

Attenuation of Cyclooxygenase and Induced Nitric Oxide Synthase and Antioxidant Properties by *Sauropus androgynus* (L.) Merr. Plant Leaves

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Abstract: *Sauropus androgynus*(SA) plant leaves contain high levels of bioactive compounds which are available in southeast Asia. The *in vitro* antioxidant activity of SA extracts has been reported but the associated anti-inflammatory properties have not been widely reported. The present study aimed to investigate the anti-inflammatory effects of SA on the pro-inflammatory enzyme, Cyclooxygenase-2 (COX-2), protein expression, its intermediate product, prostaglandin (PGE) and nitric oxide (NO) levels. SA leaves extracted using ethylacetate solvent mixtures and also to compare the effects of antioxidant, analgesic and anti-inflammatory properties. SA antioxidant activity was determined usually by oxidation of essential thiols (-SH) groups, whereas the hydrogen peroxide can cross cell membranes rapidly inside the cell and the reduction of NO with other radicals leads to the formation of more hazardous radicals such as peroxynitrite anion and hydroxyl radical. SA leaves extract also demonstrated hydrogen peroxide decomposition activity in a concentration dose of 150µg/ml showed 50.46% and 200µg/ml showed 50.90% similar to the standard 59.29% dependent manner. NO reacts more rapidly with superoxide showed of 150µg/ml showed 73.14% and 200µg/ml showed 73.38% nearer to the standard 79.89% of inhibition.

Key words: *Sauropus androgynus* • Antioxidant • COX-2 • NO • Anti inflammatory activity

INTRODUCTION

Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical and microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove irritant and set the stage for tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrating cells [1]. It is a complex process mediated by activation of inflammatory and immune cells. Acute or chronic inflammation plays a critical role in the initiation or development of many human diseases such as septic shock, rheumatoid arthritis, asthma, or cancer [2, 3, 4]. It is the central communication network and regulatory process that senses and controls threat, damage, containment and healing, which are all critical aspects in the maintenance of the integrity of an organism [5]. An uncontrolled and persistent inflammation may act as an etiologic factor for many of these chronic illnesses [6, 7].

The rapid induction of an inflammatory response is crucial for effective host defense. A wide range of proinflammatory agents ensure that this response occurs. Prominent among these are members of the prostaglandin and leukotriene family that are enzymatically derived from arachidonic acid (AA) C20:4 at the onset of inflammation, AA is metabolized via either cyclooxygenases (COX) to produce prostaglandins (PGs) and thromboxanes or lipoxygenases to generate leukotrienes (LTs) [8]. An anti-inflammatory effect is believed to result from inhibiting the formation of prostaglandins by prostaglandin H synthase, which converts arachidonic acid released by membrane phospholipids into prostaglandins. Two isoforms of prostaglandin H synthase, COX-1 and COX-2, have been identified and one variant form (COX-3) has recently been reported as well [9].

In the process, activated inflammatory cells (neutrophils, eosinophils, mononuclear phagocytes and macrophages) secrete increased amounts of nitric oxide

(NO), prostaglandin E2 (PGE2) and cytokines, such as interleukin (IL)-1 β , IL-6 and tumor necrosis factor. These substances not only induce cell and tissue damage, but also activate macrophages in rheumatoid arthritis, chronic hepatitis, etc [10]. NO is a major product and its production is controlled by the nitric oxide synthases (NOS), which include neuronal (nNOS), cytokine inducible (iNOS) and endothelial (eNOS). Most importantly, iNOS is highly expressed in macrophages; its activation leads to organ destruction in some inflammatory and autoimmune diseases. PGE2 is another important inflammatory mediator and is produced from arachidonic acid metabolites by the catalysis of cyclooxygenase-2 (COX-2) [11, 12].

Sauropus androgynus Merrill of family Euphorbiaceae which is also known as *Katuk* in Indonesia. It is widely used as tonic, antioxidant and febrifuge. The leaves are used as antitussive, tonic and soothing lungs and to relieve internal fever. The dark-green leaves provide a rich source of chlorophyll which is a valuable blood building element, cell rejuvenator and beneficial to the circulation [13, 14]. SA leaves can increase the quantity of milk production in mice [15]. It was also reported that the SA leaf extract can increase the mother's breast milk production without decreasing the quality of the breast milk [16]. SA can be useful as a dye in food colouring a delicious hot weather green vegetable, widely considered to be nutritious, approximately 10% protein content, the roots and leaves are used as medicine [17].

The literature study demonstrate the antioxidant capacity and nutritive values of SA and showed the antioxidant capacity and nutritive values that can be used to promote cultivation for both domestic consumption and commercial purposes [18]. The leafy vegetable SA is commonly used as an effective medicinal herb in the treatment of diabetics, cancer, inflammation, microbial infection, cholesterol and allergy due to its antioxidant effect [19]. SA also showed greater inhibition to *Bacillus cereus* and *Salmonella typhimurium* microorganisms [20]. SA were good antimutagens and was proposed that the compounds display the important role as antioxidant and/or antimutagens might be chlorophyll, flavonoids and other phytochemicals [21].

This present research aimed to determine the antioxidant activity, analgesic and anti-inflammatory properties of *sauropus androgynus* leaf extracts (SA). The anti-inflammatory effects of SA in RAW 264.7 celline were evaluated upon induction with an inflammatory marker as lipopolysacharride (LPS).

MATERIALS AND METHODS

Plant Material Preparation, Extraction and Isolation:

Mature SA leaves were obtained from herbal garden of PRIST University, Vallam, Thanjavur. The mature leaves were picked. The leaves of the plant were air dried at 35°C room temperature for 5days. The dried material was pulverized with an electric blender and ground to a fine powder before extraction then they were subjected to maceration with various solvent extraction. Based on its preliminary activity results the extraction with ethyl acetate was performed for further procedure. Extract was filtered and then concentrated under low pressure. With addition of 5% HCl pH of the extract was calibrated to pH 3. Acidic extract was stored in the cold at night to collapse resinous substances and they were removed by filtration. Filtered extract was subjected to chloroform extraction and chloroform extract concentrated under low pressure. Concentrated extract pH was calibrated to pH 8 with 25% NH₄OH addition. Then extraction with chloroform was repeated. Cream colored precipitate was formed during concentration procedure and this precipitate was obtained by filtration.

The anti-inflammatory effect of SA against PGE2 and COX-2 expression by the cells SA was prepared by extraction using ethyl acetate solvent and then freeze drying. The antioxidant activity was investigated using the hydrogen peroxide scavenging radical capacity and the Nitric oxide radical scavenging assay. The anti-inflammatory effects of SA in RAW 264.7 cells were evaluated upon induction with an inflammatory marker as lipopolysacharride (LPS). The anti-inflammatory effect of SA against PGE2 and COX-2 expression by the cells was accessed using Western blotting and ELISA.

Hydrogen Peroxide Scavenging Assay [22]: The ability of the extracts to scavenge hydrogen peroxide was determined according to standard procedures. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer. Different concentrations of the plant extracts (0.025 - 0.05 μ g/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and a standard compound was calculated as follows:

$$\% \text{ Scavenged } [H_2O_2] = [(A_0 - A_c - A_1A_c) / A_0 A_c] \times 100$$

where A_0 , A_c was the absorbance of the control, A_1 , A_t was the absorbance in the presence of the sample of extract and standard.

Nitric Oxide (NO) Scavenging Activity by Griess Reaction [23]: 100µl of Sodium nitroprusside was incubated with 100µl of the SA extract at different concentrations 20-200µ/ml for 60 min, at room temperature under light. All solutions were prepared in phosphate buffer. After incubation, 100 µl of Griess reagent was added to each well of the ELISA plates. The mixture was incubated at room temperature for 10 min and the absorbance of the chromophore (pink color) formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with N-apha-naphthylehlyendiamine was read at 562 nm. Three assays were performed, each one in triplicate.

Percent inhibition was determined by comparing the results of the test and control samples.

$$\text{Nitric Oxide scavenged (\%)} = [(A_0 - A_c - A_1 A_t) / A_0 A_c] \times 100$$

where A_0 , A_c was the absorbance of the control, A_1 , A_t was the absorbance in the presence of the sample of extract and standard.

Cell Culture: The RAW 264.7 is a macrophage-like cell line derived from tumors induced in male BALB/c mice by the Abelson murine leukemia virus. The RAW 264.7 cells were obtained from sigma aldrich, India.

Methyl Thiazolyl Tetrazolium (MTT) ASSAY [23]: The cells were seeded in 96-well ELISA plates at a density of 104 cells/well in 100 µl culture medium. Cells were incubated with the indicated concentrations of SA leaf extract for 1 hr before treatment with Lipopolysaccharides (LPS) for 24 h. Following 24 h incubation, the cells were treated with different concentrations of plant extracts. Each extract was screened initially for its cytotoxicity against cell lines at the concentration of standard at 50 µg/ml. The cells were washed with phosphate buffered saline (PBS) and inoculated with and without the extract. After 72 h incubation, the medium is aspirated. Followed by sample extract 50µg/ml, 150 µl of solution 5 mg/ml MTT in PBS is added to each well and the plates are incubated for 4 h at 37°C. After incubation, 800 µl of Dimethyl sulphoxide was added to the wells followed by gentle shaking to solubilize the formazan dye for 15 min. Absorbance was read at 540 nm and surviving cells fractions.

The percentage of cytotoxicity compared to the untreated cells was determined with the equation: Cell viability (%) = OD of treated cells / OD of control cells × 100. OD-Optical density.

Nitrite Measurement by Griess Nitrite Method [25]: RAW 264.7 cells (1.5×10^5 cells/ml) with LPS (1 µg/ml) for 24 h, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Amounts of nitrite, a stable metabolite of NO, were measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 µl of cell culture medium was mixed with 100 µl of Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was measured and the inhibitory rates were calculated by using a standard calibration curve prepared from different concentrations of sodium nitrite. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined.

% inhibition = (1-absorbancy of treated cells/absorbance of untreated cells) x 100.

Determination of Pro-Inflammatory Cytokines: RAW 264.7 cells and primary macrophages were cultured in 12-well flat plates at a density of 5×10^6 cells/well. The cells were then treated with various concentrations of β-actin and subsequently stimulated with LPS (100 mg/ml) at 37°C for 48 hrs in humidified air with 5% CO₂ gas. The supernatants were then collected and measured for COX2 and iNOS by an enzyme linked immunosorbent assay (ELISA) Kit method.

Isolation of Total mRNA AND RT-PCR Analysis: Total mRNA (messenger Ribonucleic acid) was prepared from LPS induced drug treated RAW -264.7 Macrophage cells using TRIzol reagent (Phenol-chloroform based extraction). First-strand cDNA was then synthesized from 1 µg of total RNA using 200 U rev transcriptase, in a 20 µl volume reaction containing 10 mM dithiothreitol, 10 U RNAs in ribonuclease inhibitor, 1 mM dNTPs and 2.5 µM random hexamers. The reaction mixture also contained 50mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂. Each sample was incubated for 45 min at 45°C, followed by 10 min at 72°C. The prepared cDNA was stored in -20°C for further use. For the PCR reaction, 2 µl of each cDNA template solution was amplified using 10 pmol of specific primer of COX 2 and iNOS genes combined with

1 pmol of β -actin. cDNA was amplified in a thermal cycle (Applied Biosystems, Foster City, USA) in a solution containing 1x PCR buffer (15 mM Tris-HCl, pH 8.0 and 50 mM KCl), 1.5 mM MgCl₂, 100 mM dNTPs with 1.5 units of Taq Polymerase and set of iNOS and COX-2. The COX 2 and iNOS set of primers. The PCR amplification was performed as follows: hot-start at 95° C for 7 min, followed by 28 cycles of amplification (denaturation: 45 sec at 94°C, annealing: 45 sec at 60° C, elongation: 45 sec at 72°C). A final extension was performed for 7 min at 72° C. The PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

Statistical Analysis: The antioxidant, anti-inflammatory analysis data are expressed as the mean \pm standard error of the mean (SEM). Group mean differences were ascertained with analysis of variance (ANOVA). Multiple comparisons among treatment means were checked with Least statistical Data (LSD) test. The results were considered significant if the probability of error was at least < 0.05. The statistical process was performed with SPSS 16 software.

RESULTS AND DISCUSSION

Many phytochemicals have antioxidant activity and reduce the risk of many diseases. It is crucial to know the type of phytochemical constituent, thus knowing the type of biological activity which might be exhibited by the plant [27]. Plant extracts with reducing properties have been shown to exert antioxidant action by breaking the free radical chain through the donation of a hydrogen atom [28], while the scavenging of H₂O₂ by the plant extracts was attributed to their phenolics, which can donate electrons to H₂O₂, thus neutralizing it to water²⁸. Hydrogen peroxide scavenging activity of the SA leaves extract on hydroxyl radical (Table 1) shows their activities at different concentration. H₂O₂ is highly important because of its ability to penetrate biological membranes. H₂O₂ itself is not very reactive, it is a weak oxidizing agent, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. SA leaves extract also demonstrated hydrogen peroxide decomposition activity in a concentration dose dependent manner.

Nitric oxide exhibits numerous physiological properties and it is also implicated in several pathological states. It is produced in various cells including neurons, endothelial cells and neutrophils by three isoforms of NO synthase enzyme encoded by a unique gene. The present results show that 200 μ g/ml aqueous leaves extract of SA

had scavenging activity of 79.13% similar to the standard activity of 79.8%. NO radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with O² radicals to form peroxynitrite, which damages biomolecules like proteins, lipids and nucleic acids [29].

In the present study 150 μ g/ml SA leaves ethyl acetate extract showed very good levels of inhibition of NO (70.72%) when compared to the standard (78.26%) whereas aqueous extract showed 65% inhibition (Table 2). Similarly 100 μ g/ml, 50 μ g/ml and 20 μ g/ml concentration of SA leaves extract was also performed and compared with standard showed 56.93<54.93<53.13% of inhibition, respectively.

Molecular Gene Expression of COX2 Gene and iNOS:

The anti-inflammatory activities of the SA leaves extract were evaluated and showed to inhibit the inflammatory response in macrophages, possess immunosuppressive activity and inhibit inflammation in mice. The immunomodulatory effects of SA leaves are usually associated with the stimulation of the immune system by a variety of bioactive compounds. These effects include maturation of dendritic cells, stimulation of natural killer (NK) cell activity and the activation of T and B lymphocytes [30].

In the present study, the accumulation of PGE₂ in RAW 264.7 cells was increased by LPS treatment. However, SA leaves extract compounds have inhibited the LPS-induced accumulation of PGE₂ in a dose-dependent manner. Figure 1. shows that, at a specific dose (4.5mg/ml) SA leaves extract markedly suppressed the PGE₂ production in LPS-stimulated RAW 264.7 cells. SA leaves compound exhibited high inhibition of PGE₂ production, but slight cytotoxicity was observed at the same concentration. Thus, we suggest that the hydroxyl moieties in the A-ring of flavonoids may play a role and exhibits differential effects on PGE₂ production in macrophages. Nitric oxide synthase catalyzes NO synthesis in biological systems.

The effect of *S.androgynus* leaves extract on treatment of macrophages caused a decrease in endogenous PGE₂ levels in RAW264.7 macrophages which was more pronounced at 100mg/ml doses of *S.androgynus* leaves extract. *S.androgynus* did not affect cell viability at the test concentration up to 4.5 μ g/ml doses as determined by MTT reduction assay. This causes induction of COX-2 and converts LPS-induced endogenous arachidonic acid to PGE₂. NO is a free radical generated through the conversion of L-arginine to

Table 1: Hydrogen Peroxide scavenging assay of SA leaves extract

Concentration (µg/ml)	Standard	Water Extract	Ethyl Acetate Extract
Control	15.06±0.16	16.46±0.28	22.73±0.23
20	13.03±0.18 [**] (13.47)	13.86±0.12** 15.79	14.4±0.26** 36.64
50	12.2±0.26** 18.99	13.26±0.24** 19.44	12.13±0.20** 46.63
100	10.93±0.08** 27.42	11.06±0.08** 32.80	11.96±0.70** 47.38
150	8.63±0.42** 42.69	10.83±0.17** 34.20	11.26±0.49** 50.46
200	6.13±0.12** 59.29	11.06±0.14** 32.80	11.16±0.14** 50.90

Results are presented as mean ±SEM, (n=3), *p<0.01, **p<0.05 Bonferroni test as compared to control, Values are expressed in % (Italic).

Table 2: Nitric oxide radical scavenging assay of SA leaves

Concentration (µg/ml)	Standard	Water Extract	Ethyl Acetate Extract
Control	15±0.18	12.46±0.28	12.4±0.20
20	7.03±0.18** 53.13	7.8±0.55** 37.39	6.16±0.68** 50.32
50	6.76±0.55** 54.93	6.63±0.58** 46.78	4.5±0.64** 63.70
100	6.46±0.50** 56.93	6.33±0.46** 49.19	3.63±0.48** 70.72
150	3.26±0.41** 78.26	4.36±0.43** 65.0	3.33±0.12** 73.14
200	3.03±0.12** 79.8	2.6±0.15** 79.13	3.3±0.15** 73.38

Results are presented as mean ±SEM, (n=3), *p<0.01, **p<0.05 Bonferroni test as compared to control, Values are expressed in % (Italic).

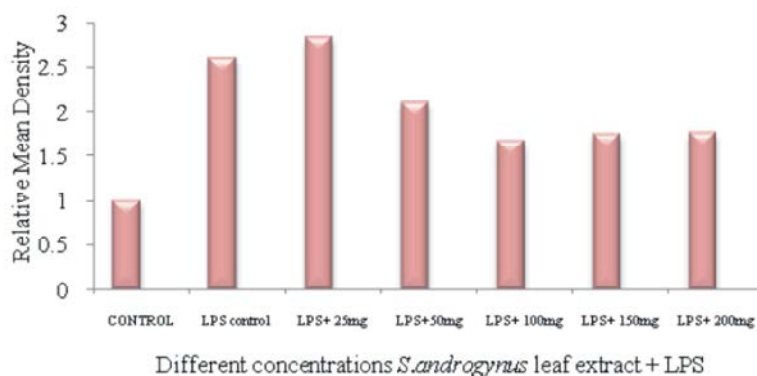


Fig. 1: Effect of *S.androgynus* leaves extract on LPS-induced COX2 expression

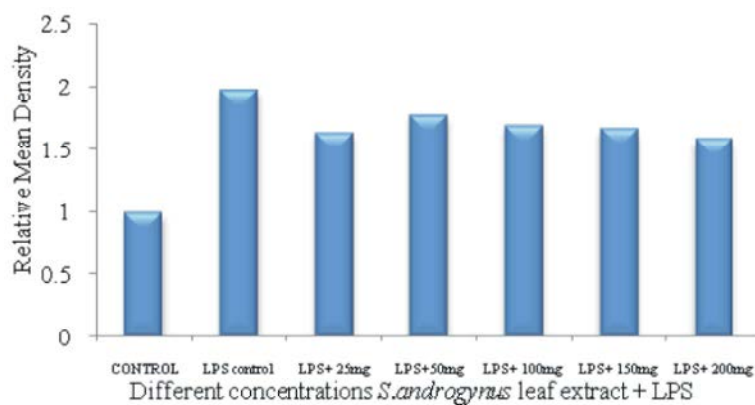


Fig. 2: Effect of *S.androgynus* leaves extract on LPS-induced iNOS expression



Fig. 3: Densitometric analysis of COX2, iNOS and β-actin

a) COX-2 gene expression and b) iNOS gene expression c) β-actin standard

citrulline, catalyzed by three isoforms of NOS, namely, the nNOS, eNOS) and iNOS isoforms. iNOS is induced in various cell types by inflammatory inducers [31]. In this study, *S.androgynus* leaves extract significantly $p<0.05$ inhibited LPS-stimulated NO and PGE2 production in a dose dependent manner (Figures 2 and 3). Consistent with these findings, *S.androgynus* leaves extract also suppressed LPS-induced expression of iNOS and COX-2 at the protein and mRNA levels in RAW 264.7 macrophages, suggesting that the observed reductions in NO and PGE2 release following treatment may have been due to the transduction and transcriptional suppression of iNOS and COX-2 genes.

Arctiin suppressed PGE2 production *via* inhibition of COX-2 enzyme activity and this is responsible for some of anti-inflammatory properties of this compound and the results also provide evidence of the bioactivity of arctiin in inflammatory diseases and suggest that arctiin may exert anti-inflammatory effect by inhibiting the pro-inflammatory mediators through the inactivation of NF- κ B [32].

Phytochemicals, especially flavonoids and phenolic acids, are of current interest because of their important biological and pharmacological properties, including reactive oxygen species (ROS) scavenging and anti-inflammatory, anticancer, anti-mutagenic and anti-carcinogenic activities [33, 34]. The potential role of phyto-compounds, including phenylpropanoids, flavonoids and phenolic acids, as important contributing factors to dietary antioxidant activity.

S. androgynus leaves extract was shown to inhibit the production of proinflammatory mediators (NO and PGE2) induced by LPS as well as their expression levels (iNOS, COX-2), through the inhibition of I κ B- α phosphorylation in LPS-stimulated RAW 264.7 macrophages. Our data indicate that *S.androgynus* leaves extract has an anti-inflammatory molecule which acts by suppressing NF- κ B activation *via* down regulating phosphorylation in RAW 264.7 macrophages. Considering these results, *S. androgynus* leaves extract may be a novel anti-inflammatory agent that could be used in the medication of inflammation-related diseases.

Hence we concluded that *S.androgynus* leaves showed potent anti-inflammatory / antioxidant inhibitory activity and also further detailed mechanistic studies as this molecule serve as lead molecule for chemoprevention

/chemotherapeutic studies. This may include benefits such as reducing pain related to inflammation and cardiovascular diseases by acting as strong COX inhibitors and antioxidants, respectively.

CONCLUSION

This study showed that SA leaves extract contain high levels of antioxidant capacity. The antioxidant activity was determined by chemical assays and anti-inflammatory activity in an *invitro* model of inflammation. The addition of SA leaves extract to LPS challenged macrophages resulted in decreased COX-2 expression, as well as in decreased PGE2 and NO levels. The results support the traditional use of this plant in inflammatory conditions and suggest the presence of biologically active components which may be worth and needs further investigation and elucidation. Due to availability of SA plant in India we have great potential to serve as an economical source of natural antioxidants for the food and nutraceutical industries.

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