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Polyphenol rich extract from *Sesbania grandiflora* (L.) Pers. bark reduces rheumatism by mediating the expression of NF kappa B in rats

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Sesbania grandiflora (L.) Pers. (Fabaceae) commonly called Agati or Vegetable Hummingbird, is autochthonal from Malaysia to North Australia; plant is cultivated in several parts of India. Root and bark paste is applied externally to relive pain and inflammation associated with arthritis. It has long been used as a traditional medicine for rheumatism. Keeping this in cognizance, the study was designed to explore the antirheumatic potential of *S. grandiflora.* The bark extracts were prepared and studied for their phytochemical study, *in vitro* antioxidant potential using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) radicals and antiarthritic activity against Complete Freund Adjuvant (CFA) induced arthritis. To probe into the causal mechanism of action, NFκB suppressing activity in paraventricular nucleus (PVN) of hypothalamus using potent extract was also studied. Polyphenol rich extract supplementation significantly normalizes the altered blood parameters and reverses the increase in paw thickness, a sign of arthritis in rats. Further, immunohistochemical analysis revealed significant reduction in the NFκB immunoreactive cells in 50% methanolic extract treated (14 days) arthritic rats (57%; $p<0.001$) as compared to control. These results consolidate the observation, that inhibition of NFκB may be a beneficial approach in the treatment of arthritis. This study corroborates the traditional use of *S. grandiflora* plant in rheumatism.

Keywords: Agati, Antioxidant, Antirheumatic, Immunohistochemistry, NFκB, Vegetable Hummingbird

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease affecting approximately 1% of the people worldwide irrespective of race. It is often accompanied by an array of articular and extra-articular manifestations[1]. Proinflammatory cytokines, notably interleukin one (IL-1) and tumor necrosis factor-α (TNF-α) plays a vital role in initiating and perpetuating inflammatory and harmful processes within the arthritic joint. Additionally, several evidences indicated an important role of nuclear factor kappa B (NFκB) within the etiology of RA. In patients with RA, activation of the NFκB in synovial cells ends up in the transactivation of an oversized variety of responsive genes that contribute to the inflammatory phenotype, together with TNF-α, chemokines and cytokines that conscript immune cells to the inflamed pannus[2]. Importantly, the genes coding TNF-α and plenty of the other factors mentioned on top are currently better known to be under the regulation of NFκB transcription factors[3] suggesting that NFκB could be one of the master regulators of inflammatory cytokine production in RA.

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The increasing interest in characterizing NFκB involvement in mediating inflammatory pain stems from the large number of genes and cellular processes that it regulates. This transcription factor contributes to controlling developmental processes, neuronal plasticity, synaptic transmission, death and cellular defense[4]. Some of the genes whose expression NFκB modulates are nitric oxide synthase, cyclooxygenase (COX), cytokines, adhesion molecules, acute-phase proteins and dinorphine[5]. Regarding inflammatory pain, it was shown that intrathecal treatment with NFκB inhibitors reduces thermal and mechanical hyperalgesia after peripheral inflammation induced by complete freund adjuvant (CFA) in rats[6]. Thus, inhibition of NFκB could reduce the expression of inflammatory genes and is a mechanism by which anti-inflammatory agents might elicit their antiinflammatory effects. Taking into account these evidences, in the present study, we investigated whether activation of NFκB is involved in the rheumatoid arthritis and subsequently reductions in their expressions alleviate the inflammation following the administration of CFA.

Sesbania grandiflora (L.) Pers. (Fabaceae), commonly called Agati or Vegetable Hummingbird,

is autochthonal from Malaysia to North Australia and is cultivated in several parts of India[7]. The plant is widely eaten for culinary purposes. It is eaten as steamed vegetable in Southeast Asia. It is mixed into soups, salads and curries called *sudhu hodhi* in Sri Lanka. It is also used in cooking for preparing recipes. Previous studies reported that the plant have been shown to possess biological activities viz. antioxidant, antiurolithiatic, anticancer, antiulcer, hypolipidemic, antibacterial, antimicrobial, anthelmintic, anxiolytic and anticonvulsive, hepatoprotective, antituberculosis and in ulcerative colitis[8]. The reported phyto-constituents were isoflavonoids and betulinic acid, sterols, saponin, tannins, polyphenol and flavonoid[9].

Traditionally, the plant has been used in the treatment of headache, catarrh, fever, jaundice and eye infections. In Ayurveda, the fruits are used as alexeteric, laxative, prescribed as medical aid for anaemia, bronchitis, fever, pain, thirst, and tumors. The flowers are used as apertif and refrigerant and for the treatment for biliousness, bronchitis, gout, nyctalopia, ozoena and quartan fever. The leaves are used as alexeteric, anthelmintic, for a epilepsy, gout, itch, leprosy, nyctalopia and ophthalmia [7]. The bark is used in treating smallpox, eruptive fevers, for dysentery, as laxative, emetic and hemoptysis. A decoction of the bark is used in small doses for diarrhoea, also for inflammation of stomach, ulceration of alimentary canal, for infantile disorders of stomach, tumours and glandular enlargements and for dispelling toxins. Externally, a paste of root is applied for painful swelling, for scabies and skin eruptions[10]. Aqueous decoctions of the powdered roots of the red-flowered variant provide relief on rheumatic swellings[7]. Root and bark paste are applied externally to relive pain and inflammation associated with arthritis and gout[11].

As most of the literature supports the use of roots and root bark of the plant for treating various ailments related to rheumatic swelling and pain inflammation. The sustainable management of roots and root bark for the commercial preparation of formulation is always questionable, since uprooting the roots will have devastating effect on the tree. Keeping this in cognizance, here, we explored the antirheumatic potential of *Sesbania grandiflora* bark extracts. The extracts were prepared and studied for their phytochemical study, *in vitro* antioxidant potential using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) radicals and antiarthritic activity against complete freund adjuvant (CFA) induced arthritis. To probe into the causal mechanism of action, NFκB suppressing activity in paraventricular nucleus (PVN) of hypothalamus using potent extract was also studied.

Materials and Methods

Chemicals and standard drugs

Indomethacin and complete Freund's adjuvant (CFA) were purchased from Sigma-Aldrich chemicals, Powai, Mumbai. NFκB p 65 Antibody (A) (cat. No. Sc-109 Santa Cruz Biotechnology, USA) was obtained from Dr. Gopal C. Kundu, National Centre for Cell Science, Pune as a generous gift.

Plant material and preparation of extract-

Sesbania grandiflora barks were collected and authenticated. The Herbarium with voucher specimen number RA 9580 was prepared, identified and deposited at Department of botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. Bark was dried and milled to a coarse powder. One kg of fresh plant material (Barks) was grounded and defatted with petroleum ether. It was extracted subsequently with ethyl acetate, acetone and methanol in a Soxhlet apparatus followed by maceration with 50% methanol for 7 days. The organic solvents were evaporated using rotary vacuum evaporator to yield ethyl acetate extract (EASG, yield: 2.5% w/w), acetone extract (ACSG, yield: 4.54% w/w), methanolic extract (MESG, yield: 10.78% w/w) and 50 % methanolic extract (HASG, yield: 11.44% w/w). Phytochemical and pharmacological evaluation of these extracts was undertaken.

Phytochemical screening-

Freshly prepared plant extracts were assessed for the presence of different phytochemical viz. flavonoid, saponins, alkaloids, carbohydrates, proteins, tannins, etc. by employing standard method described previously.

Determination of active chemical constituents

Total polyphenol contents (TP)

Total polyphenol content was determined spectrophotometrically using Folin-Ciocalteu's (FC) colorimetric method[12]. Gallic acid was used as a reference standard. Briefly, 0.1 mL of test sample in triplicate was mixed with 1.0 mL of diluted FC reagent (1:10 with distilled water). After 3 min, 1.0 mL of saturated sodium carbonate (75 g/L) solution was added and adjusted to 10 mL with

distilled water. After incubating the reaction mixture for 90 min, the absorbance was measured at 725 nm (Shimadzu UV-VIS spectrophotometer 1600) against standard blanks prepared without the FC reagent. The results were expressed as mg of Gallic acid equivalents (GAE)/g of extract.

Total flavonoids (TFA)

Flavonoid content was determined spectrophotometrically using aluminium chloride method[13]. Rutin was used as a reference standard. Briefly, 1.0 mL of test solution (1.0 mg/mL) in triplicate were mixed with 0.1 mL of 10% aluminum chloride hexahydrate ($AlCl_3.-6H_2O$), 1.5 mL of alcohol (95%), 0.1 mL of 1 M sodium acetate and 2.3 mL of distilled water in reaction flask. After incubating the reaction mixture for at room temperature for 40 min its absorbance was read at 435 nm against corresponding blanks prepared without adding $AlCl_3.-6H_2O$. The results were expressed as mg of rutin equivalents (RE)/g of extract.

Total flavanones (TFO)

Flavanone content was determined spectrophotometrically using modified 2,4-dinitrophenylhydrazine (DNPH) method[14]. Naringin was used as the reference standard. Briefly, Twenty milligrams of Naringin was dissolved in methanol and a stock solution of 500, 1000 and 2000 µg/mL was prepared. 1.0 mL of stock solution mixed with 2 mL of DNPH reagent (1%) and 2 mL of methanol were heated at 50 °C for 50 min. After cooling at 36□, the reaction mixture was mixed with 5 mL of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then, 1.0 mL aliquot of resulting mixture was diluted with 5 mL of methanol and centrifuged at 1000 rpm for 10 min to remove the precipitate. The supernatant was collected, adjusted to 25 mL and its absorbance was measured at 495 nm. All the extracts in triplicate were reacted with DNPH for determination of flavanone as described above. The results were expressed were expressed as mg of Naringin equivalents (NE)/g of extract.

In vitro antioxidant studies

Hydrogen atoms or electrons donation ability or radical scavenging capacity of *S. grandiflora* extracts was measured using DPPH and NO radicals.

DPPH radical scavenging method

The DPPH free radical scavenging activity of extracts was determined using the method described previously[15]. Ascorbic acid was used as reference standard. Briefly, test samples and standard solutions were diluted to a series of concentration (0.001-2 mg/mL). Then, 0.1 mL aliquot of sample and standard in triplicate was mixed with 4.9 mL of 50 µM DPPH solution (buffered at pH 5.5). After incubation for 30 min in dark, the absorbance was read at 517 nm. The results were expressed as IC_{50} values (concentration of extract that inhibited DPPH % formation by 50%) calculated by linear regression method.

NO radical scavenging method

NO free radical scavenging activity of extracts was determined using Griess reagent [0.1% w/v N-(1-Naphthyl) ethylenediamine + sulphanilic acid (0.33% w/v; 1:1)][16]. Ascorbic acid was used as reference standard. Briefly, test samples and standard solutions were diluted to a series of concentration (0.001-2 mg/mL). Then, 1.0 mL aliquot of sample and standard in triplicate was mixed with 4 mL of sodium nitroprusside (5 mM) and 1 mL phosphate buffer saline (pH 7.4). The reaction mixture was incubated at 25^o C for 2.5 hrs. After incubation, 4 mL of Greiss reagent was added into1 ml of the reaction mixture and allowed to stand for 30 min. The reaction produced a red-violet colored chromophore with a maximum absorbance at 540 nm. The results were expressed as IC_{50} values calculated by linear regression method.

Evaluation of anti-arthritic activity in rats

Animals-

Male Wistar albino rats (175-200 g) were used for experimental studies in compliance with the protocols approved by the Institutional Animal Ethical Committee (10/2010/CPCSEA).Animals were kept in a controlled environment and temperature (22 ± 5ºC with 12 h of light/dark cycle) with a standard pellet diet and water *ad libitum*.

Acute toxicity studies

The acute toxicity studies were done as described in the OECD manual[17]. It comprised of test and control groups (n=6). Ethyl acetate, acetone, methanolic and hydroalcoholic extracts were administered in an increasing oral dose of 1, 3 or 5 g/kg. The animals were kept under regular observation for behavioural changes, adverse symptoms and mortality for 48 h following administration of extracts. During the studies, animals were on fed state.

Induction of experimental arthritis

Arthritis was induced in rats by the method described previously[18]. Briefly, 100 µL of CFA (a suspension of heat killed *Mycobacterium tuberculosis* in mineral oil (10 mg/mL) was injected

intradermally into left hind paw. The injection day was thought of as day 0.

Experimental design and treatment schedule

Arthritic animals were randomly divided into 10 groups (n = 6 rats per group) and treated as follows: Group I, arthritis control received vehicle with (5% DMSO + PBS) with CFA; Group II, positive control received Indomethacin (10 mg/kg; *p.o.*) with CFA; Group III to X: received EASG, ACSG, MESG or HASG extract (200 and 400 mg/kg; p.o.) with CFA injected in paw. CFA was injected only once on day 0, treatment with indomethacin and extracts were administered each day starting from day 0 to 14. The left hind paw thickness of all animals was recorded on day of injection whereby on 4, 8, 14 and 21 days of treatment using Digital Vernier calliper. The severity of inflammatory response was evaluated on the basis of increase in paw thickness. The body weight of all animals was measured every day.

Hematological and biochemical parameters estimation

On the 22nd day of post-arthritis induction, blood was withdrawn through the retro-orbital vein puncture from all rats for estimation of hematological and biochemical parameters. An aliquot of collected blood was centrifuged at 4^0 and serum was separated. Total white blood cells (WBC) count, red blood cells (RBC) count, hemoglobin (Hb) concentration were assessed using SYSMEX XP 100 Haemat Analyzer. ESR was estimated by Westergren method. Biochemical parameters such as albumin was assessed by prietest TOUCH biochemistry analyser (ROBONIK, India) using Bromocresol Green end Point method, while total protein was estimated using Biuret method.

HPLC analysis of HASG extract-

HASG extract was standardized with rutin as a biomarker using HPLC system (Shimadzu, Model SPD 10 AVP, Japan) equipped with PDA detector and C_{18} column (250 × 4.6 mm, 5 µm particle). The injection volume and flow rate were kept at 10 µL and 1.0 mL min^{-1}, respectively. The mobile phase consisted of two different solutions as acetonitrile (solution A) and 50 mM phosphate buffer, pH 2.5 (solution B). The elution was started with a mobile phase composition of 5:95 (aqueous phase: acetonitrile) to 25:75 for first 15 min and for next 35 min, the composition was changed from 25:75 to 60:40 followed by re-equilibration for another 10 min, using composition of 5:95. Detecting wavelength was kept at 356 nm and the peak of rutin was identified and confirmed by comparing its retention time with that of standard[19].

Immunohistochemical evaluation-

Arthritic animals were randomly divided into 3 groups (n = 5 rats per group) and treated as follows: Group I, arthritis control received vehicle with (5% DMSO + PBS) with CFA; Group II, positive control received Indomethacin (10 mg/kg; *p.o.*) with CFA;and Group III, received HASG extract (400 mg/kg; *p.o.*) with CFA injected in paw.

After adjuvant induction, chronic activation of the HPA axis takes place and found maximum on day 14 during 7 to 21 days of study[20]. Thus, on 14th day, when the swelling appears on right hind paw, animals from above group were perfused transcardially. Brain of standard and extract treated animals, along with arthritic control were processed for immunohistochemical profiling with NFκB p65 antibody using method optimised previously[21]. Briefly, the animals were anesthetized deeply with thiopentone sodium (65 mg/kg**,** intraperitoneal), perfused transcardially with heparinized phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Coronal brains were processed for NFκB immunolabeling, primary antibody. Sections were incubated in rabbit polyclonal antibody against NFκB p65 (dilution 1:1000) diluted in 1% bovine serum albumin (BSA; Sigma) containing 0.3% Triton X-100, 0.2% Kodak Photo Flo solution and 0.08% sodium azide solution. After washing with PBS, the sections were incubated with secondary antibody biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; 1:400) for 2 h and later in ExtrAvidin-peroxidase conjugate (Sigma, St. Louis, MO, USA; 1:100) for 45 min at room temperature, respectively. After washing, NFκB-immunoreactions product (reddish brown) was developed in solution of 3-amino-9-ethyl-carbazole containing 0.03% hydrogen peroxide (AEC, Sigma).

Morphometric analysis

The quantification of NFκB immunoreactive cells was assessed using microscopic images from predetermined areas in the images captured via DFC 450 C video camera system (Leica Microsystems, Germany) in the PVN. The images (X480) were analyzed using Image J software (NIH, USA). Five measurements from predetermined fields of the PVN on both sides of each brain were taken. The data from all the animals in each group were pooled separately

and the mean ± standard error of mean (SEM) was calculated. The percentage (%) immunoreactive area occupied by NFκB was evaluated from different treatment groups using microscopic images. Sections passing through the pre-determined areas were used for image analysis. The images of the brain regions were digitized, the background was considered as threshold, and percent areas occupied by immunostained cells were measured based on individual pixel intensity in all the groups using Image J software (National Institutes of Health, Bethesda, MD). Ten measurements of both sides of the five sections of each animal were averaged (thus average reading represents an individual animal) and data from five brains in each group were collated. The images were evaluated separately by the observer blind to the treatments. The data are represented as a mean ± SEM for statistical analysis.

Statistical analysis

The results concerning, *in vitro* antioxidant activity, anti-arthritic study, changes in blood parameters and body weight were expressed as mean ± SEM Statistical comparison was carried out with treatments and the control groups using two-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni multiple comparisons test. The immunohistochemical data were analyzed by unpaired t-test. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Phytochemical screening and quantification of extracts

The results of phytochemical screening of extracts showed the presence of tannins, flavonoids, proteins, carbohydrates, tannins and saponins. Particularly, MESG and HASG extracts were good source of different phytochemical viz., proteins, carbohydrates, tannins, saponin and flavonoids. ACSG extract showed the presence of proteins, tannins and flavonoids. While terpenoids, tannins and flavonoids were detected in EASG extract. Flavonoids belongs to polyphenol group and are known for health benefits such as antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer properties[22]. Correspondingly, these extracts were quantified for the presence of polyphenol; flavonoid and flavanone content (Table 1).

The polyphenol content of different extracts was varied to great extent and ranged from 8.03 to 33.0 mg/g (GAE, mg/g of extract). HASG extract demonstrated higher polyphenol content amongst other extract and which was determined using linear regression equation of Gallic acid ($y = 0.005x + 0.075$, $r^2 = 0.998$). Amongst the extract, the highest flavonoid content was recorded in HASG extract followed by ACSG, EASG and MESG extract respectively (range from 1.39 to 4.33, RE, mg/g of extracts). It was determined using equation of rutin ($y = 0.018x - 0.001$, $r^2 = 0.990$).The flavanone content was determined using linear regression equation of naringin ($y = 0.006x + 0.020$, $r^2 = 0.995$) and added to flavonoid content for estimation of total flavonoid. The total flavonoid was found maximum in HASG extract. High phenolic content indicates the capacity of plant to cure inflammatory diseases[23].

***In vitro* antioxidant activity**

Antioxidants when present at low concentrations compared to that of an oxidizable substrate significantly delay or inhibit oxidation of that substrate[24]. Therefore, it is important to compare different analytical methods varying in their oxidation initiators and targets in order to understand the biological activity of an antioxidant. Since the free radicals are enormously produced at the inflammatory site and for that reasons, the radical scavenging activity of plant extracts using DPPH and NO methods were measured. The results of DPPH scavenging activity showed that the IC50 values of HASG extract (37.50 µg/mL) were significantly lower amongst other extracts (Table 1). This may be attributed due to strong

Table 1 — Total polyphenol, flavonoid, flavanone, total flavonoid content and antioxidant potential of different extracts of *Sesbania grandiflora*

Extracts	Total polyphenol content (TP) (GAE mg/g of extract)	Flavonoid content (TFA) (RE mg/g of extract)	Flavanone content (TFO) (NE mg/g of extract)	Total flavonoid content (TF)[#]	Antioxidant activity (IC 50 values in µg/ mL) DPPH NO
EASG	28.90 ± 0.45	3.791± 0.41	0.750 ± 0.54	4.54	56.23 620.45
ACSG	27.40 ± 0.80	4.104 ± 0.14	0.727 ± 0.12	4.83	49.26 518.71
MESG	8.033 ± 0.37	1.391 ± 0.08	0.822 ± 0.23	2.21	70.84 801.73
HASG	33.00 ± 0.91	4.338 ± 0.05	0.788 ± 0.04	5.12	37.50 396.17

[Results are mean ± SD of three replicates: GAE, RE and NE: gallic acid, rutin and naringin equivalents, respectively. [#] Total flavonoid content is determined by adding flavonoid content with flavanone content; EASG, ethyl acetate extract of *S.grandiflora*; ACSG, acetone extract of *S.grandiflora;* MESG, methanolic extract of *S.grandiflora*; HASG, hydroalcoholic extract of *S. grandiflora*]

hydrogen donating ability of polyphenol present in HASG extract to reduce DPPH and the weak abilities of other extracts[25]. Similarly, HASG extract showed significant antioxidant potential against NO radical due to its high polyphenol content. Thus, polyphenol are noteworthy contributors to measured activities. This is in accordance with previously reported results of having strong correlation between polyphenol and antioxidant potential of plant extracts.

Acute toxicity and antiarthritic efficacy

Acute toxicity studies revealed no lethality or toxic reactions upto 5000 mg/kg administered orally. After sub plantar administration of CFA in the rat paw, progressive the increase in rat paw thickness, a sign of arthritis was observed. Decrease in paw swelling is an index for the antiarthritic activity of various drugs in CFA-induced animal model of arthritis. Indomethacin was used as reference standard which significantly reduced the paw thickness as compared to arthritic control rats treated with saline.

Biphasic response was observed in extract treated animals consisting of developing and developed phases of arthritis. Both ACSG and HASG extract were found effective in developing phase of arthritis on day 4 and 8. However, polyphenol rich HASG extract showed significant activity on 14 and 21 days of study representing its effectiveness in developed phase of arthritis. Although, the polyphenol and flavonoid content of both the extracts are comparable but ACSG extract showed only 46 and 57% inhibition on 14 and 21 days while HASG extract shows 24, 40, 56.4 and 76.8% inhibition of paw thickness on day 4, 8, 14 and 21 of study, respectively (Table 2). HASG extract contain additional phytoconstituents as saponin thus synergism of these constituents with polyphenol and flavonoid may have resulted in significant antiarthritic activity in the later stages of arthritis. As reported, saponin like diosgenin reduces the production of inflammatory mediators by inhibiting NF-κB and AP-1 activation[26]. This result is in conformation with previous findings that saponin rich fraction was found effective in later phase of adjuvant induced arthritis[27].

The EASG extract was also found significant on 14 and 21 days of study. It shows 47 and 64% inhibition on 14 and 21 day. This may be attributed due to the presence of terpenoid in the extract. It was reported previously, celastrol a triterpine, was found effective when administrated both in the early and more established phase of arthritis[28]. None of the extract was found effective in both the phases except HASG extract.

Effect of extracts on haematological parameters-

Significant changes in haematological (RBC, WBC, Hb and ESR) were recorded (Fig. 1A). RBC and haemoglobin count decreased significantly, while number of WBC and ESR was increased in arthritic rats as compared to normal rats. Anemia due to abnormal storage of iron in the reticuloendothelial system and synovial tissue and the failure of bone marrow to respond to anemia is commonly observed in patients with chronic arthritis[29]. Inflammation challenges immune system against the invading pathogenic microorganism results in elevated WBC and platelet. Also, production of granulocyte and macrophages colony stimulating factors due to release of interleukins raises WBC in RA[30]. ESR is an estimate of the suspension stability of RBC in plasma. Estimation of ESR is common tool for diagnosis of inflammatory disease including RA. Elevated ESR points to increase inflammation in the body[31].

Table 2 — Effect of different extracts of *Sesbania grandiflora* on % inhibition of paw thickness in adjuvant induced arthritic rats

Treatment[#]	% Inhibition of paw thickness			
	Day 4	Day 8	Day 14	Day 21
Indomethacin (10 mg/kg)	33.031***	54.40	69.94	86.78***
EASG (200 mg/kg; *p.o.*)	9.52	24.28	34.28	42.38***
EASG (400 mg/kg; *p.o.*)	23.52	31.76	47.45***	64.31***
ACSG (200 mg/kg; *p.o.*)	6.66	16.66***	23.00	33.33***
ACSG (400 mg/kg; *p.o.*)	25.15***	39.23***	46.78***	57.84***
MESG (200 mg/kg; *p.o.*)	4.347	6.95	13.47***	30.86
MESG (400 mg/kg; *p.o.*)	16.80	25.21	37.81***	46.21
HASG (200 mg/kg; *p.o.*)	13.4***	23.71***	33.50***	43.81***
HASG (400 mg/kg; *p.o.*)	24.00	40.00***	56.4***	76.8***

[The data are expressed in mean ± S.E.M. $n = 6$ in each group; EASG, ethyl acetate extract of *S.grandiflora*; ACSG, acetone extract of *S. grandiflora;* MESG, methanolic extract of *S.grandiflora*; HASG, hydroalcoholic extract of *S. grandiflora*; [#]All groups received CFA 100 µg, (Sub Planter) at day zero and individual treatment as per groupings. * Represents statistical significance *vs*. arthritic control (P <0.05); ** Represents statistical significance *vs*. arthritic control (P <0.01); *** Represents statistical significance *vs*. arthritic control (P <0.001)]

The hematological parameters were found altered in arthritic control group, which has been remarkably countered by treatment with *S. grandiflora* extracts and standard drug indomethacin. These results may be attributed to the presence of polyphenol and saponin. Previously, it was reported that, treatment with citrus polyphenol and hot pepper extract significantly decreases ESR[32]. Also, saponin from *Achyranthes aspera* reversed the haematological changes resulting from arthritis[27]. Thus stabilizing these altered parameters to normal level is indicative of its antiarthritic potential.

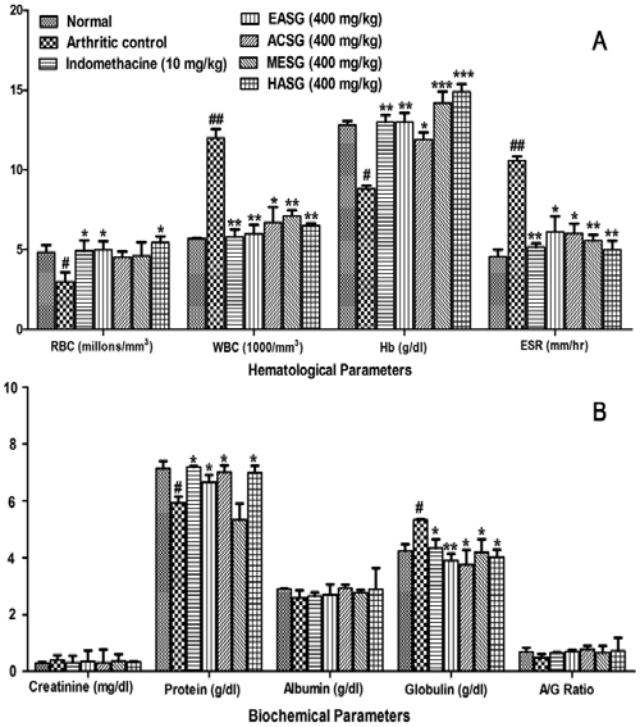

Fig. 1 — Effect of extracts on (A) hematological parameter in CFA model [On the 22nd day post-arthritis induction, hematological parameters (hemoglobin, Hb; red blood cells, RBC; white blood cells, WBC and estimated sedimentation rats, ESR) were estimated in all the treatment groups. The hematological parameters were found altered in arthritic control group, which has been remarkably countered by treatment with *S. grandiflora* extracts and standard drug indomethacin]; and (B) biochemical parameter in CFA model [On the 22nd day post-arthritis induction, biochemical parameters were estimated. Extract and standard treated rats restored these altered biochemical parameters (Creatinine, albumin, globulin and A/G ratio) to near normal level]. [The bars values are expressed as the mean ± SEM. Significant reduction in parameters was analyzed by one-way ANOVA followed by *post hoc* Bonferroni multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001*vs*. CFA control. EASG, ethyl acetate extract of *S.grandiflora*; ACSG, acetone extract of *S.grandiflora;* MESG, methanolic extract of *S.grandiflora*; HASG, 50% methanolic extract of *S.grandiflora*]

Effect of extracts on biochemical parameters and body weight

Significant changes in biochemical parameters (creatinine, albumin, globulin and A/G ratio) were recorded (Fig. 1B). Elevated creatinine and globulin fraction while reduction in albumin fraction are the manifestations found in arthritic rats reported previously[33]. However, it should be noted that mediators such as histamine and bradykinin increases permeability of vascular tissues to albumin results in decrease serum albumin levels[34]. In the present study, results revealed an alteration in biochemical parameter as mentioned above. Extract and standard treated rats restored these altered parameters to near normal thus justifying its effectiveness in arthritis.

During experimental study, it was observed that, as the disease progresses, the changes in body weight of animals also occur. It was supported with the earlier findings that the absorption of nutrients from intestine was reduced in the case of inflamed rats[35]. Here, the arthritic rats showed noticeable weight loss during developing phase followed by normal weight gain in developed phase of arthritis. Extract and standard treated rats did not show any weight loss**.** (Data is not shown).

HPLC analysis of HASG extract-

The HPLC analysis confirmed the presence of rutin in HASG extract, which was reported to be 2.7 % w/w (Fig. 2). Rutin, a natural flavonoid, inhibits NO and TNF-α productions thus attenuating the process of inflammation. Rutin also prevent the transcription of genes responsible for pro-inflammatory factors such as TNF-α in macrophages[36]. Rutin has also been reported to assuage the oxidative stress and cytokine level via reducing the expression of NFκB in CFA[37].

Effect of HASG extract on NFκB-immunoreactivity in arthritic rats

Involvement of transcription factor NFκB in the regulation of inflammatory processes of RA is widely reported. Also, NFκB regulates the expression of proinflammatory mediators such as cytokines, interleukins etc. involved in RA. Chronic activation of the HPA axis takes place after adjuvant induction suggesting the involvement of HPA axis in RA[20]. Results of previous study demonstrated that pain hypersensitivity after peripheral inflammation induced by CFA is mediated via NFκB associated pathways in central nervous system[5]. Also, results of immunofluorescence study confirmed the involvement of NF-κB and their translocation to dorsal root ganglion neurons[38]. Taking into account

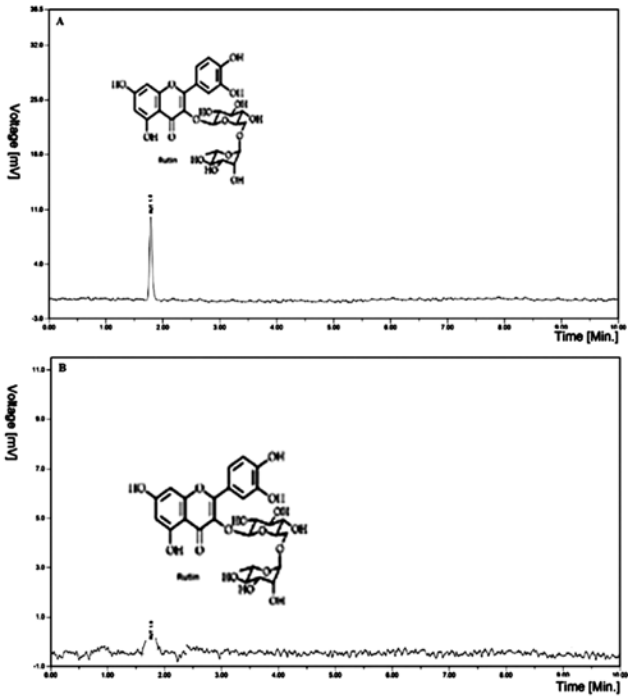

Fig. 2 — HPLC chromatograms of (A) standard; and (B) HASG extract at 365 nm showing rutin (labelled RUT). [By comparative assessment of HPLC retention time rutin was unequivocally identified using mobile phase of acetonitrile and phosphate buffer with flow rate of 1.0 mL/min]

this evidences suggests that neutralization of NFκB may provide an effective approach to the development of a novel therapeutic for RA.

Based on quantification of extracts, *in vitro* antioxidant study and *in vivo* anti rheumatic activity, polyphenol rich potent antioxidant extract, HASG were found effective in both developing and developed phase of arthritis. Also, It normalize the altered hematological and biochemical parameters indicative of its effectiveness in treatment of arthritis. Therefore, we investigated whether the antirheumatic potential of HASG extract were associated with changes in the expression of NFκB in PVN of Hypothalamus by immunohistochemical staining. In the present study, NFκB immunoreactive profile in control, standard and extract treated rat were measured. Compared to control group, significant reduction in the NFκB-immunoreactive cells was observed in the HASG extract treated rats (57%; P <0.001) on 14 day of treatment (Fig. 3). This effect was comparable with that of indomethacin treated rats in which 67% reduction of NFκB immunoreactive cells was noticed. These results may be attributed to the rutin as polyphenol and saponin as major

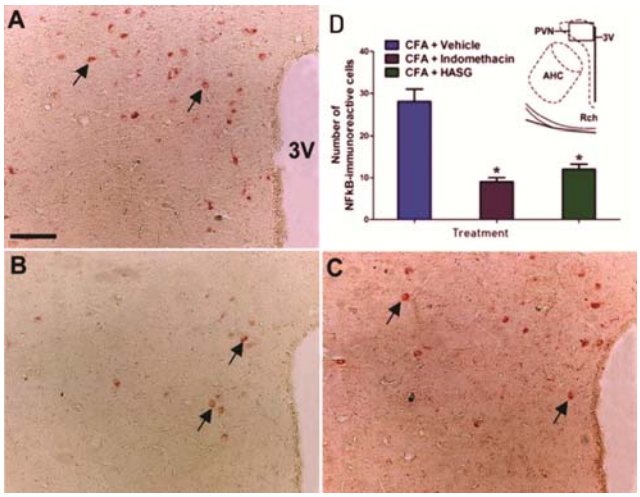

Fig. 3 — Effect of Indomethacin and HASG extract on NFκB immunoreactive cells in the paraventricular nucleus (PVN) of hypothalamus. (A) NFκB expression was observed in hypothalamic PVN at day 14 post CFA injection; Compared to control group, significant reduction in the NFκB immunoreactive cells was observed with (B) standard drug (Indomethacin); and (C) HASG extract treated rats on 14 day of treatment; and (D) Morphometric analysis of NFκB-immunoreactivity represented in bar graph. The outline of the transverse section through brain indicates the regions of the PVN at the co-ordinates −1.80 mm with respect to bregma from which the measurements were collated (square, not to scale). [3V, third ventricle; AHC, central part of the anterior hypothalamus; Rch, retrochiasmatic nucleus. Scale bar = 200 µm. The bar values are expressed as the mean ± SEM of five measurements from predetermined fields of the PVN on both the sides of each brain (n=5/group). The data were analyzed by unpaired t-test. *P <0.05, **P <0.01 *vs*. CFA control]

phytoconstituents present in extract. Different mechanism of polyphenol was suggested previously that they inhibits translocation of NFκB from cytoplasm to nucleus, transcription of pro-inflammatory cytokines and the interaction of target DNA with NFκB[39]. Thus, we can presume that, rutin in combination with other phytoconstituents attributed for the observed antirheumatic potential of HASG extract which might be due to inhibition of NFκB.

Conclusion

The present study on *Sesbania grandiflora* plant extracts exhibited significant antioxidant potential. Inhibition of inflammatory mediator NFκB is likely mechanisms underlying the modulation of complete freund adjuvant (CFA) by the HASG (50% methanolic) extract. Furthermore, plant extracts also proved to have significant role in the improvement of body weight, hematological and biochemical parameters and thus, their use might be of great value

in the treatment of rheumatism. These results consolidate the observation, that inhibition of NFκB may be a beneficial approach in the treatment of arthritis and other NFκB linked diseases. Although, these results gives the impression for the use of HASG extract for RA, however, further research is needed to determine the exact molecular mechanism involved. The present study may be considered as a major break through in identification of a lead molecule from the plant that may be responsible for the observed antirheumatic potential of the plant.

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Conflicts of interest

All the authors declare no conflict of interest.

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