

Research Article

Preventive and curative effect of the methanolic extract of *Ajuga iva* on collagen induced arthritis in rats

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ABSTRACT: **Introduction:** The medicinal plants are widely used around the world. Rheumatoid arthritis (RA) affects 1% of the adult population, and has a significant impact on physical functioning and social life. In rats, induction of arthritis by collagen II causes alterations of synovial joints similar to patients with RA. In this study the collagen induced arthritis model was used to evaluate the anti-arthritic effect of *Ajuga iva* extract. **Materials and Methods:** Arthritis was induced by collagen emulsified with complete Freund's adjuvant. Arthritic rats were treated orally with the methanolic extract of *Ajuga iva*. To study the preventive effect, 30 and 60 mg/kg of body weight, were daily administrated during the first 21 days. For the curative effect, 100 and 150 mg/kg of body weight were daily administrated during 21 days starting from the appearance of arthritis signs. Changes in body weight, swelling of legs, level of protein C, sedimentation rate, and the histopathological status of articulation were evaluated as markers of inflammation associated with arthritis. **Results:** In the preventive treatment of rats, the methanolic extract of *A. iva* inhibited significantly the markers of inflammation and arthritis. *Ajuga iva* extract, in the curative treatment study, reduced significantly the swelling of legs and other parameters of inflammation such as C reactive protein (CRP) level, erythrocyte sedimentation rate (ESR) and leukocytes. Histopathological examination showed reduced erosion and destruction of bone. **Conclusion:** Our results show that the methanolic extract of *A. iva* plays an effective role in the prevention and treatment of collagen induced arthritis. The way by which the components contained in this extract act to prevent or to reduce the inflammation is not clear but it may be due to their inhibition of the pro-inflammatory cytokines.

KEYWORDS: *Ajuga iva*, methanolic extracts, collagen induced arthritis, anti-arthritic effects, joint inflammation

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by the chronic and systemic inflammatory of the synovial membranes, and by the progressive and erosive destruction of cartilage and bone.^[1] RA affects 1% of the adult population, and has a significant impact on physical and social activities of patients.^[2] The etiology and the pathogenicity of RA are not entirely known, but an autoimmune attack on the membranes probably has a crucial role.^[3] The majority of the drugs currently used in the treatment of RA have several side effects and toxicities which prevent their long-term use. Corticoids used to

treat RA can reduce the synovitis in the short term, but in long-term cause damage to the synovial membrane.^[4] Unfortunately, they have several side effects, such as infections, osteoporosis,^[5] insulin resistance and diabetes,^[6] significant teratogenic effects.^[7] Moreover, they could increase the risk of cardiovascular disease in RA patients due to their potential noxious properties on the lipids, and the development of hypertension with/without obesity.^[8] Consequently, it is necessary to develop preventive treatments and therapeutic agents without harmful effects on the RA patients. This growing interest of the alternative medical practices clearly indicates the need to investigate their safety and effectiveness.^[9] Medicinal plants were largely used in traditional medicine to treat rheumatoid arthritis.

Ajuga iva L. (AI) Schreber (locally called Chendgoura), has been shown to display a wide spectrum of biological and pharmacological activities, which provide experimental

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support for the empiric ethnopharmacological use of this plant in folk medicine. It has numerous beneficial effects such as panacea (cure-all),^[10] gastrointestinal disorders,^[11] hypertension, diabetes,^[12] anthelmintic and anti-inflammatory.^[13] The plants of the genus *Ajuga* have been reported to have antifungal, antibacterial,^[14–15] antimycobacterial,^[16] antihypertensive,^[17] antiplasmodial,^[18] hypoglycaemic,^[19] larvae and insect antifeedant activities.^[13,20–21] Nevertheless, antiarthritic activities for *Ajuga iva* have never been proved. Thus, we considered it interesting to investigate whether there is a scientific basis for the Algerian traditional use of this plant as an anti-rheumal drug. In the present study, we evaluated the anti-arthritic effect of the methanolic extract of *Ajuga iva* on collagen induced rheumatoid arthritis using a model of the rats which is an animal model of human rheumatoid arthritis.

MATERIALS AND METHODS

Plant material

Ajuga iva (L.) Schreber was collected from Bordj Bou Arreridj, in the northeast of Algeria in June 2010, and identified by Pr. Laouar H (Department of Ecology and Vegetal Biology, University Ferhat Abbas, Setif). A voucher specimen was deposited at the Laboratory of Botany, Department of Vegetal Biology and Ecology, Faculty of Nature and Life Sciences. All reagents were purchased from Sigma Chemicals (Germany), Fluka and Prolabo.

Extraction procedure

The extraction of polyphenols was carried out according to the method described earlier^[22] with slight modifications. Dried plant material was ground in warring blender, mixed with a 10–20 volume of 85% aqueous methanol. The slurry was placed at room temperature for one week and the extract was filtered through a Buchner funnel and the methanol was removed by rotary evaporation. The dried extract was stored at -20°C temperature until use.

Animals

Male and female Wistar rats weighting 150–200 g (7–8 weeks old) were purchased from (Institut Pasteur d'Algérie, Algiers). The animals were treated under ethical conditions according to international recommendations. Rats were acclimated one week to eliminate the effect of stress prior to initiation of the experiments. They were housed five per cage in rooms maintained at $22\text{--}24^{\circ}\text{C}$ with alternating 12 h light/dark cycle. Food and water were provided *ad libitum* throughout the experiments.

Induction of Rheumatoid Arthritis methanolic extract treatment

Rheumatoid arthritis was induced in male and female Wistar rats using the method described by Rosloniec *et al.*^[23] and Charles-Schoeman *et al.*^[24] Briefly, rats were intradermally injected at the base of the tail with 300 μg of chicken type II collagen (CII) dissolved in acetic acid 0.1M, emulsified in a same volume of complete Freund's adjuvant. On day 7 all rats were boosted by injecting a further 200 μg of CII in incomplete Freund's adjuvant. The same volume of acetic acid was injected to the rats in the normal control group. A total of 60 rats were randomized into six groups of 10 rats: (G1) normal control (NCG), (G2) collagen-induced arthritis positive control (PCG), (G3, G4) *Ajuga iva* preventive treated and (G5, G6) *Ajuga iva* curative treated groups, respectively. In the preventive protocol, the treatment has began at the day of arthritis induction (day 0). CIA rats were treated daily with oral administration of different doses of AILE (30, and 60 mg/kg body weight), until day 21. In the curative treatment protocol for the established CIA, treatment with AILE, was initiated on the day of installation of arthritis (day 14) and continued daily until day 40. CIA rats were treated with oral administration of different doses of AILE, 100 or 150 g/kg of body weight.

Evaluation of arthritis development

Rats were inspected daily for the onset of arthritis characterized by edema and/or erythema in the paws. The incidence and severity of arthritis were evaluated using a system of arthritic scoring, measurement of bi-hind paw volumes and body weight every 2 or 3 days beginning on the day where arthritic signs were first visible. Lesions of the four paws of each rat (i.e., the clinical arthritic signs) were graded from 0 to 4; where, 0 = no evidence of edema and swelling; 1 = slight swelling and erythema limited to foot or ankle joint; 2 = slight edema and erythema from the ankle to the midfoot (tarsal); 3 = moderate swelling and erythema extending from the ankle to the metatarsal joints; 4 = severe swelling and erythema encompassing the ankle, foot, and digits. The score 16 is the potential maximum of the combined arthritic scores per animal for an individual scoring the highest grades for all tests.^[23]

Leukocyte counts

Peripheral blood samples were collected on day 14 and 21 for control and preventive groups and on days 14, 21, and 40 for the curative group. Blood was collected into EDTA containing tubes (Diagnostics Pasteur, France). Leucocytes were counted automatically with a blood cell counter (Coulter-Immunotech Diagnostics, Hamburg, Germany).

Histopathological analysis

The hind paws were fixed in 10% formalin for 7 days, decalcified in formic acid (15%), washed with running tap water for 3 h and dehydrated in a graded series of ethanol and xylene, then embedded in paraffin. Thin sections (4 μm) were stained with haematoxylin–eosin and studied under the light microscope. Infiltration of inflammatory cells and connective tissue hyperplasia were monitored.

Measurements of ESR and CRP levels

Blood samples were collected by retro-orbital venipuncture of animals on days 14, 21, and 40, and erythrocyte sedimentation rate (ESR) was determined by a modified method based on International Council for Standardization in Haematology (ICSH) selected methods.^[25] Briefly, 120 μl of blood sample were taken in 1.0 mm \times 100 mm capillary tube, and the rates of erythrocyte sedimentation were recorded after 60 and 120 min. Levels of CRP in serum were measured on days 14, 21 and 40 using commercially available kits for CRP (SPINREACT, S,A Giroma, Spain), according to the manufacturer's recommendations.

Statistical analysis

The data on clinical scores were analyzed with the Student *t* test and are presented as the mean \pm SD. Difference between controls and treated groups was considered significant at $p \leq 0.05$.

RESULTS

Effect of AIME on clinical signs

The rats were immunized at the base of the tail with collagen type II (CII). The inflammation started to develop after the twelfth day of the first immunization of non treated rats. The methanolic extract of *Ajuga iva* and distilled water were orally administrated to the rats each day during three weeks. For the preventive groups, the administration was carried out since the first day of the immunization, whereas, for the curative groups, the treatment began after the appearance of the signs of arthritis (day 14 until day 40). Results are shown in Figure 1. Up to 90% of the immunized animals by collagen II (the positive reference group) showed signs of arthritis; swellings and redness of the legs, after 14 days. The preventive treatment using either 30 or 60 mg/kg of body weight delayed the appearance of the clinical signs of arthritis and reduced clearly the severity of the disease (Figure 1a). Equally, the curative use of the *Ajuga iva* methanolic extract (AIME) at the doses of 100 or 150 mg/kg of the body weight,

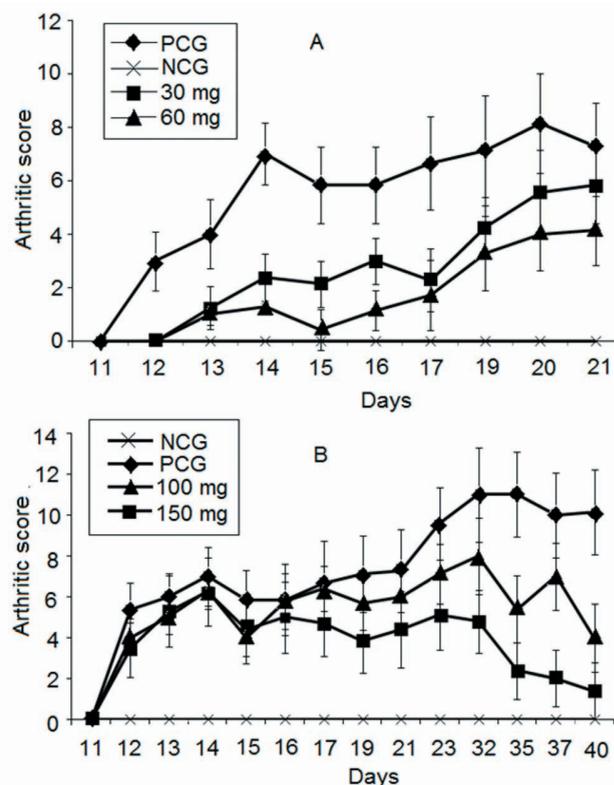


Figure 1. The development of the clinical signs (arthritic score) in rats A) preventive groups treated with 30 and 60 mg of AIME/kg of rat body weight, B) curative groups treated with 100 and 150 mg of AIME/kg of rat body weight (PCG: positive control group, NCG: negative control group).

reduced remarkably the severity of the swelling and the erythema ($p < 0.001$) (Figure 1b).

Effect of AIME on the body weight

In collagen-induced arthritis control group (PCG), the rats gradually lost their body weight after 14 days of the first immunization $p < 0.01$, in comparison with the negative control group. The extract of *Ajuga iva* leaves did not affected the body weight (Figure 2), although it effectively improved other clinical signs of arthritis such as swelling of the paws and erythema.

Effect of the AIME on the serologic parameters

Samples of the blood from the preventive and curative treated groups were taken in dry tubes. Protein C was detected using specific antibodies. The rats of the positive group showed a high level of the protein C in their serum compared to the negative group (Figure 3). AIME at doses of 100 and 150 mg/kg of body weight reduced significantly ($p < 0.05$) the rate of the protein C in the serum after 21 days, whereas, no changes were observed in the preventive groups.

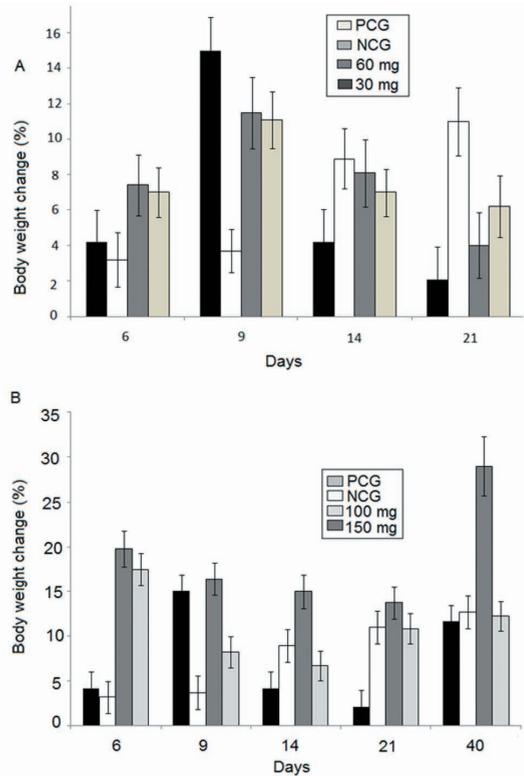


Figure 2. Body weight changes in A) preventive groups treated with 30 and 60 mg of AIME/kg of rat body weight, B) curative groups treated with 100 and 150 mg of AIME/kg of rat body weight (PCG: positive control group, NCG: negative control group).

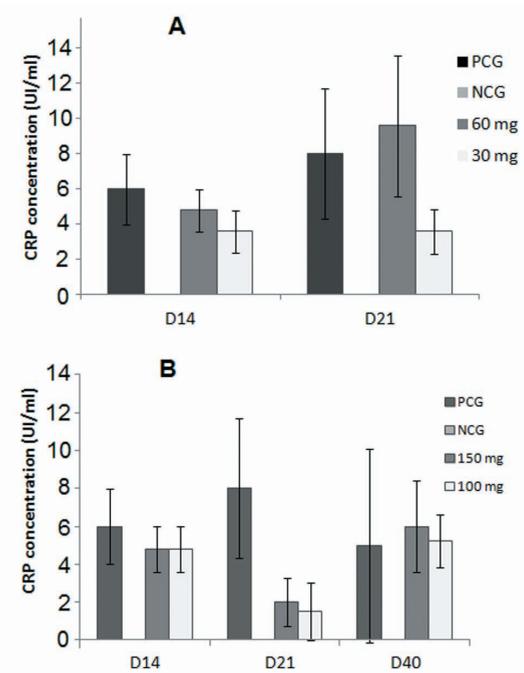


Figure 3. The rate of CRP in the serum, A) in preventive and B) curative groups compared to negative control group (NCG) and positive control group (PCG).

Erythrocyte sedimentation rate and leucocyte count

Inflammation was also reflected on the sedimentation test of the red globules, which increased slightly with the installation of arthritis at the day 14. The rats of the positive control group showed an ESR higher than the negative control group. The extract of AI at 100 and 150 mg/kg reduced the ESR after 21 and 40 days, similar result was obtained in the preventive groups (Figure 4).

After 14, 21 and 40 days of the first immunization the number of the leucocytes was evaluated automatically with a blood cell counter. We noticed a reduction in the numbers of the leucocytes in the treated rats (curative and preventive), compared to the positive control group in the twenty first and the fortieth days (Figure 5).

AIME effect on the histopathological changes

The joints of arthritic rats presented an infiltration of the granulocytes and mononuclear, which gives a synovial hyperplasia, erosion of cartilage and bone in comparison with the normal group (Figure 6a, b). The extract of *Ajuga iva* showed beneficial effects in these last pathological demonstrations (Figure 6c, d).

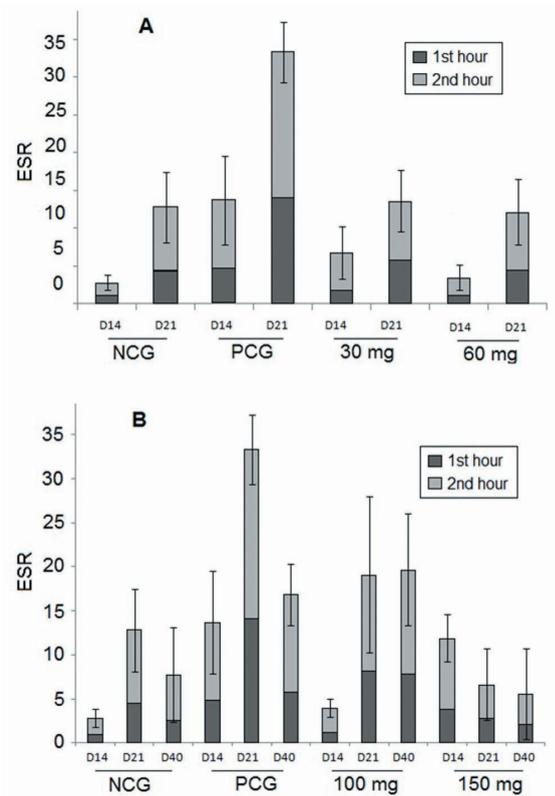


Figure 4. Erythrocyte Sedimentation Rate (ESR); A) in preventive and B) curative groups compared with negative control group (NCG) and positive control group (PCG).

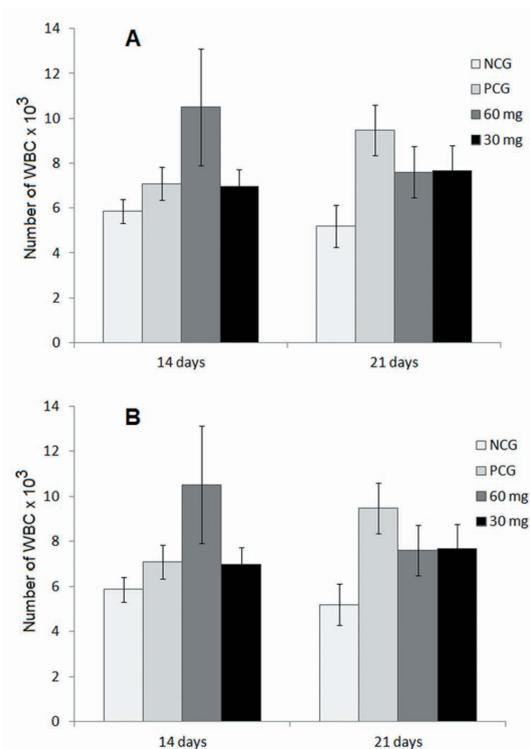


Figure 5. The rate of the white blood cell (WBC); A: preventive and B: curative groups compared to either negative control group (NCG) or positive control group (PCG).

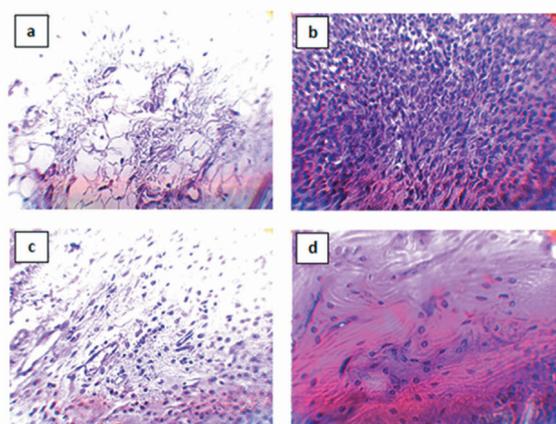


Figure 6. Histological proliferation and infiltration of the leucocytes to the joint tissues of rat paw: a: Negative control group, b: positive control group (arthritic), c: curative groups and d: preventive groups.

DISCUSSION

The use of the medicinal plants is the most widespread form of medicine today throughout the world. The recourse to the treatment by the plants as well as the search for new biological active substances presents one of the

greatest scientific concerns. So, several studies were conducted to evaluate the biological effects of the medical plants. The present study is devoted to the search for new anti-inflammatory drugs contained in methanolic extract of the air part of *Ajuga iva* using a rheumatoid arthritis model. We noticed that arthritis was induced in 90% of the non treated rats. This result prove the effectiveness of the method used in the induction of the arthritis.^[24]

The treatment with the AIME started at the first day of the immunization (day 0) appreciably prevented the beginning of arthritis. The curative treatment starting after the arthritis installation (day 14), removed clearly the progression of the disease. This inhibition seems dose dependent. These effects could be attributed to the flavonoids and polyphenolic contents of AIME which we have previously shown.^[26] In fact, the phenolic compounds have been mentioned to exert an anti-inflammatory effect.^[27–28]

The variation of the weight of the rats constitutes a significant parameter. Effectively, a difference between the growth of the arthritic and normal rats was noted. The loss of the weight is significantly high in the positive and treated groups compared to the negative group. The legs of arthritic rats swelled and rats moved hardly to reach the food. On the other, hand the loss of the weight at the treated groups could be explained by the hypoglycemic, hypolipidimiant and/or hypocholesterolemiant effects of *Ajuga iva* extract.^[29–30]

The histological study showed a significant development of arthritis by the cell infiltration and the destruction of the cartilage and the bone of the rats of the positive group, and finally a deformation of the joints. Preventive and curative treatment showed a significant reduction of the infiltration of leucocytes in the articular space, which gives a reduction of synovial hyperplasia and, then protects the cartilage and bone.

The C reactive protein is a protein of the acute phase of arthritis. We have observed an increase in the rate of this protein in the positive control group. Similar results were obtained by Mythilypriya *et al.*^[31] and Abdin *et al.*^[32] Also, Moncada *et al.*,^[33] Milovanovic *et al.*^[34] and Klimiuk *et al.*^[35] have observed high values of CRP indicative of active inflammation in the sera of patients suffering from rheumatoid arthritis. In the preventive group we did not observe any change in the rate of the serum CRP indicating that the methanolic extract of *Ajuga iva* decrease its level. Mythilypriya *et al.*^[31] and Bharadwaj *et al.*^[36] proposed that CRP can also contribute directly in the pro-inflammatory state, where it stimulates the

release of inflammatory cytokines by the monocytes such as IL-1, IL-6 and TNF α and can also act directly as a pro-inflammatory stimulant of the phagocytic cells to produce the pro-coagulant factor. The pro-inflammatory cytokines help to propagate a local or systemic inflammatory process, to induce the biosynthesis and the secretion of the metalloproteinases (MMPs) and osteoclasts that contribute in the degradation of the extracellular matrix and erosion of bone, respectively.^[37–38] In conclusion, our results indicate that the extract of *Ajuga iva* removed the common inflammatory damage of the rheumatoid arthritis, probably by reducing the production of this protein.

We have observed an increase in ESR in the rats of the positive control group compared to the negative group. Munro *et al.*^[39] also observed a positive correlation between the ESR and arthritis. Analysis of the ESR indicated, a significant difference ($p < 0.05$) between the treated groups and the positive control group. *Ajuga iva* reduced significantly the ESR in the preventive and curative groups.

The increase in the white blood cell (WBC) in rats immunized with standard collagen II confirms the results obtained by Selph *et al.*^[40] and Choi *et al.*^[41] This increase is due to the stimulation of the immune system against the pathogenic micro-organisms;^[42] that is obvious by the infiltration of the inflammatory mononuclear cells in the joint.^[31]

In the present study we found that the methanolic extract of *Ajuga iva* has an anti-inflammatory effect by the reduction in volume of edema of the legs and the markers inflammatory like CRP, it removed the overproduction of the protein C, the severity of the signs of arthritis (arthritic score), to decrease the number of the leucocytes, the levels of (ESR) were close to that of the negative reference group. However, other studies are necessary to check the effect of the extract of *Ajuga iva* on the other serologic parameters and to assess the possible mode of action of these extracts. Consequently, the administration of the extract of *Ajuga iva* orally can offer an advantage when it is combined with other drugs in the treatment of arthritis.

CONCLUSION

In conclusion, our results indicate that the extract of *Ajuga iva* prevent the common inflammatory damage of the rheumatoid arthritis, probably by reducing the production of this protein.

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