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Research article

# MOLECULAR MECHANISMS OF ANTI-INFLAMMATORY POTENTIAL OF PURIFIED PROTEASE INHIBITOR FROM *Solanum aculeatissimum* IN RAW 264.7 MACROPHAGES

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**ABSTRACT**

Plant-based biomolecules that can inhibit mutagenesis, proliferation of cells and inflammation is a fascinating area for biologists. Many promising molecules were reported as anti-inflammatory and anti-metastatic such as soybean-derived protease inhibitors, Bowman-Birk Inhibitor and Kunitz-Trypsin Inhibitor. Inflammation is a unique biological response toward injury or infection. Cytokine and other mediator are produced by macrophage during the inflammatory reactions. PI from *Solanum aculeatissimum Jacq.* (SAPI) was isolated, purified by ion, gel and affinity chromatography techniques. The molecular mass was identified by SDS-PAGE as 22.2 kDa. Subsequently, the molecular mechanism of anti-inflammatory activity of PI was analyzed in LPS stimulated-murine macrophage cell line (RAW 264.7). SAPI significantly inhibited the production of NO, LTC4, PGE2 and TNF-αin a concentration dependent manner and inhibited the expression of iNOS and COX-2 in LPS-stimulated macrophage cell lines. SAPI remarkably inhibited nuclear translocation of NF-kB by counter acting the degradation of inhibitor of KB-α as well as by blocking phosphorylation of Akt and MAPKs. These results reveal that the anti-inflammatory potentialities of SAPI are associated with the down-regulation of iNOS, COX-2, and pro-inflammatory cytokines through the inhibition of NF-kB pathway in LPS-stimulated RAW 264.7 cells. Further studies are warranted to confirm the efficacy of the PI using animal models.

**Key words:** Anti-inflammation, Solanum, MAPK, NF-kB, RAW 264.7 cells.

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## INTRODUCTION

Molecular mechanisms describing *in vivo* anti-inflammatory potentiality of herbals include antioxidative and radical scavenging potentialities, regulation of cellular functions of the inflammation-related cells: mast cells, macrophages, lymphocytes, and neutrophils, modulation of enzymes of arachidonic acid (AA) metabolizers like phospholipase A2 (PLA2), cyclooxygenase (COX), and lipoxygenase (LOX) and the nitric oxide (NO), nitric oxide synthase (NOS) (Chen S, 2011). Suppression of such enzymes by biomolecules of medicinal plants reduce the production of AA, prostaglandins (PG), leukotrienes (LT), and NO, which are potential mediators of inflammation (Khanapure SP *et al*., 2007). Thus, the inhibition of these enzymes by biomolecules reveals its role in anti-inflammatory cellular mechanisms. Many research evidences support that certain phytochemicals are the modulators of gene expression, especially the modulators of pro-

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inflammatory gene expression, thus leading to mitigation of the inflammatory response. Many herbal crude preparations are employed to treat fever and inflammation in the traditional folklore of medicine. Therefore, discovering novel therapeutic plant based drugs capable of suppressing inflammation is need of the hour. The common synthetic drugs used are belongs to non-steroidal anti-inflammatory category such as ibuprofen, naproxen, diclofenac and celebrex are known to inhibit COX2 enzyme activity with many reported side effects. (Shaikh RU *et al*., 2016) analyzed herbals like *Cissus quadrangularis*, *Plumbago zeylanica*, *Terminalia bellarica* and *Terminalia chebula* in water, ethanol and hexane extracts *in-vitro* for COX-1 and 2 inhibitory and antioxidant activities. Similarly, Murugesan and Devi (2014) reported that most of the anti-inflammatory molecules from plant origin belongs to polyphenols including flavonoids.

PIs are reducers of the physiological activity of proteases and fascinated the attention of biotechnological researchers. In the evolutionary course, plants have developed diverse adaptive mechanisms of defence against various unfavourable conditions including that of predators and pathogens. Generally, PIs possess significant number of disulfide bonds due to cysteine residues that provide them resistance to heat, extremes of pH, and proteolysis. PIs have been proven as effective defence molecules against pests and pathogens in *in vivo*experimentals and also reported their optimal expression in transgenic crops. Most PIs bond with specific active site of the proteases results in the formation of stable protease inhibitor complex and there by inactivating the catalytic reaction either by competitive or non-competitive mode of reactions or depolarization of cell membrane of the pathogens thereby inhibiting its growth and invasion. . PIs have multiple functions by interfering with the proteolytic activity of the target proteases in the organisms. Serine Protease inhibitors (SPI) represent the largest family of inhibitors distributed throughout in plants. They are low-molecular mass proteins (3–25 kDa) that inhibit trypsin and/or chymotrypsin. SPIs are classified into different classes on the basis of amino acid sequence and mechanism of interaction including α-helical, β-sheet and a/b proteins, as well as small disulfide rich proteins. SPIs from leaves were best analysed from Solanaceous species i.e., when leaves were wounded mechanically or through insects initiates the synthesis of diverse SPIs.

*Solanum* (Solanaceae), a group of annual or short lived perennial herbaceous weeds, distributed throughout the temperate and tropical regions of the world. *Solanum* contributes the largest and most complex genus with more than 2,000 species. *Solanum* species represent nearly1% of the world's angiosperm flora, which might beat tribute to its great antiquity and an extraordinary rate of speciation. This huge diversity makes *Solanum* an interesting life form in terms of its evolutionary and economic stand point of view. Examples of food plants in *Solanum* were potato, eggplant, naranjilla, jasmine nightshade and others (Edmonds JM, 1979). Many species of *Solanum* were used as medicine to cure digestive, intestinal problems like stomach-ache, diarrhoea, piles, dysentery and also for various skin problems such as sores, boils, cuts, wound, bruises, fever and malaria, headache and rheumatism. Some species were stimulants whereas others have sedative properties. Furthermore, many species were employed against respiratory tract disorders such as cough, sore throat, bronchitis, asthma and urinary problems. Most of the medicinal attributes of the species was due to the presence of steroidal glycoalkaloids (Patel K *et al*., 2007). Similarly, *Solanum*species shows insecticidal and fungicidal properties. *S. nigrum*, the black nightshade, a noxious weed but effectively inhibit the gut proteinases of pests and could potentially be used in generating insect resistant transgenic plants.

*Solanum aculeatissimum* Jacq., known as Dutch eggplant is a weedy shrub that bears small, Flowers are in few-flowered lateral clusters. Corolla star-shaped, whitish.Fruit 2-3 cm in diameter, spherical, striped or marbled green and creamy-white, turning dirty yellow when ripe. The highest concentration of alkaloids (4.4%) is found in the seeds. Nevertheless a fruit decoction, fruit sap, or sap of roasted fruit, has been recorded as traditional remedies in Africa (Welman WG, 2003). Solasonine is the major glycoalkaloid in its foliage, stems, fruit and seeds, besides minor alkaloids.

Which include solamargine, solanine and solasodine. To cure constipation fruit decoction of *Solanum aculeatissimum* is administered as an enema in Liberia and Nigeria. Warm leaves are ground in water and the extract is administered as an enema as an abortifacient or purgative. In Uganda the fruit sap is used as eye drops to treat trachoma and the sap of roasted fruits is taken to induce labour (Mugisha D and Origa S, 2007). In South Africa a root decoction is drunk to cure back pain, male impotence, snakebites, smoke from burning fruits is inhaled to cure toothache and the fruit is pressed to the forehead to remedy headache and to the skin to cure skin infections. The root extract is taken as a purgative and also to stop flatulence (Burkill HM, 2000).

## MATERIALS AND METHODS

### Plant material

*Solanum aculeatissimum* Jacq.fresh fruits were collected from Munnar hills of Western Ghats, Kerala. Taxonomic identity was carried by using flora and authenticated by matching with the herbarium of JNTBGRI, Palode, Kerala, India.

### Purification

100 g fresh fruits were homogenized with 250 ml of saline Tris buffer (20 mM Tris, pH 8.0; 0.15 M NaCl) containing 1 % polyvinyl pyrrolidone (1:6 w/v) and filtered through chilled 4-fold muslin cloth and further, centrifuged for 15 min at 10,000 x *g*. The entire protocol was carried at 4 °C. The crude PI extract was fractionated by 20-90 % $(NH_4)_2SO_4$ precipitation. The $(NH_4)_2SO_4$ was removed by the process of dialysis using the extraction buffer stirred gently with magnetic stirrer to improve solute exchange and the dialysis buffer was changed once in 3 h for 4-5 times. The dialyzed protein showing high protease inhibition activity was subjected to DEAE cellulose exchanger column, pre-equilibrated with 20 mMTris buffer with pH 8.0. 3 ml protein fractions were eluted using linear gradient of NaCl (0.02-0.50 M) at a flow rate of 0.5 ml /min. Fractions eluted with 0.18 to 0.24 M NaCl were pooled, dialyzed, lyophilized and loaded (1.0 mg/ml) to Sephadex G-50 superfine from Pharmacia column. The column fractions with SAPI activity were dialyzed, concentrated and loaded onto sepharose affinity column equilibrated with 100 mM phosphate buffer (pH 7.6) containing 100 mM NaCl. The adsorbed SAPI was eluted with 100 mM HCl. The purity was checked by reverse phase HPLC (C18 column) at a flow rate of 1.0 ml /min with 100 % solvent A (0.1 % trifluoroacetic acid (TFA) in water) for 10 min and a linear gradient (0–100 %) of solvent B (0.08 % TFA in 80 % acetonitrile) over 45 min. Apparent molecular weight was checked by Sephadex G-50 gel filtration column (0.1 M phosphate buffer, pH 7.6) calibrated with known molecular weight proteins (14.3 to 43 kDa).

### PI assay & SDS- PAGE

SAPI activity was determined by estimating the residual hydrolytic activity of trypsin and chymotrypsin towards the substrates BAPNA (N-benzoyl-L-arginine-p-nitroanilide) and BTPNA (N-benzoyl-L-tyrosyl-p-nitroanilide), respectively, at pH 8.0 after pre-incubation with inhibitor (Prasad ER *et al*., 2010). Molecular mass and purity of PI was evaluated by SDS–PAGE (Laemmli UK, 1970). The molecular mass was further compared with size elution chromatography.

### Cell culture

RAW 264.7 murine macrophage cells (ATCC, Rockville, MD, USA) were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 µg/mL) at 37°C.

### Measurement of NO, $PGE_2$, TNF-α, IL-1α and IL-6

1 x $10^6$ cells/well density of macrophage cells were plated in a 12-well plate and incubated for 24 h. The cells were treated with different doses of SAPI for 1 h, and then triggered with 1 µg/mL LPS for another 24 h. Then the cells were centrifugated at 2,000*g* for 10 min and stored at -80°C until used for analysis. Griess reaction was employed for the estimation of nitrite concentration in the cultured media as an indicator of NO production, as per the protocol of (Dewi K *et al*., 2015)**.** Levels of $PGE_2$, IL-1β, IL-6, $LTC_4$and TNF-α in cultured media were quantitated by ELISA, as per the manufacturer's protocols. Experimental sample was treated with four different concentrations of SAPI. Indomethacin was used as reference compound

### Reverse transcription-polymerase chain reaction (RT-PCR)

Murine cells plated in a 6-well plate were pretreated with SAPI for 1 h and then induced with LPS for 6 h. Total RNA from each category was isolated with the TRI-zol reagent. 5 µg of total RNA was employed for reverse transcription using oligo-dT-adaptor primer and superscript reverse transcriptase. PCR was carried out with the gene-specific primers: COX-2 sense, 5'-CAGCAAATCCTTGCTGTTCC-3'; COX-2 antisense, 5' TGGGCAAAGAATGCAAACAT-3'; iNOS sense, 5'-CACCTTGGAGTTCACCCAGT3'; iNOS antisense, 5' ACCACTCGTACTTGGGATGC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5'-GACCCCTTCATTGACCTCAA-3'; GAPDH antisense, 5'-CTTCTCCATGGTGGTGAAGA-3'. GAPDH was used as an internal standard to evaluate relative expression of COX-2 and iNOS.

### Transient transfection and luciferase assay

2 x $10^5$ RAW 264.7 cells/well was plated in a 24-well plate was transiently transfected with Lipofectamine/Plus reagents + NF-κBpromoter/luciferase DNA (1 µg) along with 20 ng control pRL-TK DNA for 40 h. Cells pretreated with 0-100 µg/mL SAPI for 1 h were triggered by LPS (1 µg/mL) for 6 h. Each well was washed thoroughly washed with cold phosphate-buffered saline (PBS), harvested in 100 µL of lysis buffer (HEPES 0.5 mM,

pH 7.8, Triton N-101 1%, 1 mM $CaCl_2$ + $MgCl_2$ 1 mM) and employed for the quantification of luciferase activity using a luciferase assay kit. Luminescence was evaluated on a Top Count microplate scintillation and luminescence counter in single-photon counting mode for 0.1 min/well, following a 5 min adaptation in the dark. Luciferase activity was normalized to the expression of control pRL-TK.

**Extraction of cytosolic and nuclear portions**

5 x $10^6$ cells/well was pretreated with SAPI for 1 h and induced with LPS for 30 min and were harvested by washing thrice with cold PBS. Cell pellets were resuspended in 300 µL of hypotonic buffer (HEPES/KOH -10 mM, KCl-10 mM, $MgCl_2$ -2 mM, EDTA - 0.1 mM, DTT- 1 mM, and phenyl methylsulfonyl fluoride - 0.5 mM at pH 7.9) and incubated on ice for 15 min. 10 s after vortexing, the homogenates were separated into cytoplasmic and nuclear fractions (supernatant and pellet) by centrifugation at 13,000 g for 15 min. The pellet was gently resuspended in 40 µL lysis buffer contain 50 mM HEPES/KOH, 50 mMKCl, 1 mM DTT, 300 mMNaCl, 1% IGEPAL CA- 630, 0.1 mM EDTA, 10% glycerol, and 0.5 mM PMSF at pH 7.9 and centrifuged for 20 min at 4°C with 13,000 g. The supernatant was employed as the nuclear component.

**Western immunoblot analysis**

Murine cells were incubated with different concentrations of SAPI for 60 min and triggered with 1 µg/mL LPS for 30 min. The cells were washed three times with cold PBS and lysed using lysis buffer (Tris-HCl - 50 mM, NaCl - 150 mM, IGEPAL CA-630 - 1%, Tween 20 - 1%, SDS - 0.1%, $Na_3VO_4$ - 1 mM, leupeptin - 10 µg/mL, NaF - 50 mM, and PMSF- 1 mM at pH 7.5) on ice for 60 min. After centrifugation for 12 min at 18,000 g, the protein concentrations in the supernatants were calculated, and 40 µg of the protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBST) with 0.1% Tween 20 for 60 min, followed by the incubation for 2 h with primary antibody in TBST containing nonfat dry milk (5%). The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST containing nonfat dry milk for 60 min, and immune complexes were visualized using an ECL detection kit.

**Immuno fluorescence analysis**

Murine cells were maintained on glass coverslips to evaluate nuclear localization of NF-κBin 24-well plates for 24 h (Sen SS *et al*., 2015). Cells treated with SAPI for 60 min were induced with 1 µg/mL LPS for 30 min. Cells were fixed in paraformaldehyde (4%) for 15 min in PBS at 28° C, and then permeabilized with Triton X-100 (0.5%) in PBS for 10 min. Cells were washed twice with PBS and blocked with 3% BSA/PBS for 30 min. Subsequently, cells were incubated with an anti-NF-KB polyclonal antibody diluted in 3% BSA/PBS for 2 h, and incubated with Alexa Fluor® 488-conjugated secondary antibody diluted in 3% BSA/PBS for 60 min. Cells were stained with 2 µg/mL DAPI and images were captured using an LSM700 laser scanning confocal microscope.

**Statistical analysis**

Values were noted as the means ± SDs from six separate experimental. The obtained results were analyzed using one-way analysis of variance. Differences were considered significant at values of $p< 0.05$. All data were carried using SPSS for Windows, version 10.07 (SPSS Inc., Chicago, IL, USA).

**RESULTS AND DISCUSSION**

As an initial part, protease inhibitor activities from different plant parts of *S. aculeatissimum* were evaluated. Fruits displayed the maximum PI activity compared to leaves, stem and seeds i.e., 54 % for trypsin and 48 % chymotrypsin inhibitory activity. SAPI was concentrated by $(NH_4)_2SO_4$precipitation with varying concentrations ranging from 0-20, 20-40, 40-60, 60-80 and 80-90 % saturation. The $(NH_4)_2SO_4$precipitation yielded 1.41 and 1.51 fold of purification compared to the crude extract. Pooled active fractions after DEAE cellulose chromatography using 0.18 to 0.24 M NaCl (fractions: 9-12) were dialyzed and showed 93.2 TlU and 90.2 ClU for trypsin chymotrypsin inhibitory activities respectively. The protein was further purified by gel and affinity chromatography. Thus, purified SAPI yielded a specific activity of 502 TIU U/mg and 433.7 ClU, with low protein content of 0.95 mg. Overall, the specific activity increased about 92.6 and 82. 9 folds with 9.8 and 8.77 % yield with respect to trypsin and chymotrypsin respectively. The purity of PI was further checked by RP-HPLC with retention time of 10 min in 50 mM Tris–HCl buffer, pH 8.0, coinciding with the protein peak (Fig.1).

**Level of NO, $LTC_4$, $PGE_2$ andTNF-α**

Generally, RAW 264.7 macrophages release low profile NO i.e., the basal value was 0.22 µM, meanwhile, LPS triggered the release of NO significantly up to 34.2 ± 0.8 µM for 18 h. Similarly,

$PGE_2$ was also enhanced from the basal value of 0.013 ± 0.001 to 0.126 ± 0.009 ng/ml by LPS treatment. Aminoguanidine, the synthetic inhibitor of nitric oxide synthase activity, inhibited the NO production (100% with100 µM. Application of NS398, the COX-2 inhibitor soundly reduced $PGE_2$ release (100% with 10 µM) (Table 1). Interestingly, SAPI also inhibited LPS triggered $PGE_2$ production effectively (Table 2) i.e., 0.068, 0.039, 0.009 and 0.001 with 5, 10, 25 and 50 µg respectively. $LTC_4$also showed a more or less similar trend in SAPI treated cells compared to LPS and synthetic drugs. Similarly, SAPI inhibited the NO synthesis also (Table 1). Here also, the inhibition level was comparable with that of aminoguanidine. TNF-α, another mediator of inflammation was released negligible levels in LPS-treated RAW 264.7 cells i.e., 2.8 ± 0.03 ng/ml, while LPS triggered its level to 20.7 ± 0.06 ng/ml at 18 h. Dexamethasone, the common drug reduced TNF-α synthesis (1 µM) (Table 1). SAPI showed remarkable effect on TNF-α level in the macrophage cells (Table 1).

**Effect of SAPI on LPS-induced iNOS and COX-2 proteins and mRNA expression**

Nitric oxides (NO) are produced by NO synthase (NOS) and are involved in many physiological events (Forstermannand Sessa, 2012). Inducible NOS (iNOS) are unique proteins triggered by cytokines as well as bacterial lipopolysaccharide (LPS) (Jana *et al*., 2007). Prostaglandins are synthesized via arachidonic acid by cyclooxygenase (COX). COX-2 an isoform is activated against stimulants like LPS and this in turn produces PGE2 that leads to inflammation (Ricciottiand FitzGerald, 2011). Treatment with SAPI effectively suppressed iNOS protein production in a concentration dependent manner; however, COX-2 protein synthesis was inhibited at concentration from 100 µg/mL SAPI, which is more or less at par with PGE2 synthesis (Fig. 3 a and b). Further, SAPI also inhibited mRNA expression of iNOS. COX-2 mRNA expression was also blocked by SAPI at 100 µg/mL onwards. Thus, the western blot analysis suggests that the SAPI induced arrest of NO and PGE2 synthesis in LPS-triggered macrophages is connected with low expression of iNOS and COX-2 at transcriptional level.

**Activity of SAPI against NF-κB stimulated by LPS**

Nuclear factor kappa B (NF-κB)/p65 the regulators of cytokines and others were visualized by confocal microscopy in the cytoplasmic region in unstimulated cells (Fig. 4). On the other hand, LPS induction leads to the translocation of NF-kB/p65 from cytoplasm into the nuclear region as seen by their intensity in the nucleus (Fig. 4). Meanwhile, p65 level was decreased in the nucleus by treating with SAPI. The results tempted to analyze the effect of SAPI on the NF-κB promoter activity in LPS induced cells. The base value of NF-κB promoter activity in the cells was 4.56 and was enhanced to 5 folds by LPS (27.6) ($p < 0.05$). Interestingly, a reduction in NF-κBpromoter activity was noticed by SAPI in LPS treated cells i.e., from 13.4 (10 µg/ml) to 4.98 (50 µg/ml).

Cells were pretreated with and without SAPI for 1 h followed by LPS stimulation for 30 min. NF-κB/p65 subunits were probed by anti-NF-κB polyclonal primary antibody and Alexa FluorW488-conjugated secondary antibody. The nuclei were stained with DAPI and the images were captured by confocal microscopy.

Molecular analysis revealed that IKKβphosphorylation increase and IkB-α degradation in LPS treated cells compared to control (non-treated) and SAPI treatment i.e., suppression of IKKβ phos-phorylation and IkB-α degradation, (regaining the control level of cytosolic IkB-α in a concentration dependent manner). As a consequence of IkB-α degradation, the enhanced NF-κB level in the nucleus after LPS induction was regained by SAPI treatment in a concentration dependent manner (Fig. 5). Meanwhile, c-Jun phosphorylation, a transcription factor component of AP-1, was not altered by SAPI treatment. Thus, the overall results suggest that the SAPI-induced inhibition of iNOS, COX-2, and pro-inflammatory cytokines synthesis were regulated mostly by the transcription factor NF-κB in LPS triggered RAW 264.7 cells.

Cells were incubated with different doses of SAPI for 1 h, and then stimulated with LPS (1µg/mL) for 30 min. Cytosolic and nuclear fractions were prepared and analyzed by Western blotting using corresponding antibodies. The results are mean of three independent trails. $p< 0.05$ suggests the level of significance.

**Influence of SAPI on LPS triggered MAPKs and Akt phosphorylation**

Down regulation of phosphorylation of JNK, p38 MAPK, ERK, and Akt proteins triggered by LPS in RAW 264.7 cells by SAPI revealing its potential role in regulating NF-κB pathway via suppressing the MAPKs and Akt proteins phosphorylation (Fig. 6b). To substantiate the SAPI's anti-inflammatory potential, phosphorylation of IκBαin LPS triggered RAW 264.7 cells were analyzed in presence of Wortmannin (PI3K/Akt pathway inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), or SB203580 (p38 inhibitor). Interestingly, PD98059,

and SP600125, were blocked the phosphorylation of IκBαtoa moderate level. Thus, it is plausible to confirm the role of SAPI on the phosphorylation of MAPKs and Akt proteins for NF-κ Binduction in response to LPS.

Synthetic drugs have been used as anti-inflammatory agents but exhibits different degrees of adverse effects (Adebayo SA *et al*., 2015). In this context, herbal molecules may be employed as novel anti-inflammatory agents with minimum side effects. These biomolecules possess ability to protect inflammatory mediators through NF-kB regulation in RAW 264.7 macrophages (Zhen *et al*., 2015). Protease inhibitors are proved as drug against many disorders however, the anti-inflammatory potential and its mode of action has not been fully utilized so far. Therefore, the role of PI of *S. aculeatissimum* at molecular level in LPS-stimulated RAW 264.7 macrophages was attempted.

Injury or infection induce diverse inflammatory mediators including pro-inflammatory cytokines synthesized by activated macrophages and act jointly with other inflammatory mediators ends in to inflammation related disorders (Liu C *et al*., 2015). Similarly, over production of pro-inflammatory cytokines plays significant role in acute and chronic inflammatory responses and diseases.Biomolecules capable to mitigate NO or $PGE_2$ level may be potential as anti-inflammatory drugs (Lee W and Bae JS, 2015). Recent studies have shown that *in vivo* or *in vitro* treatments by biomolecules are potential in retarding inflammation by the inhibition of pro-inflammatory cytokines, which may pacify inflammatory issues like atherosclerosis, cancer, and inflammatory arthritis (Lee W and Bae JS, 2015). The present results reveals that inhibiting NO, $PGE_2$ and $LTC_4$synthesis by SAPI in LPS-stimulated cells followed by expression levels of iNOS and COX-2 proteins and genes suggest the transcriptional inhibition of iNOS and COX-2 by SAPI.

The present study was undertaken to examine the anti-inflammatory effect of SAPI on LPS-stimulated murine macrophage cells. To further understand the molecular mechanisms of SAPI, we investigated the effects of SAPI on the secretion of NO, $PGE_2$, TNF- α, IL-1 β, and IL-6, the expression of iNOS and COX-2, and the activation of NF-κB. Our results indicated that SAPI effectively inhibited the secretion of NO, $PGE_2$, TNF- α, IL-1 β, and IL-6 through a blockade of the NF-KB and MAPK pathways in LPS-stimulated macrophages. The inhibitory effect of SAPI on the expression of inflammatory mediators suggested one of the mechanisms responsible for its anti-inflammatory action and its potential for use as a therapeutic agent for treating inflammatory diseases.

NF-κB family of transcription factors (transcriptional induction of iNOS and COX-2 gene) has crucial role in inflammation and innate immunity and crosstalk with multiple signaling molecules and pathways (Hoesel and Schmid, 2013). NF-κB induces cytokines that regulate TNFα, IL-1, IL-6 and IL-8 (innate response) as well as adhesion, which progress to the recruitment of leukocytes to inflammation sites (Oeckinghaus A and Ghosh S, 2009). (Song YA *et al*., 2011) studied that regulation of NF-κB via ubiquitin-proteosome mechanism by polyphenols of black tea through phosphorylation, ubiquitination, and subsequent degradation of IκB. Pathway of SAPI on NF-κB regulations is still untraced but the present results reveals that SAPI controls proteolytic degradation of IκB - α and NF-κB promoter-driven luciferase expression triggered by LPS in murine cells. Therefore, iNOS, COX-2, and other pro-inflammatory cytokine expression in macrophage cells are connected with activation/deactivation of NF-κBpathway. In addition, NF-κBis also controlled by MAPKs (ERK, JNK, and p38 signaling kinases) and Akt signals (Joung E *et al.,* 2012). Phosphorylation of MAPKs in response to LPS was suppressed by SAPI (Fig.6). Further, activation of Akt, a downstream regulator of PI3K, was also suppressed by SAPI in response to LPS signal in the macrophage cells. (Kim AR *et al*., 2011) reported that phlorofucofuroeckol A, which showed a correlation between ROS and PI3K/Akt pathway in regulations of inflammatory genes. So it is possible to speculate that the anti-inflammatory potential of SAPI may be related to blocking of Akt phosphorylation. Thus, it is possible to suggest that suppression of MAPKs and Akt phosphorylation by SAPI may leads to the SAPI-mediated inhibition of NF-κB pathway in LPS-stimulated murine cells lines.

Doumas S *et al.,* 2005 analyzed anti-inflammatory roles of secretory leukocyte (SL) PIs i.e., SLPI inhibits the downstream portion of the nuclear factor κB (NF-κB) pathway by protecting I-κβ (inhibiting factor of NF-κB) from degradation by the ubiquitin-proteosome pathway. Thus, SLPI renders macrophages unable to release proinflammatory cytokines and nitric oxide. (Shigetomi H *et al*., 2010) evaluated anti-inflammatory actions of serine PIs containing the Kunitz domain contribute to termination of the inflammatory process, including modulation of cytokine expression, signal transduction and tissue remodeling. Similarly, Bermudez H and Motta *et al*., 2015 proved that serine PIs protect optimally IL-1β and TGF-β anti-inflammatory cytokines against mouse colitis when delivered by recombinant lactococci i.e., a key regulator of immunological homeostasis and inflammatory responses.

**Table 1. Potentiality of SAPI in inhibiting TNF-α and NO-release from RAW 264.7 cells stimulated with LPS (1 µg)**

| Group | TNF-α (ng/ml) | Nitrite release (µM) |
|---|---|---|
| LPS (1 µg/ml) | 20.7 ± 0.06 | 34.2 ± 0.8 |
| NS398 (10 µM) | - | - |
| Amino guanidine (100 µM) | - | 0.00 ± 0.0 |
| Dexamethasone (1 µM) | 0.01± 0.001 | - |
| 5 µg | 11 ± 0.28 | 28.7 ± 0.55 |
| 10 µg | 8 ± 0.041 | 19.6 ± 0.03** |
| 25 µg | 4.2 ± 0.03* | 10.9 ± 0.12** |
| 50 µg | 1.2 ± 0.09** | 3.2 ± 0.019* |

**Table 2. Role of SAPI in Inhibiting $PGE_2$ (COX-1) and $LTC_4$-release (5-LOX) from RAW 264.7 cells stimulated with LPS**

| Group | $PGE_2$ (ng/ml) | $LTC_4$ (ng/ml) |
|---|---|---|
| LPS (1 µg/ml) | 0.126 ± 0.009 | 0.621 ± 0.04 |
| Indomethacin (25 µM) | 0.00 ± 0.001** | 0.011 ± 0.001** |
| 5 µg | 0.068±0.02 | 0.230.005 |
| 10 µg | 0.039±0.04 | 0.14±0.003 |
| 25 µg | 0.009±0.001 | 0.072±0.002 |
| 50 µg | 0.001±0.001 | 0.035±0.001 |

**Fig 1. RP-HPLC Chromatogram of purified SAPI using C-18 column.**

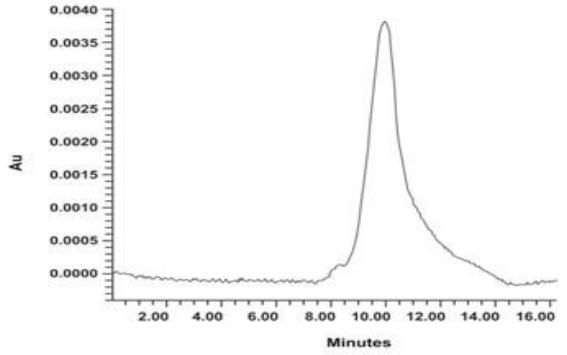



**Fig 2. SDS PAGE ofpurified SAPI**

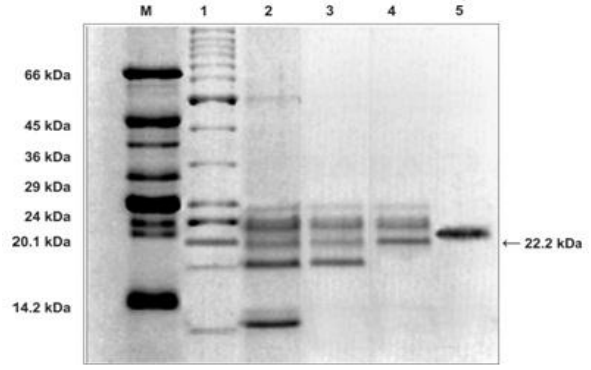
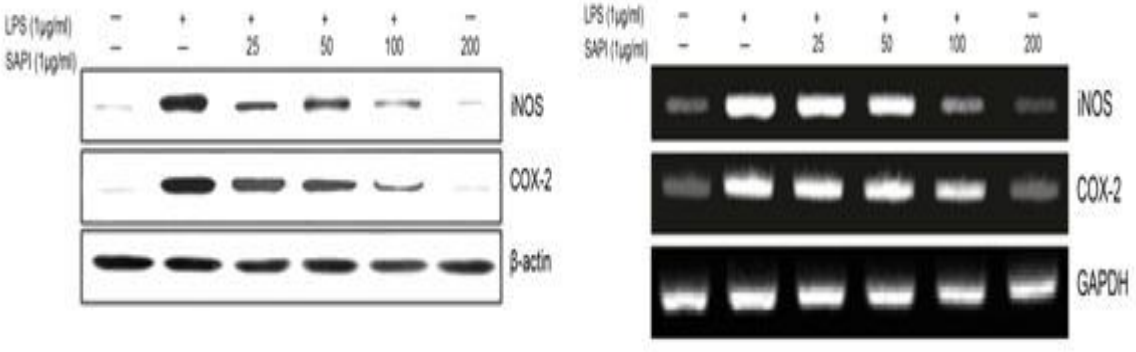



**Fig 3. Effect of SAPI on LPS-induced iNOS and COX-2 protein and mRNA expression in RAW 264.7 cells**




(a) Cells were pretreated with different concentration of SAPI for 1 h and stimulated with LPS (1 µg/mL) for 16 h. 40 µg of proteins were subjected to 10% SDS-PAGE. The expression of iNOS, COX-2, and β-actin protein was identified by Western blotting employing corresponding antibodies. (b) Cells were pretreated with SAPI for 1 h and induced with LPS for 6 h, and then total RNA was prepared for RT-PCR. The results presented as mean of three independent trails. $p< 0.05$ indicates significant level

**Fig 4. Effect of SAPI on activation and translocation of NF-κB in RAW 264.7 cells.**

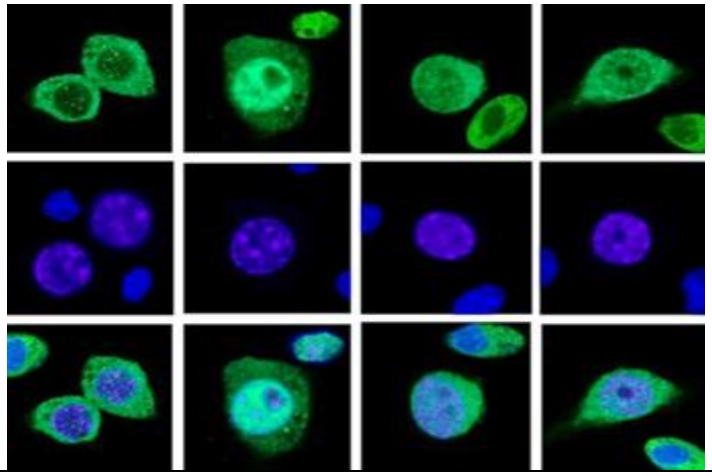
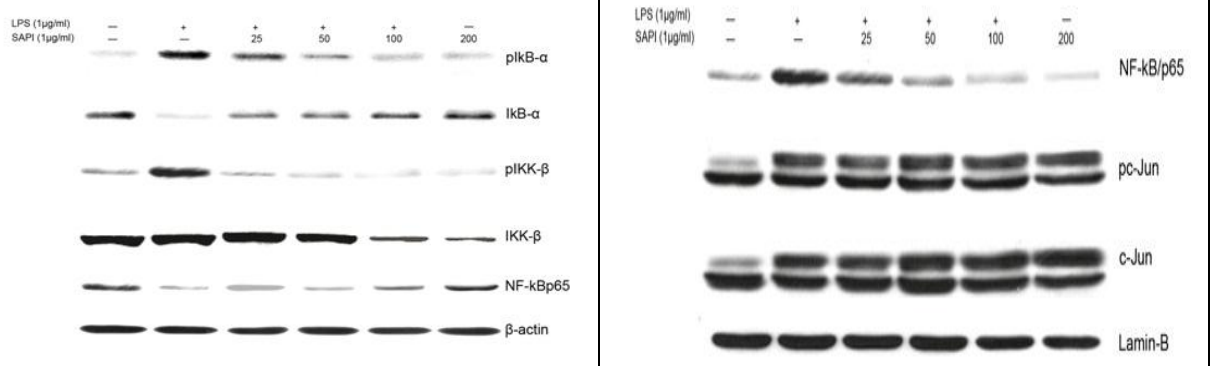
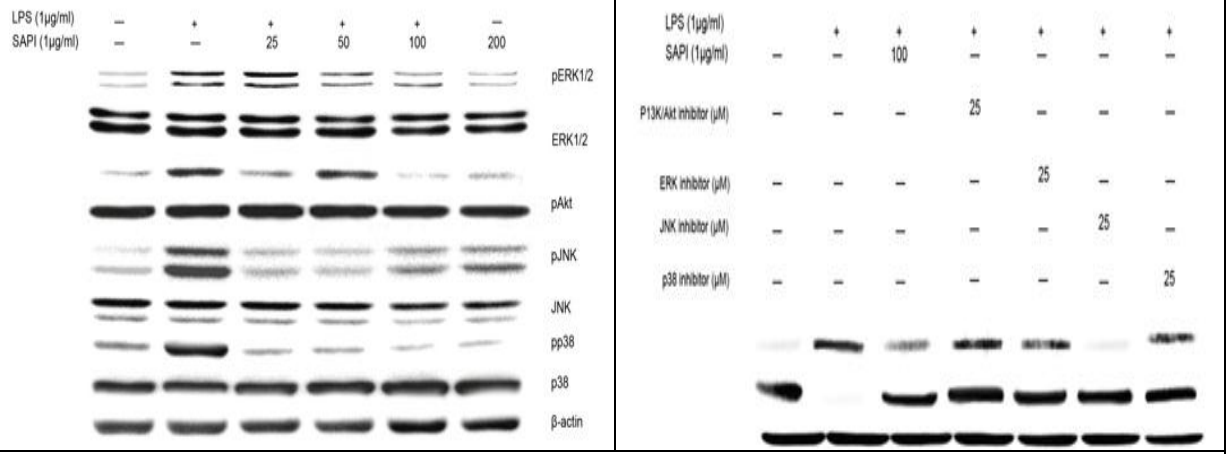


**Fig 5. Inhibitory effect of SAPI on the degradation of IκB-α and the activation of NF-κB in LPS-stimulated RAW 264.7 cells.**




Cells were incubated with different doses of SAPI for 1 h, and then stimulated with LPS (1 μg/mL) for 30 min. Cytosolic and nuclear fractions were prepared and analyzed by Western blotting using corresponding antibodies. The results are mean of three independent trails. $p < 0.05$ suggests the level of significance.

**Fig 6. a) Impact of SAPI on the phosphorylation of MAPKs and Akt in RAW 264.7 cells. Cells pretreated with indicated doses of SAPI for 1 h were induced with LPS (1 μg/mL) for 30 min. (b) Cells pretreated with indicated concentrations of SAPI or inhibitors for 1 h were stimulated with LPS (1 μg/mL) for 30 min. Whole cell lysates (40 μg) were evaluated by Western blotting using specific antibodies. The results are mean of three independent experiments**




$p < 0.05$ level of significance.

## CONCLUSION

In conclusion, the present study confirmed the *in-vitro* anti-inflammatory effects of PIs of *S. aculeatissimum*. SAPI inhibited the release of inflammatory mediators like NO and PGE2 and also the expression of iNOS and COX-2 in LPS triggered macrophages. These findings suggest that SAPI decreases NO and PGE2 levels by down regulating iNOS and COX-2 respectively. LPS stimulate MAPK the signaling cascade molecules such as p38, ERK, and JNK (Kim HK *et al*., 2014) and therefore, SAPI mediated anti-inflammatory reactions were analyzed by Western blotting. SAPI reduced the phosphorylation of p38, ERK, and JNK in LPS triggered RAW 264.7 macrophages. Thus, the present study gives a new insight for unraveling the mode of action involved in anti-inflammatory effect by PIs as well as substantiates for using the species in alternative and complementary therapies in the future.

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