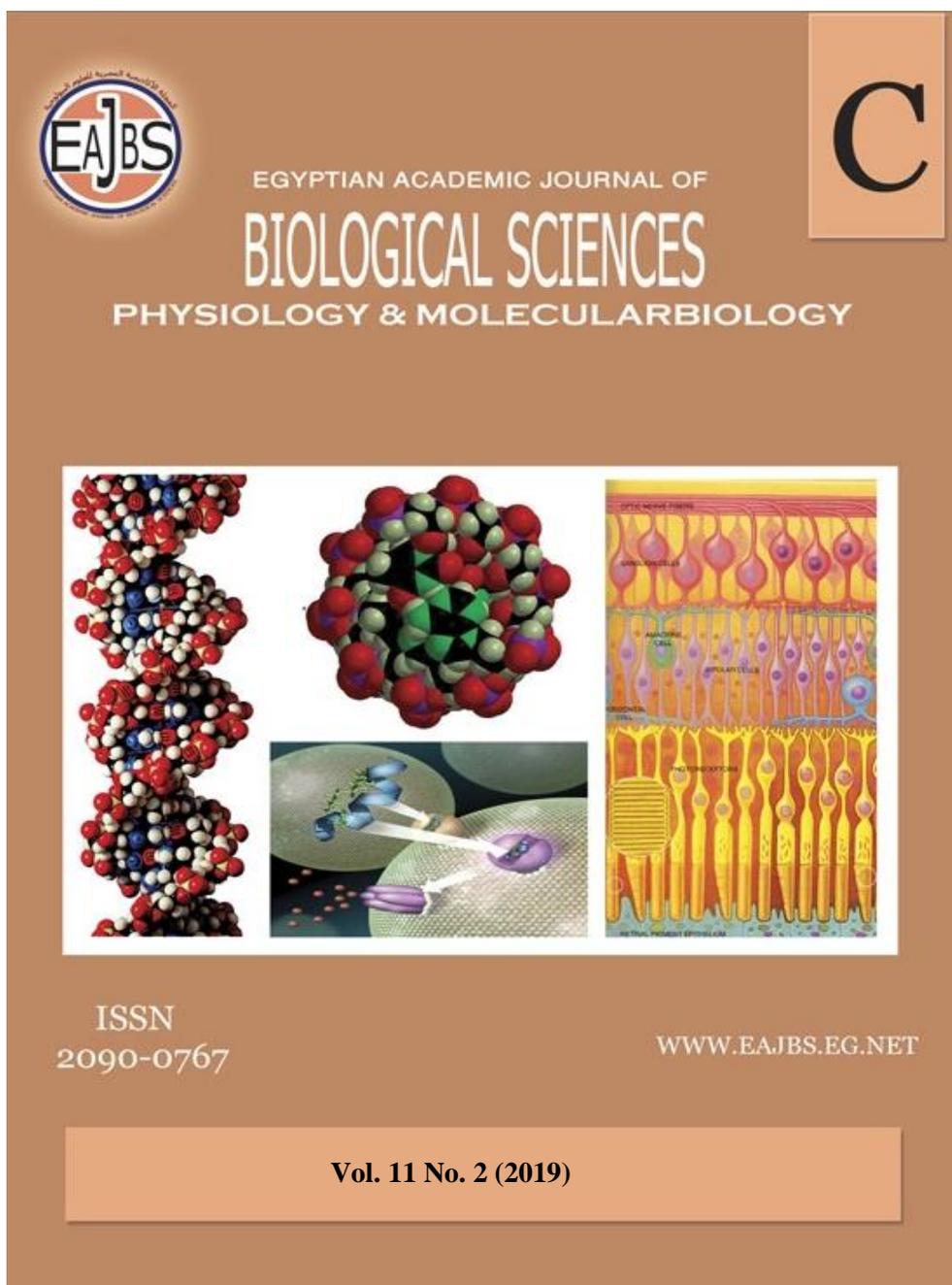


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## Potential Anti-Inflammatory Effects of the Egyptian Scorpion (*Androctonus amoreuxi*) Venom in Rheumatoid Rat Model

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### ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disorder, which takes place in the synovial tissues. This study aims to assess the possibility of using the Egyptian scorpion *Androctonus amoreuxi* venom (SV) on the treatment and management of RA. The venom was collected from scorpions using electrical stimulation then processed to get crude which was subcutaneously injected to arthritis induced rats. RA was induced in the rats by a single injection of 0.1 ml of Complete Freund's Adjuvant (CFA) at the footpad of the right hind paw of 32 adult male albino rats. Animals were divided into five groups, normal control, RA none treated, SV treated with low and high doses, and methotaxate treated. Results exhibited that SV (0.24 and 0.48 mg/kg) treatment relived pain, improved movement and reduced paw edema and joint swelling volume in arthritic rats. SV had diminished tissue damage in which malondialdehyde (MDA), lactate dehydrogenase (LDH), and creatine kinase (CK) were significantly decreased compared to arthritic rats without side effects in liver, blood, and kidney parameters. Histological examination showed marked improvement in the joint structure after SV treatment. Immunohistochemical staining showed a significant decrease in the expression of nuclear factor-kappa B Cell 65 (NFκB-p65), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in the arthritic joint after treatment with SV. In conclusion, SV possesses therapeutic properties in RA treatment and management due to its analgesic, antioxidant, and anti-inflammatory effects without noticeable side effects.

### INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease associated with chronic inflammation, the primary expression of which occurs in the synovial membranes and can lead to gradual joint and bone destruction (Erickson *et al.*, 2017). So, RA is related to functional disability, increasing depression and death rate, all of which showing an increase in social burden. The disease is characterized by pain, swelling and redness of the affected joints, and stiffness of the surrounding muscles that causes damage to the cartilages and bones with substantial loss of functioning and mobility (Bellucci *et al.*, 2016). RA is a complicated chronic disease affecting approximately

0.25-1% of the general population worldwide (Wedekind *et al.*, 2017). Most immunological and inflammatory components have a vital role in the disease management as T and B lymphocytes, neutrophils, monocytes and endothelium of vessels (Turner *et al.*, 2014). Rodent models of RA act as important tools to estimate the underlying mechanisms at early, intermediate and late stages of RA (Williams, 1998).

Scorpion is a predatory arthropod animal belonging to order Scorpionida in Arachnida's class. *Androctonus amoreuxi* (*A. amoreuxi*) is a scorpion which is widely spread in Egypt (Balozet *et al.*, 1975). Scorpion venoms are recognized as a source of inhibitor peptides, some of these peptides are able to depolarize human T cells, inhibiting inflammatory and proliferating responses, and thus might play an important role in the treatment of autoimmune diseases (Balozet *et al.*, 2013). Therefore, there is an urgent need to develop a new potent anti-inflammatory agent for RA treatment with no or less adverse effects. The current study aimed to assess the potential role of *A. amoreuxi* scorpion venom treatment in the control and management of induced RA in rat model.

## MATERIALS AND METHODS

### Collection of *A. amoreuxi* and Venom Preparation:

Two hundred *A. amoreuxi* scorpions were collected from the Western Mediterranean Coastal Desert of Alexandria, Egypt in August 2016. Each scorpion was kept separately in a container containing sand and fed on cockroaches once a week. The venom was collected by using electrical stimulation of the scorpion telson and then milked (Al-Asmari *et al.*, 2016). The venom was lyophilized (Lab Conco Freeze Dry System, model 77500) and the obtained crude was stored at -10°C until used.

### Determination of Subcutaneous Lethal Dose 50 (LD<sub>50</sub>) and Treatment Doses of *A. amoreuxi* Venom:

The LD<sub>50</sub> of *A. amoreuxi* venom for subcutaneous injection to the rats was calculated according to the method described by Meier and Theakston (Barsante *et al.*, 2005). The obtained LD<sub>50</sub> was 2.4 mg/kg, 1/5 and 1/10 LD<sub>50</sub> were chosen as subcutaneous treatment doses into induced arthritic rats.

### Animals and Experimental Design:

Forty adult male albino rats (180-200 g) were purchased from the Animal Unit, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt and were housed in plastic cages at Animal House, Zoology Department, Faculty of Science, Port Said University, and kept at room temperature (12 hrs light/dark cycle). All procedures on care and maintenance of the experimental animals were in accordance with the International Guiding Principles for Animal Research. The rats were divided into 5 groups (8 rats for each group) according to the following design:

Group I: Negative control or normal group, was injected subcutaneously with an isotonic saline solution (0.9% NaCl) until the end of the study period (30 days).

RA was induced in the other four groups (II, III, IV, and V) by injection of a single dose 0.1 ml of Complete Freund's Adjuvant CFA (Sigma-Aldrich, St. Louis, MO, USA) in the footpad of the right hind paw of rats (Barsante *et al.*, 2005). Treatment was subcutaneously initiated on the 12<sup>th</sup> day after CFA injection unless the clinical signs of arthritis were obviously detected, three times weekly till the 30<sup>th</sup> day (Makhlouf *et al.*, 2013).

Group II: Arthritic group (positive control), left untreated, just was injected subcutaneously three

times weekly with (0.9% NaCl). Group III: SV low dose treated group, was subcutaneously treated three times weekly at a dose of 1/10 LD<sub>50</sub> (0.24 mg /kg). Group IV: SV high dose treated group was treated subcutaneously three times weekly at a dose of 1/5 LD<sub>50</sub> (0.48 mg/kg). Group V: Methotrexate (MTX) treated group was treated subcutaneously with MTX (Orion Pharma, Espoo, Finland) three times weekly at a dose of 0.3 mg/kg (Bauerova *et al.*, 2010).

#### **Body Measurements and Behavioral Tests:**

##### ***Assessing Swelling of the Right Hind Paw:***

Swelling assessment of the right hind paws was done by measuring their mean thickness once a week by the electric caliper, paws were photographed on the first and last day of the experiment.

##### ***Scoring of Arthritic Dorsal Flexion Pain Test:***

The ankle joint was gently flexed dorsally until toes touched the front of the leg for 5 times with an inter-test interval of 5 seconds. The pain was scored (0) when the animal showed neither squeaking nor quick leg-withdrawal, scored (1) when either reaction appeared and scored (2) if both reactions appeared. A total score between 0-10 was obtained for each test session (Wang *et al.*, 2000).

##### ***Motility Test:***

The motility patterns of rats were observed for a period of 5 minutes and scored (0) if the rat walked with difficulty and avoided touching the toes of the inflamed paw to the floor, scored (1) if walked with little difficulty when toes touching floor and scored (2) if walked easily (Amdekar *et al.*, 2012). Observations were scored once a week each 6<sup>th</sup> day; the day prior treatment (Shen *et al.*, 2017), observation time was fixed at 8.00 - 12.00 A.M. at room temperature (25 ± 5 C°).

#### **Biochemical Assays and Hematological Parameters:**

Blood samples were collected from retro-orbital sinus via heparinized capillary tubes under light isoflurane anesthesia into two tubes. The first tube was contained EDTA as an anticoagulant for hematological assays, the second was plain gel tube and were centrifuged at 5000 rounds per minute for 10 min, serum was pipetted off then stored at -20°C until used for estimation of biochemical parameters.

##### ***Estimation of Biochemical Parameters:***

Serum alanine transferase (ALT) and aspartate transferase (AST) activities, albumin, creatinine, urea and uric acid levels were measured in plasma using the colorimetric method as described by the manufacturer (EGY- CHEM for Lab Technology, Egypt). Serum alkaline phosphatase (ALP), creatine kinase (CK) and lactate dehydrogenase (LDH) activities and total protein content were estimated according to the commercial kit purchased from (AMS, U.K, Ltd., Galgorm, Co.Antrim). Serum catalase activity and MDA tissue content were estimated by colorimetric methods described with the manufacturer (Bio Diagnostic Co., Egypt).

##### ***Estimation of Hematological Parameters:***

Hematological parameters were determined by using cell counting equipment (HumaCount 5L, HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Germany).

#### **Histological Studies:**

##### **Tissues Collection and Specimen's Preparation:**

All animals were sacrificed by a cervical decapitation on the 30<sup>th</sup> day of the experiment after light isoflurane anesthesia. Hind paws of the right limbs were removed from 5 rats of each group and routinely processed to produce paraffin blocks. Ankle joints were isolated and fixed in 10% buffered formalin, decalcified with 5% nitric acid, dehydrated, cleared and embedded in paraffin wax. Obtained

blocks were sectioned by a microtome at 5  $\mu\text{m}$  thickness, stained with hematoxylin and eosin (H&E) and examined under the light microscope.

**Immunohistochemical Staining of Nuclear Factor-KappaB-p65 (NF-kBp65), Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2):**

Paraffin sections of right joints were dewaxed with xylene, hydrated with gradient ethanol. Sections were blocked with an appropriate reagent and incubated with antibodies against NF-kBp65, iNOS and COX-2 according to the methodology steps of Chou *et al.*, (2011) and Wang *et al.*, (2013).

All obtained histopathological and immunohistochemical sections were examined by digital microscope camera (Tucsen ISH1000) using Olympus® CX21 microscope, with a resolution of 10 MP (megapixels) "IS Capture" software for capture and image enhancements. All slides were captured at 400 x magnifications, UIS optical system (Universal Infinity System, Olympus®, Japan).

**Statistical Analysis:**

The result values were expressed as means  $\pm$  standard error (SE) for eight rats (n=8) of each group. Tabulation and graphics were designed using Microsoft Excel software. Data were statistically analyzed using the Statistical Package for Social Science (SPSS) version 18 software. One-Way

Analysis of variance (ANOVA) test was performed for determining the statistically significant differences between groups followed by t-test. Data were considered statistically significant when the P values were  $\leq 0.05$ .

**RESULTS**

**Body Measurements and Behavioral Tests:**

***Swelling Test of the Right Hind Paw, Dorsal Flexion Pain Score and Motility Score:***

The swelling of the right hind rat paw was significantly increased ( $P < 0.05$ ) in all RA induced groups before treatment compared to the normal control group. In the SV low and high doses and MTX treated groups, swelling was significantly decreased ( $P < 0.001$ ) compared to non-treated arthritic group. The dorsal flexion pain was significantly increased ( $P < 0.001$ ) in the rheumatoid induced rats compared to the normal control group and significantly decreased ( $P < 0.001$ ) after treatment with SV and MTX when compared with the non-treated arthritic group. Motility score was significantly decreased ( $P < 0.001$ ) after rheumatoid induction compared to the normal rats and significantly increased ( $P < 0.001$ ) after treatment with SV and MTX when compared with the non-treated arthritic group. Data were summarized in Table (1).

**Table (1)** Effect of SV and MTX treatments on the rat paw swelling volume, dorsal flexion pain and motility score in different treated groups on 6<sup>th</sup>, 12<sup>th</sup>, 18<sup>th</sup>, 24<sup>th</sup> and 30<sup>th</sup> days of experiment.

Days Groups	6 <sup>th</sup> day	12 <sup>th</sup> day	18 <sup>th</sup> day	24 <sup>th</sup> day	30 <sup>th</sup> day
<b>Normal control</b>					
Swelling volume	3.75± 0.19	3.81± 0.15	3.78± 0.17	3.9 ± 0.16	3.82± 0.19
Flexion pain	9.61± 0.38	9.58± 0.41	9.71± 0.43	9.3 ± 0.39	9.54± 0.41
Motility	1.33± 0.19	1.33± 0.21	1.32± 0.18	1.31± 0.19	1.36± 0.17
<b>Arthritic control</b>					
Swelling volume	7.5 ± 0.39	8.6 ± 0.59	8.7 ± 0.59	8.8 ± 0.62	9.14± 0.54*
Flexion pain	9.7 ± 0.40	9.41 ± 0.42	8.50 ± 0.42	8.33 ± 0.27	8.16 ± 0.11*
Motility	1.33 ± 0.21	0.83 ± 0.16	0.5 ± 0.22	0.5± 0.22	0.33± 0.21*
<b>SV low dose</b>					
Swelling volume	7.49 ± 0.3	8.0 ± 0.28	7.1 ± 0.26	6.5 ± 0.62	6.13± 0.49**
Flexion pain	9.58±0.30	9.16 ± 0.28	7.3± 0.26	6.6 ± 0.62	4.0 ± 0.49**
Motility	1.33±0.21	0.66 ± 0.21	0.8± 0.22	1.2 ± 0.16	1.66 ± 0.21**
<b>SV high dose</b>					
Swelling volume	8.12 ± 0.26	8.47 ± 0.29	7.38 ± 0.51	6.6 ± 0.39	6.06± 0.41**
Flexion pain	9.58 ± 0.27	8.5 ± 0.42	6.25± 0.30	5.1± 0.32	2.91 ± 0.5**
Motility	1.33 ± 0.21	0.5 ± 0.22	0.83 ± 0.16	1.5±0.22	1.66 ± 0.2**
<b>Methotrexate</b>					
Swelling volume	7.17 ± 0.24	8.05 ± 0.37	6.5 ± 0.39	6.3 ± 0.43	5.9 ± 0.43**
Flexion pain	9.58± 0.44	9.16±0.51	7.66 ± 0.51	5.2 ± 0.21	2.5 ± 0.32**
Motility	1.33± 0.21	0.66 ± 0.21	1.16 ± 0.16	1.6 ± 0.21	1.83± 0.16**

Values are represented as means ± SE (n=8).

\* refers to the significant difference compared with the non-treated arthritic group when (P<0.05).

\*\* refers to the significant difference compared with the normal control group when (P<0.05).

**Biochemical assays and Hematological Parameters :**

***Effect of SV on Serum Alkaline Phosphatase (ALP) Activity:***

Regarding ALP activity, table (2), it was significantly increased (P<0.001) in the non-treated arthritic group as compared with the normal control rats, significantly decreased (P<0.001) after treatment with SV two doses and MTX compared with the non-treated arthritic group.

**Tissue Damage and Oxidative Stress Parameters:**

***Effect of SV Treatment on Serum LDH Activity and Synovial Tissue MDA Level.***

Results are given in table (2) revealed a significant increase

(P<0.001) in LDH activity and MDA level in the non-treated arthritic group compared with the normal control group, a significant decrease (P<0.001) in SV low and high doses and MTX treated groups compared with the non-treated arthritic group.

***Effect of SV Treatment on Serum Catalase Activity:***

Data presented in the table (2) indicated a significant decrease (P<0.001) in catalase activity in the non-treated arthritic group in comparison to the control group, a significant increase (P<0.001) in SV low and high doses and MTX treated group compared to the arthritic non-treated control group.

**Table (2)** Effect of SV and MTX treatments on serum ALP, LDH, CAT, CK activities and the synovial tissue MDA content in the control and different treated groups after 30 days of the experiment.

Groups Parameter	Control	Arthritic	SV low dose	SV high dose	MTX
ALP (U/L)	54.1±4.5	203±6.6*	97±3.2**	76.3±8.7*	82.5±5.6*
LDH (U/L)	1998±35	4924±265*	3100±177**	2950±218	2311±153*
MDA	18±1.2	65±3.7*	24±1.1**	22±0.5**	28±1.9**
Catalase (U/L)	878±54	399±49*	761±56**	659±29**	635±51**
CK (U/L)	448 ±82	1837±130*	1051±160**	849±105*	1192±160*

Values are represented as means ± SE (n=8).

\* refers to the significant difference compared with the non-treated arthritic group when (P<0.05).

\*\* refers to the significant difference compared with the normal control group when (P<0.05).

### Liver Function Parameters:

#### *Effect of SV Treatment on Serum Total Protein and Albumin Contents and Liver Enzymes ALT and AST Activities.*

There were no significant differences (P>0.05) between the non treated arthritic group and the normal control group in AST and ALT activities, significant changes (P>0.05) in SV low dose treated group compared with the arthritic group. Conversely, there was a significant increase (P<0.01) in serum AST and ALT in the high dose SV and MTX treated groups compared with the control group. No significant

differences (P>0.05) in serum total protein and albumin contents between control, non-treated arthritic groups, and all treated groups were reported, table (3).

### Kidney Function Parameters:

#### *Effect of SV Treatment on Serum Creatinine, Urea and Uric Acid Concentrations:*

As shown in the table (3), there were no significant changes (P>0.05) in creatinine, urea and uric acid levels between the control group, non-treated arthritic group, and all treatment groups.

**Table (3)** Effect of SV and MTX treatments on serum AST, ALT, total protein, albumin, creatinine, urea, and uric acid concentrations in different treated groups after 30 days of the experiment.

Groups Parameters	Control	Arthritic	SV low dose	SV high dose	MTX
AST (U/L)	22±1.5	32.2±2.6	24.7±2.7	46.8±3.0*	63.2±3.0*
ALT (U/L)	20.5±2.05	31.3±2.5	29.8±1.7	44.5±3.2*	60.9±3.3*
Total protein (g/dl)	9.3±0.8	8.8±0.4	8.7±0.2	7.5±0.15	9.4±0.7
Albumin (g/dl)	3.9±0.15	3.5±0.14	3.3±0.52	3.08±0.12	2.7±0.25
Creatinine(mg/dl)	0.45±0.03	0.43±0.02	0.41±0.02	0.53±0.02	0.51±0.03
Urea (mg/dl)	43±2.5	45±4.1	43±2.5	54±3.2	79±2.5
Uric acid (mg/dl)	2.7±0.34	3.1±0.47	2.5±0.27	2.1±0.26	2.8±0.38

Values are represented as means ± SE (n=8).

\* refers to the significant difference compared with the non-treated arthritic group when (P<0.05).

\*\* refers to the significant difference compared with the normal control group when (P<0.05).

### Effect of SV Treatment on Hematological Parameters:

No significant differences (P>0.05) was reported in the red blood cells (RBCs) count and hemoglobin (Hb) level between non treated arthritic,

normal control, SV low and high doses treated groups. Conversely, there was a significant decrease (P< 0.001) in RBCs count and Hb level between MTX treated group and non-treated arthritic group, table (4).

Scorpion venom has a potent analgesic anti-inflammatory and antioxidant properties.

White blood cells (WBCs) count was significantly increased ( $P < 0.05$ ) in the non-treated arthritic group compared with the normal control group, no significant difference ( $P > 0.05$ ) in WBCs count in SV low and high doses and MTX treated groups compared with the non-treated arthritic group. Nevertheless, monocytes and granulocyte percentages revealed significant increase ( $P < 0.001$ ) in the non-treated arthritic group compared with the normal control group,

conversely, significantly decreased ( $P < 0.01$ ) in SV low and high doses and MTX treated groups compared with the non-treated arthritic group. Lymphocyte percentage showed a significant decrease ( $P < 0.001$ ) in the arthritic group compared with the normal control group, while, significantly increased ( $P < 0.01$ ) after treatment with SV low and high doses and MTX compared with arthritic group, table (4).

**Table (4)** Effect of SV and MTX treatments on hematological parameters in the different treated groups after 30 days of the experiment.

Parameters \ Groups	Control	Arthritic	SV low dose	SV high dose	MTX
WBCs $\times (10^3/\text{ml})$	6.68 $\pm$ 0.90	11.8 $\pm$ 1.91*	11.0 $\pm$ 0.5	10.6 $\pm$ 1.72	9.5 $\pm$ 0.35
Lymphocytes %	91.7 $\pm$ 0.96	75.3 $\pm$ 1.63*	85.6 $\pm$ 2.24**	88 $\pm$ 1.29**	91.9 $\pm$ 1.02**
Monocytes %	6.58 $\pm$ 0.40	17.3 $\pm$ 1.23*	9.93 $\pm$ 0.80**	8.63 $\pm$ 0.78**	6.5 $\pm$ 0.69**
Granulocytes %	2.01 $\pm$ 0.27	7.3 $\pm$ 0.53*	4.36 $\pm$ 0.72**	3.66 $\pm$ 0.66**	2.0 $\pm$ 0.33**
Platelets $\times (10^3/\text{ml})$	388 $\pm$ 56	572 $\pm$ 54	866 $\pm$ 122	671 $\pm$ 76	900 $\pm$ 213
RBCs $\times (10^6/\text{ml})$	7.71 $\pm$ 0.36	7.75 $\pm$ 0.35	6.93 $\pm$ 0.25	7.11 $\pm$ 0.41	4.18 $\pm$ 0.67**
Hb (g/dl)	13.0 $\pm$ 0.58	12.1 $\pm$ 0.33	12.9 $\pm$ 0.32	13.0 $\pm$ 0.50	7.7 $\pm$ 1.09**

Values are represented as means  $\pm$  SE (n=8).

\* refers to the significant difference compared with the non-treated arthritic group when ( $P < 0.05$ ).

\*\* refers to the significant difference compared with the normal control group when ( $P < 0.05$ ).

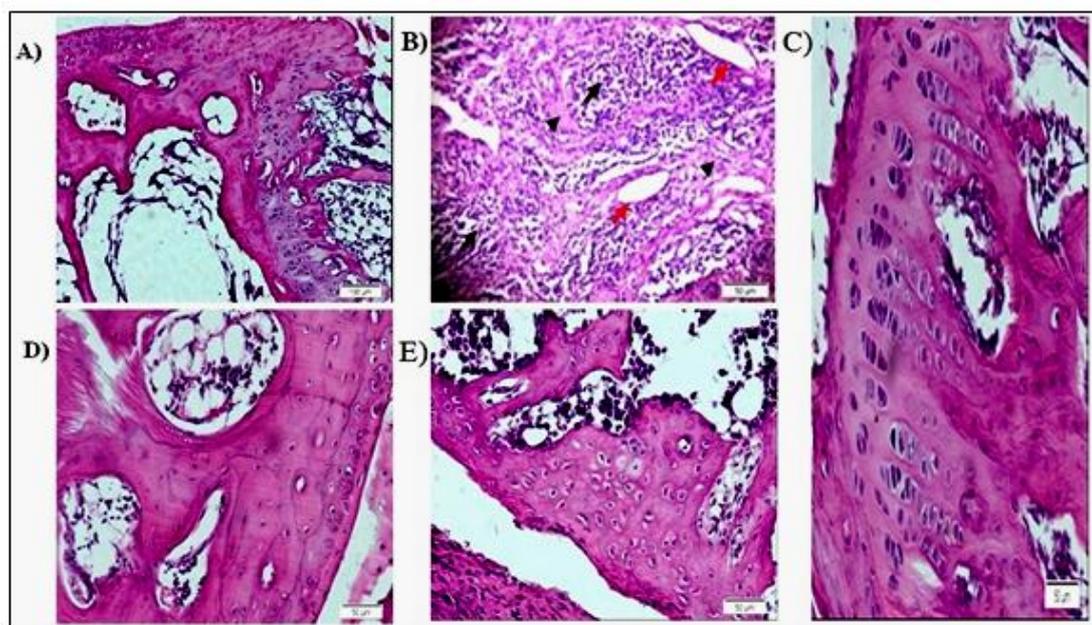
### Histopathological Examination:

Sections microscopic examination revealed that joints of the normal control group as shown in figure 1 (A) have a uniform mature bone surface covered by hyaline cartilage with smooth surfaces. The underlying connective tissue had adipocytes, capillaries, and some collagen fibers. Arthritic group (B) showing a presence of pannus, composed of diffused inflammatory infiltrate, macrophages, and lymphocytes with scattered blood vessels in a fibrous stroma. SV low dose treated group (C) showing a loss on the surface of the articular cartilage with irregularity and erosions. In SV high dose treated group (D) the joint space was restored with little inflammatory exudate and the articular cartilage had a uniform smooth outer surface with few scattered degenerated

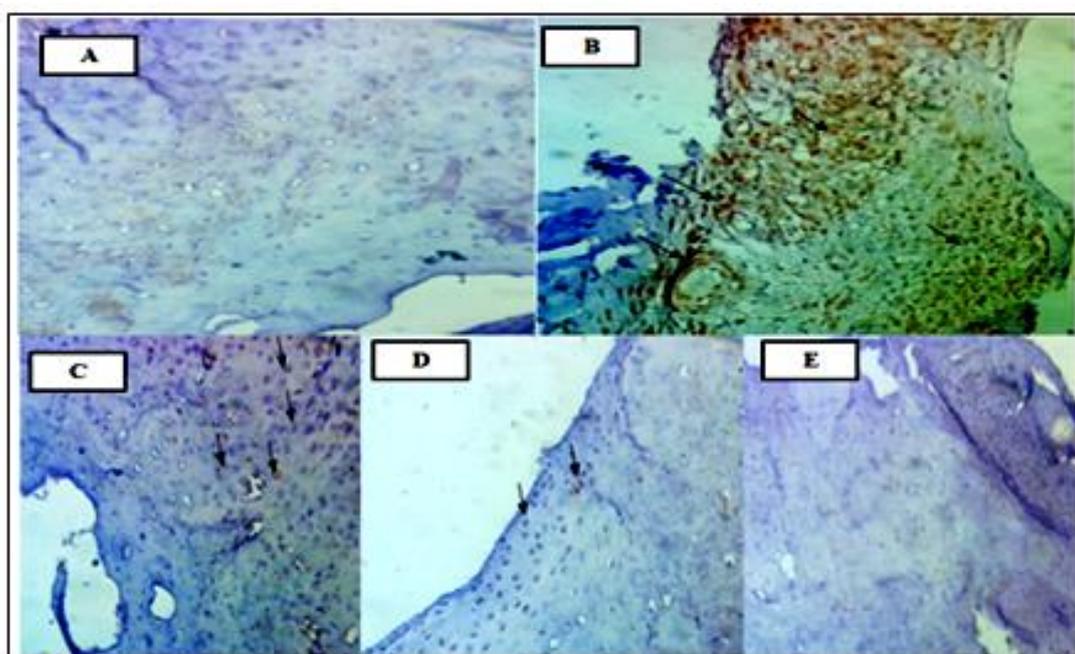
chondrocytes. MTX treated group (E) showing synovial fibroblastic proliferation with mild inflammatory cell infiltrate and restored joint space.

### Immunohistochemical Examination:

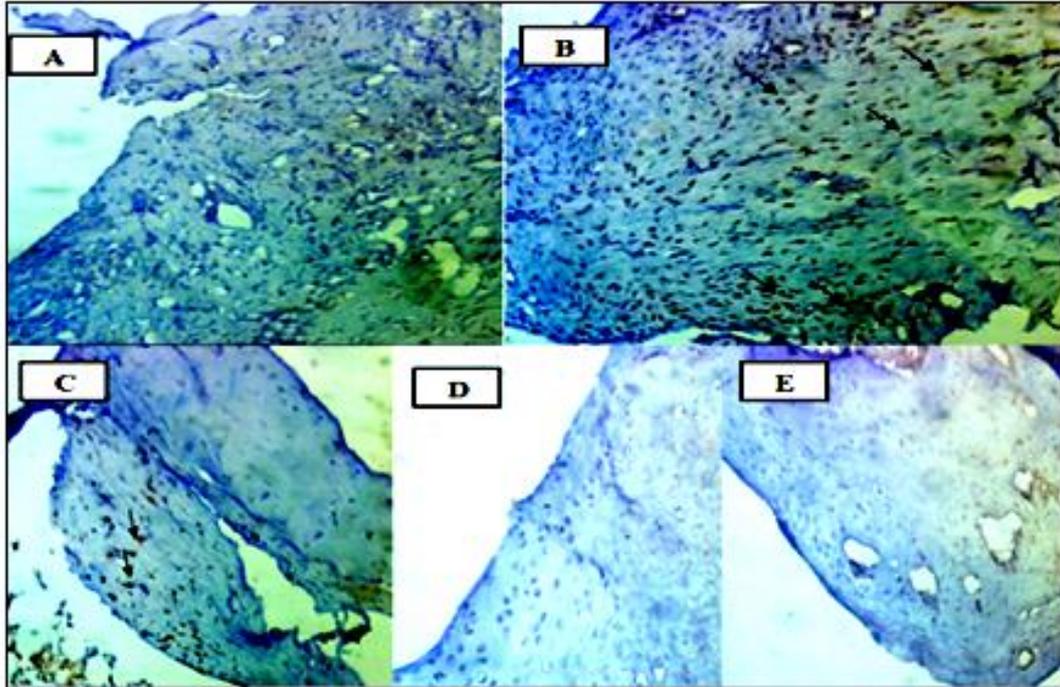
Immunohistochemical staining for iNOS, NF $\kappa$ B-p65 and Cox2 were illustrated in figures (2,3 and 4 respectively). The normal control group showed a negative immunostaining expression for the three inflammatory mediators while non treated arthritic group showed a significant expression when compared with the normal control group. The SV low and high doses groups showed a weak positive reaction for iNOS while MTX group showed a negative reaction. On the other hand, the SV low dose group showed a weak positive reaction for Cox2 while the SV high dose and MTX groups showed a negative reaction for Cox2.



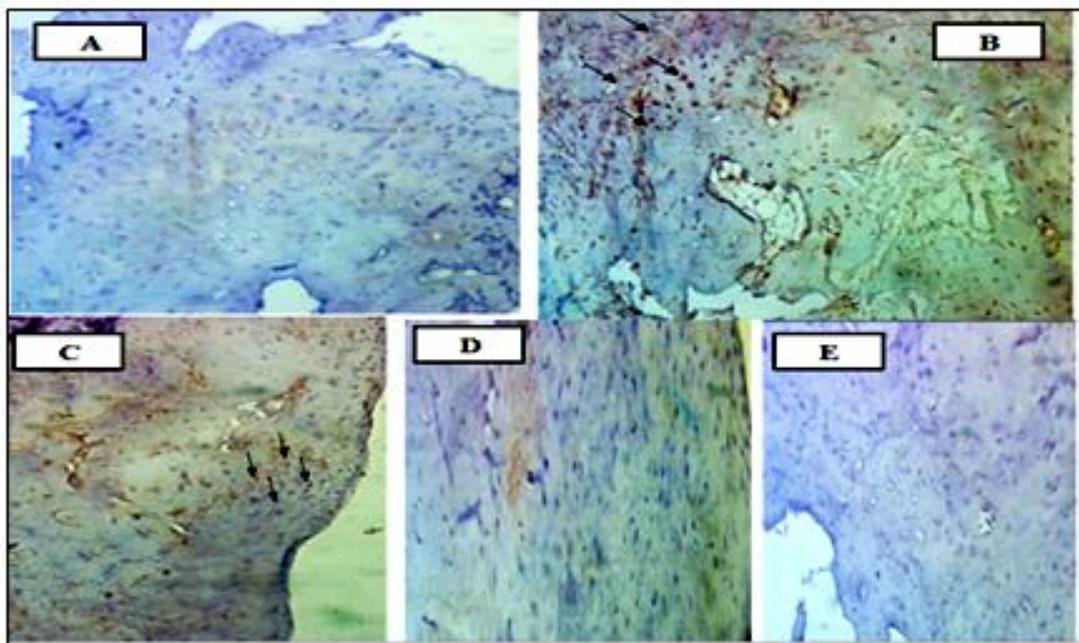
**Fig (1).** Histopathological effects of scorpion venom and MTX treatments on the articular joints in CFA induced rats. **A)** Normal control. **B)** Arthritic non treated group, black arrows indicates pannus, which is composed of diffuse chronic inflammatory infiltrate, scattered blood vessels (red arrows) and fibrous stroma (arrow heads). **C)** SV low dose treated group. **D)** SV high dose treated group. **E)** MTX treated group. Hematoxylin and eosin (H&E) stain, 5 $\mu$ m thickness at magnification X400



**Fig. (2).** Immunohistochemical staining for iNOS in the joint sections of normal control, arthritic, and different treated groups. (A) Normal control group, (B) non treated arthritic group, (C) SV low dose treated group, (D) SV high dose treated group and (E) MTX treated group.



**Fig. (3).** Immunohistochemical staining for NFκB-p65 in the joint sections of normal control, arthritic, and different treated groups.. (A) Normal control group, (B) Non treated arthritic group, (C) SV low dose treated group, (D) SV high dose treated group and (E) MTX treated group.



**Fig. (4).** Immunohistochemical staining for Cox2 in the joint sections of normal control, arthritic, and different treated groups.. (A) Normal control group, (B) Non treated arthritic group, (C) SV low dose treated group, (D) SV high dose treated group and (E) MTX treated group.

## DISCUSSION

Treatment of RA may be efficient if starts early, at the same time early and accurate diagnosis may protect the patients from aggressive therapies with potential toxicity (Arnett *et al.*, 1988). The induction of RA with CFA enhances T-lymphocytes to trigger a strong immune response in the rat paws (Billiau and Matthys, 2001). Hence, CFA rheumatoid induction in rats has been commonly used to evaluate possible therapeutic methods that can be used in RA treatment (Asquith *et al.*, 2009). Many studies have been demonstrated that scorpion venom is a rich source of polypeptides and enzymes in addition to a variety of other biologically active components (Garcia *et al.*, 1994). Different species of scorpions contain many peptide toxins that block potassium channels in T lymphocytes that could treat a variety of diseases associated with the autoimmune system in animal models (Garcia *et al.*, 1994).

Inflammation in RA could be documented by measuring swelling in diseased rat's paws that reflects impairment in synovial membrane, joints, and bones (Cai *et al.*, 2007). The present study showed that treatment with SV in RA induced rats at low and high doses showed a marked reduction in right hind paw swelling volume when compared with the non treated RA induced group. These findings may be due to that SV may suppress the migration and accumulation of leucocytes to inflamed joints of arthritic rats (Nipate *et al.*, 2014). The dorsal flexion pain score and motility test depend on the movement of joints which more greatly affected by arthritis. Treatment with SV and MTX have significantly diminished the pain sensation and improved motility. Scorpion  $\alpha$ -toxins are belonging to the sodium channel inhibitor family, thereby blocking neuronal transmission (Nipate *et al.*, 2014). A study by Shao

*et al.* (2014) demonstrated that scorpion neurotoxins have a strong analgesic and anti-tumor activities against both visceral and somatic pain.

Synovial tissue is a possible source of ALP, especially bone-type ALP, so it considered as a characteristic feature of RA persistent synovitis (Nanke *et al.*, 2002). In the current study, the RA non-treated group showed an enormous elevation in ALP activity compared with the normal control group. Increased activity of ALP may be due to the inflammatory effect of cytokines (Thompson *et al.*, 1990). On the other hand, ALP activity showed a significant decrease after treatment with SV and MTX, which explains their therapeutic role in the depletion of inflammatory cytokines.

The synovial fluid of the inflamed joints in RA is swarmed with inflammatory cells such as activated neutrophils, which produce large amounts of highly reactive radicals leading to an increase of oxidative stress, lipid peroxidation and tissue damage (Gutteridge *et al.*, 1981). In the current study, a massive elevation in MDA content in joint tissues of RA induced rats accompanied by depletion in plasma CAT activity was reported. Treatment with SV high and low doses in addition to MTX showed a significant decrease in MDA content in synovial tissues with a significant increase in CAT activity compared with the non-treated arthritic rats. Elevated CAT activity in venom-injected experimental animals was reported with Da Silva *et al.* (2011). In the fact, CAT is an effective enzyme that plays an important role in the enzymatic antioxidant defense system via the decomposition of hydrogen peroxide ( $H_2O_2$ ) to water and oxygen to protect cells against  $O_2$  toxicity and lipid peroxidation (Kalpakcioglu and Şenel, 2008).

CK is an enzyme identified in many tissues like brain, heart and

skeletal muscles. Elevated levels of CK in blood may indicate inflammation and muscle damage (Callegari *et al.*, 2017). In the present study, CK, LDH and AST levels were elevated in RA non-treated rats in a comparison with the normal control group. These enzymes may be liberated into the bloodstream following muscle damage (Howell *et al.*, 2018). Moreover, LDH is elevated in the disease progression and in acute or chronic tissue damage as joints of patients with rheumatoid arthritis (Dawes *et al.*, 1986).

AST is an enzyme found in the liver and heart at high concentration while ALT considered to be a specific liver enzyme. The current study revealed that SV high dose only slightly increases AST and ALT levels. On another hand, liver enzymes were significantly increased in the MTX treated group compared to the control group. This elevation considered to be a side effect during MTX treatment and this finding was in agreement with Kasper *et al.* (2015) who reported that the most serious side effect in patients receiving MTX therapy is liver toxicity.

A statistically significant increase in WBCs count in RA non-treated group was reported in the current study in a harmony with the study of Ahmadi *et al.* (2009). They demonstrated a moderate rising in WBC count in the arthritis conditions that could be due to the release of IL-1B inflammatory response which increases the production of granulocytes and macrophages colony-stimulating factor. MTX, an immunomodulatory drug, has many commonly reported hematological adverse effects such as leukopenia, pancytopenia, anemia, megaloblastic anemia (Chan and Cronstein, 2002). In the present study, RBCs count was in normal range values in all groups except for MTX treated group, there was a significant decrease in RBCs count and Hb level. MTX is retained

within cells as polyglutamates which inhibit folate metabolism then blocking the enzymes dihydrofolate reductase and thymidylate synthase, thereby inhibiting the synthesis of purines and pyrimidines and decreasing DNA and RNA synthesis, subsequently decreases RBCs production (Tunali-Akbay *et al.*, 2010). MTX also increases bone marrow adiposity in both short and long term of MTX therapy (Georgiou *et al.*, 2012).

Infiltration by immune cells as macrophages and PNLs was considered to be an early manifestation of adjuvant-induced arthritis (Kennedy *et al.*, 2010). In the present study, histological examination of the arthritic non-treated rat joint tissues showed a proliferation of the synovial tissues with variety of inflammatory cells, mainly polymorphonuclear leukocytes (PNLs), and infiltration with macrophages, lymphocytes, and irregular multinucleated cells, this finding was in agreement with Bauerova *et al.* (2010) and Makhoulouf *et al.* (2013). These cells can discharge a great amount of ROS into the tissue, leading to deterioration of cartilage and damage to joints (Xie *et al.*, 2013). Pannus is a mass of synovium that rich in numerous inflammatory cells and synovial fibroblasts that can grow over the articular cartilage, promote its destruction and causing ankylosis (Căpitănescu *et al.*, 2011). Angiogenesis is reported in RA, and this process facilitates the activated monocytes that attack the synovial tissues and spread into the pannus, leading to cartilage and bone deterioration (Nanke *et al.*, 2002). Treatment with SV plays an important role in the inhibition of angiogenesis by inhibiting vascular endothelial growth factor (VEGF) (Sadick *et al.*, 2005) which enhances many other events fundamental for angiogenesis (Cao *et al.*, 2013).

In the current work, the pannus formation was observed in the RA

non-treated group, and osteoclasts were detected in the interface between synovium and bone. Treatment with SV and MTX has markedly reduced the infiltration of inflammatory cells into synovial membranes, improved joint architecture and protected cartilage from destruction and bone erosion, these results were in a harmony with the study of Nipate *et al.* (2014).

NO is a singling molecule which plays a vital role in various physiological and pathological processes. iNOS is one of the NO synthase group that has a role in NO synthesizes from l-arginine (Zamora *et al.*, 2000). High levels of NO lead to the formation of reactive nitrogen oxide species NO•, which has a role in chronic inflammatory disorders (Ersoy *et al.*, 2002) NO levels in serum and synovial fluid have been reported to be increased in patients with RA (Mohan *et al.*, 2013). Likewise, NO might be also responsible for synovial hyperplasia through stimulating of hypoxia via reducing cell respiration by binding to cytochrome c oxidase (Natarajan *et al.*, 2003). Hypoxia, in turn, promotes synovial angiogenesis that subsequently guides proinflammatory cytokines into the synovium along with the newborn blood vessel (Ng *et al.*, 2010). Suppression of NO production by blocking iNOS expression may be a strategy for the treatment of chronic inflammation (Yap and Lim, 2015). In the present work, immunohistochemical observations in joint sections showed a strong positive reaction for iNOS in the RA non-treated group. This finding was in a line with Chou *et al.* (2011). Overexpression of iNOS is associated with an increase of apoptotic cells in the articular cartilage of RA patients (Narendhirakannan and Limmy, 2012).

NFκB-p65 is a key transcription factor that regulates the expression of plenty of genes that activated in RA and produce

proinflammatory mediators such as cytokines, chemokines and angiogenic factors leading to pannus formation and arthritic joint destruction (Bottini and Firestein, 2013). In the present study, SV and MTX treatments inhibited NFκB-p65 expression which was observed in RA induced rats. These results were in a line with Al Asmari and Khan (2016) who demonstrated that scorpion venom inhibited NFκB-p65 activation in rat models.

Prostaglandins play important roles in the inflammatory response, they are produced by the effect of COX enzymes and leukotrienes (Capdevila *et al.*, 1990). There are two COX isoforms, COX-1 and COX-2. Moreover, COX-1 is abundant in the gastric mucosa, kidney, platelets, and vascular endothelial cells while COX-2 expressed mainly during inflammation, especially in macrophages and monocytes (Dubois *et al.*, 1998). In the present study, immunohistochemical results showed a massive expression of COX-2 in joint tissues of RA the non-treated group. This result was also reported with Doss *et al.* (2016). On the other hand, it was significantly inhibited after treatment with SV high dose and MTX, while weakly expressed after treatment with SV low dose. Depletion in COX-2 activity inhibits synovial inflammation and joint degradation and inhibited pannus formation in arthritis induced animal models (Lai *et al.*, 2008). Several studies reveal that arthropods venom extracts play an effective role in the control of different chronic inflammatory diseases, rheumatoid arthritis is one of them, through immune suppression of many inflammatory cofactors, such as TNF, COX-2, cytokines and NO reactive species (Gomes *et al.*, 2011).

#### **Conclusion:**

This study concluded that the Egyptian scorpion (*Androctonus amoreuxi*) venom possesses an anti-inflammatory effect due to its ability in

inhibiting the expression of NF- $\kappa$ B p65, COX-2 and INOS in addition to its antioxidant properties. SV also was dimensioned the cardinal symptoms of inflammation in RA induced rats, this was supported by the biochemical and histopathological findings. In the future, further studies could provide more details of the underlying mechanisms to determine the most effective peptides or molecules in SV that could have these therapeutic activities with minimum side effects.

“All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.”

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## ARABIC SUMMARY

التأثيرات المضادة للالتهاب المحتملة لسلم العقرب المصري أندروكتونص أموريوكسي في نموذج للجرذان المصابة بالروماتويد

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التهاب المفاصل الروماتويدي (RA) هو أحد أمراض المناعة الذاتية، والذي يحدث في الأنسجة الزلالية. تهدف هذه الدراسة إلى تقييم إمكانية استخدام سم العقرب المصري *Androctonus amoreuxi* (SV) في علاج والتغلب على أعراض التهاب المفاصل الروماتويدي. تم جمع السم من العقارب وحلبها باستخدام التحفيز الكهربائي ثم تجفيفه ثم حقنه تحت الجلد في الجرذان المستحثة الاصابة بالتهاب المفاصل الروماتويدي عن طريق حقنها بجرعة واحدة 0.1 مل من معاملة فرويند الكامل Complete Freund's (Adjuvant CFA) في وسادة القدم الخلفي الايمن للجرذان. تم استخدام عدد 40 من ذكور الجرذان البالغين في هذه الدراسة. تم تقسيم الحيوانات إلى خمس مجموعات كل منها يتكون من ثمانية جرذان وتم تقسيمها كالتالي: الضابطة الطبيعية، المصابة والغير معالجة، المصابة والمعالجة بسم العقرب بجرعة منخفضة المصابة والمعالجة بالجرعة العالية، و المصابة والمعالجة بعقار الميثوتريكسات. أظهرت النتائج أن العلاج بسم العقرب بالجرعتين المنخفضة والعالية (0.24 و 0.48 ملجم / كجم) تحسنا في الاحساس بالألم وتحسن الحركة وخفض تورم المفاصل. كما أن SV قلل من تلف الأنسجة من خلال انخفاض تركيز المونديالديهيد (MDA) بشكل ملحوظ، و انزيمات (LDH)، والكرياتين كيناز (CK) مقارنة بالجرذان المصابة بالروماتويد الغير معالجة وقد تم ذلك بدون حدوث آثار جانبية في دلالات وظائف الكبد والكلى و أيضا مكونات الدم. كما أظهر الفحص النسيجي تحسنا ملحوظا في بنية المفاصل بعد العلاج بسم العقرب. كما أظهر الفحص النسيجي المناعي انخفاضا كبيرا في ظهور العامل النووي (NFκB-p65)، (COX-2) ومخلق أكسيد النيتريك (iNOS) في المفاصل بعد العلاج بسم العقرب. وعلى ذلك فان لسلم العقرب SV خصائص علاجية في علاج التهاب المفاصل الروماتويدي والحد من آثاره نتيجة لخصائصه المضادة للأكسدة وتأثيره المضاد للالتهاب وذلك دون حدوث آثار جانبية ملحوظة.