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A randomized, double blind placebo controlled study of efficacy and tolerability of *Withaina somnifera* extracts in knee joint pain

G.S.H. Ramakanth*, C. Uday Kumar, P.V. Kishan, P. Usharani

Dept. of Clinical Pharmacology & Therapeutics, Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India

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ABSTRACT

Background: Root extracts of *Withania somnifera* (Ashwagandha) are known to possess analgesic, anti-inflammatory and chondroprotective effects. An aqueous extract of roots plus leaves of this plant has shown to yield higher percentages of withanolide glycosides and, accordingly, may possess better analgesic, anti-inflammatory and chondroprotective effects than root alone extracts.
Objectives: To evaluate efficacy and tolerability of a standardized aqueous extract of roots plus leaves of *W. somnifera* in patients with knee joint pain and discomfort.
Material and methods: Sixty patients with knee joint pain and discomfort were randomized in a double-blind manner to *W. somnifera* 250 mg, *W. somnifera* 125 mg and placebo, all given twice daily. Assessment was done by Modified WOMAC, Knee Swelling Index (KSI), Visual Analogue Scale (VAS) at baseline and at the end of 4, 8, 12 weeks. Tolerability was assessed by incidence of adverse effects in treatment groups. Student's 't' test and ANOVA were used to compare mean change from baseline within and between the study groups. A $p < 0.05$ was considered significant.
Results: At the end of 12 weeks, compared to baseline and placebo, significant reductions were observed in mean mWOMAC and KSI in *W. somnifera* 250 mg ($p < 0.001$), *W. somnifera* 125 mg ($p < 0.05$) groups. VAS scores for pain, stiffness and disability were significantly reduced in *W. somnifera* 250 mg ($p < 0.001$), *W. somnifera* 125 mg ($p < 0.01$) groups. *W. somnifera* 250 mg group showed earliest efficacy (at 4 weeks). All treatments were well tolerated.
Conclusions: Both the doses of an aqueous extract of *W. somnifera* produced significant reduction in outcome variables, with the 250 mg group showing significantly better response. In addition, the therapeutic response appears to be dose-dependent and free of any significant GI disturbances.

1. Introduction

1.1. Background

Knee joint pain and discomfort are the most prevalent of the chronic rheumatic symptoms and is a leading cause of disability in most countries worldwide [1].The prevalence of joint pain and discomfort due to osteoarthritis (OA) increases with age and more so with female gender, though males are also affected. OA contributes to a higher disease burden in men below the age of 50 and in women over the age of 50 [2]. Most of the disability arising due to OA is due to involvement of hip and knee joints [3]. Knee OA is likely to become the fourth most important global cause of disability in women and eighth most important in men [4].

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs for the symptomatic treatment of pain in OA. However, NSAIDs are associated with serious gastrointestinal adverse effects which limit their use in many patients [5,6]. Other drugs like opioids and non-opioid analgesics and intra-articular steroids may not be effective in all patients [5,6].

* Corresponding author.
E-mail address: grkclinpharma@gmail.com (G.S.H. Ramakanth).
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Hence, there is a specific need for effective and safe drugs in the treatment of OA.

Herbal medicines have been explored for their usefulness in OA for a long time. *Withania somnifera* (Ashwagandha), a plant belonging to the family Solanaceae, is widely used in Ayurvedic medicine for this purpose. It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g., arthritis, rheumatism), and as a general tonic to improve overall health [7]. Roots of the plant reportedly exhibit anti-inflammatory, anti-tumour, anti-stress, antioxidant, immunomodulatory, haematopoietic and rejuvenating properties [8]. There is evidence of effectiveness of *W. somnifera* in various rheumatologic conditions due to its anti-inflammatory properties [9]. In a randomized, double-blind, placebo-controlled, cross-over study in patients with OA, treatment with roots of *W. somnifera* produced a significant drop in severity of pain and disability score. It also acts as an analgesic that soothes nervous system from pain response [10]. Chemical composition of *W. somnifera* extracts vary widely depending on which part of the plant is used as well as the extraction solvent and procedure, and thus different extracts are expected to elicit different clinical response. Sensoril® is an aqueous extract of *W. somnifera* roots plus leaves and contains withanolide glycosides, Withaferin-A and oligosaccharides as the major components. There are very few human studies evaluating the effects of *W. somnifera* root extracts, in combination with other herbal products, in patients with symptoms of knee joint pain and disability and there are no human studies reported with an aqueous extract of roots plus leaves of *W. somnifera*.

2. Objectives

To evaluate the efficacy and tolerability of a standardized aqueous extract of roots plus leaves of *W. somnifera* using Modified WOMAC index score, pain relief as assessed by Visual Analogue Scale (VAS) and changes in Knee Swelling Index (KSI) in patients with pain and discomfort of knee joint.

3. Methods

3.1. Study design

The study was a prospective, randomized, double-blind, placebo-controlled trial with 1:1:1 allocation ratio of the participants in to the 3 study groups. The study was approved by the local Institutional Ethics Committee.

3.2. Study participants

3.2.1. Eligibility criteria

The patients were screened for their eligibility to participate in the study during the screening visit (Visit 1).

a. Inclusion Criteria

Patients with knee joint pain and discomfort of either gender aged between 40 and 70 years, for at least 6 months duration and meeting the American Rheumatology Association (ARA) functional class I to III and who recorded baseline pain scores of at least 40 mm on the VAS monitored at baseline visit were enrolled. Patients who discontinued all current analgesic therapy, including NSAIDs, over the counter pain medications and topical analgesics for 7–10 days prior to the start of the study were randomized into the study.

b. Exclusion Criteria

Patients with severe OA (ARA functional class IV), on alternative system of medicine, any psychiatric disorder or who have been using systemic/intra-articular steroids within 12 weeks of study and hyaluronic acid in the last 9 months, or potential candidates for imminent joint replacement and patients with uncontrolled hypertension or diabetes, hepatic or renal impairment, pregnant or lactating females, or with a recent trauma of the involved knee were excluded from the study.

3.3. Study interventions

The study medications included capsules of *W. somnifera* in the strengths of 125 mg and 250 mg and identical placebo capsules, supplied by Natreon, Inc, New Jersey, USA.

A *W. somnifera* capsule consists of standardized aqueous extract of roots and leaves of *W. somnifera* (Sensoril®) containing not less than 10% Withanolide glycosides, not less than 32% oligosaccharides and not more than 0.5% of Withaferin- A and is standardized by HPLC (Fig. 1). The excipients used in these capsules include microcrystalline cellulose, croscarmellose sodium, silicon dioxide, magnesium stearate and gelatin from the capsule shell.

3.4. Study procedure

The study was conducted in the Department of Clinical Pharmacology and Therapeutics. The patients were randomized by the principal investigator using a computer generated simple randomization sequence with a block size of 20 patients per group. Case record numbers and sequentially numbered containers were used for random allocation sequence. The study was performed in a double-blinded manner, with both the study patients and the investigator blinded to study interventions. The participant flow chart is shown in Fig. 2.

A run-in period was allowed between screening visit (Visit 1) and randomization visit (Visit 2) to ensure that the were weaned off all medications 7–10 days prior to randomization. At the baseline/randomization visit (Visit 2, Day 0), all eligible were randomized to receive either *W. somnifera* 250 mg or *W. somnifera* 125 mg or identical placebo capsules for 4 weeks, with one capsule of the study medication to be taken twice daily after food with a glass of water. Paracetamol 650 mg tablets were used as and when required as rescue medication. The subsequent visits were scheduled at 4 weeks interval (Visit 3-after 4 weeks of treatment initiation, Visit 4 -after 8 weeks of treatment initiation and Visit 5- after 12 weeks of treatment initiation). The study and rescue medications were dispensed at visits 2, 3 and 4 and compliance to study medications was checked by pill count method during the subsequent visits. The total duration for which the patients received study medications was 12 weeks.

All the efficacy variables, pill count, use of rescue medication and Physician's Global Assessment were evaluated in the subsequent visits. Adverse reactions/serious adverse effect (ADR/SAE) monitoring was done throughout the course of study. Safety lab were done before and after treatment and as and when required.

3.5. Outcomes

The primary outcome measure was percentage change in the Modified Western Ontario and McMaster University Osteoarthritis Index (mWOMAC, Ref. www.copcord.org/images/WOMAC.pdf) score at the end of 12 weeks from baseline.

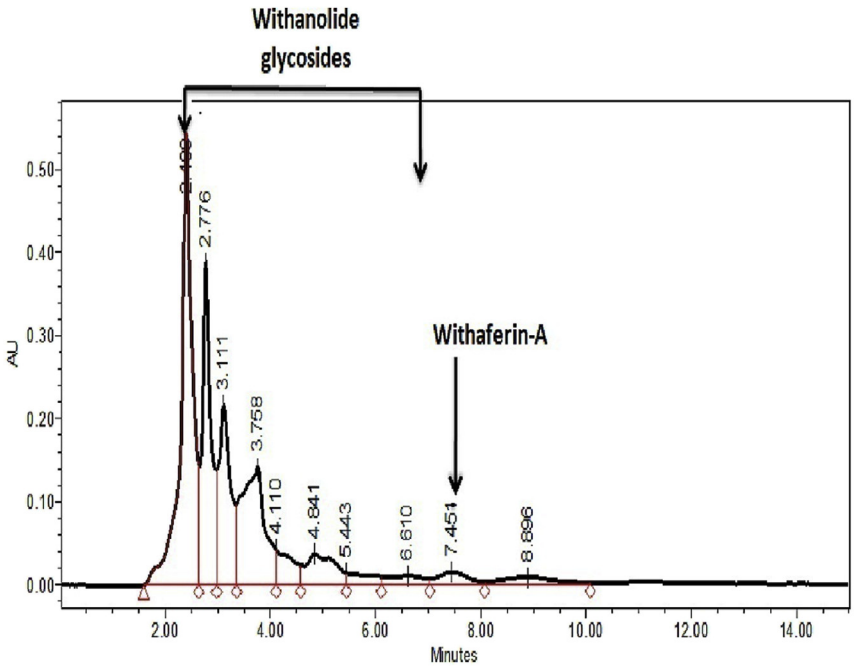

Fig. 1. HPLC Chromatogram of Sensoril®.

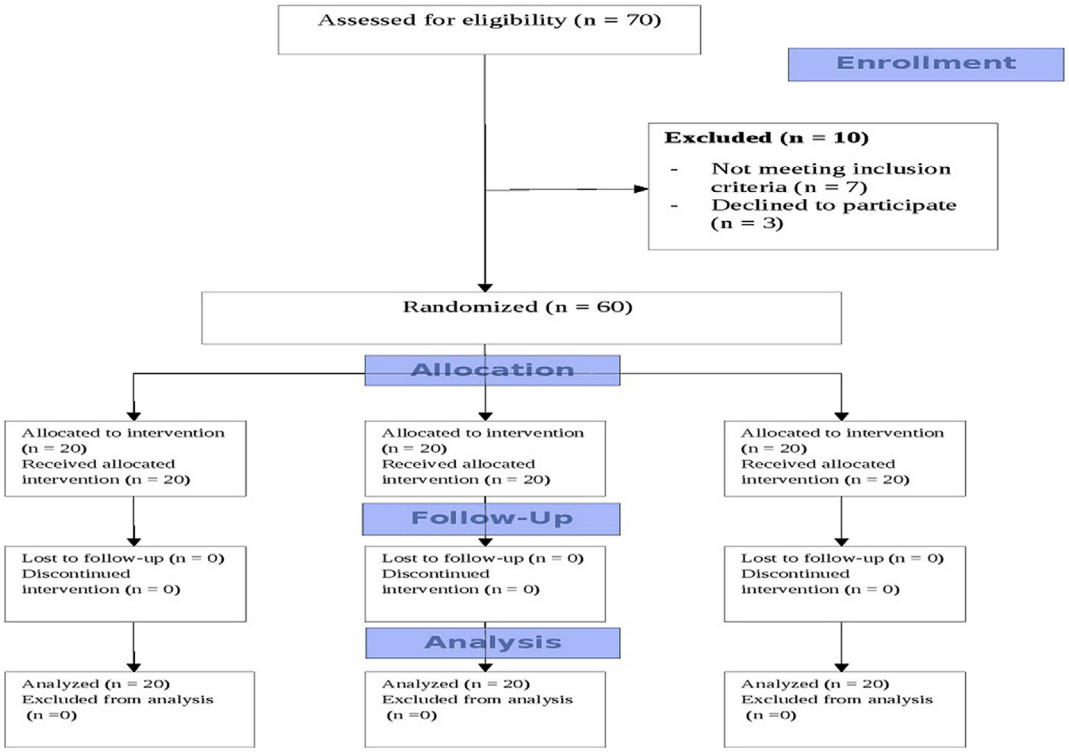

Fig. 2. Participant flow chart.

The secondary outcome measures were percentage changes in mWOMAC score at the end of 4 and 8 weeks, Knee Swelling Index (KSI) as measured by joint circumference (mm) and VAS for pain, disability and stiffness at the end of 4, 8 and 12 weeks, extent of use of rescue medication in treatment groups, Physician Global Assessment scale and tolerability. The Physician Global Assessment was used to classify the patients based on symptoms. Accordingly, the 5 categories were Excellent (complete relief of symptoms), Good (partial relief of symptoms), Fair (minimal relief of symptoms), Poor (no relief of symptoms) and Very poor (worsening of symptoms). Tolerability was assessed by 3 categories, viz., Good (no side effects), Fair (mild to moderate side effects) and Poor (severe side effects and withdrawal of therapy).

3.6. Sample size

The sample size calculation was based on the assumption that there will be a decrease of 10% in total mWOMAC score from baseline to end of treatment. A sample size of 60 evaluable cases would provide an 80% power to estimate the reduction of total mWOMAC score at 5% level of significance at the end of the study. Anticipating 15% dropouts, we enrolled 70 to get 60 evaluable cases for the study.

3.7. Statistical analysis

Primary and secondary end points were analyzed as average change in the response from baseline. Student's 't' test and ANOVA were used to compare the mean change from baseline to post-treatment period within and between study medications and placebo groups, respectively, at 80% power. A $p < 0.05$ was used to test the significance. All statistical analysis was performed using the GraphPad Prism Software 4 (GraphPad Software Inc., San Diego, California, USA).

4. Results

A total of seventy patients were recruited and screened over a period of 10 months (4/7/2014 to 16/5/2015), out of which 60 eligible patients (43 males, 17 females) with a mean age of 57.78 ± 4.49 years were enrolled into the study. Patients were divided into three groups of 20 each and were randomized to receive *W. somnifera* 250 mg, *W. somnifera* 125 mg or identical placebo capsules, one capsule twice daily for 12 weeks. All patients completed 12 weeks of treatment.

The demographic characteristics of all the three study groups are shown in Table 1. There were no significant differences between the treatment groups in baseline characteristics including age, weight and body mass index, indicating a homogenous population.

The mWOMAC scores at the end of 12 weeks are shown in Table 2(a) and the mean percentage reduction in mWOMAC scores are shown in Fig. 3. The baseline values of the mWOMAC score were comparable in all the three groups without any statistically significant difference between them. There was a significant reduction in the mWOMAC score at the end of 12 weeks from baseline in *W. somnifera* 250 mg group (A) ($p < 0.001$) and *W. somnifera* 125 mg group (B) ($p < 0.05$). The mean percentage reduction in the mWOMAC score at the end of 12 weeks showed significant differences between *W. somnifera* 250 mg (A) and *W. somnifera* 125 mg groups (B) ($p < 0.001$), *W. somnifera* 250 mg (A) and placebo groups (C) ($p < 0.001$) and *W. somnifera* 125 mg (B) and placebo groups (C) ($p < 0.01$).

The KSI scores at the end of 12 weeks are shown in Table 2(a) and mean percentage change in KSI scores are shown in Fig. 4. The baseline values of the KSI scores were comparable in all the three groups without any statistically significant difference between them. There was a significant reduction in the KSI score at the end of 12 weeks from baseline in *W. somnifera* 250 mg group (A) ($p < 0.001$) and *W. somnifera* 125 mg group (B) ($p < 0.05$). The mean percentage reduction in the KSI scores in all the three groups at the end of 12 weeks showed a significant difference between *W. somnifera* 250 mg (A) and *W. somnifera* 125 mg (B) groups ($p < 0.001$) and *W. somnifera* 250 mg (A) and placebo (C) groups ($p < 0.001$).

The VAS score for pain, stiffness and disability are shown in Table 2(b) and the mean percentage changes in these scores are shown in Figs. 5–7, respectively. The baseline values for pain, stiffness and disability measured by VAS were comparable in all the

Table 1
Demographic data.

	W. somnifera 250 mg (A)	*W. somnifera* 125 mg (B)	Placebo (C)
No. of Subjects	20	20	20
Gender (M/F)	13/7	14/6	16/4
Age (Yrs)	58.92 ± 6.07	55.42 ± 3.69	58.95 ± 3.73
BMI(Kg/m^2)	23.79 ± 2.99	23.82 ± 1.86	24.15 ± 2.17

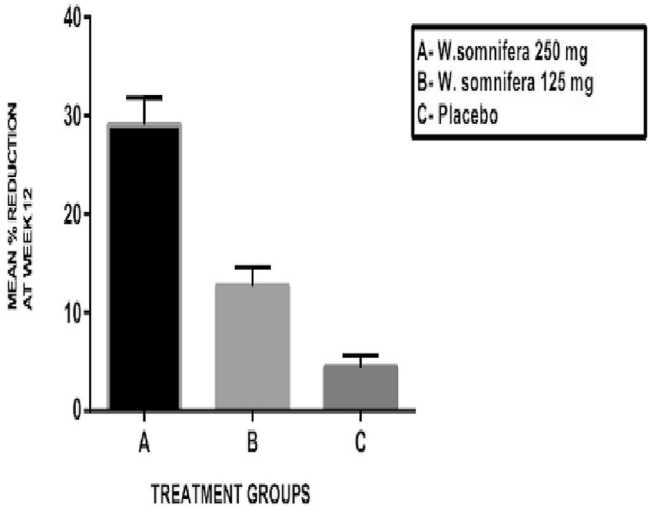

Fig. 3. Mean percentage change in modified WOMAC score at the end of 12 weeks.

Table 2a
Summary of results – I.

	mWOMAC score			Knee swelling index		
	W. somnifera 250 mg (A)	*W. somnifera* 125 mg (B)	Placebo (C)	*W. somnifera* 250 mg (A)	*W. somnifera* 125 mg (B)	Placebo (C)
Baseline	53.1 ± 2.90	51.15 ± 2.39	50.45 ± 2.11	423.25 ± 9.63	415.5 ± 20.76	408.5 ± 15.05
End of 4 weeks	48.6 ± 2.41#	49.95 ± 2.11	49.97 ± 2.04	415.25 ± 9.66#	413.75 ± 19.65	404.6 ± 15.30
End of 8 weeks	43.4 ± 2.47*	47.4 ± 1.91	49.31 ± 2.11	404.75 ± 10.44*	409.25 ± 19.61	401 ± 15.61
End of 12 weeks	37.65 ± 2.41*	44.6 ± 1.42@	48.24 ± 2.31	396.25 ± 10.98*	405.75 ± 19.21@	399.5 ± 15.55
Absolute change at end of 12 weeks	15.45 ± 1.73*	6.5 ± 1.23#	2.15 ± 0.75	27 ± 6.76*	9.75 ± 3.43^{NS}	9 ± 2.62
Mean percentage change at end of 12 weeks	29.07 ± 2.73*	12.7 ± 1.96#	4.27 ± 1.46	6.37 ± 1.58*	2.34 ± 0.78^{NS}	2.2 ± 0.65

@ p value < 0.05; #p value < 0.01; *p value < 0.001 compared to baseline; NS- Non-significant.
Absolute change in mWOMAC score: * A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).
Mean percentage change in mWOMAC score:* A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).
Absolute change in KSI: * A vs B, A vs C ($p < 0.001$),^{NS} B vs C.
Mean percentage change in KSI: * A vs B, A vs C ($p < 0.001$), ^{NS} B vs C.

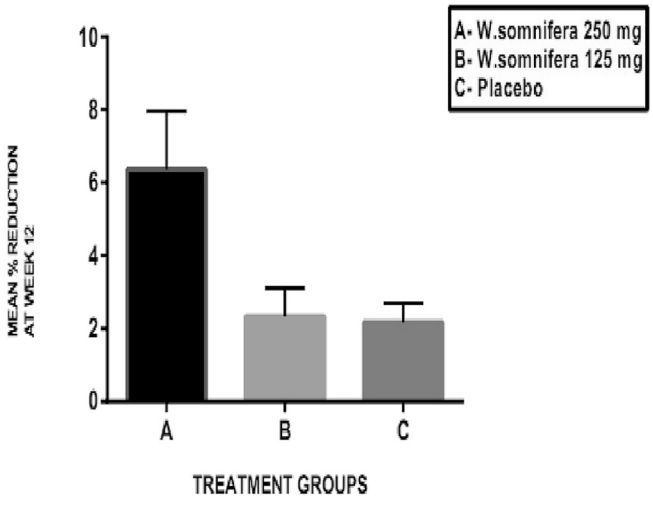

Fig. 4. Mean percentage change in knee swelling index score at the end of 12 weeks.

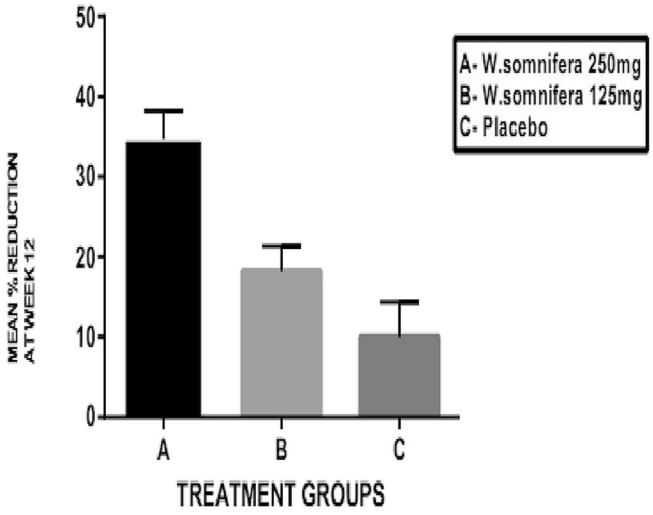

Fig. 5. Mean percentage change in VAS – pain score at the end of 12 weeks.

three groups without any statistically significant differences between them. In *W. somnifera* 250 mg group (A), there was a significant reduction in pain, stiffness and disability ($p < 0.001$) at the end of 12 weeks from baseline. In *W. somnifera* 125 mg group (B), there was a significant reduction in pain ($p < 0.01$), stiffness ($p < 0.01$) and disability ($p < 0.05$). The mean percentage reduction in the pain, stiffness and disability in all the three groups at the end of 12 weeks showed a significant difference between *W. somnifera* 250 mg (A) and *W. somnifera* 125 mg (B) groups ($p < 0.001$) and *W. somnifera* 250 mg (A) and placebo (C) groups ($p < 0.001$) and *W. somnifera* 125 mg (B) and placebo (C) groups ($p < 0.01$).

5. Rescue medication usage

The mean number of rescue medication tablets (Paracetamol 650 mg) was 10, 13 and 17 in *W. somnifera* 250 mg (A), *W. somnifera* 125 mg (B) and placebo (C) groups, respectively.

6. Physician Global Assessment

The Physician Global Assessment was done at the end of 12 weeks. In *W. somnifera* 250 mg group, 15 patients were assessed to be excellent and 5 were assessed as good by the Physician Global Assessment scale.

In *W. somnifera* 125 mg group, 17 patients were assessed to be good and 3 were assessed as fair and in the placebo group, 1 patient was assessed to be fair and 19 patients were assessed as poor.

7. Results at 4 and 8 weeks

At the end of 4 weeks, patients treated with *W. somnifera* 250 mg twice daily showed statistically significant reductions in the mWOMAC score ($p < 0.01$), KSI ($p < 0.01$), pain ($p < 0.01$), stiffness ($p < 0.01$) and disability ($p < 0.01$) as measured by VAS when compared to baseline. However, patients treated with *W. somnifera* 125 mg twice daily did not show any significant changes at the end of 4 weeks compared to baseline.

At the end of 8 weeks, patients treated with *W. somnifera* 250 mg showed statistically significant reductions in the mWOMAC score ($p < 0.001$), KSI ($p < 0.001$), pain ($p < 0.001$), stiffness ($p < 0.001$) and disability ($p < 0.001$) as measured by VAS, compared to baseline. *W. somnifera* 125 mg (B) showed significant

Table 2b
Summary of results – II.

	VAS, pain			VAS, stiffness			VAS, disability		
	W. somnifera 250 mg (A)	*W. somnifera* 125 mg (B)	Placebo (C)	*W. somnifera* 250 mg (A)	*W. somnifera* 125 mg (B)	Placebo (C)	*W. somnifera* 250 mg (A)	*W. somnifera* 125 mg (B)	Placebo (C)
Baseline	67.25 ± 4.48	65.6 ± 4.56	63.8 ± 2.78	60.25 ± 4.11	62.55 ± 4.43	61.8 ± 2.71	50.45 ± 4.35	63.95 ± 4.71	58.6 ± 4.30
End of 4 weeks	60.95 ± 4.22#	63.05 ± 4.67	60.5 ± 3.24	55.8 ± 3.83#	59.45 ± 4.13	58.6 ± 2.85	46 ± 3.98#	61 ± 5.03	55.35 ± 4.06
End of 8 weeks	52.9 ± 3.37*	59.5 ± 4.07	58.65 ± 3.56	49.4 ± 3.28*	56.3 ± 4.05 @	56.7 ± 2.96	40.35 ± 3.64*	57.75 ± 5.03	53.5 ± 4.42
End of 12 weeks	43.9 ± 3.30*	53.3 ± 4.02#	57.4 ± 3.68	41.3 ± 3.54*	50.1 ± 4.12#	55.2 ± 2.98	33.55 ± 3.79*	52.6 ± 5.37@	51.87 ± 4.48
Absolute change at end of 12 weeks	23.35 ± 3.04*	12 ± 2.31#	6.4 ± 1.57	18.95 ± 3.08*	12.45 ± 2.08#	6.6 ± 1.82	17.4 ± 3.08*	11.35 ± 1.56#	6.5 ± 1.79
Mean percentage change at end of 12 weeks	34.73 ± 3.49*	18.29 ± 3.13#	10.08 ± 2.71	31.4 ± 4.26*	19.9 ± 3.15#	10.67 ± 2.93	33.7 ± 3.37*	17.74 ± 3.13#	11.15 ± 3.18

@ p value < 0.05; #p value < 0.01; *p value < 0.001 compared to baseline.
Absolute change in VAS scores:
a) Pain: * A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).
b) Stiffness: * A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).
c) Disability: * A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).
Mean percentage change in VAS scores:
a) Pain: * A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).
b) Stiffness: * A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).
c) Disability: * A vs B, A vs C ($p < 0.001$); #B vs C ($p < 0.01$).

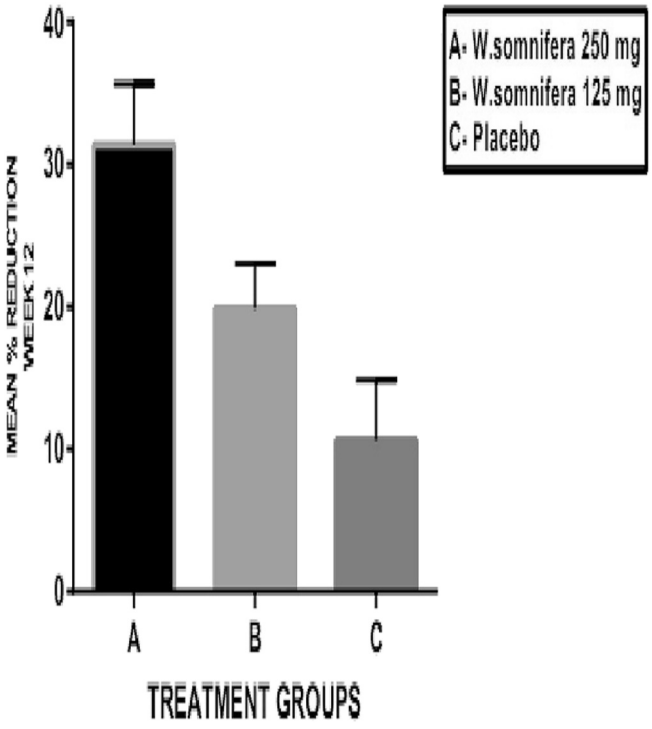

Fig. 6. Mean percentage change in VAS – stiffness score at the end of 12 weeks.

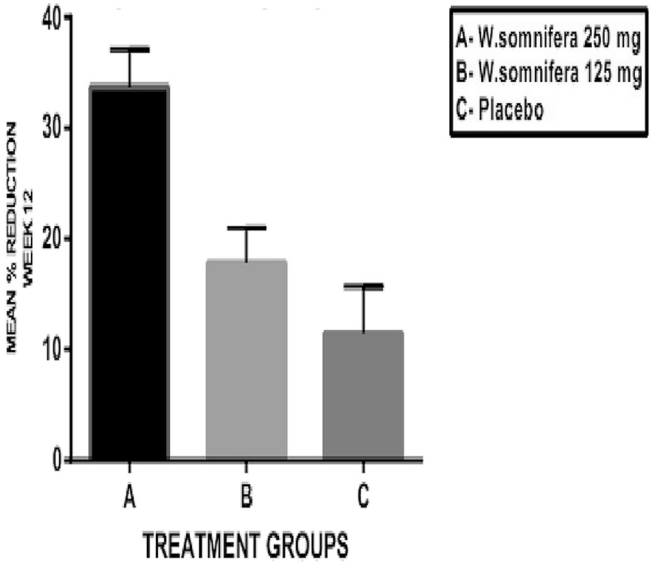

Fig. 7. Mean percentage change in VAS – disability score at the end of 12 weeks.

change from baseline only in VAS stiffness at the end of 8 weeks ($p < 0.05$).

There were no significant changes in the efficacy variables in the placebo group at the end of 4 and 8 weeks. The results at the end of 4 and 8 weeks are summarized in Tables 2(a), 2(b).

8. Safety and tolerability

All safety hematological and biochemical variables were within normal limits in all the three treatment groups at the baseline recording. Both *W. somnifera* 250 mg and *W. somnifera* 125 mg were well tolerated. In *W. somnifera* 250 mg group, 4 patients complained of nausea and 1 patient developed mild gastritis. In *W. somnifera* 125 mg group, 2 patients complained of nausea and mild headache. None of the patients in placebo group had any adverse effects. No patients in any of the groups discontinued the study.

9. Discussion

W. somnifera has multiple bioactive components which contribute to it's biological activity as an analgesic, anti-inflammatory, anti-arthritic and chondroprotective [11]. The chemical analysis of *W. somnifera* shows its main constituents to be alkaloids and steroidal lactones. The biologically active alkaloids include withanine, somniferine, somnine, isopelletierine and anferine, and the steroidal lactones include withanolides and withaferins [12,13]. The roots and leaves of *W. somnifera* have been shown to be rich in withanolides, which resemble steroids in their action and are considered to account for the biological activities of *W. somnifera*. Other components include saponins like sitoindoside VII and VIII and iron. Much of *W. somnifera*'s pharmacological activity has been attributed to Withaferin A and Withanolide D [13]. However, chemical composition of *W. somnifera* extracts vary widely depending on which part of the plant is used as well as the extraction solvent and procedure, and thus are expected to elicit different clinical response. Sensoril® is an aqueous extract of *W. somnifera* roots plus leaves and contains withanolide glycosides, Withaferin-A and oligosaccharides as the major components.

In the present study, the mWOMAC score was reduced significantly at the end of 12 weeks from baseline in both *W. somnifera* 250 mg and *W. somnifera* 125 mg groups, with the reduction being greater in *W. somnifera* 250 mg group. This reduction in mWOMAC scores by *W. somnifera* may be attributed to its analgesic and chondroprotective effects. The analgesic activity of *W. somnifera* is mainly mediated by Withaferin A which has been shown to block the cyclo-oxygenase (COX) pathway involved in the production of prostaglandins, the endogenous pain mediators [14]. The analgesic activity is also due to its action of soothing the nervous system from pain responses [15]. Also it is suggested that serotonin may be involved in the analgesic effects of *W. somnifera* [16]. An experimental study has demonstrated the chondroprotective activity of *W. somnifera* in human cartilage tissue [17].

Another important biological effect of *W. somnifera* is its anti-inflammatory activity which can be determined by its effect on the KSI. The KSI scores were significantly reduced at the end of 12 weeks from baseline in both *W. somnifera* 250 mg and *W. somnifera* 125 mg groups. However, greater reduction was seen in *W. somnifera* 250 mg group. This anti-inflammatory effect of *W. somnifera* has also been attributed to Withaferin A [18]. The possible role of anti-oxidant activity of *W. somnifera* on its anti-inflammatory properties has also been proposed. An experimental study in rats has shown that the anti-inflammatory activity of *W. somnifera* may be due to the inhibition of biological changes like increase in levels of lipid peroxides and glycoproteins and decreased antioxidant status and bone collagen in the affected joint [19]. In the affected joint, reactive oxygen species are known to activate a number of intracellular signaling pathways such as NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), which further activate the transcription of various pro-inflammatory cytokines (interleukins and TNFα), cell adhesion molecules and COX 2 [20]. Some of the constituents of *W. somnifera* like flavonoids and phenolic acid such as gallic acid, rutein, vanillic acid, quercetin and kaempferol block the distinct signal transduction events necessary for NF-kB activation and thus inhibit transcription factors such as

NF-kB, activating protein-1 (AP-1) and nuclear factor-erythroid 2-related factor 2 (Nrf2) [21].

VAS scores for pain, stiffness and disability were used to assess the physical function of the knee joint. *W. somnifera*, in both 250 mg and 125 mg twice daily doses, significantly reduced the pain, stiffness and disability at the end of 12 weeks. However, the extent of reduction of these variables was higher with *W. somnifera* 250 mg twice daily dose. Also, the mean percentage reduction in all the three variables at the end of 12 weeks was significant with *W. somnifera* 250 mg, when compared to *W. somnifera* 125 mg and placebo groups.

The clinical assessment of patients with knee joint pain and discomfort was made by the Physician Global Assessment scale. Accordingly, patients in *W. somnifera* 250 mg group were assessed to have performed better in terms of clinical improvement compared to the other two groups.

Rescue medication in the form of Paracetamol 650 mg was allowed as and when required during the course of the study. We evaluated the usage of rescue medication as one of the outcomes measures of efficacy of *W. somnifera* treatment. The usage of rescue medication was the least in *W. somnifera 250 mg* group and the highest in the placebo group, indicating effectiveness of *W. somnifera* in reducing the symptoms of knee joint pain and discomfort.

The efficacy was also evaluated at 4 weeks and 8 weeks to determine the earliest onset of action. At the end of 4 and 8 weeks, patients treated with *W. somnifera* 250 mg twice daily showed statistically significant reductions in all efficacy variables, compared to baseline. However, patients treated with *W. somnifera* 125 mg twice daily did not show any significant changes at the end of 4 weeks compared to baseline and showed only significant change in VAS stiffness ($p < 0.05$) at the end of 8 weeks, compared to baseline. This suggests that treatment with *W. somnifera* 250 mg twice daily produces earlier and better symptomatic relief thus increasing patient compliance and satisfaction. In addition, the response with *W. somnifera* seems to be linearly dose-dependent.

The safety of *W. somnifera* was evaluated by monitoring the occurrence of any adverse effects. Both *W. somnifera* 250 mg and *W. somnifera* 125 mg were well tolerated and our study did not report any serious adverse effects with either of the doses of *W. somnifera*. This is consistent with the available literature on safety of *W. somnifera* [22]. Gastritis, nausea and headache that were reported were treated symptomatically with standard care of treatment.

10. Conclusions

Treatment with *W. somnifera* 250 mg and *W. somnifera* 125 mg, both taken twice daily in patients with knee joint pain and discomfort for a period of 12 weeks showed significant reduction in the outcome variables of efficacy and safety, when compared to baseline and placebo. On further analysis, *W. somnifera* 250 mg produced better reduction in outcomes when statistically compared to *W. somnifera* 125 mg. Also, this effect was seen earlier (in 4 weeks) in *W. somnifera* 250 mg group than in *W. somnifera* 125 mg group. The need for rescue medication (Paracetamol 650 mg tablets) was the least with *W. somnifera* 250 mg group suggesting its analgesic and anti-inflammatory effects. All the study medications were well tolerated and mild gastrointestinal adverse effects like nausea and gastritis were observed in few patients. None of the patients discontinued the study suggesting the favorable safety profile of *W. somnifera*. Further studies with *W. somnifera*, hence, are needed to confirm its therapeutic potential in patients with knee joint pain and discomfort and other painful rheumatologic conditions.

Registration of the trial

The trial was registered in the Clinical Trial Registry-India (CTRI) with the reference number REF/2014/08/007385.

Conflict of interest

None.

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Withania somnifera inhibits NF-κB and AP-1 Transcription Factors in Human Peripheral Blood and Synovial Fluid Mononuclear Cells

Divya Singh[1], Amita Aggarwal[1], Rakesh Maurya[2] and [a]Sita Naik[1]*

[1]Department of Immunology, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Lucknow 226 014, India
[2]Medicinal and Process Chemistry Division, Central Drug Research Institute, Lucknow 226 001, India

Withania somnifera **(WS) is an important herb with known antiinflammatory activity. Its molecular mechanism of action has not been investigated. The effect of a WS crude ethanol extract was studied on peripheral blood mononuclear cells of normal individuals and rheumatoid arthritis (RA) patients and synovial fluid mononuclear cells of RA patients *in vitro*. The WS extract significantly suppressed lipopolysaccharide (LPS) induced production of proinflammatory cytokines TNF-α, IL-1β and IL-12p40 in normal individuals and RA patients, but had no effect on IL-6 production at the protein and transcript level. WS also suppressed LPS activated nitric oxide production in the mouse macrophage cell line, RAW 264.7. The extract inhibited nuclear translocation of the transcription factors NF-κB and AP-1 and phosphorylation of IκBα in normal and RA patients' mononuclear cells. HPLC analysis of the crude extract showed the presence of withaferin A and pure withaferin A also inhibited NF-κB translocation.**

The study demonstrated that the WS crude ethanol extract suppressed the production of proinflammatory molecules *in vitro*. This activity is partly through the inhibition of transcription factors NF-κB and AP-1 by the constituent withanolide. The role of additional constituents needs to be studied. Studies on the mechanism of action of the extract may yield potentially useful compounds for the treatment of inflammatory diseases.

Keywords: natural products; immunomodulation; proinflammatory cytokines, rheumatoid arthritis; antiinflammatory agents, nitric oxide; IκB.

INTRODUCTION

Withania somnifera (WS) Dunal (Family Solanaceae) known as Ashwagandha is traditionally used by Ayurveda practitioners for the treatment of arthritis. WS has also been used for its antistress effect and as a tonic, hypnotic and diuretic (Begum and Sadique, 1988; Archana and Namasivayan, 1999; Mishra *et al.*, 2000; Andallu and Radhika, 2000). The root extract of WS has been shown to cause a significant increase in haemoglobin concentration, red and white blood cell counts and body weight in three myelosuppressed mouse models (Ziauddin *et al.*, 1996). It decreased the chemotactic activities of macrophages and TNF-α and IL-1 production in mice treated with ochratoxin, a fungus derived tumor promoter (Dhuley, 1997). The aqueous extract of WS roots prevented lipid peroxidation in stress-induced animals (Dhuley, 1998). Antiinflammatory and immunomodulatory properties have also been reported (Davis and Kuttan, 2000).

Over 35 biologically active chemical constituents of WS have been isolated and characterized (Mishra *et al.*, 2000). Withanolides are ergostane-type steroids with atoms C-22 and C-26, bridged by a D-lactone functionality and an oxidized C-1 position. Recently, two new withanolides with cholinesterase inhibiting activity have been isolated from the whole plant of WS (Choudhary *et al.*, 2004). These compounds are specific to the Solanaceae family, and in particular, to the genus *Withania*, and thus they are used as marker compounds. The biological activities of withanolides, especially of the dominant withaferin A has been studied and reported from the roots and leaves of both species of *Withania* (Shaila *et al.*, 2006). Withanolide D has antitumour activity (Leyon and Kuttan, 2004) and sitoindosides VII and VIII are antioxidants (Bhattacharya *et al.*, 1997). Other withanolides, including their glycosylated products, are reported to have immunomodulatory and other activities (Zhao *et al.*, 2002). The active principles of WS have been shown to be sitoindosides VII–X and withaferin A. Withaferin A isolated from WS roots and leaves have been shown to inhibit NF-κB activity and NF-kB regulated genes (Ichikawa *et al.*, 2006; Kaileh *et al.*, 2007). Recently, a bioactive dimeric thiowithanolide, ashwagandhanolide, isolated from the roots of *Withania somnifera* was found to have inhibitory effects on lipid peroxidation and COX-2 activity (Subbaraju *et al.*, 2006).

So far, few studies have addressed the molecular mechanism of action of WS. Thus, the present study was designed to evaluate the immunomodulatory properties of a crude ethanol extract of WS. It was shown that the WS extract acts on intracellular signaling

* Correspondence to: Professor Sita Naik, Department of Immunology, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Raebareli Road, Lucknow 226014, India.
E-mail: sitanaik@sgpgi.ac.in
Contract/grant sponsor: Department of Biotechnology, Government of India.
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factors thereby showing significant antiinflammatory activities *in vitro*. The extract as well as its isolated compounds should be evaluated as possible therapeutic agents for inflammatory diseases.

MATERIALS AND METHODS

Preparation of ethanol crude extract. The roots of WS were collected from areas around the city of Varanasi and were correctly identified at Banaras Hindu University, Varanasi, India. The dried and powdered roots were suspended in ethanol (95%) (Sigma, St Louis, MO, USA). After constant, gentle shaking for 18 h (overnight) at 37 °C, the alcohol extract was aspirated and stored. This process of extraction was repeated four times and the combined alcohol extract was centrifuged at 1000 rpm for 10 min. The supernatant was retained as an ethanol extract and stored at –20 °C until used.

Sample preparation for HPLC. The 4.5% alcohol extract of WS root powder was prepared by soaking the root powder in absolute alcohol for 18 h with constant shaking at 37 °C. The extracts were filtered through Whatman filter paper No.1. A stock solution of 20 mg/mL of standard withaferin A (Natural Remedies Pvt. Ltd, Bangalore, India) was used.

Chromatography. The standard compound withaferin A from WS was analysed using Shimadzu HPLC system consisting of a pump LC-10ATVP, an automatic sampling unit (Autosampler), SIL-10ADVP, a column oven CTO-10ASVP, a diode array detector SPD-M10AVP and system controller SCL- 10AVP version 5.40. Shimadzu Class VP software version 6.10 was used for data analysis and data processing. The mobile phase consisted of acetonitrile/H_2O; 1:1, v/v). Peaks were assigned by spiking the samples with purified compounds of WS and comparison of the UV-spectra and retention times (Shaila *et al.*, 2006).

Cell culture. Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy volunteers and rheumatoid arthritis (RA) patients by the density gradient centrifugation method (Boyum, 1968) using commercial Lymphoprep (Pel-Freez Clinical Systems, LLC, Norway). Synovial fluid mononuclear cells (SFMC) were isolated from synovial fluid of RA patients. All patients in the study fulfilled the American College of Rheumatology Classification Criteria for diagnosis of RA. Cells were resuspended in RPMI 1640 (Invitrogen, Maryland, USA) supplemented with 25 mM HEPES, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% heat inactivated fetal bovine serum (FBS) (Sigma, St Louis, MO, USA) and stimulated with LPS [(*E. coli* strain 055:B5) (Sigma, St Louis, MO, USA)] in the presence and absence of different doses of WS crude ethanol extract. Control cultures were also set up using the vehicle. Culture supernatants and cells were harvested after 18 h of incubation at 37 °C, 5% CO_2 for ELISA and RNA extraction. The cell viability was assessed by mitochondria-dependent reduction of a yellow tetrazolium dye 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (Sigma, St Louis, MO, USA) to insoluble purple formazan by dehydrogenases (Mosmann, 1982) at the end of 18 h culture. Doses that showed more than 90% cell viability, that is 1.0, 0.5 and 0.2 mg/mL, were included in the study. All glassware/plasticware materials used for the study were treated with E-Toxa-Clean (Sigma, St Louis, MO, USA) to ensure that there was no endotoxin contamination.

Cytokine ELISA. Sandwich ELISAs for the cytokines TNF-α, IL-1β, IL-6 and IL-12p40 were performed according to the manufacturer's instruction (BD Biosciences, Pharmingen, San Diego, CA, USA).

Measurement of nitric oxide. NO was measured as nitrite released from mouse macrophage cells, RAW 264.7, obtained from the National Centre for Cell Science, Pune, India, using Griess reagent (Pandey *et al.*, 2005).

RT-PCR. RNA was isolated by the TRIzol method (Sigma, St Louis, MO, USA) and reverse transcribed using 10 U of AMV reverse transcriptase (Invitrogen, Maryland, USA). PCR was performed using primers (25 pmol) shown in Table 1. GAPDH was used as housekeeping gene.

Electrophoretic mobility shift assay (EMSA). PBMC (4×10^6 cells/well) plated in 6-well tissue culture plates were stimulated with LPS (10 ng/mL) in the presence or absence of varying doses of WS extract for 2 h at 37 °C,

Table 1. Sequences of primers used and expected product size

Target	Primers sequences (5′–3′)	Annealing temperature (°C)	Product size (bp)
TNF-α	F-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA R-GCAATGATCCCAAAGTAGACCTGCCCAGACT	66	444
IL-1β	F-CAGAGAGTCCTGTGCTGAAT R-GTAGGAGAGGTCAGAGAGGC	66	234
IL-6	F-GAACTCCTTCTCCACAAGCG R-GAATCCAGATTGGAAGCATCC	58	316
IL12p40	F-GGCCCAGAGCAAGATGTGTCACCA R-TCTCCAGGGGCATCCGGATACCAA	65	144
GAPDH	F-GAA GGT GAA GGT CGG AGT C R-GAA GAT GGT GAT GGG ATT TC	60	225

F-*Forward*; R-*Reverse*.

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5% CO_2. Nuclear extracts were prepared and EMSA was performed as described previously (Pandey *et al.*, 2005) using following probes.

NF-kB 5′-AGT TGA GGG GAC TTT CCC AGG C-3′
3′-TCA ACT CCC CTC AAA GGG TCC G-5′

AP-1 5′-CGC TTG ATG AGT ACG CCG GAA-3′
3′-GCG AAC TAC TCA GTC GGC CTT -5′

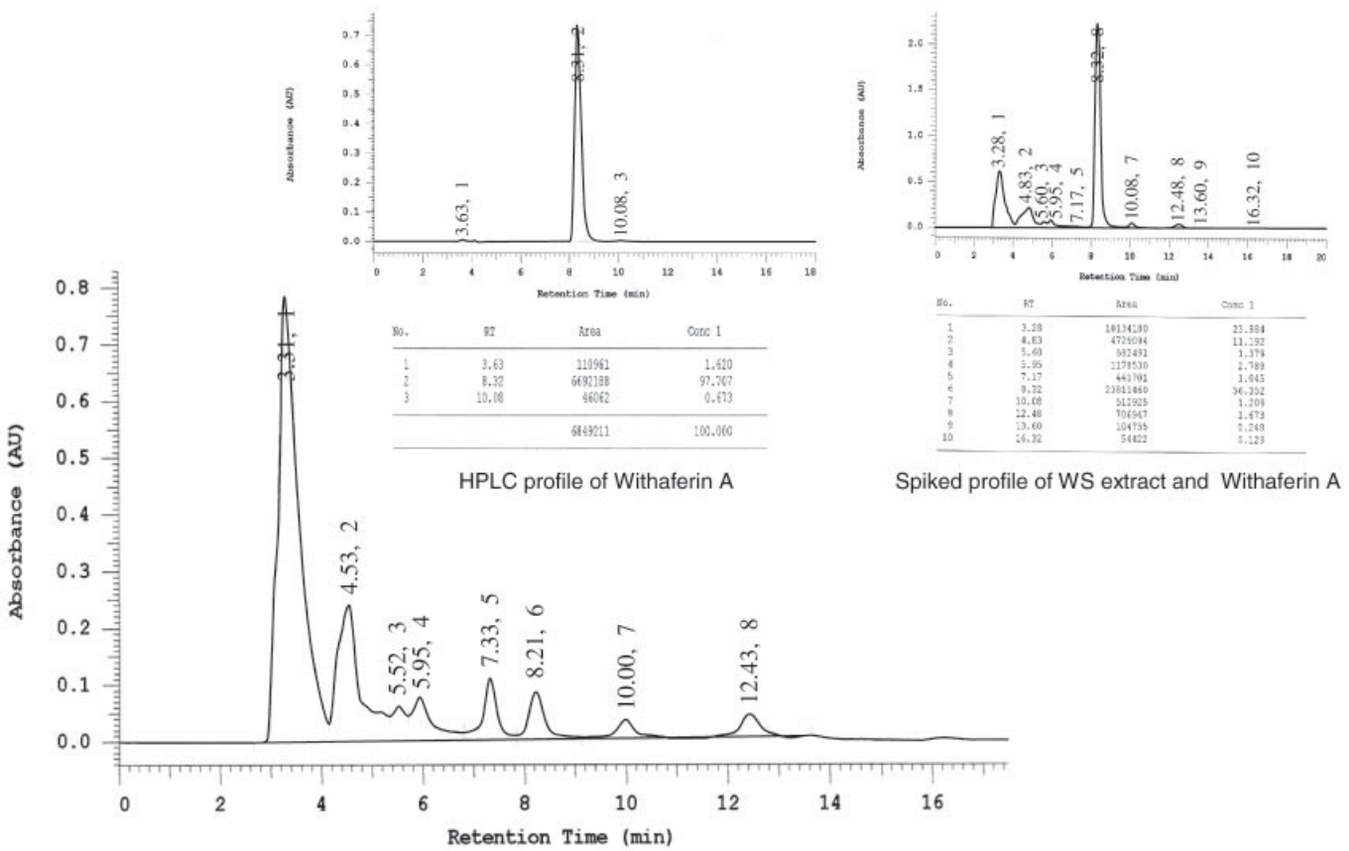
Western blotting. Protein samples (25 μg) were separated on 10% SDS polyacrylamide gels and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were blocked in 5% dry skimmed milk in TBST (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) for 1 h, washed and incubated with human PCNA (1:1000, Sigma, USA) and Phospho-IκBα antibodies (1:1000, Promega, Madison, US) overnight at 4 °C. Membranes were then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (1:1000, Promega, Madison, US). The protein bands were detected by chemiluminescence detection system according to the manufacturer's instruction (Roche Diagnostics, Mannheim, Germany).

Statistical analyses. A non-parametric, Wilcoxon signed ranks test was employed for comparing the untreated group with extract treated groups using statistical software SPSS 9.0. Student's *t*-test was employed for comparing unstimulated with stimulated NO production in RAW 264.7 cells. Results were considered significant at $p < 0.05$.

RESULTS

HPLC analysis of WS crude extract

The HPLC profile of standard withaferin A at an optimum wavelength of 225 nm, showed a mean area of 6692188, at a mean retention time (RT) of 8.32 min The HPLC profile of WS extract showed eight peaks. Spiking of pure reference standard in the extract was used for peak identification. The spiking analysis confirmed the presence of withaferin A in the extract which was observed at a retention time of 8.32 min, with an area of 23811460 at a wavelength of 225 nm (Fig. 1).



No.	RT	Area	Conc 1
1	3.31	11667494	56.856
2	4.53	3842202	18.723
3	5.52	595571	2.902
4	5.95	1176822	5.735
5	7.33	1144953	5.579
6	8.21	974727	4.750
7	10.00	547574	2.668
8	12.43	571926	2.787
		20521269	100.000

Figure 1. HPLC chromatogram of WS extract in comparison with HPLC profile of purified withaferin A.

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Effect of WS extract on LPS activated and spontaneous cytokine production

A dose dependent response of WS extract was observed on spontaneous and LPS activated TNF-α, IL-1β and IL-12p40 production (data not shown) and since the maximum effect was seen with the dose of 1.0 mg/mL, only data for this dose are shown. A significant suppression of LPS stimulated TNF-α, IL-1β and IL-12p40 production was seen with WS extract while IL-6 remained unaffected in normal PBMC. There was also significant suppression of spontaneous production of TNF-α, IL-1β and IL-6, but not of IL-12 p40 production by normal PBMC (Fig. 2). In RA PBMC, LPS activated TNF-α, IL-1β and IL-12p40 production was suppressed while no effect was seen on IL-6 production; the extract suppressed spontaneous production of all four cytokines. In SFMC, the extract suppressed LPS induced IL-1β and IL-12p40 production and showed no effect on TNF-α and IL-6 production; it also suppressed spontaneous production of all four cytokines (Fig. 3).

Effect of WS extract on TNF-α, IL-1β, IL-6 and IL12p40 mRNA expression

Gene expression of all the proinflammatory cytokines was up-regulated by LPS stimulation. The WS extract at a 1 mg/mL dose reduced the LPS induced and spontaneous expression of TNF-α and IL-1β. Basal IL-12p40 was undetected and LPS induced IL-12p40 was suppressed in the presence of the extract; there was no effect of the extract on LPS induced IL-6 expression, while spontaneous IL-6 was suppressed (Fig. 4).

Similar to the effect of the extract in normal PBMC, in RA PBMC and SFMC, WS suppressed the LPS activated TNF-α, IL-1β and IL-12p40 m-RNA. While there was no effect of the extract on LPS activated IL-6, spontaneous expression of m-RNA of TNF-α, IL-12p40 and IL-1β as well as of IL-6 was also suppressed (Fig. 5).

Effect of WS extract on nitric oxide production in RAW 264.7 cells

The WS extract at a 1 mg/mL dose showed significant suppression of LPS induced NO production. However, the 0.5 mg/mL and 0.2 mg/mL doses of the extract had no effect on NO production (Fig. 6). The extract alone showed no effect on NO production (data not shown).

Effect of WS extract and withaferin A on transcription factors NF-kB and AP-1

LPS activated NF-kB and AP-1 were suppressed in normal PBMC in a dose dependent manner. The extract also inhibited spontaneous AP-1 nuclear translocation, however, it showed minimal effect on spontaneous NF-kB (Fig. 7). In RA PBMC and SFMC also, only LPS activated NF-kB and AP-1 were inhibited, while no effect of the extract was seen on the basal levels of these transcription factors (Figs 8 and 9). Withaferin A also inhibited spontaneous and LPS activated transcription factor NF-kB in a dose dependent manner (Fig. 10).

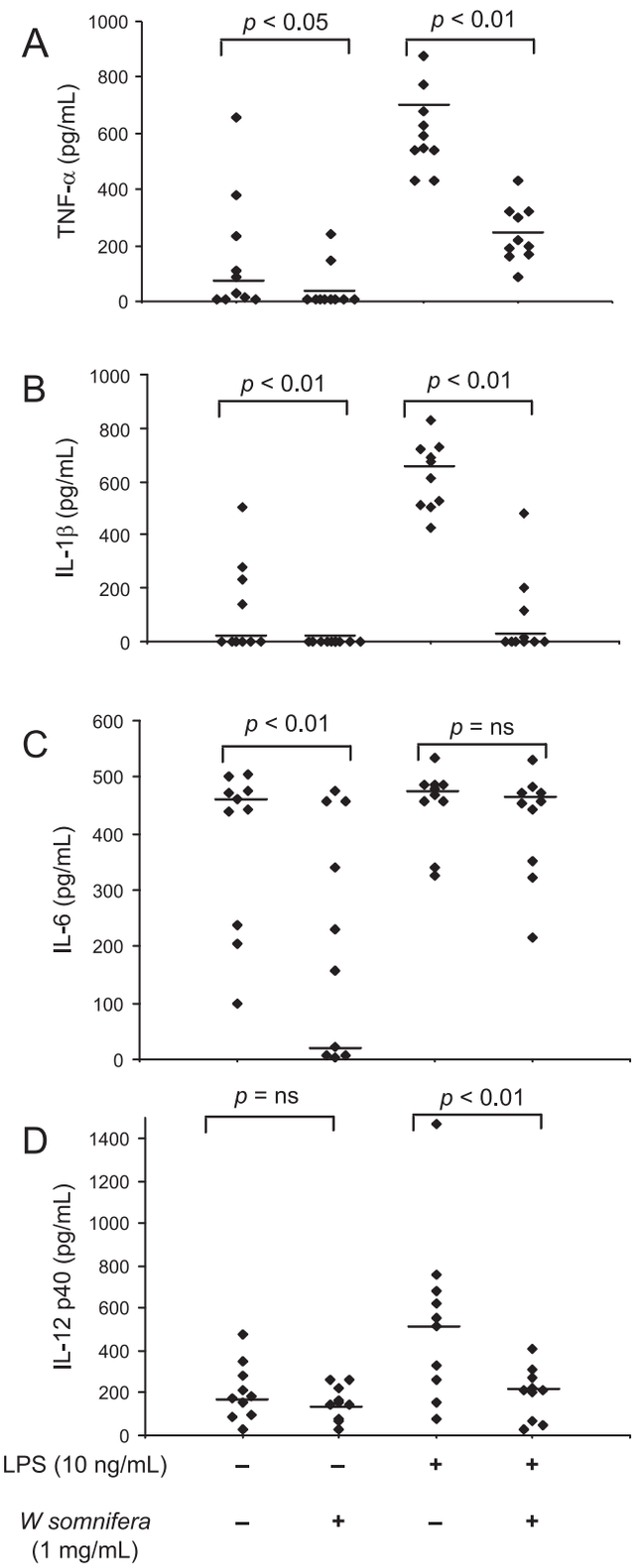

Figure 2. Effect of WS extract on spontaneous and LPS activated production of TNF-α (A), IL-1β (B), IL-6 (C) and IL-12p40 (D) in PBMC of healthy individuals ($n = 10$). (ns = not significant).

Effect of WS extract on phosphorylation of IκBα

Stimulation of normal PBMC with LPS resulted in phosphorylation of IκBα that was seen after 15 min and was maximum at 30 min after stimulation. This phosphorylation was inhibited in the presence of WS extract at 30 min (Fig. 11).

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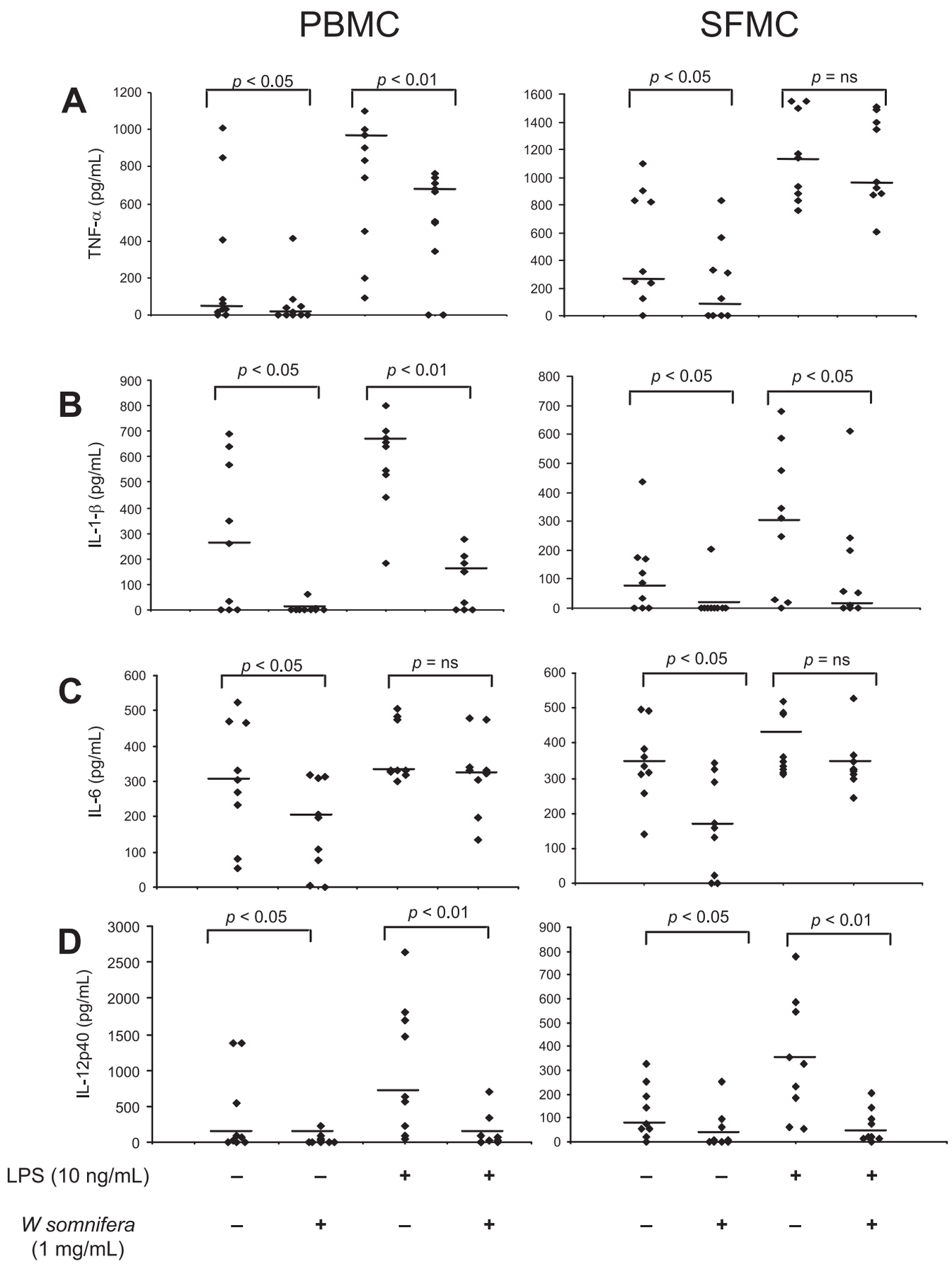

Figure 3. Effect of WS extract on spontaneous and LPS activated production of TNF-α (A), IL-1β (B), IL-6 (C) and IL-12p40 (D) in PBMC and SFMC of RA patients (n = 10). (ns, not significant).

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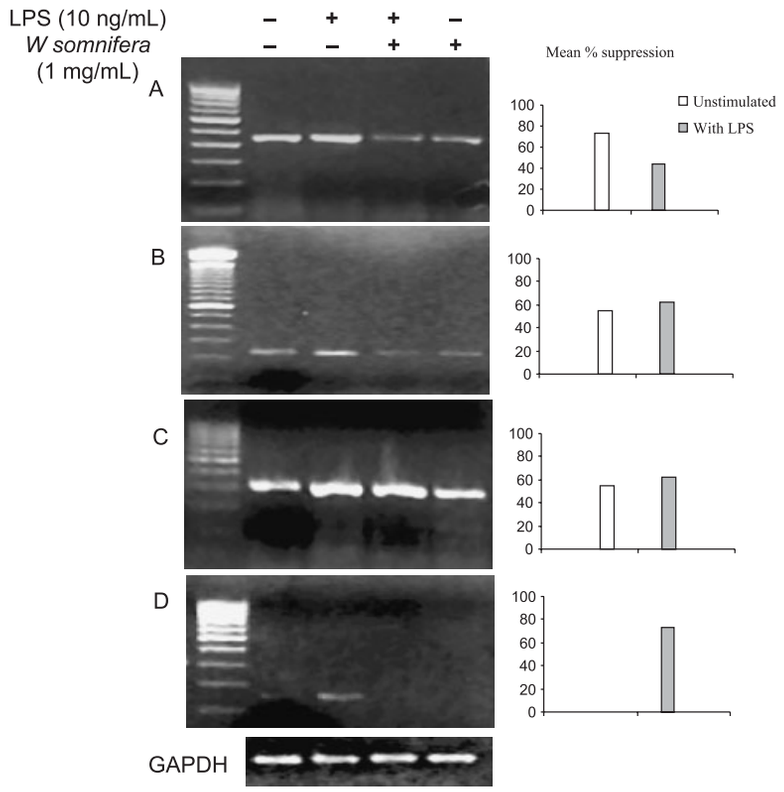

Figure 4. Effect of WS extract on m-RNA expression of TNF-α (A), IL-1β (B), IL-6 (C) and IL-12p40 (D) in PBMC of healthy individuals. Densitometric analysis of three independent experiments showing mean % suppression is shown adjacent to the respective figures.

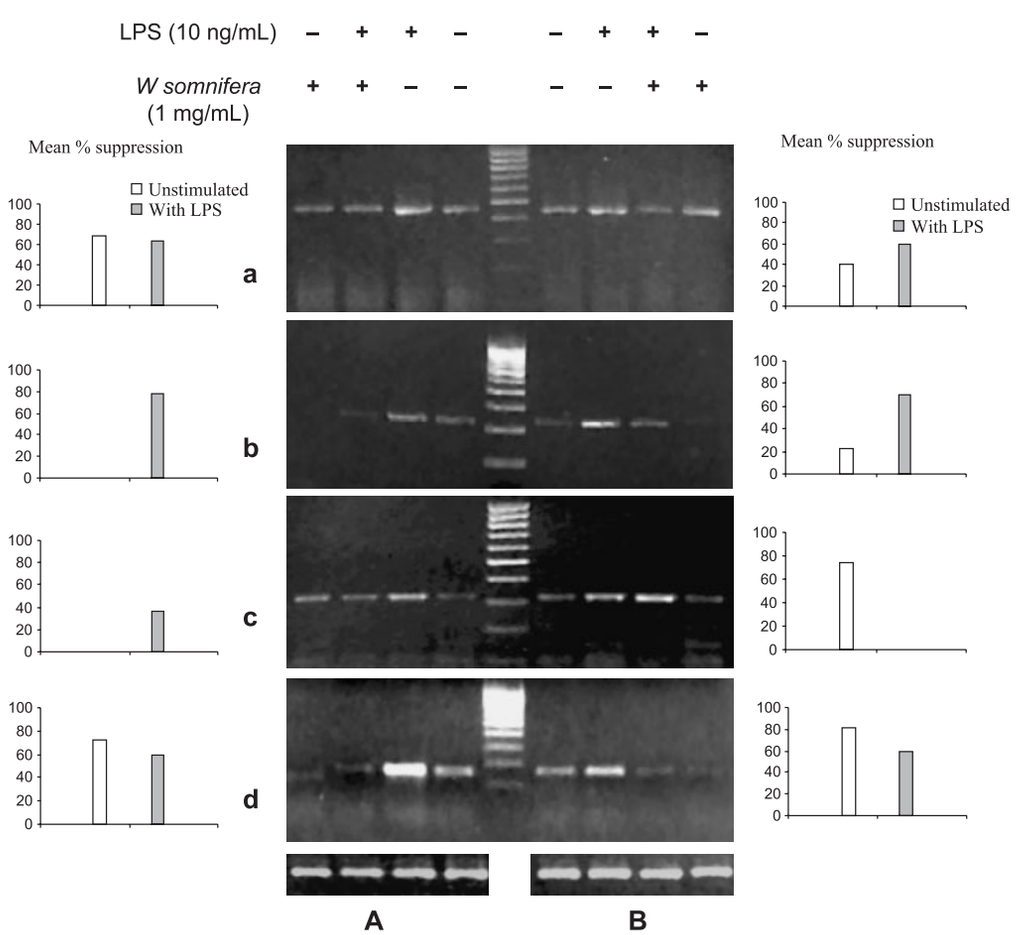

Figure 5. Effect of WS extract on m-RNA expression of TNF-α (a), IL-1β (b), IL-6 (c) and IL-12p40 (d) in SFMC (A) and PBMC (B) of RA patients. Densitometric analysis of three independent experiments showing mean % suppression is shown adjacent to the respective figures.

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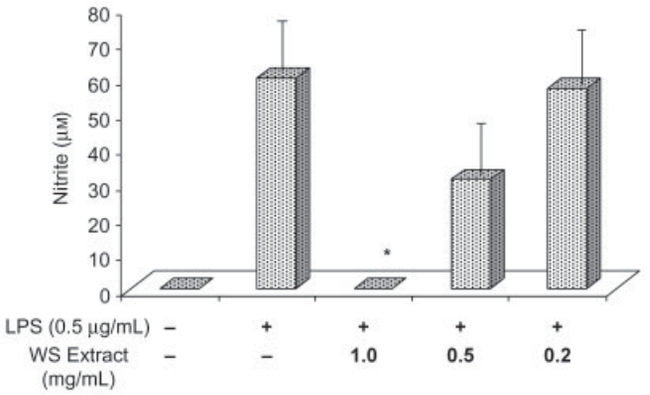

Figure 6. Effect of WS extract on LPS activated NO production in RAW 264.7 cells ($n = 3$). Data are expressed as mean ± SD of three independent experiments. All p values are in comparison with LPS stimulated cultures (* $p < 0.05$).

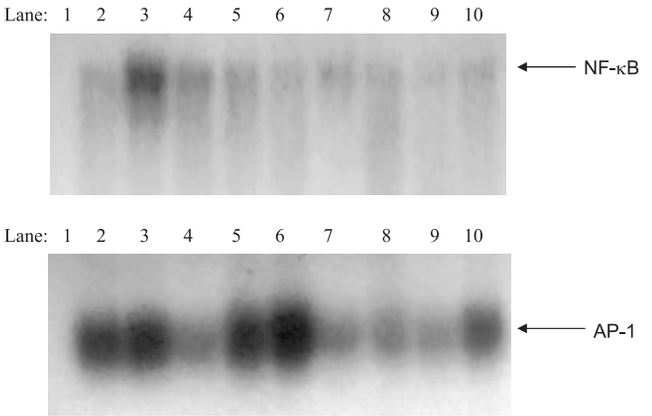

Figure 7. (A) Effect of WS extract on transcription factor NF-κB. Lane 1: Cold Competition; Lane 2: Untreated; Lane 3: LPS (10 ng/mL); Lane 4: WS (0.2 mg/mL) + LPS; Lane 5: WS (0.5 mg/mL) + LPS; Lane 6: WS (1 mg/mL) + LPS; Lane 7: WS (0.2 mg/mL); Lane 8: WS (0.5 mg/mL); Lane 9: WS (1 mg/mL); Lane 10: Vehicle control. (B) Effect of WS extract on transcription factor AP-1. Lane 1: Cold Competition; Lane 2: Untreated; Lane 3: LPS (10 ng/mL); Lane 4: WS (1.0 mg/mL) + LPS; Lane 5: WS (0.5 mg/mL) + LPS; Lane 6: WS (0.2 mg/mL) + LPS; Lane 7: WS (1.0 mg/mL); Lane 8: WS (0.5 mg/mL); Lane 9: WS (0.2 mg/mL); Lane 10: Vehicle control. The result is representative of three independent experiments.

DISCUSSION

The data show the immunosuppressive activity of a crude ethanol extract of WS roots on spontaneous and LPS induced production of proinflammatory cytokines

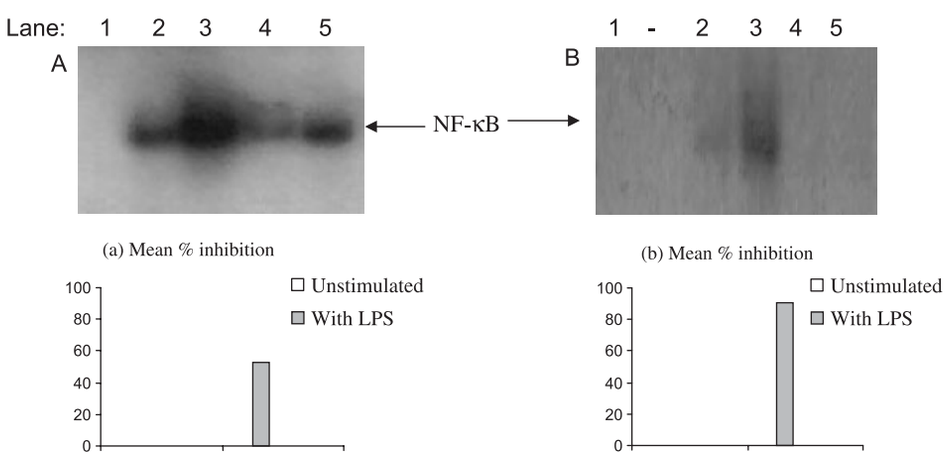

Figure 8. Effect of WS extract on transcription factor NF-κB in PBMC (lanes 1–4) and (lanes 6–9) SFMC of RA patients. Cells were treated with WS extract with or without LPS for 2 h. Lane 1: Cold Competition; Lane 2: Untreated; Lane 3: LPS (10 ng/mL); Lane 4: WS (1 mg/mL) + LPS; Lane 5: WS (1 mg/mL). Densitometric analysis of (a) RA PBMC and (b) RA SFMC showing mean % inhibition of two independent experiments.

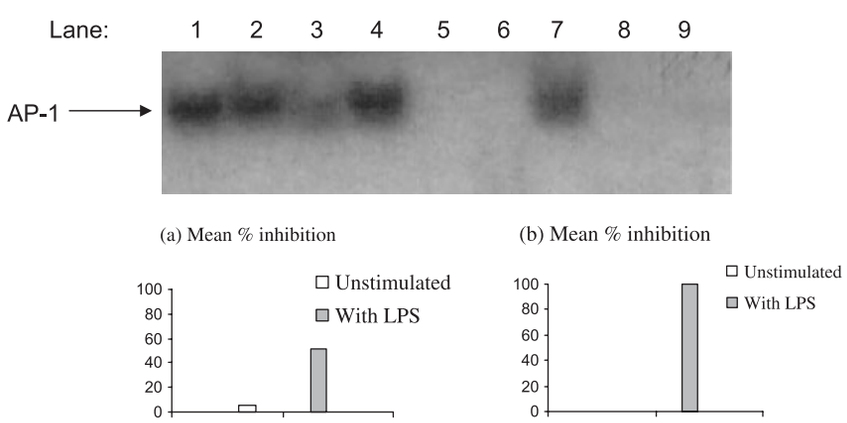

Figure 9. Effect of WS extract on transcription factor AP-1 in (A) PBMC and (B) SFMC from RA patients. Cells were treated with WS extract with or without LPS for 2 h. Lane 5: Cold Competition; Lanes 1 and 6: Untreated; Lanes 2 and 7: LPS (10 ng/mL); Lanes 3 and 8: WS (1 mg/mL) + LPS; Lanes 4 and 9: WS (1 mg/mL). Densitometric analysis of (a) RA PBMC and (b) RA SFMC showing mean % inhibition of two independent experiments.

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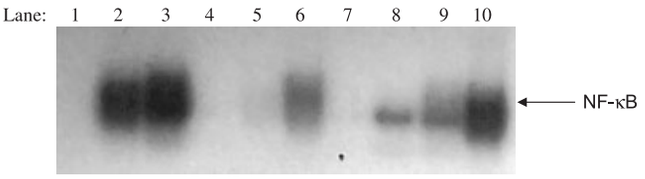

Figure 10. Effect of withaferin A (WfA) on transcription factor NF-κB in PBMC from healthy individual. Lane 1: Cold competition; Lane 2: Untreated; Lane 3: LPS (10 ngm/L); Lane 4: WfA (10 μg/mL) + LPS; Lane 5: WfA (1.0 μg/mL) + LPS; Lane 6: WfA (0.1 μg/mL) + LPS; Lane 7: WfA (10 μg/mL); Lane 8: WfA (1.0 μg/mL); Lane 9: WfA (0.1 μg/mL); Lane 10: Vehicle control. The result is representative of three independent experiments.

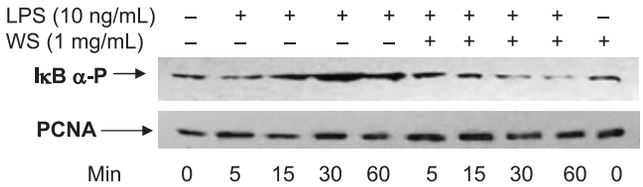

Figure 11. Effect of WS extract on phospho-IκBα. The result is representative of two independent experiments.

at transcript and protein levels, in normal individuals and RA patients. Furthermore, this effect was mediated by the inhibition of nuclear translocation of transcription factors, NF-κB and AP-1, both of which are important for the regulation of proinflammatory cytokines. It was also found that the extract inhibited phosphorylation of IκBα. Withaferin A, which is an important withanolide present in WS, also inhibited the transcription factor NF-kB in a dose dependent manner.

TNF-α and IL-1β are pivotal proinflammatory cytokines involved in the pathogenesis of rheumatoid arthritis (Vassalli, 1992; Dinarello, 1996). Their role in the disease is confirmed by the successful therapeutic use of inhibitors of these cytokines such as Infliximab, Etarnecept (Shahnan and Clair, 2002) and Anakinra (Bresnihan *et al.*, 1998). The proinflammatory cytokines, IL-12p40 and IL-6 are also involved in RA pathogenesis (Feldmann *et al.*, 1996). Here, it was shown that WS extract suppressed spontaneous and LPS activated TNF-α, IL-1β and IL12p40 production. However, only spontaneous production of IL-6 was suppressed in PBMC and SFMC. The extract reduced LPS activated m-RNA expression of TNF-α, IL-1β and IL-12p40 but not of IL-6 in PBMC and SFMC and also suppressed the spontaneous production of all cytokines and this activity was not at the post-translational level. These observations suggest that the extract has differential activity on the production of proinflammatory cytokines.

In this study, it was also shown that the WS extract inhibited LPS activated nitric oxide accumulation in the mouse macrophage-like cell, RAW 264.7. Therefore, suppression of NO by the extract may be of additional benefit in its use as antiinflammatory agent.

NF-κB and AP-1 are involved in the regulation of matrix metalloproteinases (MMPs), cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) that are important mediators of disease in RA (Ghosh *et al.*, 1998; Xie *et al.*, 1994; Karin *et al.*, 1997). It was also shown that WS extract inhibited the translocation of transcription factors NF-κB and AP-1 in LPS activated PBMC and SFMC. The inhibition of NF-κB translocation by the extract is supported by the inhibition of LPS induced phosphorylation of IκBα.

Although anti-TNF-α and anti-IL-1β therapies have been effective in the treatment of RA, their use is limited by their cost and the side effects. Moreover, these therapies are not recommended to be used in conjunction with each other as their combined side effects may be unacceptable (Goldblatt and Isenberg, 2005). Development of natural compounds that cause effective and specific abrogation of inflammatory mediators maybe more cost effective and thus can be exploited as additional strategies for disease arrest. The presence of withaferin A in the extract was confirmed by HPLC analysis and it was shown that pure withaferin A suppressed transcription factors in the mononuclear cells in a dose dependent manner. Thus, the action of the crude extract could be in part through withaferin A. The study suggests that WS and its constituents may be developed for this purpose.

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