis activity by suppressing the induced anemia. The increase in WBC is well known in inflammatory conditions [35]. This may be attributed to the stimulation of immune system against the invading pathogenic agents [36]. The significant reduction of this hematological parameter, in treated rats, revealed the immunomodulation effect of *A. strigosa* extracts and suggests the anti-artheritic activity of this plant.

Preliminary phytochemical investigations on the aqueous and methanol extracts, of the whole plant of *Anchusa strigosa*, were noted the presence of a variety of constituents as like as carbohydrates, alkaloids, flavonoids, saponins, steroids, and glycosides. Many of these Phytochemicals were already reported for their anti-artheritic activity. Therefore they may be accounted for the anti-artheritic activities observed in this study.

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### Table 2: Alterations in the values of some hematological parameters, at the completion of the experiment (21 days), following the treatment of induced arthritis in rats by *A. strigosa* extracts.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose mg/kg</th>
<th>Parameters</th>
<th>CTRL</th>
<th>CFA</th>
<th>BTM</th>
<th>ME</th>
<th>AgE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hb (gm/100ml)</td>
<td>RBC (million/ml)</td>
<td>WBC (thousands/cmm)</td>
<td>ESR (mm/hr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td></td>
<td>13.8±0.35</td>
<td>7.19±0.43</td>
<td>7.80±0.29</td>
<td>1.47±0.35</td>
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<tr>
<td>CFA</td>
<td></td>
<td>8.07±0.29***</td>
<td>3.37±0.32***</td>
<td>16.54±0.96***</td>
<td>36.20±4.72***</td>
<td></td>
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<tr>
<td>BTM 3</td>
<td></td>
<td>12.65±0.33*</td>
<td>6.45±0.23</td>
<td>12.61±0.58***</td>
<td>6.79±0.52***</td>
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<tr>
<td>ME 250</td>
<td></td>
<td>10.2±0.47***</td>
<td>4.23±0.37***</td>
<td>13.95±0.42***</td>
<td>10.25±1.23***</td>
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<tr>
<td>ME 500</td>
<td></td>
<td>11.85±0.29**</td>
<td>5.87±0.41</td>
<td>12.93±0.78***</td>
<td>9.89±0.74***</td>
<td></td>
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<tr>
<td>AgE 250</td>
<td></td>
<td>10.27±0.42***</td>
<td>4.19±0.28***</td>
<td>13.87±0.65***</td>
<td>10.42±1.05***</td>
<td></td>
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</tr>
<tr>
<td>AgE 500</td>
<td></td>
<td>12.05±0.35**</td>
<td>5.92±0.19*</td>
<td>13.08±0.54***</td>
<td>9.55±0.33***</td>
<td></td>
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</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=6); *p<0.05, **p<0.01 and ***p<0.001 compared with normal control group (CTRL); ##p<0.01 and ###p<0.001 compared with CFA arthritic control group (CFA).
Conclusion
The present study shows that both aqueous and methanol extracts of Anchusa strigosa have anti-arthritic effects on Complete Freund’s adjuvant-induced arthritis in rats. The anti-arthritic effects of A. strigosa may be due to its ability to attenuate paw edema, arthritis index, body weight loss, and to decrease the elevated serum WBC level in CFA-induced rats. It suggests that A. strigosa may be a useful treatment of RA. However, the precise mode of action and the most effective constituent in this anti-arthritic activity remain to be further investigated.

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References


