Phytochemical screening and evaluation of the antiarthritic potential of *Ammoides pusilla* aqueous extract on Freund's adjuvant-induced rheumatoid arthritis

Hamza Belkhodja 1*, Khadidja Belhouala 2, Soumia Nehal 2

1Laboratory of Bioconversion, Microbiology Engineering and Health Safety, University of Mustapha Stambouli, Mascara, Algeria.

2Department of Biology, University of Mustapha Stambouli, Mascara, Algeria.

**Running title:** Antiarthritic potential of *Ammoides pusilla* aqueous extract

*Corresponding author:* BELKHODJA Hamza

**Postal address:** 32, Bendimered city, Sig, Mascara, ALGERIA.

**Email:** hamza.belkhoja@univ-mascara.dz, hamzabelkhodja@yahoo.fr,

**Phone:** 00213558437634

**ORCID ID:** https://orcid.org/0000-0002-1937-2897
ABSTRACT

Background: *Ammoides pusilla* plant is a species of therapeutic interest which used in traditional medicines. This work aims to valorize this plant by the characterization of their bioactive components and the evaluation of the *in vivo* anti-inflammatory potential against a severe disease affecting the bone structure and the stability of the articular cartilage. Methods: First, the phytochemical screening of the polyphenolic extracts of *A. pusilla* was carried out. The second part of our study is devoted to the evaluation of the in vivo anti-inflammatory activity of the aqueous extract of *A. pusilla* based on the method of the Freund’s adjuvant-induced Rheumatoid arthritis respectively. Results: Phytochemical tests demonstrated the richness of extract with flavonoids, tannins, coumarins, anthocyanins and triterpenes. Whereas, the quantitative determination reveals that the aqueous extract of *A. pusilla* is the richest with bioactive components with contents of total polyphenols, flavonoids and tannins equal to 9.52 ± 0.11 mg GAE / g, 4.75 ± 0.05 mg QE / g and 8.64 ± 0.02 mg CE / g respectively. The results of the anti-inflammatory activity showed that the aqueous infused extract of *A. pusilla* has an interesting antiarthritic potential on Freund’s adjuvant-induced Rheumatoid Arthritis in mice. It is manifested by a weight gain; a normal arthritic index and biochemical parameters close to those of Diclofenac®. Conclusion: The aqueous infused extract of *A. pusilla* is therefore of considerable therapeutic interest as an alternative compound for the prevention of inflammation and for the improvement in bone structure.

Keywords: *Ammoides pusilla*; Rheumatoid Arthritis; Anti-inflammatory; Polyphenols; Toxicity.

Introduction

Plants alongside their capacity to produce primary metabolites (carbohydrates, proteins, lipids and nucleic acids), they also synthesize a very important arsenal of other metabolites indirectly essential to the life of plants, called "secondary metabolites". Aromatic plants, such as those from Apiaceae families produce a large number of bioactive components which have demonstrated several therapeutic properties, mainly antifungal, antioxidant and anti-inflammatory. Inflammation is defined as a complex biological response of vascular tissue to stimuli caused by injury, environmental agents, infection, and cellular modifications. It is a protective mechanism by the body to remove the injurious stimuli as well as initiate the healing process for the tissue. So, it represented as one of the body’s self-defense systems that is classified as part of immunity system. Rheumatoid arthritis (RA) is a chronic, autoimmune
inflammatory disease. Basically, and according to some estimates, it affects 0.7 to 1% of the Algerian population. Just over 300,000 people are affected by RA with a clear predilection for women. For the treatment of inflammation, relief of this pain and stability of joint function, Non-Steroidal Anti-Inflammatory Drugs and classic drugs are available. However, their annoying side effects (peptic ulcers, acute renal failure and even heart complications) prompt people to consider the use of more natural alternatives: medicinal plants.

Algeria has a rich and diverse plant flora. In the perspective of researching, and developing, anti-inflammatory phytomedicines from these plants, our choice focused on the species Ammoides pusilla, Known as Ajwain. It is a dicotyledonous plant that belongs to the Apiaceae family that grows spontaneously in northern Africa (Algeria, Morocco and Tunisia) as well as in Asia (India, Pakistan, Afghanistan). Ammoides pusilla have several therapeutic potentials including analgesic, diuretic, antiasthma effects. As a traditional medicine, the decocted fraction of this plant is used for curing amoebiasis, diarrhea and stomach disorders. However, as yet, its analgesic potential of alcoholic extracts of Ajwain has not been scientifically evaluated. The species Ammoides pusilla was described in the literature as being a plant rich in polyphenolic compounds, having a marked antioxidant and anti-inflammatory potential and could play an interesting function in the prevention of inflammatory diseases. In this context, this present work was aimed to the valuation of the species Ammoides pusilla by the phytochemical characterization of the aqueous extract followed by the in vivo anti-inflammatory activity.

Materials and methods

Plant Material
The aerial part (leaves and flowers) of the species A. pusilla were harvested in Ain oussara, Djelfa region (Algeria) during February 2019. The plant was identified by botanists of the Department of Biology of the University of Mustapha Stambouli, Mascara, Algeria.

Experimental animals
The albino mice "Mus musculus", with an average body weight of 35 ± 5 g were provided by the pet of the University of Mustapha Stambouli, Mascara. The mice were divided into batches and were kept at a temperature of 20 ± 1 °C with a natural photoperiodic cycle. They were fed with food pellets and water ad-libitum with care and treatment conditions in accordance with the guidelines of the Organization for Economic Cooperation and Development.
Preparation of aqueous extract
After boiling 500 ml of distilled water, 50 g of plant A. pusilla has been added. After 15 minutes, the solution is filtered and the filtrate was concentrated in a vacuum evaporator at a temperature of 55 °C followed by drying in the oven and the extract was collected in sterile and hermetically sealed bottles.\textsuperscript{12}

Phytochemical screening

Qualitative analysis
This is a qualitative study aimed to find the main chemical groups (alkaloids, flavonoids, coumarin, tannins, etc.). Characterization tests were based on precipitation and complexation reactions with the formation of insoluble and colored complexes. The observed coloration was caused by the use of an appropriate reagent and is generally due to the reaction between molecules.\textsuperscript{13}

Quantitative analysis

Total Phenolic Content (TPC)
The determination of total polyphenols was carried out according to the Folin-Ciocalteu (FC) method.\textsuperscript{14} 1.25 ml of Folin-Ciocalteu reagent was added to 1 ml of sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}) 2\% and 0.25 ml of aqueous extract, incubated at room temperature 90 min. The absorbance is measured at 760 nm with Spectrophotometer SHIMADZU UV-1280. The results are expressed in mg gallic acid equivalent / g of dry vegetable matter (mg GAE / g) with reference to the gallic acid calibration curve.

Total Flavonoids (TF)
The determination of total flavonoids was carried out by the aluminum trichloride (AlCl\textsubscript{3}) method according to the protocol of Dewanto et al.\textsuperscript{15} 1 ml of diluted extract (0.01 g / 10 ml of the same extraction solvent), added to 0.3 ml of sodium nitrite (NaNO\textsubscript{2}). After 5 min added 0.3 ml aluminum trichloride (AlCl\textsubscript{3}). Then, 2 ml of sodium hydroxide (NaOH) was added. The absorbance was measured at 510 nm with Spectrophotometer SHIMADZU UV-1280. The results are expressed in mg Quercetin equivalent / g of dry plant matter with reference to the Quercetin calibration curve.

Condensed Tannins (CT)
The content of condensed tannins was determined using the vanillin spectrophotometric method. A volume of 0.5 ml of polyphenolic or standard extract (catechin) added to the mixture of 3 ml to vanillin 4%, 1.5 ml of hydrochloric acid and then homogenized. The mixture was left for 15 min at room temperature. Then, the absorbance of each extract was calculated at 500 nm with Spectrophotometer SHIMADZU UV-1280. The total content of condensed tannins was calculated as mg equivalent of catechin (mg CE / g) using the equation obtained from the calibration curve.

**Acute toxicity test**

A total of 60 mice (male and female) weighing between 36 - 40 g were used. Acute toxicity was estimated using the method described by Tahraoui et al, which consisted of dividing the mice into 06 groups of 10 mice each. The mice were previously deprived of food for 18 h. Then, each animal was identified and weighed. The first group (control batch) subjected to a gavage of 10 ml / kg of saline and the other groups receiving the different doses of the aqueous infused extract of *A. pusilla* (1 ml / 100 g). The concentrations were: 250, 500, 1000, 1500 and 2000 mg / kg of body weight. The different doses were administered orally to the different groups. After the administration of the extracts, the mice were observed individually every 30 minutes for 6 hours, the first day and every day for 15 days. The number of dead mice as well as the behavior and symptomatic disorders were recorded.

**In vivo anti-inflammatory activity**

**Rheumatoid arthritis induction**

The induction was carried by intra-plantar injection of 100 µl of Complete Freund's Adjuvant (CFA) into the left hind paw of mice. This inflammatory pain model caused the same pathological aspects of RA present in human patients. A total of 25 mice were selected for this study. The mice were divided into five groups of three mice each and the experimental protocol was carried out according to the method of Mubashir et al. The groups thus formed were: One group used for the control, receiving saline solution (10 ml / kg). An arthritic group used for the negative control receiving no treatment. A group receiving the infused extract of *A. pusilla* (250 mg / Kg) 2 hours before the injection of the mice by CFA. A standard group receiving the infused extract of *A. pusilla* (250 mg / Kg) on the 10th day after induction. A standard group receiving the reference drug Diclofenac® (20 mg / Kg) on the 10th day after induction. The different treatments were administered orally daily for 19 days.
Evolution of Body weight
The physiological situation of animals influenced their nutrient intake, as well as their body weights. A decrease in these can therefore reflect the presence of certain functional problems in mice. Throughout the period of the experiment, monitoring of the body weight of the animals was carried out regularly using an analytical balance. The initial and final weights of each group were recorded.

Monitoring of edema
Synovitis or swelling of the paw injected with Freund's adjuvant after the onset of the disease was the main symptom of RA. Measuring the diameter of the edema was therefore, an important step in monitoring this disease as well as evaluating the effect of the treatments tested. The evolution of the swelling of the paw injected before induction and during treatment was determined by measuring the thickness of the paw (mm) using a caliper having an accuracy of 0.01-150 mm. The measurements were taken at an interval of 02 days.

Arthritis index
In animal experiments, RA was also assessed by index. At the end of treatment, an Arthritic index was calculated for each animal as the sum of the scores. The average of this index in the different treated mice was then compared with that obtained in arthritic mice.

Relative mass of organs
At the end of the experiment, all the mice were deprived of food for 12 hours and were euthanized. A sample of blood and some organs was taken. As a systemic disease, RA can cause serious problems not only in the joints but also in other organs. The treatments administered (standard and extract) can have undesirable effects and thus affect the functioning of the internal organs. Determining the weights of a vital organs therefore remained important. The organs removed were the heart, liver, kidneys, lungs, spleen. The relative weight of each organ was calculated in relation to body weight (g / 100 g body weight).

Biochemical parameters
Blood samples were taken from the abdominal Aorta. The blood sample was then collected in tubes containing an anticoagulant (heparinized) and was centrifuged at 4000 rpm for 10 min. The plasma was used for the following analyzes: CRP, ALP and Calcium. The estimation of this biochemical parameters was carried out using a Spinreact reagent kit (Spain).
Radiographic analysis
In human clinics, conventional X-ray radiography now accounts for the largest percentage of medical imaging examinations. As in humans, imagery of the small animal was first performed on radiological film before devices dedicated to the animal were developed with digital sensors. Radiography is an essential step in the diagnosis and monitoring of the development of RA. The characteristic elementary signs that can be determined by this approach are peri-articular erosion, joint pinching reflecting cartilage destruction and increased radiological transparency. This is a study of 15 mice that were induced by RA and then treated with the infused extract of *A. pusilla* and the drug Diclofenac®. All of the feet of mice injected with CFA, in addition to the left hind paw of mice given saline solution, were removed and immediately were stored in 10% formalin. The radiographic analyzes were thus carried out by an X-ray device. Radiographs in charge of the pathological foot profile were performed according to the following steps: The radiographic analysis of the paw of the treated and untreated mice was performed. The Agfa 24/30 cassette form was divided into different zones. Then, it was deposited on the table of a Shimadzu conventional device. After thorough disinfection, the knees were placed on the tape. Using an X-ray tube, an X-ray beam was directed vertically towards the cassette. The tape will be bombed with constant Kv: 48, weight: 200, Sec 6.3. An Agfa processor ensures high quality development for silver bromide plates with a width not exceeding 36 cm. Then, the pictures were developed after printing.

Statistical analysis
The values were expressed as mean ± standard deviation (Mean ± SD). The results were analyzed by ANOVA single factor for multiple comparisons. The P values less than 0.05 (p <0.05) were considered statistically significant.

Results and discussions
Qualitative analysis
Phytochemical screening tests helped to detect the different groups of secondary metabolites existing in the plant by qualitative reactions of colored characterization or precipitation by specific chemical reagents. The results of the phytochemical analysis revealed the richness of the aqueous extracts of *A. pusilla* in flavonoids, tannins, coumarins, alkaloids, leucoanthocyans, saponosids, triterpenes and quinones with the complete absence of Iridoids.
Our results were in agreement with those reported by the work of Toubal et al\textsuperscript{10} on phytochemical tests of \textit{A. pusilla} aqueous extract which certified the presence of tannins, anthocyanins, coumarins and especially flavonoids in significant quantities. These results were also in agreement with those reported by the work of Felidj et al\textsuperscript{27} and Benaissa\textsuperscript{28}, which confirmed the presence of tannins, flavonoids and triterpenes. These results were confirmed by the work of Mohamed Said and Benmansour\textsuperscript{29} and Hafiane and Ounnas\textsuperscript{30} where they recorded the presence of 06 chemical groups: flavonoids, tannins, gallic tannins, catechin tannins, anthocyanins and coumarins. It has already been noted that this plant contained a large amount of tannins.\textsuperscript{31,32} Our study revealed the existence of catechic tannins in the extract. Tannins were held as good remedies in the treatment of respiratory and inflammatory diseases. They have a strong antioxidant activity to inhibit the formation of superoxide radicals.\textsuperscript{33} Flavonoids and tannins have antioxidant, anti-inflammatory activities and played a positive role in the treatment of cardiovascular and neurodegenerative diseases. In some cases, they were known for their antiviral, antimicrobial and anti-tumor activity.\textsuperscript{34}

**Quantitative analysis**

The results showed a total polyphenol content equal to 9.52 ± 0.11 mg EAG/g. The study of Hafiane and Ounnas\textsuperscript{30} indicated lower total polyphenol contents in the aqueous extract of \textit{A. pusilla} (2.5 ± 0.07 mg GAE / g). While the results obtained by Haouli\textsuperscript{35} recorded total polyphenol contents which were very low (0.12 mg GAE / g). However, Popovici et al\textsuperscript{36} found that the content of phenolic compounds was linked to the maturation phases of the plant; the younger the plant, the higher the level of polyphenols present in it. The differences observed in the total polyphenol contents could be due to: the geographic region, the harvest period, the extraction protocol and the method used for the determination and even the extraction solvent. For Total Flavonoids content, the infused extract recorded a level of flavonoids which was equal to 4.75 ± 0.05 mg QE / g). These results were compared and were appeared to be clearly superior to that obtained by Hafiane and Ounnas\textsuperscript{30} whose found low levels of flavonoids (1.875 ± 0.013 mg QE / g). While, the quantification of the condensed tannins revealed that the infused fraction contained the contents of condensed tannins with a value of 8.64 ± 0.02 mg CE / g.

**Acute toxicity test**
After administration of the aqueous extract of *A. pusilla* in gradual doses, the behavior and the appearance of symptoms of toxicity in mice was monitored. Observations during a period of the experiment did not show any serious clinical symptoms of suffering despite some clinical signs observed such as hypoactivity. They were reversible and appeared in mice for a short time (2-4 hours) then they returned to its activity. These results, which mark the absence of mortality in the various groups of exposed mice, were in accordance with surveys conducted at the National Pharmacovigilance Center, among herbalists specialized in the ethno-pharmacological study of Bentahar, which revealed no toxicity reported in *A. pusilla*. So, this evaluation revealed that the doses 250, 500, 1000, 1500, 2000 mg/kg were tolerated by mice. This showed that the aqueous extract of *A. pusilla* was relatively non-toxic and harmless. A study by Pankaj et al. was in perfect agreement with these results. In fact, in order to monitor the toxicological effect of extracts of *A. pusilla*, their study showed that the LD$_{50}$ can reach a value more than 4500 mg/kg in mice. According to O’Neil and Azizi et al., the toxicity of *A. pusilla* was linked to the presence of certain bioactive elements in the group of monoterpenes such as pinene and cimene and even carvacrol. They assigned an LD$_{50}$ value of 3700, 4750, 810 mg/kg for these three molecules. According to his observations, no significant behavioral side effect was observed in mice treated with these doses.

Another study by Yadav et al. where they administered orally to fasting albino mice two different doses of 1000 and 2000 mg/kg of aqueous extract of *A. pusilla*. The animals were observed continuously for the initial 4, 6, 24 and 48 h for a total of 14 days for behavioral changes and mortality. The behavior observed was hyperactivity, sedation, loss of reflex and increased breathing. However, the recorded symptoms remained reversible for a short time. This indicated that the 2000 mg/kg dose of aqueous extract of *A. pusilla* appeared safe and did not present any acute toxicity.

The same result was confirmed by the in vivo study of Merhfour studying the acute toxicity of the aqueous extract of *A. pusilla*, administered intraperitoneally to mice. No mortality of mice was observed up to the dose of 6 g/kg which made it possible to conclude that *A. pusilla* was a slightly toxic plant, according to the classification of Diezi and that it could be considered non-toxic in traditional medicine provided that it was used properly and rationally.

**In vivo anti-inflammatory activity**

**Evolution of Body weight**
The change in body weight was used as a general indicator of the undesirable effects of chemical compounds.\textsuperscript{44} Regular monitoring of the body weight of normal control mice treated with the aqueous extract of \textit{A. pusilla} during a period of 29 days allowed us to obtain the results represented in the figure 1.

The body weight monitoring results showed a significant reduction (P < 0.05) in body weight in the mice injected with the CFA compared to the control group (Figure 1). This could be explained by the direct effect of the trauma to the lesion and the pathophysiological factors of the inflammatory process of RA which required a several organic and biological systems and functions. There was also the division and growth of different participating cell strains and / or involved in the post-traumatic pathophysiological process.\textsuperscript{45} This reduction in weight may be associated with a reduction in daily food intake after administration of the CFA.\textsuperscript{46} This was the case with untreated arthritic mice, whose weight loss was probably due to the inflammation caused by Freund's complete adjuvant.

On the other hand, it was recorded a marked improvement in body weight in the first days of the administration of the aqueous extract in the preventive and curative group (250 mg / kg) and even with the drug Diclofenac\textregistered. It was also noted that the mice in the preventive group showed stability of the body mass during the period of induction of RA (about 07 days) so that it could continue in gaining weight with the other treated groups. While the group of untreated arthritic mice continued weight loss. The normal evolution of the body mass of arthritic animals having received the different substances tested testified to the capacity of these substances to keep the weight of the mice constant. RA was generally associated with weight loss known as rheumatoid cachexia which led to decreased physical activity and muscle strength.\textsuperscript{47} Some researchers have reported that impaired metabolism reduced the intestinal absorption of glucose. It was explained by the dose / absorption interactions and by the decrease in the amount of food absorbed.\textsuperscript{48} The gain in body weight noticed in the present study may be due to the restoration of the absorption capacity of the intestine and therefore to the management of rheumatoid cachexia.

**Monitoring of edema**

The CFA injection caused the release of several chemical mediators which increased the permeability of the capillaries and blood circulation to the interstitial space. Therefore, it was a localized edema, which, in turn, compressed the nerve endings and thus determined a sensation
Since the first day of CFA induction, arthritic mice have experienced swelling and redness of the tissue in the joints of the injected foot. They showed maximum intensity on the 7th day. According to the results recorded, the edema formed in arthritic mice caused the ankles to swell with an average thickness equal to 4.95 ± 0.03 mm compared to the healthy control group (Figures 2 and 3). Whereas after the administration of the different treatments, it was observed a significant reduction (p <0.05) in the thickness of the paw of 3.65 ± 0.01, 5.56 ± 0.03, 5.67 ± 0.04 mm from day 9 at a thickness of 2.51 ± 0.01, 2.59 ± 0.007, 2.71 ± 0.007 mm for the preventive, curative and Diclofenac® groups, respectively. The results revealed that the aqueous extract of A. pusilla appeared effective in reducing the responses induced by the CFA.

Compared with the reference medicine, it was found that oral administration of 250 mg / kg of the aqueous extract of A. pusilla reduced the volume of edema from 5.56 ± 0.03 to 2.59 ± 0.007 mm for the curative test and from 4.76 ± 0.01 to 2.51 ± 0.01 mm for the preventive test. While the administration of 20 mg / Kg of Diclofenac® reduced the volume of edema as a symptom of inflammation, going from an average thickness of 5.67 mm to 2.71 mm, or 52.20%. Pain can be relieved and edema can be effectively reduced by non-steroidal anti-inflammatory drugs that inhibited cyclooxygenase (COX-1 and COX-2) which then inhibited the transformation of arachidonic acid.50

It was noted that in the preventive group, the recovery of the volume of edema began on the 3rd day following the CFA injection. These results showed and confirmed the early action of the aqueous extract of A. pusilla in this type of treatment. Therefore, the mechanism of action of the aqueous extract of A. pusilla studied as an anti-inflammatory implied a reduction of all inflammatory mediators to different degrees in the model of inflammation by CFA.51 The inhibition of the inflammation took place during the time intervals during the experiment carried out.

A study by Asif et al52 showed that the aqueous extract had a significant anti-inflammatory effect. Its anti-inflammatory activity could be due to an increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during the formation of granuloma tissue.53 Landoulusi and these collaborators54 studied different extracts from species in the Apiaceae family where they showed anti-inflammatory activity in vitro and in vivo. These extracts inhibited the synthesis of cytokine TNFα (tumor necrosis factor α), one of the most important pro-inflammatory cytokines, and the synthesis of nitric oxide generated by inducible nitric
oxide synthase (INOS) in murine endothelial cells without affecting cell viability. In vivo, the anti-inflammatory activity of its extracts administered orally at a dose of 200 mg / kg has been proven to inhibit mouse paw edema.

The anti-inflammatory activity exerted by the aqueous extract of A. pusilla suggested that it has an effect on kinin, synthesis of prostaglandins, bradykinins and lysozyme. The presence of triterpenes, glycosides and steroids in plants have been found to exert active anti-inflammatory effects.\textsuperscript{55,56}

**Arthritis index**
Assessment of the arthritis index was important in monitoring the degree of inflammation and the severity of RA.\textsuperscript{57} The arthritic index was calculated for each group based on the sum of the scores related to the symptoms observed in the ears, nose, tail, forelegs and hind legs. The average of this index in the various mice treated with the infused extract of A. pusilla was then compared with that obtained in untreated arthritic mice. The results were illustrated in the figure 4.

At the end of treatment with the infused extract of A. pusilla and the drug Diclofenac\textregistered, the results showed that Diclofenac\textregistered presented a significant reduction (p <0.05) in the arthritic index to a value of 1. Although the extract of A. pusilla in the preventive and curative group attenuated the evolution of the inflammation with a sum of scores identical to that of Diclofenac\textregistered (arthritic index = 1). However, the group of untreated arthritic mice had an arthritic index score of 6. This explained the symptoms linked to the inflammation of RA. The arthritis index was an essential parameter in the evaluation and clinical follow-up of inflammation during RA.\textsuperscript{58} So, the reduction in this index was therefore directly linked to the suppression of the inflammation.

**Relative mass of organs**
Given the action of the inflammatory process of RA on the physiological state of different systems of the body\textsuperscript{59}, it was essential to follow the evolution of the relative masses of the organs removed. Figure 5 represented the variation of relative weight of the organs in the different groups.

According to the figure 5, the weight gain of the organs showed that the weights of the organs of the Preventive, Curative and Diclofenac\textregistered groups were quite similar compared to the healthy control group apart from the liver and the spleen of the mice of the Diclofenac\textregistered group which
were slightly heavier than those of other groups. This increase in relative weight was one of the side effects of NSAID drugs and can be attributed to the increased demands for detoxification of xenobiotic compounds. In addition, it referred to the increase in cell mass or cell density. With regard to the group of untreated arthritic mice, the results showed a significant increase (p <0.05) in the relative mass of the liver, lungs, hearts and spleen. This increase was due to the absence of treatment which has stimulated the complications of RA and amplified the manifestations of inflammation which affected all organs. One study indicated that enlargement of the liver may be linked to the maintenance of normal liver functional capacity. Gebhard et al\textsuperscript{60} claimed that the changes in the relative mass of the spleen were attributed to the action of cytokines released during the inflammatory process of RA which explained the resulting imbalance in the immune system.

This was in perfect agreement with the results of Patel\textsuperscript{61} where they showed that the arthritic mice induced by Freund's adjuvant presented a significant increase in the relative mass of the spleen. While treatment with plant formulations has improved this value. Therefore, it can be deduced that herbal preparations can have a protective effect against the spleen and other organs. The spleen together with the thymus represented the major immune organs in the body, the thymus functioning as the central immune organ and the spleen as the peripheral immune organ. Changes in the thymus and spleen indices were considered to reflect the overall immune function of the body.\textsuperscript{62,63}

Biochemical parameters

Reactive Protein C (CRP)

CRP was a protein of the inflammatory reaction with fast kinetics. Its biological half-life was short, on the order of 8 to 12 hours.\textsuperscript{64} The CRP values obtained after a period of 20 days of treatment in the different groups were shown in the figure 6. The histogram (Fig. 6) showed that there was a significant increase (p <0.05) in the synthesis of CRP in the group of untreated arthritic mice with a value of 1200 μg / ml compared to the treated groups which reported a value of 600 μg / ml. Therefore, it was assumed that the recovery of CRP was corrected by the dose of 250 mg / kg of aqueous extract of A. pusilla and by Diclofenac®. Therefore, it can be
concluded that treatment in the preventive and curative mode with aqueous extract of *A. pusilla* was able to stabilize the CRP concentration.

Given the known relationship between CRP and IL-6, it was suggested that the IL-6 produced in the affected joint may be partly responsible for the elevations in systemic CRP observed. IL-6 was known to be the main regulator of CRP production and may have a role in the inflammatory process. IL-6 was known for its direct effect on chondrocytes and stimulates T cells. Another potential stimulus for hepatocyte production of CRP was IL-1. This cytokine seemed to work in synergy with IL-6 to promote the production of CRP. During an inflammatory syndrome, CRP increased earlier than the rate of sedimentation. At the end of inflammation, CRP decreased faster than Sedimentation speed. Some authors believe that a very high CRP was linked to an infectious process. However, such increases can also be seen during major inflammatory syndromes as a symptom of RA. The action regulating the serum level of CRP by the aqueous extract with *A. pusilla* tested may be due to the inhibition of inflammation and the synthesis of chemical mediators. It was attributed to the richness of the extract in bioactive substances with anti-inflammatory power.

**Alkaline phosphatase (ALP)**

Alkaline phosphatases (ALP) were enzymes found in many parts of the body such as the liver, kidneys, digestive tract and bones. About 90% of ALP were found in the liver and bones. Its rate, expressed in international units per liter of blood (IU / L). ALP was the most frequently used biomarker in the detection of osteoblastic bone formation. ALP concentrations in the liver and bones have been applied in the diagnosis of osteoarticular pathologies. The results obtained showed that the Preventive, Curative and Diclofenac® groups have normal physiological values compared to the reference value ([ALP] = 70 ± 38 IU / L). It was recorded average values equal to 71.75, 87.5 and 73.5 IU / L for the Preventive, Curative and Diclofenac® groups, respectively. Although these values were significantly high compared to the control group considered to be healthy. On the other hand, the group of untreated arthritic mice had a value which is clearly higher (162.75 IU / L) than the physiological reference values. So, the aqueous extract of *A. pusilla* was able to exhibit a certain physiological stability of ALP in the preventive and curative mode compared to the reference medicine. ALP levels have been reported to be elevated in the serum of RA patients which was correlated with disease activity assessed by erythrocyte sedimentation rate and serum CRP levels. The increased activity of
ALP can be induced by inflammatory cytokines such as interleukin-1 (IL-1), since IL-1 has been shown to correlate with acute phase reagents. Interference with these enzymes led to biochemical disturbances, tissue damage and loss of cell function. The increase in alkaline phosphatase was associated with an increase in gamma-glutamyl-transpeptidase as a sign of bone damage. In addition, the level of ALP indicated that there may be active bone formation, since ALP was a byproduct of osteoblast activity. Corrado et al. have shown in bone cells that excessive production of alkaline phosphatase was linked to cell proliferation of erosive arthropathy.

**Calcium**

To determine the correlation between the serum calcium concentration and the inflammatory reactions induced by RA and to test the regulatory action of the aqueous extract of A. pusilla and the drug Diclofenac®, we determined the serum calcium concentration after a 20 days treatment period. Based on the results recorded and comparing with the reference value for calcium (10 mg / dl), it was noticed a significant hypercalcemia (P < 0.05) in all study groups with different values. The group of untreated arthritic mice had the highest value (17.65 ± 1.36 mg / dl). In contrast, the Diclofenac® and healthy control groups had almost the same values 15.91 ± 0.04 and 15.71 ± 0.03 mg / dl respectively. These results were significantly higher than that of the Preventive and Curative groups, which had the values 13.91 ± 1.69 and 14.82 ± 0.02 mg / dl respectively. This increase was linked to bone or thyroid pathology because the three main mechanisms responsible for hypercalcemia were the massive release of calcium by bone tissue, the decrease in its renal excretion and the increase in digestive absorption of calcium. Osteoarticular disorders and primary hyperparathyroidism are the most common causes of hypercalcemia. Treatment with Diclofenac® and the aqueous extract of A. pusilla tested after installation of the disease led to a recovery and stability in the concentration of serum Calcium. A study by Saptarini et al. on the determination of serum calcium levels in patients with RA showed that it was lower than in normal controls. Calcium absorption in RA was reduced due to a primary malabsorptive process, but the mechanism remains unknown. However, some authors suggested that hypocalcemia resulted from drugs used in the treatment of RA. Glucocorticoids given to patients with RA can cause bone loss. Glucocorticoids inhibited the resorption of calcium in the renal tubules and the absorption of calcium in the intestine through mechanisms dependent on vitamin D. The regulation of calcemia mainly depended on three
organs: the kidney, the bone tissue and the intestine, and on two hormones, parathyroid hormone and vitamin D.69

Radiographic analysis
In order to observe the modifications recorded on the bone structures of the mice during the period of treatment against RA, the radiographic examination was carried out on the arthritic mice and treated with the aqueous extract of *A. pusilla* and Diclofenac®. The images of the radiographic image described the bony structures visible on an X-ray of the Ankle-Tibia-Femur. The results of the X-ray analysis presented in figure 7 showed different groups:

1) Control group: the interpretation of radiography showed Femorotibial and coxofemoral joint of normal radiographic appearance and normal opacity, normal bicondylar aspect. Normal tibial-tarsal joint, normal opacity with no sign of erosion or bone destruction phenomenon, slope, external malleolus and metastasis of normal radiographic appearance, fibula of normal radiographic appearance.

2) Arthritic Untreated group: it showed an irregular-looking fibula with the presence of bone demineralization, especially in the epiphyseal region with signs of erosion and thickening at the point of insertion at the femorotibial joint, caudal and tibiotarsal region and coxofemoral (red arrows). Among the signs recorded: Thickening of the soft parts (important ++++) with very visible major synovial swelling especially in the Tibiotarsal articular region. It is diffuse extensive and extended to the tarsometatarsal region. There is also an Early, large and diffuse line spacing. The Tibial and femoral epiphyseal was demineralized in the distal region. Marginal erosions ++, central, bulky was determined in the Talus region, external malleolus reaching the metatarsus with symptoms of ankylosis (especially at the tarsus and the femoro-iliac junction). So, these Radiographic signs were compatible with erosive arthropathy such as RA.

3) Preventive, curative and Diclofenac® group: They showed a femorotibial and coxofemoral joint of normal radiographic aspect and heterogeneous opacity, normal bicondylar aspect although. There is Thickening of the soft parts with very visible major synovial swelling, especially in the group treated with Diclofenac® in the articular region Tibiotarsian, coxofemoral, extensive diffuse. The Pinching of the line space was early, important and diffuse especially in the healing group. It was noticed a Slight distal tibial epiphyseal demineralization (preventive and curative group). Some Marginal erosions ++, central, bulky was determined in the Talus region, external malleolus reaching the metatarsus with ankylosis (especially at
tarsus) (healing group). Thus, there is some Negative signs: no bone proliferation, no bone demineralization on the radiograph (Diclofenac® group). So, the Radiographic signs were compatible with destructive arthritis of the RA type (in the curative group), good resolution in the Diclofenac® group and particularly in the preventive group.

The results of the radiographic analysis revealed the potential of the aqueous extract of *A. pusilla* to inhibit the onset (preventive action) and / or slow down the arthritic progression (curative action) compared to the reference drug (Diclofenac®) reducing inflammation and preserving the metabolic functions. This anti-inflammatory potential was explained by the action of the biomolecules present in the aqueous extract of *A. pusilla* participating in the reduction of the level of cytokines, TNF-α and other pro-inflammatory factors.

**Conclusion**

Qualitative analyzes have shown the presence of a multitude of varieties of bioactive compounds. The acute toxicity test of the infused extract on mice showed that the aqueous extract of the species *A. pusilla* was relatively non-toxic and harmless. Whereas, the results of the *in vivo* study indicated that the group of mice treated with the aqueous extract of *A. pusilla* at a dose of 250 mg / kg (preventive) marked a good improvement during the follow-up of the edema, body weight and biological parameters. All of these results showed that the aqueous infused extract of *A. pusilla* has an interesting anti-inflammatory activity on the method of Freund's adjuvant RA and was therefore of considerable therapeutic interest as an alternative compound for the prevention and treatment of inflammation.

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Figure 1. Evolution of Body weight
Figure 2. Evolution of Body weight
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Figure 3. Chronological photograph of the evolution of edema in the preventive group.
Figure 4. Arthritic index of mice at the end of treatment.

Figure 5. Relative mass of organs
Figure 6. Variation of CRP, ALP and Calcium concentration in control and treated mice.
Figure 7. X-ray of the feet of treated and untreated mice.