Assessment of Anti-Inflammatory and Anti-Arthritic Potential of Ethanolic Extract of *Solanum nigrum L.* Leaves in CFA-Induced Arthritic Rat Model

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Abstract

Objective: To investigate the anti-inflammatory and anti-arthritic properties of *Solanum nigrum* (SN) leaf extract in rats induced with complete Freund's adjuvant (CFA).

Methods: 30 male Wistar albino rats were used in a 4-week pre-clinical experimental trial, which were split up into 5 groups; Group-1 Negative (healthy) control (0.9% normal saline), Group-2 positive (diseased) control (0.9% normal saline), Group-3 Standard (Methotrexate 1.5mg/kg), Group-4 (*Solanum nigrum* 100mg/kg), Group-5 (*Solanum nigrum* 200mg/kg). To develop rheumatoid arthritis, 0.1mL of Complete Freund's Adjuvant was administered intraarticularly in the right knee joints of all groups except Group-1 at day 0. Knee joint circumference was assessed by using a Vernier caliper once a week. On the 29th day, pentobarbital 100mg/kg was injected intraperitoneally to induce anesthesia in all animals, and a cardiac puncture was done to extract 8 ml to 10 ml of blood for further investigations. 4 to 5 ml of that blood was centrifuged and serum was separated to perform an Enzyme-linked immunosorbent assay (ELISA) to analyze the pro-inflammatory mediators including IL-1, IL-2,
IL-6, TNF-á, and prostaglandin E2. SPSS version 22 was used to analyze the results, and ANOVA was applied for intergroup and intragroup comparisons. A P-value less than 0.05 was considered significant at a 95% confidence interval.

Results: Knee joint circumference was significantly decreased in both standard and herbal groups when compared with the diseased controls, exhibiting SN efficacy as an anti-inflammatory agent. The ELISA showed a substantial rise in all pro-inflammatory cytokines in the positive control group. Both herbal (G-4 & G-5) and standard (G-3) groups considerably reduced the levels of pro-inflammatory cytokines, while the G-5 group showed maximum decline in inflammatory markers.

Conclusion: *Solanum nigrum* can be used as an effective adjunctive with standard DMARDs like MTX to increase efficacy in the treatment of RA.

Keywords: *Solanum nigrum*, Rheumatoid arthritis, CFA

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Introduction

Amongst the various contributors to the major disability burden worldwide, arthritis acquires the prime position. Arthritis, the inflammation of joints, is one of the common causes of multiple joint pain in elderly people. It is a blanket term that covers various arthritic conditions including rheumatoid arthritis, osteoarthritis, gout, psoriatic arthritis, ankylosing spondylitis, septic arthritis, and many more. Multiple types of arthritis have been studied but the most debilitating type is rheumatoid arthritis[1]. Rheumatoid arthritis is a long-standing autoimmune disease distinguished by the destruction of cartilage, bone, and synovial membrane leading to joint damage and possibly permanent disability. Other typical characteristics of RA include symmetric polyarthritis, particularly of small joints along with hyperplasia and bone damage[2]. The global prevalence of RA is 1% which is quite notable and the male-to-female ratio is 1:3[3]. The increasing prevalence of RA is a serious issue of concern, es-

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pecially in developing countries like Pakistan. Various etiological pathways of RA are described in the literature but the exact pathogenesis of RA is still unclear and needs to be investigated. According to the literature, the etiology of RA is based on genetic variability along with a few triggering factors such as infection or environmental influences. Among genetic risk factors, the shared epitope alleles HLA-DRB1 *01, *04, and *10 are strongly connected to RA susceptibility and about 60% of the risk of RA development is due to genetic alterations[4]. The eminent characteristic features of active RA include synovitis, joint damage, and swelling which are the outcomes of complicated inflammatory and autoimmune processes involving innate and adaptive immune systems. In RA, the synovium of the joint capsule transforms into a brutal, tumor-like organ known as a pannus. The pannus then invades and erodes the joints causing further tissue damage. Besides the articular damage, many patients may develop extra-articular manifestations which include respiratory system complications (pulmonary nodulosis), skeletal complications (osteoarthritis), and most importantly cardiovascular-related morbidities and mortalities[5]. Therapeutics prescribed and used for RA do not cure the disease entirely but either may modify the progression of the disease (DMARDs - Disease-modifying anti-rheumatic drugs) or provide symptomatic relief (Steroids & NSAIDs - Non-steroidal anti-inflammatory drugs). Long-term medicinal use of DMARDs and NSAIDs has very common and frequently reported adverse effects including gastrointestinal toxicity, hepatic and pulmonary fibrosis, and cardiovascular disorders[6]. Therefore, there is a dire need for time to discover better therapeutic agents with the least adverse effects.

In many regions of the world, medicinal plants have been in use for various ailments since ancient times. Extracts of different herbs and plants, which have biologically active constituents, are now replacing synthetic drugs to avoid many adverse effects and drug resistance. *Solanum nigrum* (SN), one of the traditional therapeutic herbs, belongs to the *Solanaceae* family which has almost 2000 species that are typically herbs. Commonly it is known as 'black nightshade' or '*makoh*' and has been in use for joint pain for a long time[7]. Various researchers have discovered multiple pharmacological effects of SN which include its hepatoprotective, anti-inflammatory, antinociceptive, antioxidant, antitumor, antiulcerogenic & antipyretic effects[8]. In recent times, this plant has become the center of attention owing to its marked antitumor potential. Previously, it has been used for the treatment of tracheitis, asthma, edema, and hepatic damage[9]. Previous studies have reported various biologically active components of SN that have been shown to have anti-inflammatory and antioxidant properties such as Polyphenols, alkaloids, tannins, flavonoids, and glycosides[10]. Additionally, this herb can be used in combination with conventional treatment for RA in the future due to its affordability and accessibility.

As mentioned earlier, the standard drugs used for RA treatment like DMARDs can just alleviate the symptoms of the disease to a certain extent and are also associated with severe toxicities of various organs. Besides DMARDs, steroids and NSAIDs are also being used for symptomatic treatment but long-term use of these drugs leads to severe nephrotoxicity and non-compliance from the patients. Herbs possess great pharmaceutical significance owing to an abundance of natural phytochemicals and have been an invaluable source of medicines. Apart from their therapeutic effects, herbs are easily available and low-priced. In line with such background, this study is designed to evaluate *Solanum nigrum* as an antiarthritic and anti-inflammatory agent for the treatment of RA in albino rats.

Methodology

It was a pre-clinical experimental study conducted from November 2021 to May 2022. Plant extraction was done in the Department of Pharmacognosy, Karachi University while animal handling was done in the animal house of the College of Pharmacy, Ziauddin University. ELISA and histology were done in the MDRL-1 and MDRL-II labs of Ziauddin University.

Solanum nigrum (SN) was purchased from the local nursery and authentication was done from the Karachi University herbarium. The leaves were washed and air-dried at room temperature away from the sunlight and ground into coarse particles. This powder was soaked in absolute ethyl alcohol for 10 days and stirring was done daily 2 to 4 times. To obtain the material the solution was filtered through Whatman filter paper 1. Rotary evaporation was done to concentrate the extract and then stored in a refrigerator in an air-tight flask.

Complete Freund's adjuvant (CFA) (F5881-10ML, batch number 100333309) was ordered from Sigma Aldrich, Germany. Phenobarbital, dimethyl sulfoxide (DMSO), and alcohol were ordered from Laboratory Scientific Supplies Pvt. Limited, Karachi. Methotrexate vials were purchased from a local pharmacy.

30 Male Wistar albino rats, weighing 200±20g, were used in the study. Animals were kept in the animal house of the College of Pharmacy, Ziauddin University. The animals were retained under standard conditions (25 ± 5 f C) in their conventional cages in a 12/12-hour light-dark cycle with ad libitum access to water and food at room temperature. All the investigational procedures done in the study were approved by the Animals ethics committee (Protocol number: 2021-004/MM) and were performed according to the "Canadian Council on Animal Care- Revised on April 2020".

To induce arthritis, 0.1mL of CFA was injected intraarticularly in the right knee joint of all rats except the negative control group (G-1) at day 0. This was followed by intraperitoneal injections of the standard drug MTX (in G-3) and SN extracts (in G-4 and G-5) on days 0,7,14 and 21 according to the following groups.

The rats were randomly assigned to 5 groups (n = 6). Group-1: Negative control (0.9% normal saline); Group-2: Positive control (0.9% normal saline); Group-3: Standard group (1.5mg/kg MTX); Group-4: SN 100mg/kg; Group-5: SN 200mg/kg.

Following induction of arthritis, Group-1 and Group-2 were treated with 0.9% normal saline on days 0, 7, 14, and 21. Group 3 was treated with an intraperitoneal injection of 1.5mg/kg methotrexate as a standard dose on days 0, 7, 14, and 21. Group-4 and Group-5 were treated with intraperitoneal injections of ethanolic extract of SN at 100mg/kg and 200mg/kg, respectively at the same intervals. These doses were selected based on different preliminary studies that were done to evaluate anti-arthritic activity in rats[11,12].

Knee joint circumference was measured to assess edema by a manual Vernier caliper at day 0 before induction of arthritis and on days 7, 14, 21, and 28.

On the 29th day, after overnight food deprivation, all the animals were injected intraperitoneally with 100mg/kg pentobarbital to anesthetize the animals (American Veterinary Medical Association AVMA) as per Institutional Animal Ethics Committee (IAEC) guidelines 1998. A cardiac puncture was done to collect 8 to 10ml of blood that was transferred into vacutainer tubes. About 4 to 5ml of that blood was then immediately centrifuged at 3000rpm for 10 minutes. After centrifugation, the serum was separated to quantify levels of pro-inflammatory markers such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-á) and prostaglandin-E2 (PG-E2) through ELISA.

SPSS software version 22 was used for statistical analysis. For numeric variables mean and standard deviation were calculated. According to Shapiro Wilk's analysis, the data was found to be parametric. Hence, ANOVA followed by post hoc Tukey's test was applied for inter and intra-group comparison. P-value <0.05 was considered significant at a 95% confidence interval.

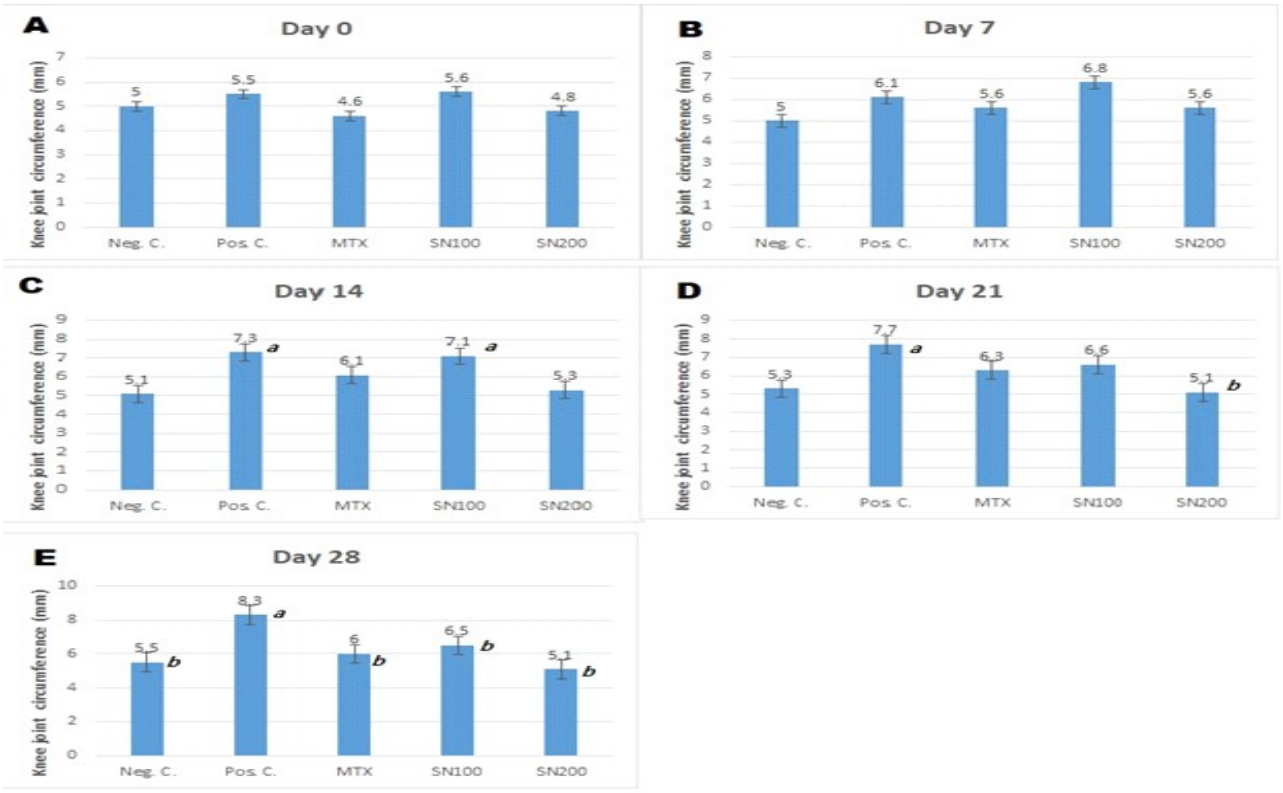
Results

Knee joint edema of all the Wistar albino rats was measured on days 0, 7, 14, 21, and 28. On day

0, there was no significant difference in joint edema of both treated and untreated groups (Fig.

1A). While on day 7, a significant increase in knee joint edema in all the arthritic induced-groups (Positive control, MTX, SN100, and SN200) was observed (Fig. 1B). Measurements taken at the last day of study, day 28, revealed continuous increment in joint edema of the positive control group (Fig. 1E). On the same day, there was no notable increase in joint edema size of the negative control group. When the MTX group was compared with the diseased (positive) control group, it expressed a significant decrease in edema size nearly equal to that of the negative (non-diseased) control group (Fig. 1E).

Similarly, On the 28th day, the herbal group SN100 also expressed a significant decrease in edema size as compared to the positive control, almost equally effective as the MTX group. The second herbal group SN200 showed a remarkable decrease in edema size when compared with the positive control while a non-significant difference exists between SN200 and the MTX group (Fig. 1E).

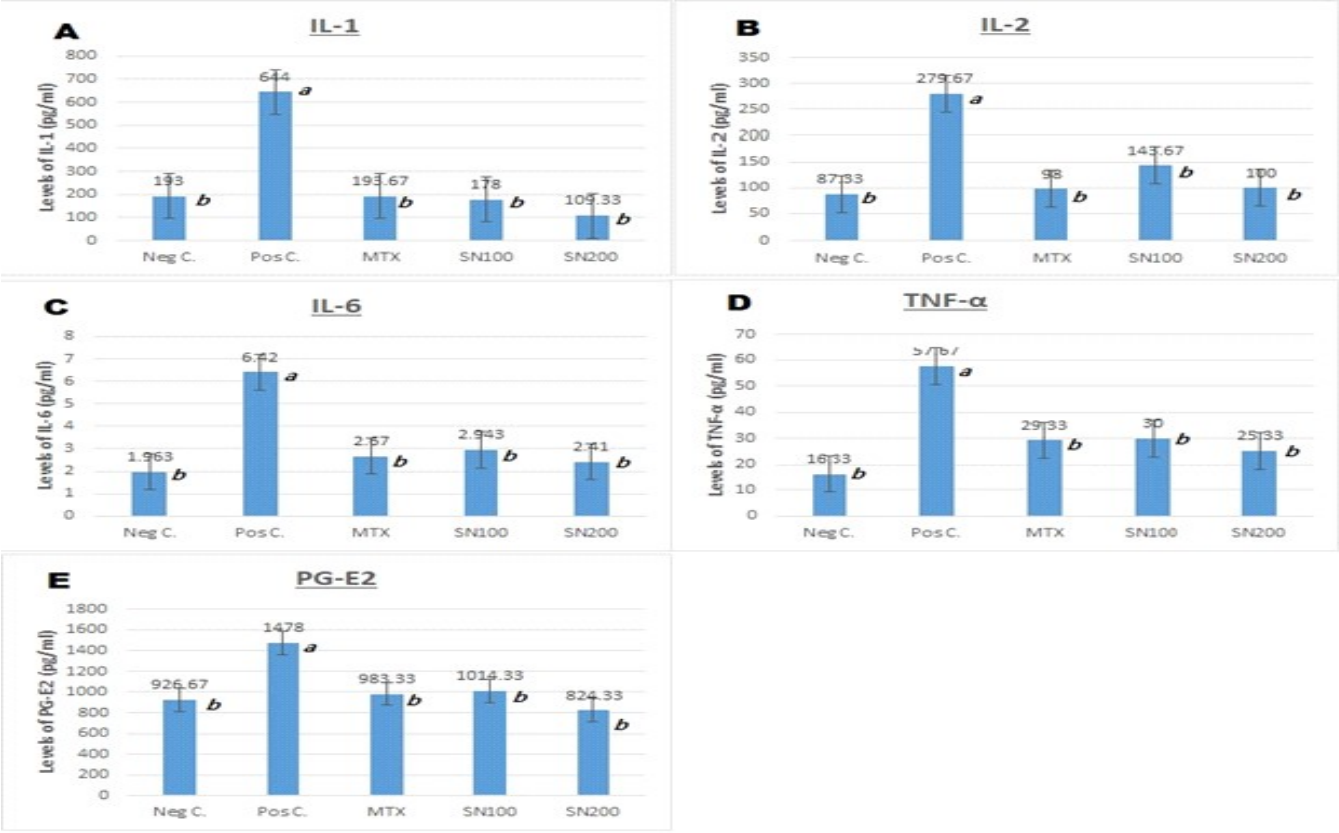


a shows a significant (p = <0.05) difference exists between negative control and other groups b shows a significant (p = <0.05) difference exists between positive control and other groups

Fig 1. Post-treatment effects of SN on knee joint circumference in CFA-induced arthritic

rat model

Serum ELISA of all 5 groups was done after the completion of the study. It showed a remarkable increase in pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-á) and prostaglandin E2 (PG-E2) levels in the positive-control group. MTX and SN100 groups showed low levels of IL-1 when compared with the positive controls and it was equal to the negative-control group. While SN200 expressed a much greater decrease in IL-1 levels even lower than the negative controls (Fig. 2A). MTX and SN200 showed equal levels of reduction in IL-2 levels when compared with the positive controls. The SN100 group also had reduced IL-2 levels but was not as effective as the MTX and SN200 groups (Fig. 2B).



a shows a significant *(p = <0.05)* difference exists between negative control and other groups *b* shows a significant *(p = <0.05)* difference exists between positive control and other groups

Fig 2. Post-treatment effects of SN on IL-1. IL-2, IL-6, TNF-á, and PG-E2 production in

CFA-induced arthritic rat model on day 28

MTX and SN200 groups resulted in a nearly equal decrease in IL-6 levels when compared with the positive controls. SN100 also showed a significant reduction but slightly less than the other standard and herbal treatment groups (Fig. 2C). Regarding TNF-á, the SN100 group showed equal effectiveness as the standard group. While SN200 showed more effectiveness in reducing TNF-á levels (Fig. 2D). Results of PG-E2 analysis showed that all the treatment groups decreased the PG-E2 levels when compared with the positive controls. SN100 showed a minimum decrease in between all other treatment groups. A maximum decrease in PG-E2 levels was expressed by SN200 which was even lower than the negative control PG-E2 levels (Fig. 2E). SPSS version 22 software was used to analyze the results and for intergroup and intragroup comparison ANOVA was applied. P-value <0.05 was considered significant at a confidence interval of 95%.

Discussion

Complete Freund's adjuvant has been reported as a gold standard adjuvant for inducing cell-mediated immunity in research models of autoimmune diseases especially experimental autoimmune encephalomyelitis and rheumatoid arthritis. It is composed of inactivated and desiccated *mycobacterium butyricum/tuberculosis* suspended in sterile non-metabolizable paraffin oil[13]. The CFA-triggered RA exhibits chronic synovial and cartilaginous damage and it is mostly used to induce and study arthritis in rats[14]. The most widely recognized mechanism of CFA is depot formation at the injection site causing antigen trapping. This prolonged release of antigen from the depot site causes a constant stimulation of the immune system leading to the induction of cytokines, recruitment of immune cells, enhancement of antigen uptake and presentation, and production of antibodies leading to a chronic inflammatory reaction[15].

In our study, CFA was induced in all treatment groups except the non-diseased control Group 1. At days 0, 7, 14, 21, and 28 knee joint circumference was measured to assess the edema as an indicator of inflammation. In the first two weeks, a continuous increment of knee joint circumference was observed in all groups except the negative controls. A maximum increase in knee joint edema was observed in the diseased-control group which was not treated with any drug or herb. Various other studies have also reported a significant increase in knee joint size due to inflammatory and edematous effects produced by CFA[16]. MTX, a standard DMARD used in RA, caused a notable reduction in joint edema size in Group 3 showing its anti-inflammatory and anti-arthritic activity. Various other studies have reported the suppressive effect of MTX on edema size[17]. The herb used in our study, *Solanum nigrum*, was used at two different doses that were 100mg/kg and 200mg/kg. Both doses had shown a noteworthy decline in inflammatory edema size but the higher dose (200mg/kg) resulted in more significant suppression of joint edema and performed even better than the standard drug MTX. Another study reported a significant decrease in edema size when treated with SN herbal extract. Steroidal alkaloid Solanine A, flavonoids, and polyphenols isolated from SN have also shown notable suppression of inflammatory edema[18].

Targeting pro-inflammatory cytokines in the treatment of RA is a major mechanism of action of most disease-modifying antirheumatic drugs (DMARDs). The major inflammatory mediators and cytokines found to play a vital role in the pathogenesis of RA are interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-á) and prostaglandin E2 (PG-E2). The proliferation of these mediators causes the activation and production of further several cytokines required for the progression of RA. In our study, the results obtained from ELISA have revealed that CFA-induced arthritis caused a remarkable increase in the levels of all pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-á) and PG-E2, as evident in Group 2 of our study. Multiple other studies have reported that CFA induction causes an increase in pro-inflammatory cytokines [19, 20]. As documented in the literature, our study has also shown a halting effect of MTX on rising pro-inflammatory cytokines and PG-E2 (17). When compared with the diseased control, both 100mg/kg and 200mg/kg doses of SN reported downregulation of the pro-inflammatory cytokines and PGE2 but the SN200 group has shown more efficacy. Previous literature has also reported the immunomodulatory effect of SN and down-regulation of pro-inflammatory cytokines[21]. Phytosterols, steroidal saponins, and alkaloids isolated from SN have reported their anti-inflammatory activity by their suppressive action on pro-inflammatory cytokines[22].

Regarding toxicity, SN has some reported dose-dependent adverse effects including nausea, vomiting, fever, headache, tachycardia, and mental confusion. These adverse effects are majorly attributed to the plant's natural defense glycoalkaloids like solanine, solasonine, and solamargine[23]. Regarding its toxicity profile, a 14-day-long study reported that no toxic effects on rats were observed even at 2000mg/kg of aqueous and alcoholic extracts of *Solanum nigrum* [24].

Conclusion

Solanum nigrum has shown obvious activity in reducing joint edema and suppressing the proinflammatory cytokines. Moreover, the high-dose group of *Solanum nigrum* has been more effective in producing anti-inflammatory effects and reduced the number of pro-inflammatory cytokines more effectively. Thus, we can summarize that *Solanum nigrum* can be used as an effective adjunctive with standard DMARDs like MTX to increase efficacy in the treatment of rheumatoid arthritis. After further validation by clinical trials, this herb can be come up as integrated medicine in the future.

Extended pre-clinical trials should be carried out to evaluate the long-term adverse health consequences, of long-term use, of *Solanum nigrum* at different doses before showcasing their effectiveness for holistic management of RA in clinics.

Conflict Of Interest: None

Disclaimer: None

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