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Research Article

# Evaluation of *In Vitro* and *In Vivo* Anti-Arthritic and Xanthine Oxidase Inhibitory Activities of Thymoquinone: Applied to Collagen-Induced Rheumatoid Arthritis in Male Rats

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**ABSTRACT**

The aim of the present study is to evaluate the *in vitro* anti-arthritic effect of thymoquinone (TQ), the possible protective effects of TQ against collagen II-induced rheumatoid arthritis, in male rats with influence of orally TQ treatment in rheumatoid arthritis score and its effect on the accompanying xanthine oxidase activity, in plasma, liver and spleen homogenates.*In vitro* anti-arthritic activity was evaluated using BSA denaturation inhibition test. Rheumatoid arthritis was induced in rats by intradermal injection of collagen II at dose of 03 mg/kg, emulsified in Complete Freund's Adjuvant (V/V), the arthritic rats were treated with TQ for 40 days, using two doses (05 and 10 mg/kg). Then, xanthine oxidase activity was estimated in both plasma and tissue homogenates of arthritic and TQ-treated rats, using spectrophotometry methods. This study revealed a significant in vitro anti-arthritic activity in dose dependent manner. Collagen II-induced rheumatoid arthritis is accompanied by a significant increase of xanthine oxidase activity in plasma, liver and spleen. In addition, TQ treatment led to restore xanthine oxidase activity in a dose dependent manner. Thymoquinone possesses significant anti-arthritic activity which expressed by the decreased arthritic score, a delay in the onset of the disease and inhibition of xanthine oxidase inhibition activity, in both plasma and tissue homogenates.



## INTRODUCTION

Rheumatoid arthritis (RA) is one of the most inflammatory diseases. It is a chronic autoimmune disease, characterized by bilateral and symmetrical joint inflammation [1], synovial hyperplasia, cellular proliferation of the synoviocytes and neo-angiogenesis leads to formation of pannus stroys the articular cartilage and the bone, causing swelling and deformation of the affected joints. Several studies [2-6] provide evidences for the involvement of xanthine oxidase and ROS in the pathogenesis of rheumatoid arthritis. Lipid peroxidation mediated by ROS is considered to be the major mechanism of cell membrane destruction and cell damage.

Thymoquinone (TQ), 2-Isopropyl-5-methyl-1, 4-benzoquinone, is the major compound of the volatile oil derived from Nigella sativa seeds. It has several pharmacological properties;such as anti-inflammatory, antioxidant and anti-arthritis activities [7-10].

Xanthine oxidase (XO; EC 1.1.3.22) is one of the endogenous source of Reactive Oxygen Species (ROS), through oxidation of hypoxanthine to xanthine and xanthine to uric acid. It requires the use of molecular oxygen as an electron acceptor, producing superoxide anion and hydrogen peroxide [11, 12]. XO also catalyzes the reduction of nitrate to nitrite and NO• [12]. It has become the subject of great interest because of its involvement in several human pathologies. It is one of the most enzymes involving in inflammation, several studies have been published to investigate the role of xanthine oxidase in inflammatory diseases [13-16].

The aim of this study is to evaluate the *in vitro* anti-arthritic effect of TQ, and to examine potential change in xanthine oxidase activity, in the case of collagen induced rheumatoid arthritis, in plasma and tissue homogenates (liver and spleen). The other objective is to

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determine the influence of orally TQ treatment in rheumatoid arthritis score and the accompanying xanthine oxidase activity.

## MATERIALS AND METHODS

### Chemicals

Thymoquinone, Complete Freund's Adjuvant, Incomplete Freund's adjuvant, Bovine serum albumin (BSA), xanthine substrate and all others products were purchased from Sigma Aldrich.

### Experimental Animals

Twenty-eight male Wistar rats (200–250 g) were purchased from the Animal House of Pastor institute Alger, Algeria. The animals were acclimatized for one week and maintained under standard conditions of temperature (23 ± 2°C), humidity (60 ± 10%) and 12 hours light/dark cycle. The rats were fed with a standard diet and water.

### Evaluation of the anti-arthritic effect in vitro

The *in vitro* anti-arthritic effect of thymoquinone is determined using the BSA denaturation inhibition test according to the protocol of Williams and his collaborators [17]. Briefly, 500 µl of 0.2% of the BSA prepared beforehand in tris-HCl buffer (20 mM, pH 6.8) are added to 500 µl of the different concentrations of thymoquinone or diclofenac acid (250 µg / ml). This mixture is incubated at 37 ° C for 20 min and then at 65 ° C for 10 min. After cooling, the absorbances are measured at 660 nm and the percent inhibition is calculated using the following equation:

% inhibition = [(Abs control - Abs test) / Abs control] x 100

### Effect of Thymoquinone on Rheumatoid Arthritis

### Induction of Rheumatoid Arthritis in Male Rats

Arthritis was induced in male rats according to the method previously described by Xu and his collaborators [18] and Zhang and his collaborators [19]. Briefly, rats were divided into four groups: **group1,** a normal control group in which the rats received normal saline solution; **group 2,** an arthritis control group in which the rats were induced arthritis using collagen II emulsified with Complete Freund's Adjuvant to a final concentration 0,3 mg/kg b.w. and left untreated; **group 3,** 5 mg TQ -treated group, in which the rats were treated with thymoquinone at a dose of (05 mg/kg b. wt) orally and **group 4,** 10 mg TQ -treated group, in which the rats were treated with thymoquinone at a dose of (10 mg/kg b. wt) orally.

The process which used to induce arthritis in rats of different groups was the following:

**On Day 0:** Immunization of the rats (arthritic groups, treated with 05 mg and 10 mg) is performed by intradermal injection, 03 mg / kg of collagen II emulsified in complete Freund V / V adjuvant. This injection is performed under anesthesia using urethane at a dose of 01 g / kg injected intraperitoneally. Urethane injection is performed in the control group.

Treatment of rats is effected daily for 40 days, orally as follows:

The rats of groups 05 and 10 mg are treated orally with TQ (of 05 and 10 mg / kg / day, respectively). Normal and arthritic group rats are left without treatment. However, the rats in the control group are treated with NaCl gavage containing 0.1% tween 80 (the vehicle in which the TQ is dissolved).

**In Day 07:** A booster immunization of rats is performed by intradermal injection of 1.5 mg / kg collagen II, emulsified in incomplete Freund V / V adjuvant. This injection is performed under the same conditions as the first injection.

**In 40$^{th}$ day:** the animals were sacrificed under diethyl ether anesthesia by cervical dislocation, Blood samples were collected from the retro-orbital sinus of the eye by ocular puncture into heparinized tubes for biochemical analyses. Plasma was separated by centrifugation at 3000 rpm for 10 min, at 4°C. Rat livers were quickly excised and perfused with chilled 1.15% (w/v) KCl solution in order to remove all traces of hemoglobin. The livers were blotted dry, weighed and a portion was used to prepare homogenate and stored at - 80°C pending analysis.

### Evolution of Rheumatoid Arthritis

### Disease Score

The arthritic score is determined from 0 to 4 following the evolution of the swelling of the anterior paws in the wrist and the middle and also in the ankle and the middle of the hind paws where: 0 = no arthritis, 01 = extreme redness and / or slight swelling, 02 = medium swelling, 03 = severe swelling and 04 = severe swelling with swelling of the thumb of the paw that can no longer support the weight of the animal. The arthritic score at the metacarpophalangeal and

interphalangeal and metatarsophalangeal joints of the first four fingers of each leg is evaluated as follows: 0 = no swelling, 01 = swelling. The total score is the sum of the score of all the joints mentioned above. The score that expresses the maximum severity of the disease is 20 / leg it is 40/2 legs and 80 / animal [20].

**Preparation of Plasma and Tissue Homogenates**

The animals were sacrificed under diethyl ether anesthesia by cervical dislocation Blood samples were collected from the retro-orbital sinus of the eye by ocular puncture into heparinized tubes for biochemical analyses. Serum was separated by centrifugation at 3,000 rpm for 10 min, at 4°C. Liver and spleen homogenates (10% w/v), was prepared by homogenizing 0.5 g of tissue samples with 5 ml of ice-cold 0.15 M KCl, the homogenizing buffer. The homogenate was centrifuged at 4000 rpm for 10 min, at 4 °C to obtain a supernatant for analyses of xanthine oxidase activity. Homogenate and plasma were stored at -80°Cpending analysis.

**Effect of Thymoquinone on Xanthine Oxidase**

The determination of xanthine oxidase is carried out according to the protocol of Bergmeyer and his collaborators [21] following the increase in the production of uric acid at 290 nm formed following oxidation of xanthine in the presence of an enzymatic source (plasma or homogenate). Briefly, 333 µl of xanthine (0.15 mM) are added to 666 µl of potassium phosphate buffer (50 mM, pH 7.5) and 33 µl of serum or homogenate in a quartz vat. Uric acid production is monitored by measuring the change in absorbance for 1 min against a blank that contains all reagents except the sample which is replaced by distilled water. The enzymatic activity of xanthine oxidase is calculated according to the following equation:

U / ml enzyme = (d A290 nm / min sample-dA290 nm / min White) (1) / (12.2) (0.033)

1 = the total volume.
12.2 = Milli-molar extinction coefficient of uric acid at 290 nm.
0.033 = the volume of the sample.

**RESULTS**

***In Vitro* Anti-Arthritic Effect**

To evaluate the *in vitro* anti-inflammatory effect of TQ, the ability of TQ to inhibit the induced denaturation of BSA was estimated. The results obtained show that TQ has an inhibitory effect against the denaturation of BSA caused by the incubation of BSA at a temperature of 37 ° C for 20 min and then at 65 ° C for 10 min. This effect is significantly (p≤0.001) lower than that of the positive control diclofenac acid (Dic. ac) which expresses a percentage inhibition of 94.14 ± 0.92% at a concentration of 250 µg / ml. However, the inhibition percentages obtained with the TQ are 37.09 ± 4.07, 21.53 ± 3.05, 10.21 ± 1.5 and 10.05 ± 3.26% at concentrations of 200, 100, 50 and 25 µg / ml, respectively (Fig. 1). TQ concentrations above 200 µg / ml exert a denaturing effect on BSA (Fig. 1).

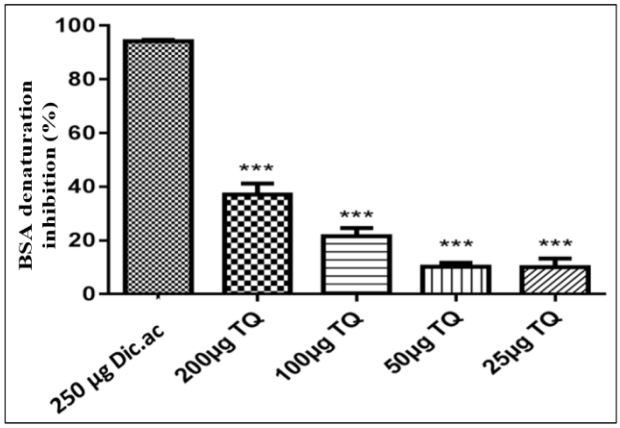


**Figure 1:** BSA denaturation inhibition effect of TQ and standard (diclofenac acid). Each value represents the average of three trials ± SD, ***: p≤0.001.

**Evolution of Rheumatoid Arthritis Induced in Rats**

**Plasma Level of Markers of Inflammation**

To diagnose inflammation in rheumatoid arthritis, plasma levels of rheumatoid factors and ASLOs were determined. The qualitative and quantitative determination of the CRP and the determination of the formula and blood count (FNS) were also performed. The results obtained are shown in Fig. 2. Rheumatoid arthritis is accompanied by a significant increase (p≤0.001) of rheumatoid factors (RF) and ASLOs, compared with the control group. The qualitative and quantitative results of CRP were negative. Treatment of the rats with TQ allowed to restore the level of these parameters to a level similar to that recorded in the rats of the control group. The results of the formula and blood count showed a significant increase (p≤0.05) in white blood cells (WBC), platelets (PLT) and lymphocytes (LYM). A highly significant increase (p≤0.01) of granulocytes (GRAN), MID and MID% was recorded in arthritic rats compared with rats in the normal control group. However,

treatment with TQ for 40 days decreased these parameters in a dose-dependent manner, whose treatment with 10 mg / kg / day allowed to restore them and maintain values similar to those of the control group (Table 1).

**Table 1:** Effect of thymoquinone on formula and blood count

| Parameters | Control | RA group | RA + 5 mg | RA+ 10 mg |
|---|---|---|---|---|
| **WBC ($10^{12}$/L)** | 8.0 ± 0.28 | 7.12 ± 1.44 | 7.23± 0.70 | 7.71 ± 0.81 |
| **PLT ($10^9$/L)** | 524.60 ± 43.92 | 700 ± 39.8 * | 675.7 ± 49.8 | 666.60± 102 |
| **RBC (109/L)** | 7.30 ± 0.88 | 10.39 ± 1.59 * | 9.30 ± 9.30 | 7.08 ± 1.21 ## |
| **HGB (g/dl)** | 14.52 ± 0.38 | 12.2 ± 2.2 | 13.0 ± 1.2 | 14.0 ± 1.00 |
| **LYM ($10^9$/L)** | 5.4 ± 0.7 | 6.5 ± 1.3 * | 6.1 ± 1.2 | 5.2 ± 0.9 ## |
| **GRAN ($10^9$/L)** | 1.1 ± 0.2 | 2.4 ± 0.5 ** | 2.0 ± 0.5 | 1.1 ± 0.2 ## |

Values are expressed asmean ± SEM, (n = 7); *: p≤0.05, **: p≤0.01 a significant difference compared to rat control group, ##: p≤0.01 a significant difference from the group of arthritic rats.

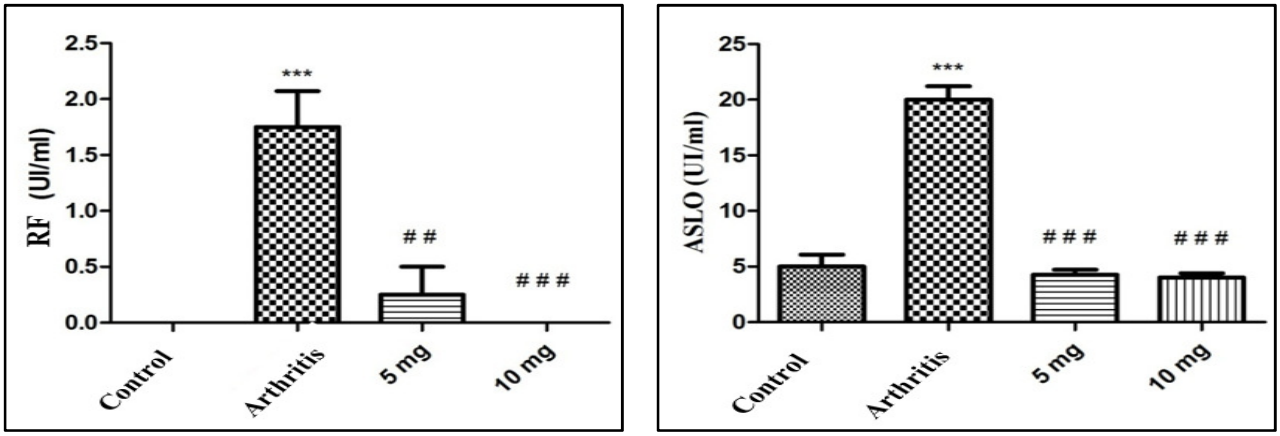


**Figure 02:** Plasma levels of rheumatoid factors (RF) and ASLO in arthritic and treated rats. The values are expressed as mean ± SEM, (n = 7); ***: p≤0.001 a significant difference over the control group of rats, ###: p≤0.001 a significant difference compared to the group of arthritic rats.

**Evolution of Arthritic Score**

To validate this model of experimental arthritis in rats, Fig. 3 shows the evolution of total score, the average score of the different rats in the group. The arthritic score of the legs posterior is presented independently of that of the forelegs. With regard to the hind legs, the results show that the first signs of arthritis started on the 14 day in arthritic group rats (untreated patients) (n = 7). The best arthritic score of this group was 36 ± 3 joints in both legs (one writes 36 ± 3/2 paws). This score is recorded on the 28th day. Then, it decreased with time until score of 21 ± 3/2 legs on the 40th day. This decrease is accompanied by an increase in rigidity and deformation of the legs.

Treatment of rats with TQ resulted in a highly significant decrease (p≤0.001) arthritic score, compared with that of the arthritic group, this decrease was dose-dependent. The rats treated with 05 mg / kg / day showed a delay in the appearance early signs of joint inflammation, these signs were observed on the 16th day. The arthritic score evolved over time until waiting for the value of 15 ± 7/2 paws on the 30th day. Then, this arthritic score decreased with time until having a value of 8 ± 4/2 legs, in the 40th day. Similarly, treatment of rats with 10 mg / kg / day caused a delay in the appearance of the first signs of joint inflammation, which are observed on the 18th day. The arthritic score evolved over time until the value of 05 ± 3/2 legs 29th day. This score decreased with time until the value of 02 ± 2/2 legs, the 40th day (Fig. 3A). For the forelegs, the results obtained reveal that the first signs of arthritis were observed on the 15th day in rats from the arthritic group (n = 7), then the score evolved to achieve the highest score which was also 36 ± 3/2 paws. This score is recorded on the 28th day then it decreased with time until having a score of 24 ± 4/2 legs, the 40th day. The Treatment of rats with TQ revealed a highly significant (p≤ 0.001) decrease in score arthritic in a dose-dependent manner (Fig. 3B). The rats treated with 05 mg / kg / day showed a delay in

the appearance of the first signs of joint inflammation. Their signs of joint inflammation were observed on the 22nd day. The arthritic score had evolved with time until reaching the best score, which is 03 ± 1/2 paws, recorded on the 31st day. Similarly, treatment of rats with 10 mg / kg / day caused a delay in manifestation of the first signs of joint inflammation. These signs are observed on the 27th day. Then, the arthritic score evolved over time until waiting for the value of 01 ± 1/2 paws the 31st day (Fig. 3B). These results express the effectiveness of TQ in the protection and therapy of the rheumatoid arthritis induced immunization of rats with collagen II emulsified in Freund's adjuvant.

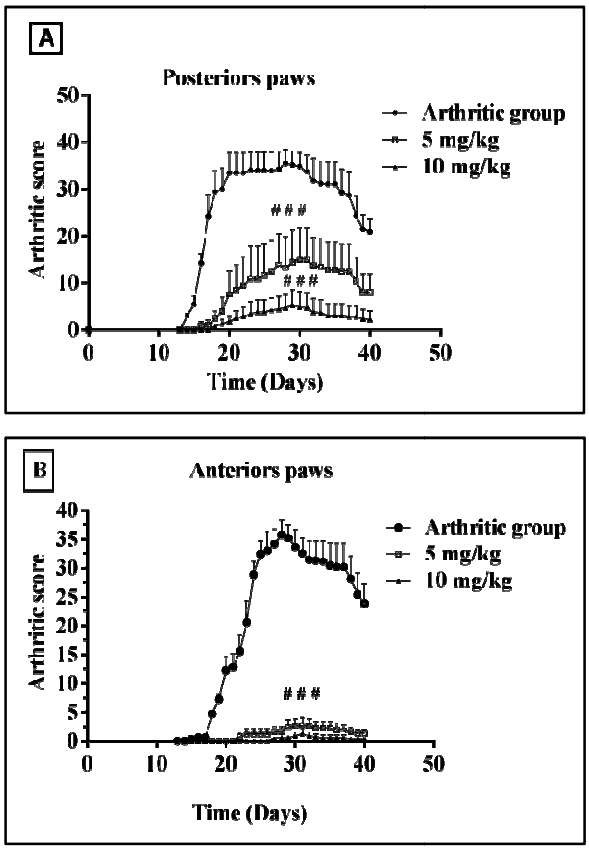


**Figure 3:** Effect of TQ on the evolution of the arthritic score of rats. The values are expressed as mean ± SEM, (n = 7); ### : p≤ 0.001 a significant difference from the group of arthritic rats.

**Effect on Xanthine Oxidase Activity**

The results of xanthine oxidase (XO) activity studied in the case of arthritis in homogenates (liver and spleen) and in plasma are shown in figure 04 and Table 2. They show that arthritis is accompanied by a significant increase in XO activity (p≤0.01 and p≤0.001, respectively) in comparison with the control group. The results show that the spleen homogenate expresses an enzyme activity of the XO comparable to that recorded at the level of the liver homogenate. The treatment of arthritic rats with 05 and 10 mg / kg / day of TQ caused a dose-dependent decrease in XO activity, whose dose of 10 mg / kg / day restored it and maintained a similar activity to that recorded in the control group.

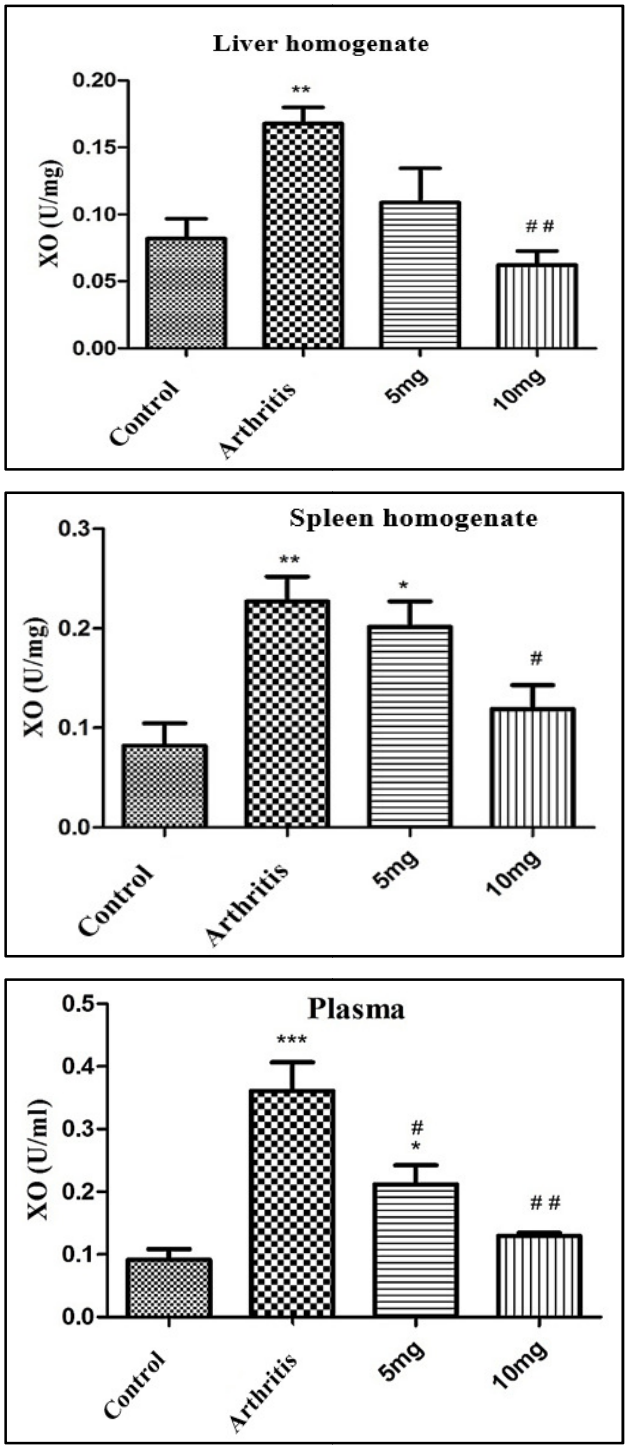


**Figure 4:** Effect of TQ on xanthine oxidase in cases of arthritis. Values are expressed as mean ± SEM, (n = 7); *: p≤0.05, **: p≤0.01, ***: p≤0.001 a significant difference compared torat control group, #: p≤0.05, ##: p≤0.01 a significant difference from the group of arthritic rats.

**Table 2:** Effect of TQ on xanthine oxidase activity in the case of rheumatoid arthritis

| Treatments | Plasma XO (U/ml) | Liver homogenate XO (U/mg) | Spleen homogenate XO (U/mg) |
|---|---|---|---|
| **Control** | 0.091±0.017 | 0.082± 0.015 | 0.082± 0.022 |
| **Arthritic group** | 0.361±0.046 *** | 0.168± 0.012 ** | 0.227±0.025** |
| **TQ (05 mg/kg)** | 0.212±0.031 *, # | 0.109± 0.026 ns | 0.201±0.026* |
| **TQ (10 mg/kg)** | 0.129±0.005 # # | 0.062±0.01 # # | 0.119± 0.024 # |

The values are expressed as means ± SEM, (n = 7); *: p≤0.05, **: p≤0.01, ***: p≤0.001 compared to control group, #: p≤0.05, # # : p≤0.01 compared to arthritic group.

## DISCUSSION

In this study the collagen-induced arthritis (CIA) model was chosen. This model is the best known and certainly the most appreciated because it involves immunization with a component of cartilage: collagen II (CII). The obtained results show that rheumatoid arthritis is one of disease which characterized by the contribution of innate immune cells, this could be explained by the elevation of white blood cells and granulocytes numbers. The severity of rheumatoid arthritis is manifested by the very high obtained arthritic score. TQ is a significant *in vitro* and *in vivo* anti-arthritic agent, this due to its anti-inflammatory effect which is manifested by decreasing in number of white blood cells and granulocytes, the anti-inflammatory effect of TQ was confirmed by several researchers; Umar and his collaborators which demonstrated that TQ inhibits the accumulation and activation of polymorphonuclear cells and maintains homeostasis in cytokine imbalance by lower levels of TNF-α, IL-1β, IFN-γ and IL-6 and the level of IL-10 [22], Vaillancourt and his collaborators [23] also reported that TQ (05 mg / kg / day) significantly reduced serum IL-1 and TNF-α levels, El-Gazzar and his collaborators [24] have shown that TQ has the ability to maintain pro-and anti-inflammatory cytokine balance in the case of inflammatory diseases, by increasing the production of anti-inflammatory cytokines and by decreasing the production of pro-inflammatory cytokines. The results are consistent with other studies that have demonstrated the efficacy of TQ as a potent anti-arthritic agent [7, 21, 22, 23,25]. These effects can be explained by the anti-inflammatory and immunomodulatory effect of TQ, demonstrated in rheumatoid arthritis models induced in animals.

Xanthine oxidase (XO) activity assay results show that the activity of XO in plasma, in the case of arthritis is higher than in the homogenate of the liver and the spleen, Although the natural distribution of Xanthine oxidoreductase (XOR) is predominant in the liver[1]and the highest levels of transcription are in the liver and intestine [26]. This can be explained by the transformation of XOR into XO. Once released into the plasma, the XOR is rapidly converted to the XO oxidase form potentially generating species reactive oxygen [27]. The presence of other sources of the XO is obvious, because XOR mRNA is detected in most tissues. Benboubetra and his collaborators [2] located the enzyme in the synovial membrane. During inflammation, XOR is activated by various inflammatory mediators, such as TNF-α, which cause the conversion of XDH into XO [28]. Neutrophils activated also cause irreversible conversion of XDH form to XO form in endothelial cells [29]. The decreasing of xanthine oxidase activity after treatment of arthritic rats with TQ could be explained by its anti-inflammatory effect, as previously described by several researchers [7, 22, 23, 25] which demonstrate the ability of TQ to decrease the pro-inflammatory cytokines levels, IL1, IL6 and TNFα. The last one is responsible to the conversion of XDH form to XO form.

## CONCLUSION

Thymoquinone is a significant *in vitro* anti-arthritic agent demonstrated by its remarkable inhibition of BSA denaturation. Collagen-induced rheumatoid arthritis in male rats accompanied by increased xanthine oxidase activity in both, plasma and tissues homogenates (liver and spleen), the severity of rheumatoid arthritis was expressed by the decreased arthritic score. Treatment with thymoquinone allows us to suggest that TQ has the ability to improve significantly the xanthine oxidase activity in plasma and tissues, accompanied by a delay in the onset of the disease and a decrease in its severity are also recorded by decreasing in arthritic score. These results explain the significant *in vitro* and *in vivo* anti-arthritic effect through xanthine oxidase inhibition property of thymoquinone.

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