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Anti-Inflammatory Activity of *Withania Somnifera* Root Extract in Complete Freund′S Adjuvant-Induced Arthritis in Male Albino Rats

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ABSTRACT
Background: A very high rate of morbidity and mortality is linked to the condition known as rheumatoid arthritis (RA).
Objective: The current study has aimed to examine the impact of daily delivery of *Withania somnifera* (*W. somnifera*) or Ashwagandha extract (ASHE) and standard drug (Diclofenac potassium) on oxidative stress and inflammatory biomarker in adjuvant-induced arthritis (AIA) in male albino rats.
Materials and methods: Animals were divided into ten groups (n=5). Group 1 acted as control, group 2 presented the RA rats and groups 3, 4, 5 were treated with different doses of ASHE (50, 100 and 200 mg/kg, respectively) daily for 3 weeks, Group 6 received standard drug (Diclofenac potassium) at a daily dose of 10 mg/kg for 3 weeks. Groups 7, 8 and 9 represent CFA-induced RA rats, which received tested extract at 50, 100 and 200 mg/kg respectively: daily for 3 weeks. The tenth group represented RA group received standard drug (Diclofenac potassium).
Results: The changes induced in AIA rats were evaluated by measurement of oxidant and antioxidant biomarkers as well as interleukins-1β (IL-1β) and tumor necrosis factor alpha (TNF-α), in addition to cyclooxygenase-1 (COX1) and cyclooxygenase-2 (COX2) measurement. *W. somnifera* dose-dependently alleviated the severity of the disease based on the reduction of lipid peroxidation and ceruloplasmin and improved the GSH, SOD, CAT and TAC as compared to AIA-rats. ASHE reduced the serum level of IL1β, TNF, as well as COX1 and COX2 as compared to AIA-rats.
Conclusion: The results of the experiments showed that *W. somnifera* might be useful for management of RA.
Keywords: *Withania somnifera*, Rheumatoid arthritis, Cytokines, Oxidative stress.

INTRODUCTION

Rheumatoid arthritis, or RA, is a classic example of an autoimmune disease that is caused by a combination of causes. This illness primarily affects the smaller joints and manifests as an erosive and destructive polyarthritis [1]. The prevalence of RA is believed to be between 0.5 and 1% of the global population [2].

Rheumatoid arthritis (RA), which causes joint swelling, degeneration of cartilage affecting juxta-articular bone has been linked to reduced bone mineral density (BMD) and bone mass. This disease causes progressive joint degeneration as a result of its persistent nature, resulting in considerable locomotor handicap and a decline in quality of life [3].

Numerous investigations, have found that inflammatory cytokines such IL-1, IL-6, and TNF-α are crucial in the pathophysiology of RA [4, 5]. Massive synovial growth and inflammatory cell infiltration occur as a consequence, and when angiogenesis and inflammatory cell infiltration are coupled, cytokines are produced. At least in animal models of arthritis, cytokines are implicated in every stage of the pathogenesis of rheumatoid arthritis, including the induction of autoimmunity, the maintenance of chronic inflammatory synovitis, and the degeneration of joint tissue that is nearby the afflicted joint. Thus, cytokines combine the immune-regulatory and tissue-destructive processes that underlie the clinical presentation and progression of rheumatoid arthritis [6].

Non-steroidal anti-inflammatory medications (NSAIDs), such as ibuprofen, and straightforward analgesics were once used to treat rheumatoid arthritis (RA). Both NSAIDs and disease-modifying anti-rheumatic medications (DMARDs) are linked to significant toxicities, including hepatotoxicity, idiopathic lung responses, and bone marrow suppression[7].

Plants with medicinal properties are utilized all over the world as a kind of alternative or supplemental treatment. Discovery on these medicinal plants, including pharmacological and toxicological analyses, is necessary for the development and research of new pharmaceuticals [8]. Ashwagandha, sometimes known as Indian ginseng, is the common name for the plant *Withania somnifera* (*W. somnifera*), which belongs to the family Solanaceae and is an annual evergreen shrub that grows in India, the Middle East, and some areas of Africa [9]. There are a few additional species in the genus Withania that have a similar overall appearance[10].

Even though it is offered as a nutritional supplement in many different countries and is regarded to be valuable as a medicinal plant in Ayurveda, there is inadequate scientific data to suggest that it is either safe or effective for treating any ailment. It is currently not advised for the treatment of any condition because there is insufficient evidence that it is effective, and it may have adverse effects.

The goal of the current study was to assess the antioxidant and anti-inflammatory properties of an aqueous extract of Ashwagandha roots in rat in-vivo arthritic models.

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MATERIALS AND METHODS

1. Animals:

Male albino rats were purchased from the Lab Animal House of the Faculty of Veterinary Medicine, Suez Canal University in Ismailia. 2 weeks prior to the experiment, the animals were allowed to acclimatize. Rats weighing 120–130 g and between 2 and 3 months of age were housed in polystyrene cages with sawdust-covered floors to reduce the likelihood of painful contact with a hard surface. Rats were exposed to a 12-hour light-dark cycle and had unrestricted access to food and drink. The temperature in the room was 25 °C.

2. Experimental groups:

This work was carried out on 50 healthy male albino rats. Animals were randomly divided into ten groups, 5 rats in each group. Group 1: Normal negative control group, intraperitoneally received isotonic saline (0.9% NaCl). Group 2: CFA-induced arthritic rats (received single dose of CFA 20 µl for each rat subcutaneous at the planter surface of hind paw). Group 3: Ashwagandha extract (ASHE)-treated group at low dose (ASHE 50 mg/kg). Group 4: ASHE-treated group at moderate dose (ASHE 100 mg/kg). Group 5: ASHE-treated group at high dose (ASHE 200 mg/kg) by daily intragastric administration for 3 weeks. Group 6 received standard drug treatment (Diclofenac Potassium) at a daily dose 10 mg/kg for 3 weeks. Groups 7, 8 and 9 represent CFA-induced RA rats and intragastrically received tested extract at 50, 100 and 200 mg/kg respectively. The tenth group represented RA group receive standard drug.

3. Determination of reduced Glutathione content (GSH):

Using the method described by **Srivastava and Beutler** [11] the amount of reduced glutathione (GSH) was measured by spectrophotometry at 412 nm. The GSH levels were determined using a standard curve. The results were given in terms of nanomoles of GSH per ml.

4. Determination of malondialdehyde (MDA) level:

Malondialdehyde (MDA) and thiobarbituric acid (TBA) interact at 95°C for 30 minutes in an acidic solution to produce thiobarbituric acid reactive product. The absorbance of the resultant pink product may be determined at 534 nm [12].

5. Determination of superoxide dismutase and catalase activities:

The superoxide dismutase (SOD) activity was measured at 505 nm and 37 °C, as specified by the instructions provided by the producer of the commercial kit (Randox Lab., UK). The activity of the SOD was measured in terms of units per milligram of protein [13]. The catalase activity was determined by utilizing a commercial kit (Randox Lab., UK) and measuring it at 340 nm and 37 °C in accordance with the instructions provided by the manufacturer [14].

6. Determination of total antioxidant capacity:

Using an ELISA kit, TAC in rat serum was measured (Cat. No. MBS1600693, My BioSource, San Diego, USA). Kit plate is pre-coated with Rat TAS antibody, per manufacturer. Sample TAC binds to well-coated antibodies. Then biotinylated Rat TAC Antibody binds sample TAC. Streptavidin-HRP binds to biotinylated TAS antibody. Washing unbound Streptavidin-HRP after incubation. The amount of Rat TAS determines the color of the substrate solution. Add acidic stop solution and measure absorbance at 450 nm.

7. Inducible Nitric Oxide Synthase (INOS):

MyBioSource, Inc.'s commercial kit was used to detect serum inducible nitric oxide synthase (Cat. No. MBS723326, USA). The INOS ELISA kit uses an INOS-HRP conjugate and a monoclonal anti-INOS antibody to perform a competitive enzyme immunoassay.

8. Serum Ceruloplasmin:

The oxidase activity of ceruloplasmin was determined using the method that determine the absorbance of the 540-nm wavelength of o-dianisidine hydrochloride. The serum or fractions eluted from the column were incubated for 5 and 15 minutes at 30 °C in 1 ml of acetate buffer containing 7.88 mM o-dianisidine hydrochloride. By subtracting the 5 minute absorbance from the 15 minute absorbance, the oxidase activity may be calculated [15].

9. Determination of IL-1 in rat serum (µl/ml):

Using a solid phase sandwich ELISA test kit purchased from Immuno-Biological Laboratories Co. Ltd., Japan, the interleukin 1 (IL-1) concentration in serum of blood was measured (Catalog no. 27193).

10. Determination of TNF-α in rat serum (pg/ml):

Using a solid phase sandwich ELISA test kit purchased from Immuno-Biological Laboratories Co. Ltd., Japan, the concentration of tumor necrosis factor-alpha (TNF- α) in blood serum was measured (Catalog no. 27194).

11. Cyclooxygenase 1 and 2 (COX1 and COX2):

Following the manufacturer's instructions, the levels of Cyclooxygenase 1 and 2 in the serum were determined using ELISA kits. The solid phase sandwich ELISA test kit was purchased from MyBioSource Company (Southern California, San Diego) (USA).

Ethical approval:

All the experimental procedures were carried out according to the principles and guidelines of the Ethics Committee of the of Faculty of science, Suez University, Egypt conformed to "Guide for the care and use of Laboratory Animals" for the use and welfare of experimental animals, published by the US

National Institutes of Health (NIH publication No. 85–23, 1996).

Statistical analysis

To compare the means of the various groups, one-way ANOVA and Duncan's test were employed, and statistical analysis was carried out using SPSS for Windows version 20.0. All data were presented as mean values with standard deviation, and significance was determined using a p-value ≤ 0.05.

RESULTS

1. Acute Toxicity Study

At the maximal dosage of 5000 mg/kg provided, the *Withania somnifera* extract did not result in any fatalities. As a result, the study's doses of 50, 100, and 200 mg/kg were chosen.

2. Effect of *Withania somnifera* extract on oxidative stress markers of CFA-induced Arthritic Rats

The results of the study on how ASHE affected the parameters of oxidative stress in adjuvant-induced arthritic albino rats showed that the rats' exposure to Complete Freund's adjuvant caused a significant ($p <0.05$) elevation in the serum concentrations of MDA, CP, and NO, as well as a significant ($p <0.05$) decrease in the levels of GSH and TAC, as well as the activities of SOD and CAT compared to the normal control. Treatment with 50, 100, and 200 mg/kg of ASHE, however, considerably reduced ($P <0.05$) the content of MDA, CP, and NO while significantly increasing ($P< 0.05$) GSH and TAC as well as the activity of SOD and CAT. When compared to the effects of the untreated arthritic rats, this impact was somewhat comparable to that of the reference medicine, diclofenac potassium (Table 1).

Table (1): Effect of daily administration of Ashwagandha- extract for 3weeks on oxidative stress parameters in male rats with AIA – adjuvant induced arthritis.

Groups	GSH (nmol/mg protein)	MDA (nmol/L)	SOD (U/mg protein)	CAT (U/mg protein)	NO	TAC (mmole /mgprotein)	CP (µg/ml)
Control	164.53±4.61	11.87±0.92	147.37±1.66	132.47±1.71	43.27±3.24	51.73±1.53	113.43 ±6.13
RA	65.77±1.47[a]	34.37±1.49 [a]	44.20±2.13 [a]	23.57±2.25 [a]	153.50±1.15 [a]	15.10±2.22 [a]	180.70±3.89 [a]
ASHE 50mg/kg	160.40±1.51	12.53±0.72	147.53±1.63	131.87±3.68	52.13±1.30	52.20±1.56	110.10±2.57
ASHE 100mg/kg	159.13±2.19	13.77±0.29	133.13±5.12	105.00±2.40	52.37±4.34	51.50±3.04	114.43±3.19
ASHE 200mg/kg	157.87±5.31	14.60±0.44	134.27±2.46	100.73±1.55	52.43±1.51	52.37±5.21	109.13±8.58
Standard drug (SD)	159.43±2.81	12.00±0.87	124.03±4.38	125.33±3.28	47.53±1.22	36.80±1.90	120.40±4.73
ASHE 50mg/kg	123.43±2.37[b]	24.63±0.66	98.63±0.81 [b]	79.87±1.91 [b]	111.83±2.81	26.00±1.97	139.23±4.04 [b]
ASHE100 mg/kg	130.77±1.51 [b]	20.47±0.61 [b]	106.70±2.77 [b]	77.77±1.60 [b]	103.63±2.57 [b]	29.80±1.73 [b]	115.80±10.62 [b]
ASHE 200mg/kg	134.40±1.25 [b]	15.57±0.48 [b]	120.97±1.01 [b]	90.70±0.78 [b]	79.37±1.28 [b]	30.40±0.84 [b]	110.38±6.84 [b]
RA+ SD	97.37±1.34[b,a]	44.50±2.23	82.53±2.53 [b]	35.00±2.88 [b]	123.57±1.05 [b]	20.30±2.36 [b]	167.32±3.15

Values are expressed as mean ± S.E. for five rats in each group .$^{a}P< 0.05$ denotes value significantly different from control using one way ANOVA followed by Duncan. $^{b}P<0.05$ experimental groups compared with RA group using one way ANOVA followed by Duncan. Other values proved no significance.

3. Effect of *Withania somnifera* extract on inflammatory markers of CFA-induced Arthritic Rats

Figures (1, 2, 3, and 4) showed the effects of ASHE on interleukin-1 beta (IL-1ß), TNF-α, cyclooxygenase-1 and cyclooxygenase-2 activity in rats given an adjuvant-induced arthritic condition. Rats given the adjuvant showed a substantial increase (p 0.05) in IL-1ß and TNF-a levels as well as COX-1 and COX-2 activity when compared to the healthy control group. After 21 days of oral treatment of ASHE, a drug identical to the reference drug diclofenac potassium, the levels of IL-1 and TNF-α as well as the activity of COX-1 and COX-2, significantly decreased ($P < 0.05$) as compared to untreated arthritic albino rats.

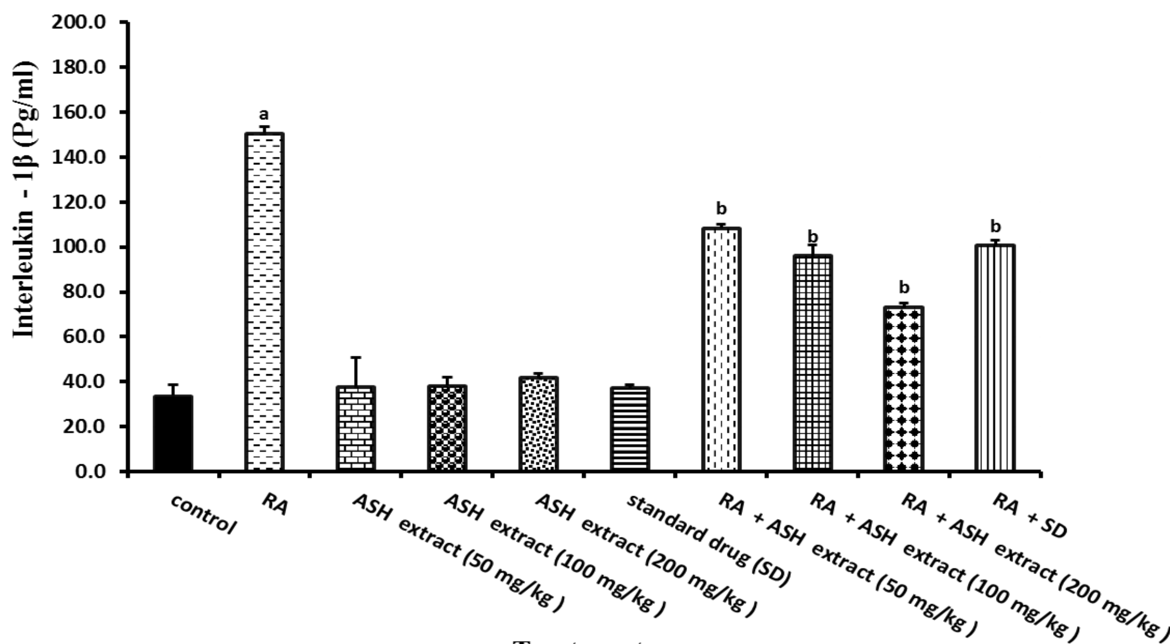

Fig. (1): Effect of Ashwagandha -extract, daily administration for 3 weeks, on Interleukin - 1β (Pg/ml) level in AIA of male rats. Data are presented as the mean ± SE. **a** and **b** indicate significant change from control and RA model group respectively using One Way ANOVA test followed by Duncan multiple comparison tests ($P < 0.05$).

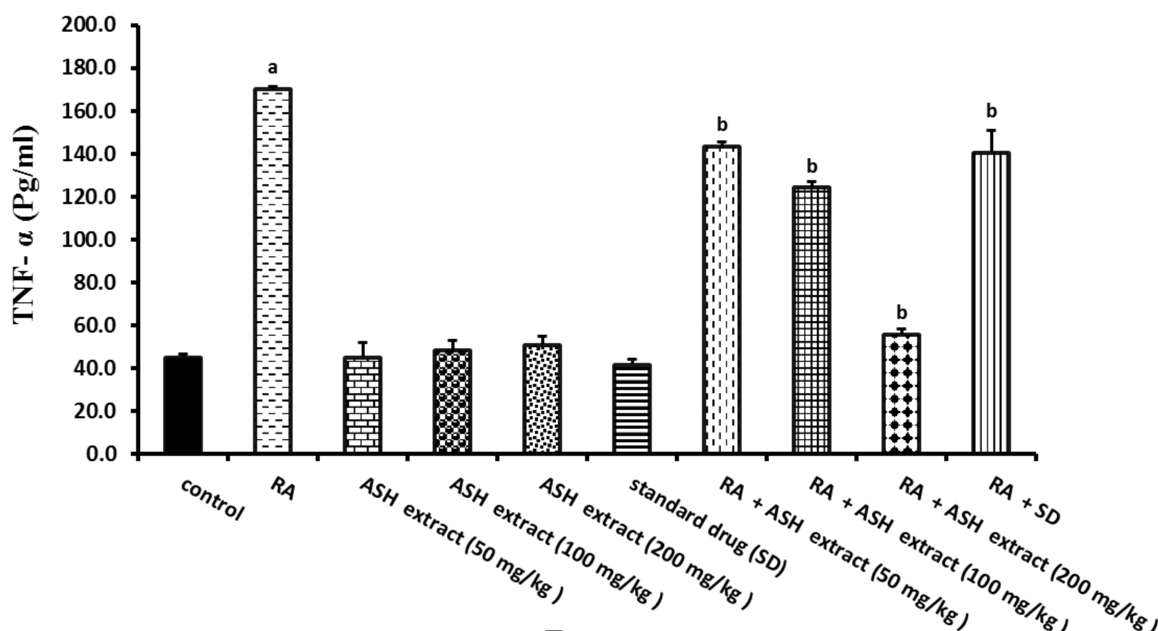

Fig. (2): Effect of Ashwagandha -extract, daily administration for 3 weeks, on TNF- α (Pg/ml) level in AIA of male rats. Data are presented as the mean ± SE. a and b indicate significant change from control and RA model group respectively using One Way ANOVA test followed by Duncan multiple comparison tests ($P < 0.05$).

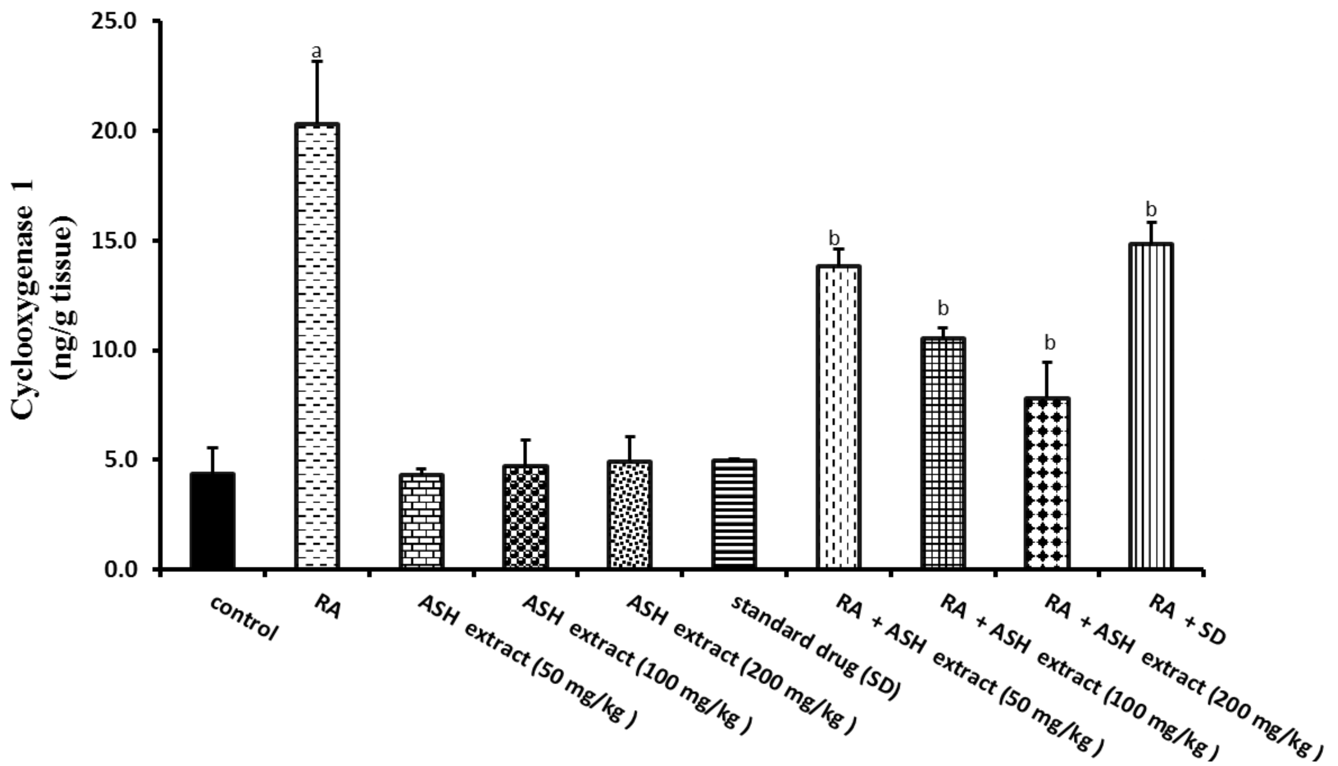

Fig. (3): Effect of Ashwagandha -extract, daily administration for 3 weeks, on Cyclooxygenase -1 activity in AIA of male rats. Data are presented as the mean ± SE. a and b indicate significant change from control and RA model group respectively using One Way ANOVA test followed by Duncan multiple comparison tests ($P < 0.05$).

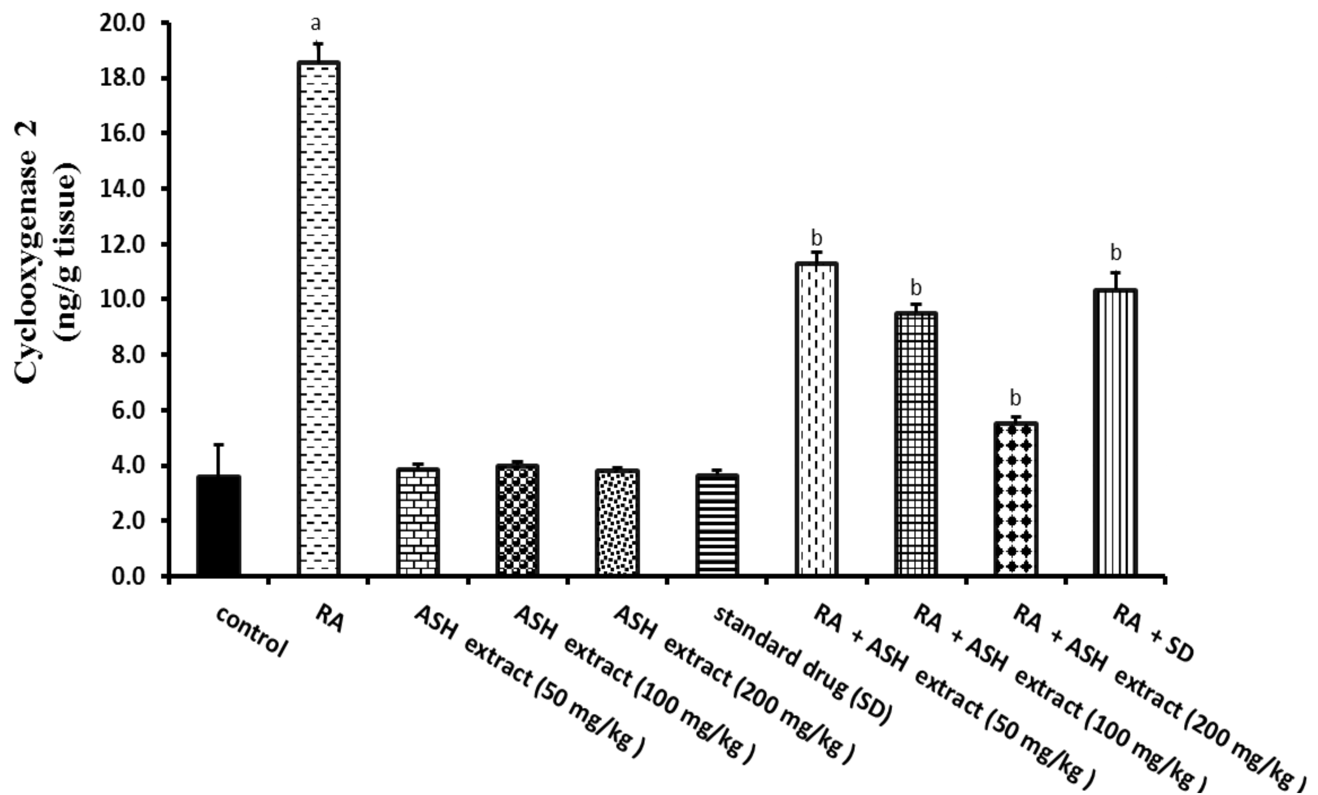

Fig. (4): Effect of Ashwagandha -extract, daily administration for 3 weeks, on Cyclooxygenase -2 activity in AIA of male rats. Data are presented as the mean ± SE. a and b indicate significant change from control and RA model group respectively using One Way ANOVA test followed by Duncan multiple comparison tests ($P < 0.05$).

DISCUSSION

Rheumatoid arthritis (RA) is an autoimmune condition that causes inflammation of the articular tissues [16]. It is characterized by increasing synovitis, articular and cartilage devastation, and it can be caused by a variety of different factors. It affects middle-aged people, most frequently and eventually leads to disability as well as early death [17, 18].

Non-steroidal anti-inflammatory medicines (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs) have long been used to treat rheumatoid arthritis [19]. NSAIDs decrease inflammation by preventing the production of prostaglandins by inhibiting cyclooxygenase 1 and 2 (COX-1 and COX-2) in cells [20].

As a result of severe side effects, including gastrointestinal distress, ulceration, and other possible toxicities, NSAIDS and DMARDS have limited usage in the treatment of RA [21]. Rheumatoid arthritis treatment needs a new therapeutic method that is less harmful and more inexpensive in light of these side effects [22]. Herbal treatment is becoming more popular to treat RA disease progression and extra-articular symptoms related to cardiovascular difficulties, humoral abnormalities, gastrointestinal and respiratory illnesses and immunodeficiency, and blood disorders [23].

W. somnifera, or "Ashwagandha" is an important medicinal herb in India. Over a period of 3,000 years, it has been consistently employed in Indian systems of medicine to cure a variety of ailments, either by itself or in conjunction with other plants. The Withania genus and family Solanaceae include the ashwagandha plant (*W. somnifera*). It contains a wide range of phytochemicals, each of which has a distinct biological activity. Anti-diabetic, anti-microbial, anti-stress/adaptogenic, neuroprotective and cardioprotective as well as hepatoprotective and immunomodulatory effects of *W. somnifera* that have all been established [24].

The purpose of this experiment was to investigate the anti-arthritic potential of ashwagandha extract by analyzing the mechanism of action in reducing the severity of polyarthritis in rats. In the blood of rats, the measures of oxidative stress as well as the impact of the tested extract on the regulation of pro- and anti-inflammatory cytokine were evaluated. At this point in time, the CFA-induced arthritic model of RA with chronic inflammation is the best model that can be accessed. CFA-induced arthritis, which is a model of chronic polyarthritis, possesses features that are comparable to those of RA [25].

As a consequence of the current study, acute toxicity test for ashwagandha extract revealed no mortality was seen at any of the tested dosages of Ashwagandha extract. It was observed that there were no changes in overall appearance during the experimental period, showing that Ashwagandha extract was well tolerated in rats when administered orally at doses of up to 5,000 mg/kg body weight, which was consistent with previous investigations [26].

A large part of the pathology of rheumatoid arthritis may be traced back to oxidative stress. Neutrophils and macrophages create excessive levels of reactive oxygen (ROS) and nitrogen (RNS) species [27-29], which injure joints mostly by increasing matrix metalloproteinase activity and activating osteoclasts [30]. The pathological process of RA is linked to the generation of free radicals, particularly reactive oxygen species, and a decrease in antioxidant scavenging potentials [29].

In this study, higher MDA levels in RA albino rats suggested that CFA-induced oxidative stress in rat. Our results are in accordance with **Anyasor *et al.*** [31] and **Sharma *et al.*** [32] both found elevated levels of MDA as a result of membrane lipid peroxidation when treated with CFA. When there is an imbalance between the activities that create free radicals, and those which protect against them, this lipid peroxidation is likely to occur (oxidative scavengers). MDA levels in RA rats were reduced by the Ashwagandha extract, this may be due to antiperoxidative properties of Ashwagandha extract [33].

Notably, lipid oxidation stimulates macrophages to produce ROS [34]. Antioxidant enzymes, such as SODs and CAT, are part of the cell's defense mechanism against oxidative stress from external and endogenous causes [35]. For this reason, Treatment of oxidative damage necessitates reducing ROS levels or enhancing antioxidant enzyme activity [35].

Based on the research conducted on rheumatoid arthritis rats treated with *W. somnifera* (Ashwagandha extract), it was found that tested extract with high dose (200 mg/Kg BW) is capable of scavenging induced free radical generation by decreasing the levels of MDA and improve antioxidant system in rats for rheumatoid arthritis and showed reduction in proinflammatory cytokine as well as COX1 and COX2. It thus appears that the drug may prove useful in treating/preventing inflammatory response to some extent.

CONCLUSION

The present study demonstrated that Ashwagandha extract had an anti-inflammatory effect, which is exerted by reducing proinflammatory cytokines.

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