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The effect of crude alcoholic extract of *Withania somnifera* leaves in experimentally induced arthritis in mice

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Abstract

The alcoholic extract 70% of the leaves of *Withania Somnifera* was dried and percentage of extract 8.3 were used .Incomplete freunds adjuvant was used to induce arthritis in mice 0.1ml of this adjuvant was injected in the right tarsal joint. The mice were divided into four groups, the first group were treated with alcoholic extract 500mg/kg body weight (T1), the second group was treated with diclofenac sodium 3mg/kg body weight (T2) and third group was treated with distilled water (T3), the fourth group used as a control group (T4) non induced non treated.Vernier calipers was used for measuring the diameter of the joint. The mice in T1group showed a significant decrease in the diameter of tarsal joint of right leg as compared with mice in T2, T3 and T4 groups. The hematological results of mice in T1 group showed significant increase as compared with T4 group. While inT2 and T3 groups showed a significant decrease as compared with T4 group .The histological results showed that the joint of mice in T1 group showed no inflammatory response in the membrane of joint. The joint of mice in T2 group showed inflammatory cell infiltration while the joint of mice in T3 group showed fibrous connective tissue proliferation with mononuclear cell infiltration in the membrane of the joint .

تاثير الخلاصة الكحولية الخام لاوراق سم الفراخ في التهاب المفاصل المحدث تجربيا في الفئران

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الخلاصة

تم تجفيف الخلاصة الكحولية ٧٠% لأوراق نبات سم الفراخ إذ بلغت نسبة الخلاصة 8.3 . تم استحداث مرض التهاب المفاصل وذلك زرقincomplete freund's adjuvant بمفصل الكاحل (tarsal joint) الأيمن وأعيد الزرق بعد ٧ أيام حيث ظهر المرض بعد ١٤ يوم من أول زرق . قسمت الفئران (٢٨ فارة) إلى أربعة زمر الزمرة الأولى معالجة بالخلاصة الكحولية ٥٠٠ملغم /كغم من وزن الجسم والزمرة الثانية معالجة بالفولتارين ٣ ملغم /كغم من وزن الجسم والزمرة الثالثة معالجة بالماء بينما تركت الزمرة الرابعة كزمرة سيطرة . استخدم مقياس (vernier calipers) لقياس قطر المفصل، فقد أظهرت الدراسة إن الخلاصة الكحولية سببت تثبيط وضمور مرض التهاب المفاصل من خلال وجود فروق معتدة ($p<0.05$) بالمقارنة مع الزمر المعالجة بالماء والمعالجة بالفولتارين. أظهرت دراسة الصورة الدموية زيادة واضحة للهيموغلوبين وحجم خلايا الدم المرصوصة والكريات البيض وزيادة اللمفاويات وزيادة العدلات بالنسبة للزمرة المعالجة بالخلاصة الكحولية مقارنة بزمرة السيطرة أظهرت الزمرة المعالجة بالفولتارين والزمرة المعالجة بالماء المقطر انخفاض واضح لخضاب الدم وخلايا الدم المرصوصة والكريات البيض مقارنة بزمرة السيطرة وزيادة خلايا العدلات والحمضات وانخفاض اللمفاويات والوحيدة النواة والقعدات بالنسبة للزمرة المعالجة بالفولتارين مقارنة بزمرة السيطرة . لم يظهر المفصل للفئران المعالجة بالخلاصة الكحولية استجابة للالتهاب في غشاء المفصل اظهر المفصل للفئران المعالجة بالفولتارين ارتشاح الخلايا الالتهابية بينما لوحظ تكاثر النسيج الليفي الرابط مع ارتشاح خلايا وحيدة النواة في غشاء المفصل للفئران المعالجة بالماء المقطر.

Introduction

Withania somnifera was widely used in Ayurvedic medicine, the traditional medical system of India. It was prescribed for a variety of treatment musculoskeletal condition (e.g.; arthritis, rheumatism) which was reduced the severity of pain and disability scores of arthritis (3). Because of many studies have been found various constituents of *Withania somnifera* exhibit a variety of therapeutic effect with little or no associated toxicity so this herb was considered as potential therapeutic effect to treat arthritis without side effect (11). Arthritis was defined as a group of conditions that effect on the health of the bone joints in the body. It was considered one of the most prevalent chronic health problems worldwide. To treat arthritis we may use non-steroidal anti-inflammatory e.g., Diclofenac sodium and this groups cause side effect like gastrointestinal , bleeding and ulceration ,or treatment by using corticosteroids that cause diabetes, cataracts, osteoporosis gastric ulcer and high blood pressure(18). This study was performed to study the following aims : Treatment of induced arthritis in mice by using 70% crude alcoholic extract of *Withania somnifera* and Comparison between treatment of arthritis of the *Withania somnifera* extract and nonsteroidal anti-inflammatory drug e.g., Diclofenac sodium .

Materials and Methods

- **Extraction of leaves of *Withania Somnifera* :**

The powder of *Withania Somnifer* leaves was extracted with 70% ethyl alcohol. One hundred grams of the leaves powder were placed in 1 liter Arelmyer's flask with 700 ml (70% ethyl alcohol). A tefon magnet was placed in the solution. The flask was placed on hot plate magnetic stirrer. The temperature of extraction was 40-45c° . The solution was left stirring for 72 hours and then sieved by using sterial guaze to get rid of coarse particules. The solution then filtered through Whitmann fliter. The filtrate was poured in clean and sterile petridishes (12x2 cm) and kept in incubator at temperature of 45c° until dryness (20).

- **Experimentally Animals:** Twenty eight albino Swiss mice weighting 30g were obtained from the animal house of Biotechnologycenteral-Al-Nahrain university . mice were housed plastic cage 30x10x10 cm placing in the room until the beginning of experiments.Standard rodent diet (commercial feed pellets) and Tap *ad.lib*. water were freely available .

- **Induction of arthritis:** Incomplete fruend's adjuvant was used to induce arthritis. Twenty eight mice were used in this experiment 0.1ml of incomplete fruend's adjvant was injected in the right tarsus joint of each animal. The injection was repeated after 7 days. The arthritis was developed after 14 days (19).

- **Pilot study:** In order to determine the appropriate dose of treatment of arthritis a pilot study was conducted on mice with experimentally induced arthritis. Two doses were chosen, 300, 500mg/kg of the alcoholic extract were treated in two groups. Three mice were used in each group. First group was treated with 300mg/kg a daily dose of extract plant orally. Second group was treated with a daily dose of 500mg/kg extract plant orally. The treatment continued for 14 days. The dose of 500mg/kg showed the best results and was chosen for consequent experiments.

- **Experimental design:** Twenty eight mice were divided into four groups(7 mice in each one) :

1. 1-The first group(T1) was treated by daily dose of 500mg/kg body weight of *Withania somnifera*
2. given orally by stomach tube for 14 days .
3. The second group(T2) was treated with a daily 3mg per kg body weight of Diclofenac sodium given orally by stomach tube for 14 days .
4. The third group(T3) was treated by distilled water given orally by stomach tube for 14 days
5. The fourth group (T4) was (control group).

Parameters used in this experiment:

- **Measurement the radius of joint :** The radius of both right and left tarsus joint were measured by vernier calipers instrument in each animal in each groups every three days before and during 14 days of treatment .
- **Blood tests :**

- **Haemoglobin test:** the procedure was according to the method mentioned by Varley *et.al*, (23).
- **Packed Cell Volume (P.C.V.) :** This is according to the method mentioned by Archer, (1) .
- **White Blood Cells Counting:**the procedure was according to the method mentioned by colse, (7) .
- **Differential Counting of WBCs:** The method is mentioned by (7).

• **Histological study:** All groups were taken the parts from bone after killed it. These samples were taken for histological study and these were kept in 10% formalin solution until the time of sections (17). The sections were worked in the dental medicine college, university of Baghdad.

• **Statistical analysis:** The ready program SAS (21) was used in statistical analysis for study the effect of different treated in adjective studies and the significant between means was compared with less significant LSD.

Results

• **Plant extraction:** The plant leaves of *Withania somnifera* was extracted by preprating alcoholic extract 70% and percentage of extract 8.3 were used , the crude alcoholic extract was obtained the resulted was taken to full dryness to form dark green crystals.

• **The results of measurement of the diameter of tarsal joint:** The results showed a significant increase ($P<0.05$) diameter of tarsal joint of right leg as compared with tarsal joint of mice in T4 group (negative) which considered as control group as listed in table (1) . In the pilot study the results showed that dose of 500mg/kg body weight of animal caused better reduction of the tarsal joint than dose 300mg/kg body weight of animal as listed in table (2) . The results of the diameter tarsal joint showed in table (3) , after 15 days of treatment the diameter of right tarsal joint of mice (T1) showed no a significant difference from the mice in control group T4 . The diameter of tarsal joint of right leg of mice in T1 group showed a significant decrease ($P<0.05$) after 15 days of treatment from that of mice in T2 group (treated with diclofenac sodium). After 15 days both groups of mice in T1, T2 groups there was a significant decrease ($P<0.05$) (2.2 , 2.5) mm respectively as compared with that mice in T3 group (treated with distilled water) 3.2 mm . After 15 days the diameter of tarsal joint of mice in T3 group showed a significant increase ($P<0.05$) as compared with that mice in T4 group.

Table (1)The effect of Incomplete freunds adjuvant in the diameter (mm) of the right tarsal joint

Groups	The right leg induced	The control group (non induced, non treated)
1	3.4±0.04 a	2.2±0.04 b
2	3.2±0.04 a	2.2±0.04 b
3	3.3±0.06 a	2.2±0.04 b
4	3.2±0.04 a	2.2±0.04 b

*Data taken as mean ±SE *W. S.: Withania somnifera

*Small letters mean column comparison

Table (2) The effect of alcoholic extract of *Withania somnifera* on diameter (mm) of the right tarsal joint at (300,500) mg/kg doses.

Weeks	500mg/kg dose of extract of W. S	300mg/kg dose of extract of W.S
1	2.8±0.34 Aa	3.5±0.31 Bb
2	2.2±0.20 Bb	3.1±0.30 Aa

*Data taken as mean ±SE *W. S . : Withania somnifera

*Capital letters mean row comparison *Small letters mean column comparison

Table (3) The compared of diameter in millimeter of the tarsal joint between treatments for all groups daily

Days	T1	T2	T3	T4
3	3.0±0.14 aA	3.1±0.03 aA	3.3±0.03 aB	2.2±0.04 aC
6	2.8±0.13 bA	3.0±0.04 aB	3.3±0.03 aC	2.2±0.04 aD
9	2.5±0.04 cA	2.8±0.08 bB	3.2±0.02 aC	2.2±0.04 aD
12	2.3±0.05 dA	2.7±0.08 bB	3.2±0.08 aC	2.2±0.04 aA
15	2.2±0.04 dA	2.5±0.08 cB	3.2±0.08 aC	2.2±0.04 aA

*Data taken as mean ±SE *Capital letters mean row comparison .

*Small letters mean column comparison

Table (4) Effect of alcoholic extract of *Withania somnifera* , Diclofenac sodium and Distilled water on P.C.V ,Hb and total WBCs count after 15 days of the treatment.

Groups	PCV %	Hb g/deciliter	WBCs cell /cm³	N %	L %	M %	E %	B %
T1	38.2 ± 0.86 a	11.1 ± 0.33 a	7880 ± 13.50 a	55.0 ± 0.58 a	40.2 ± 0.37 a	4.6 ± 0.24 a	3.2 ± 0.37 a	0.6 ± 0.20 a
T2	25.6 ± 3.17 b	7.2 ± 1.09 b	3110 ± 8.18 b	55.2 ± 2.52 a	35.4 ± 1.56 b	3.6 ± 0.92 b	4.0 ± 0.37 b	0.4 ± 0.24 b
T3	27.4 ± 2.42 c	9.0 ± 0.85 c	8820 ± 7.83 c	56.6 ± 0.81 b	44.6 ± 0.50 c	4.8 ± 0.37 a	3.5 ± 0.20 a	0.6 ± 0.24 a
T4	35.8 ± 2.65 d	10.5 ± 0.35 d	6270 ± 8.31 d	51.0 ± 1.20 c	36.8 ± 1.56 d	4.2 ± 0.58 a	3.4 ± 0.4 a	0.6 ± 0.24 a

*Small letters mean column comparison *Data taken as mean ±SE.

• **Hematological parameters:** The hematological parameters are shown in table (4),the packed cell volume (P.C.V.) % and hemoglobin(Hb) g/deciliter of mice in T1 group showed significant increase ($p<0.05$) as compared with that mice T2 and T4 groups . The P.C.V. % and Hb g/deciliter of mice in T2group showed a significant decrease ($p<0.05$) as compared with that mice in T4 group . P.C.V. % and Hb g/deciliter of mice in T3 group showed a significant decrease ($p<0.05$) as compared with that mice in T4 group . The total white blood cells count (WBCs) and lymphocytes percentage of the mice in T1 group showed a significant increase ($p<0.05$) as compared with that mice in T2 and T4 groups . The WBCs count and lymphocytes percentage of mice in T2 group showed a significant decrease ($p<0.05$) as compared with that mice in T4 group . The WBCs count and lymphocytes percentage of mice in T3 group showed a significant increase ($p<0.05$) as compared with that mice in T4 group . Neutrophil percentage of mice in T1 and T2 groups showed no a significant deference whereas both groups (T1 and T2) showed a significant increase ($p<0.05$) as compared with that mice in T4 group . Monocyte, eosinophil and basophil percentages of mice in T1 group showed no important significant as compared with that mice in T4 group. Eosinophil percentage of mice in T2 group showed a significant increase ($p<0.05$) as compared with that mice in T4 group, basophil and monocyte percentages of mice in T2 showed a significant decrease ($p<0.05$) as compared with that mice in T4 group.

• **Histopathological changes :** The joint of mice in T1 group , showed no inflammatory response in the membrane of joint or the bone figure (1) . The joint of mice in T2 group showed inflammatory cells infiltration fibrous connective tissue in the membrane of joint figure (2). The joint of mice in T3 group showed fibrous connective tissue proliferation with mononuclear cells infiltration in the membrane of joint figure (3).

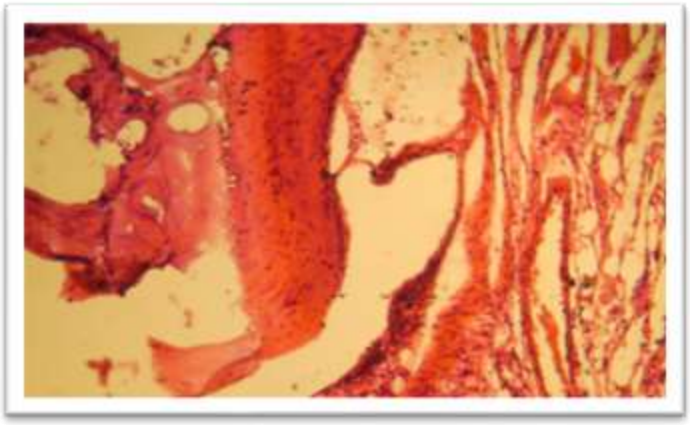

Figure: (1) Tissue section in the joint of one mouse treated with alcoholic extract of *Withania somnifera* showed no inflammatory cell reaction in the region of the joint. H and E (20 x 2)

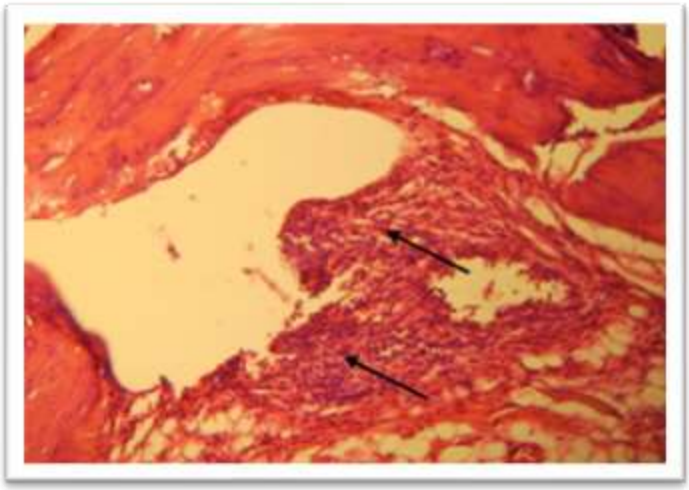

Figure: (2) Tissue section in the joint of one mouse treated with diclofenac sodium showed inflammatory cell infiltration and proliferation of fibrous connective tissue in the membrane of the joint . H and E(20 x 1.6)

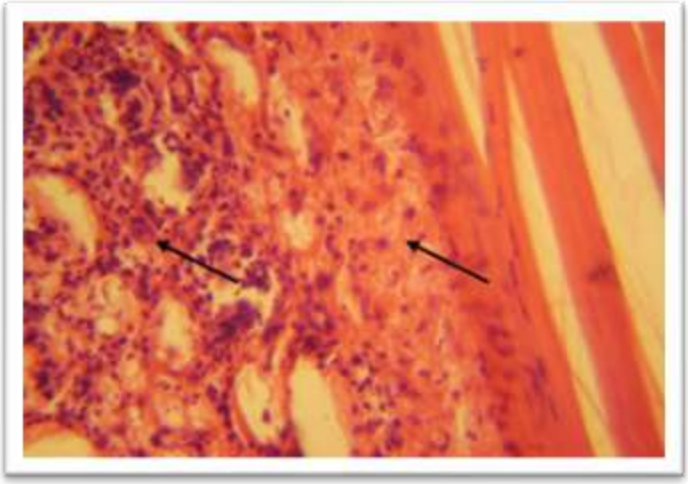

Figure: (3) histological section in the joint of one mouse treated with distilled water explains fibrous connective tissue proliferation around the bone with mononuclear cells infiltration. H and E (40 x 1)

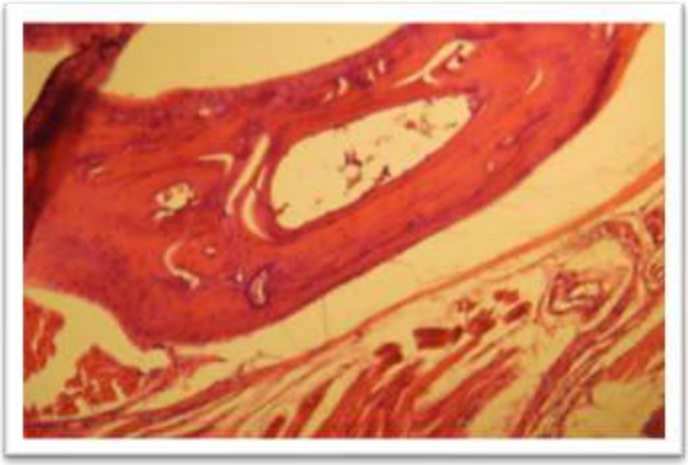

Figure: (4) histological section in the joint of one mouse (negative control group) showed normal histological section. H and E (40x1.2)

Discussion

The extract of *Withania somnifera* may have active constituents to inhibit inflammatory process with low toxicity as compared with steroidal and non-steroidal anti-inflammatory drugs (11), to take advantage of presence of many active ingredients which may have synergistic affect and the extract can be obtained easily with low cost . In addition the extract doses not need sophisticated apparatus and complicated procedure for the above mention reasons *Withania somnifera* was chosen to treat arthritis. In this study, injection of incomplete freund's adjuvant in synovial cavity of tarsal joint of right leg in mice to induce arthritis caused puffy swelling of the joint and some symptoms appeared on mice such as limbing and redness of the joint, this results agreed with results reported by (15) they mentioned that incomplete freund's adjuvant can cause arthritis and facilitate autoimmune response. After 15 days of arthritis induction the blood picture showed a significant decrease in Hb and P.C.V. with increase in total white blood cells count the decrease in PCV and Hb parameter regarded to reticulocytosis and immunostimulatant and this results agreed with results reported by (15,5) . After 15 days of daily oral treatment with *Withania somnifera* crude extract the diameter of tarsal joint of right leg showed no significant difference as compared with diameter of tarsal joint of mice in T4 group , the inhibitory effect may be regarded to an increase in WBCs , neutrophil and lymphocyte which considered very important to heal arthritis , this results agreed with that results were reported by (10,24) they enhanced that leaves of *Withania somnifera* had immunomodulatory effect and it was more capable for protecting the bone marrow from injury and chemotherapy preventing the severe decrease in WBCs count. In another study recorded by (2,6) they mentioned that the alcoholic extract of *Withania somnifera* had been high action to inhibition the growth of micro-organism and reduce the inflammation as a result of increase the neutrophil . In study by Davis and kuttan (9) were mentioned that extract of plant caused increase in cytotoxic lymphocyte production.The increase in P.C.V. % and Hb of mice in T1 group may be regarded to the effect of extract leaves plant which contain a high percentage of the ferrous element leading to increase production of these parameters this results agreed with results reported by Asthana and Raina (2).This results supported by histopathological study of the tarsal joint of mice in T1 group showed no pathological changes, this regarded to the effect of alcoholic crude extract of *Withania somnifera* in stimulation of stem cell proliferation in the bone as a results of active substance in *Withania somnifera* attributed in reducing the inflammation such as withaferin A (9) also this results supported by (22) . After studying blood picture of mice in T2 group showed a significant decrease in Hb and PCV% as compared with control group which could be due to inhibition of stem cell synthesis in the bone marrow or because acute immune hemolytic anemia that resulted from dicolfenac metabolites specially the 4-hydroxylated glucuronide ester of dicolfenac that

bound the Hb resulting in hemolysis of these cells, this results agreed with (4). The WBCs count of mice in T2 group showed a significant decrease as compared with WBCs count of mice in T4 group , this results agreed with results reported by (4,14) . they refered to the effect of drug on bone marrow that inhibited the stem cell synthesis and decrease WBCs count.Neutrophil and esinophil cells recorded significant increase in all treated groups as compared with that of control group, this is may be due to the presence of dicolfenac metabolites in blood mainly 3-hydroxy and 4-hydroxy diclofenac that induce rising in neutrophil percentage in blood as a macrophage cells, while response of the body to inflammatory process leading to stimulate esinophil in the beginning of treatment arising their level in blood (8).Lymphocyte, monocyte and basophil recorded for T2 group showed minor significant decrease in treated groups proportional with the doses because of rapid migration from circulatory system to the areas of tissue injury especially liver and kidney in which they adhere to the endothelium and immigrated to the perivascular areas (8). After 15 days of induction arthritis the histopathological change of joint induced with arthritis showed fibrous connective tissue proliferation with mononuclear cell infiltration in the membrane of joint due to disability and significant joint damage may result from early progressive inflammatory arthritis and later secondary degenerative changes in joint this results agreed with results reported by (16). Whereas after studying histopathological change of joint mice in T2 group we showed inflammatory cell infiltration and fibrous connective tissue in the membrane of joint this may regarded to the side effect of voltaren in delayed healing of bone , this results agreed with results reported by (14) when they mentioned that voltaren cause delayed in the healing because the mechanism of action in inhibition of Cox2 leading to inhibit prostaglandins which essential for bone healing by increase osteoblast activity and new bone formation .The conclusion is the alcoholic extract 70% of leaves of *withania somnifera* plant lead to treat the arthritis without side effect.

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