Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 6 Issue 2 February 2023

Research Article

A.paniculata extracts reverse LPS induced inflammation Via Suppression of Serum Amyloid A and NLRP3 Mediated Inflammasome Pathway

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DOI: 10.31080/ASMI.2023.06.1203

Received: January 09, 2023

Published: January 19, 2023

Abstract

Serum amyloid A (SAA) is known to be one of the earliest acute phase response proteins to be elevated in infections. Its role in LPS induced inflammatory conditions is being studied for devising strategies to modulate acute phase response. Present study involves analyzing the effect of Gram negative bacterial LPS on the expression of SAA and role of a traditional herb *Andrographis paniculata* on this expression. Plant extracts have been traditionally used since centuries as a means of treating infections. *A. paniculata* is one such herb extensively utilized in multiple continents for treating inflammatory conditions, infections and even cancer. While testing LPS from *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella typhi*, it was observed that *K. pneumoniae* LPS showed maximum induction of SAA expression. On screening of patients suffering with bacterial sepsis, a general trend of high SAA expression was observed in patients showing poor outcome. *K. pneumoniae* LPS induced SAA levels were observed to be reversed by *A. paniculata (*Ap*)* methanolic extracts. Interestingly, the extracts also reduced LPS induced inflammatory cytokines- TNF, IL-1β and IL-6 levels, NLRP3, Caspase-1 and Caspase-8 activation, indicating, reversal of apoptotic and pyroptotic cell death. The extracts also reduced the expression of the inflammatory enzyme Cyclooxygenase-2. Upon immunoblotting, it was observed that activation of NFκB and phosphorylation of ERK were significantly inhibited by Ap extracts, indicating that this might be the mechanism of reversal of pyroptosis and apoptosis. In conclusion, these findings are useful to state the importance of *A. paniculata* extracts in alleviating adverse effects of the immune response and host survival.

Keywords: Serum Amyloid A; Inflammation; *Andrographis paniculata*; TNF-α; IL-1β

Abbreviations

LPS: Lipopolysacharide; Ap: *Andrographis paniculate*; SAA: Serum Amyloid A

Introduction

A major diagnostic challenge in sepsis is prompt and accurate diagnosis for timely patient treatment. Clinicians have sought reliable markers to detect sepsis in early stages for a long time and to exclude diseases of non-infectious origin [1,2]. Early diagnosis and treatment of sepsis are vital to improve patient outcome. Since sepsis has high incidence of mortality (30-40%), early diagnosis and aggressive supportive care may reduce mortality rates. Procalcitonin (PCT) is the current gold standard for bacterial sepsis, followed by blood culture, which has several drawbacks

such as low sensitivity, high incidence of false-negative results and prolonged time period required for reporting [3,4]. PCT is a 116-amino acid peptide that is involved as a precursor in calcium homeostasis and widely used as useful marker for the diagnosis of sepsis. A large number of markers such as C-reactive protein (CRP) have been proposed for early diagnosis of sepsis [3]. CRP is an acute-phase protein found in blood, that is produced by the liver because of infection or tissue injury [4-7]. However, the specificity and value of CRP as a marker has several drawbacks.Thus, search for an accurate and reliable biomarker for sepsis remains a challenge for researchers even today [8].

Serum amyloid A (SAA) is a precursor protein in inflammation-associated reactive amyloidosis, whose level in the blood increases up to 1000 fold in response to infection and is synthesized in the liver. SAA is also an acute phase reactant like PCT and CRP and has been proven to be a prognostic marker in late-onset sepsis in preterm infants [9-11]. Arnon., *et al*. reported that SAA had an overall better diagnostic accuracy than CRP for predicting early onset of sepsis [12]. They also showed that SAA is a useful inflammatory marker during late-onset sepsis in preterm infants [11].

Among the various plants used, *Andrographis paniculata* (Burm. f.) Nees, (Ap) a traditional medicinal plant is known to demonstrate several biological activities such as anti-inflammatory, immunomodulating, antibacterial, and antiviral both in vivo and in vitro. The anti-inflammatory properties of Ap are based on observations where it was found to suppress production of inflammatory mediators including IL-6, NO, TNF-α, IL-12, and PGE2 in activated macrophages [13,14]. Hence, using medicinal plants with therapeutic properties is a valuable alternative and a promising source for pharmaceutical agents [15]. Plants are rich in a wide variety of secondary metabolites and phytochemical elements like alkaloids, flavonoids, tannins and terpenoids which act against different diseases [16-18]. On the whole, phytomedicines may prove to be a cheaper and more efficient source for treatment than chemotherapeutic agents [19]. As the therapeutic options available for diseases are significantly reduced, the search for new chemotherapeutic agents that may be applied for the treatment of such acute and chronic diseases currently has high potential [20,21]. The impact of plant extracts on several bacteria have been studied by a significant number of researchers in different parts of the world [22]. Considerable amount of work is being done on ethnomedicinal properties of plants in India as well [23].

IL-1β is a key cytokine that has been implicated in the pathogenesis of several inflammatory disorders, such as gout, rheumatoid arthritis (RA), and atherosclerosis [10,11]. The production of mature IL-1β is tightly regulated. Two signals are required for the secretion of biologically active IL-1β. First, transcription of the IL-1β gene and production of cytosolic pro-IL-1β are dependent on the activation of NF-κB via, for example, TLRs. The second signal is needed to activate the pathway that leads to cleavage of pro-IL-1β by caspase-1 and secretion of the mature IL-1β cytokine. Caspase-1 is activated by inflammasomes, cytoplasmic multiprotein complexes capable of sensing stress and danger signals [24]. Upon activation, NLRP3 inflammasome assembly cleaves cytosolic pro-IL-1β and pro-IL-18 into the mature proinflammatory cytokines IL-1β and IL-18 by activated caspase-1. Innate immune response, particularly Inflammatory cytokines play a critical part in the pathophysiology of several diseases and triggers macrophage activation to produce interleukin (IL)-1β, tumor necrosis factor (TNF)-α and nitric oxide (NO) [25]. Thus, by manipulating the inflammatory responses we may help prevent of the disease or its symptoms., Significant effort has been focused on exploring the molecular mechanisms of inflammatory responses to find therapeutic targets in order for developing strong and safe anti-inflammatory drugs to prevent or reverse many kinds of inflammation-related diseases. In this study, we have explored the effect of *Andrographis paniculata* extracts on inflammation and effect on SAA, a proinflammatory protein.

Materials and Methods

Materials

Methanol from Qualigens (Thermo Fisher Scientific), Phosphate buffered saline (PBS), DEPC water MTT(TC191), RPMI 1640 Medium, DMEM Medium, penicillin-streptomycin were purchased from Hyclone(G.E Health care Life Sciences, Logan, Utah), LPS (*Klebsiella pneumoniae*, Sigma-Aldrich, USA), Fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA), SYBR Green Master Mix from Applied Biosystems(Thermo Fisher Scientific, Vilnius, Lithuania), Primers from Integrated DNA Technologies,

Collection of plant material

Ap is known locally as Nilavempu, Kalamegh, nilavempu, commonly known as King of Bitters, Creat or Green Chiretta in English, finely powdered plant leaves sold in sealed plastic bags

were purchased from Wonder herbals with batch no10 which were prepared following Good Manufacturing Practices (GMP). The components were checked for the presence of any visible contaminant such as harmful foreign matter, sand, stones, and poisonous chemical residues. The powder was also found to be entirely free from moulds or insects, as microbial contaminants can produce toxins, TLC was performed to detect contamination from other plants using original plant leaf extract as control. The plant name has been checked with https://www.theplantlist.org on 27.11.2020 from Wonder Herbals and stored in an airtight container until further use.

Preparation of Experimental plant extracts

The plant extract from the powder was extracted in methanol. The consecutive extractions were performed using a cold maceration process for 72 h with continuous agitation [26]. The extracts were filtered using Whatman (No.1) filter paper and concentrated under reduced pressure in a vacuum using a rotary flask evaporator and dried in a hot air oven. The methanolic extract yields a dark greenish solid residue weighing 5.750 g (23.0% w/w). The concentration (mg/ml) of the extract, was obtained by evaporation of the solvent used and the dry weight of the extracts was noted. These crude extracts of methanol were used for further investigation. The extract was preserved in sterile tubes under refrigerated conditions (2 to 4°C) until further use.

LPS purification

LPS was purified by TRIzol extraction protocol [27]. Lyophilized bacterial cells (1-10 mg) were suspended in 0.2 ml of Tri-Reagent. The cell suspension was then incubated at room temperature for about 10-15 min for complete cell homogenization. After incubation, 20 ml of chloroform per mg of cells was added to create a phase separation. The mixture was then vigorously vortexed and incubated at room temperature for an additional 10 min. The resulting mixture was centrifuged at 12000g for 10 min to separate the aqueous and organic phases. The aqueous phase was transferred into a new 1.5 ml centrifuge tube. Distilled water (100 ml) was added to the organic phase. The mixture was vortexed, incubated at room temperature for 10 min, and centrifuged at 12000g for 10 min. The upper aqueous phases from both steps were combined. Two additional water extraction steps were repeated to ensure complete removal of LPS.

Blood culture

Undiluted whole blood (WB) was cultured in flat-bottomed 24-well culture plates (Thermo Scientific NuncTM, USA) at a volume of 900 µL per well prior to the addition of test agents. All agents were diluted with RPMI 1640 medium (Invitrogen, USA) supplemented with 0.1 % heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin (all chemicals were purchased from Invitrogen, USA) and prepared freshly from the stock concentration for each experiment. Blood Cultures were incubated at 37 °C in a 5% CO_2 atmosphere. At each indicated incubation time point, cultured cells were harvested, collected for RNA isolation followed by cDNA synthesis.

Exposure to Differential Bacterial LPS

Gram-negative bacteria *K. pneumoniae* (ATCC700603), *E. coli* (ATCC25922), *S. typhi* (ATCC25567), and *P. aeruginosa* (ATCC27853) were obtained from ATCC. The bacterial cultures were cultured and maintained in nutrient agar/broth (Himedia, India). The concentration of isolated LPS was determined by Standard KDO method(Sigma, India) [28,29]. For WB treatments, THP-1 cells were cultured separately in 24 well plates and100ng of each isolated bacterial LPS diluted in RPMI were added per mL blood and incubated for 12 h.

Cell culture

Human monocytic leukemia cell line THP-1 was purchased from NCCS, Pune The cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin. For monocyte-macrophage differentiation, THP-1 cells were divided on 6-well plates (1.5×10^5 cells/ml/well), and PMA was added to the final concentration of 50 nM for 24-48 h.

LPS induced THP-1 Cells

THP-1 cells were grown in suspension in RPMI +Glutamax supplemented with 10% (v/v) FBS in a humidified 37°C, 5% CO_2 incubator. Cells were plated in 6-well tissue culture treated plates (Nunc, Thermo) in the presence of phorbol 12-myristate-12 acetate (PMA, Sigma-Aldrich) PMA was removed after 2 days of treatment. Cells were treated with *Klebsiella pneumoniae* LPS from Sigma-Aldrich (100 ng/mL, Sigma) for 30 min followed by treated with 50 and 100µg/mL Ap extract for 24 h.

Analysis of mRNA by semi-quantitative reverse transcriptase (RT) and real-time polymerase chain reaction

The levels of mRNA expression of inflammatory mediators, was determined by extracting the total RNA from LPS-treated blood and THP-1 cells with TRIzol Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The extracted RNA was stored at −80°C until use. Semi-quantitative RT reactions were conducted as described previously. Quantification of mRNA was performed by real-time RT-PCR with SYBR Master mix according to the manufacturer's instructions (Applied Biosystems, Forster, CA, United States) using a real-time thermal cycler StepOnePlus (Applied Biosystems, Forster, CA, United States) as described previously and The program was set as follows Denaturation at 95 °C for one minute followed by 40 cycles of initiation at 95°C for 10 s, annealing at 56 °C for 15 s, elongating at 72 °C for 20 s and a single final step at 58 °C for 90 s. At the end of the program, the melting curve was checked and the data were analysed by calculating the Cycle Threshold.

Three biological replicates were performed. Relative expression was normalized to the internal control gene GAPDH using $2^{-\Delta\Delta CT}$ method. The primers used are listed in Table.

The sequences of the primer pairs used for q-PCR were as follows:

Gene	Forward primer	Reverse primer
GAPDH	ATGGATGAGGAAATCGCTGCC,	CTCCCTGATGTCTGGGTCGTC
IL-6	CCAGAGCTGTGCAGATGAGT	CTGCAGCCACTGGTTCTGT
TNF-α	ACCAGGCCTTTTCTTCAGGT	TGCCCAGTCTGTCTCCTTCT
IL-1β	AAGCTGATGGCCCTAAACAG	AGGTGCATCGTGCACATA AG
Cox-2	GAATGGGGTGATGAGCAGTT	CAGAAGGGCAGGATACAGC
SAA	CCCTTTTGGCAGCATCATAG	AGCCGAAGCTTCTTTTCGTT

Table a

Measurement of supernatant cytokine levels

Supernatant levels of tumor necrosis factor (TNF-α) and IL-1β were measured using human ELISA kits (Applied-Biosystems, USA), according to the manufacturer's instructions. These cytokines were measured because they are known to be increased in sepsis [30].

Western blotting

For Western blotting of cell-culture media, human or mouse macrophages were incubated overnight at 37°C in fresh RPMI 1640 medium supplemented with 10 mM HEPES, 1% l-glutamine, and 1% penicillin-streptomycin treated with LPS and plant extracts. After stimulation, the cells were lysed in RIPA buffer and subjected to 12% SDS-PAGE, followed by immunoblotting with Abs for caspase-1, Caspase-3, Caspase-8, pERK, ERK, SAA and NLRP-3 (dilution 1:500).

Statistical analysis

Statistical analyses and visualization were performed using GraphPad Prism software (version: 7.0). The differences between groups was compared using One-way ANOVA. The results expressed as the mean ± SD, and * P value < 0.05 was considered as being statistically significant.

Results

SAA expression is elevated in response to bacterial LPS

Serum amyloid A is known to be elevated in infection and is one of the earliest markers of acute phase response. LPS from Gram negative bacteria is an important inducer of infection and bacterial sepsis. To understand the effect of LPS isolated from various Gram-negative bacteria on expression of SAA, healthy human blood samples were treated with 100 ng of LPS isolated from *K. pneumoniae, P. aeruginosa* and *S. typhi*. All the three types of LPS elevated SAA transcription by almost 8-fold at 12 h incubation, *K. pneumoniae* LPS showing higher effect compared to the remaining two bacterial species (Figure 1). Interestingly, the elevation in SAA expression was observed from 4 h itself, indicating its importance even in early stages of infection. Since SAA response was observed

to slightly higher in case *K. pneumoniae*, it was used in further analysis to detect change in the inflammatory cytokine IL-6. *K. pneumoniae* LPS showed more than 4-fold increase in IL-6 levels from 6 hours post treatment itself, which reduced at the end of 24 h, whereas SA levels increased at a slower rate comparatively and showed more than 10-fold elevation at 12 hours (Figure 2).

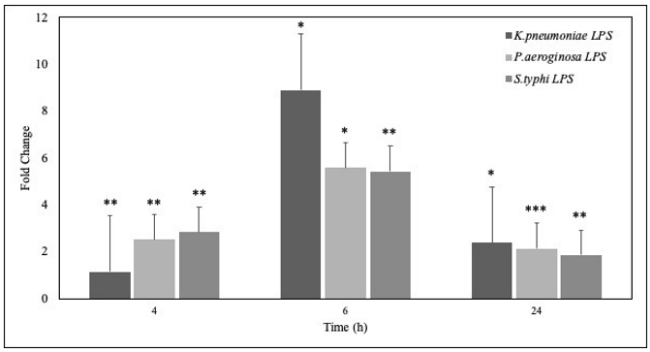

Figure 1: Expression of SAA in response to LPS isolated from Gram negative bacteria.

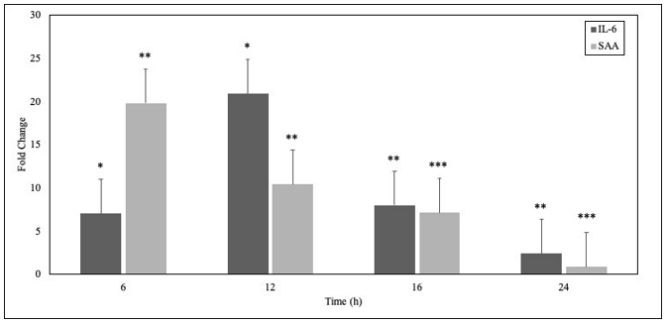

Figure 2: SAA and IL6 expression levels in response to *K. pneumoniae* LPS.

Whole blood samples were treated with 100 ng LPS isolated from three bacterial strains, i.e., *K. pneumoniae, P. aeruginosa and S. typhi*. The samples were lysed and levels of SAA were analysed by qRT-PCR. The graph depicts fold change in SAA expression with reference to GAPDH. Values represent the mean ± SD of three independent experiments.

THP1 cells treated with 100 ng of *K. pneumoniae* LPS were lysed and processed for analysing SAA and IL-6 expression by qRT-PCR. Graph depicts time dependent changes in SAA and IL-6 expression with reference to GAPDH. Values represent the mean ± SD of three independent experiments *P < 0.05, **P < 0.01 and ***P < 0.001.

SAA may serve as a marker for severity of sepsis

To understand the effect of infection and role of SAA, patient sera were collected from bacterial sepsis patients from a local hospital (Prasad Hospitals, Hyderabad). The samples were divided into Survivor and Non-survivor category based on the outcome of treatment. Interestingly, out of two survivors and two non-survivors screened for SAA expression by immunoblotting and qRT-PCR which showed a general trend of higher levels of SAA expression in both protein and mRNA levels (Figure 3 A and B). Further analysis on a larger cohort of patients will help to understand the applicability of SAA as an early marker of sepsis and severity of sepsis.

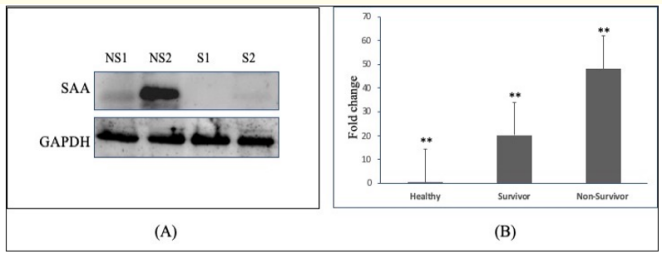

Figure 3: LPS induces the transcription of Inflammatory markers in human THP-1 cells. THP-1 cells were incubated with LPS (100 ng/ml) for 4h followed by Ap for 24 h. The cells were lysed and analyzed for IL-6, TNF-, COX-2, IL-1β, SAAand GAPDH for changes in protein and mRNA levels. Values represent the mean ± SD of three independent experiments. A) Immunoblots showing changes in protein levels; B) Graph showing changes in RNA levels.

Identification of components by GCMS

The GC-MS data was interpreted using the database from National Institute Standard and Techniques (NIST) which comprises of more than 62,000 patterns. The relative percentage of each component was calculated by comparing their average peak areas to the total area. The molecular weights, structure, and names of the components in the test materials were ascertained by comparing the spectrum of the unknown component with the spectrum of the known component found in the NIST library.

A total of 18 natural compounds were identified in the methanolic extract of Ap through the GCMS analysis. The retention time (RT), molecular formula, molecular weight (MW), and % concentration in the methanolic extract of Ap are presented in Table 5. Among the identified compounds, 2-Methyl-6-(5-methyl-2-thiazolin-2-ylamino) pyridine (7.33%) was predominant, followed by 9,12,15-Octadecatrienoic acid (6.78%), 2-nonadecanone (6.56%). 3,7-Diazabicyclo[3.3.1]nonane, or bispidinone (5.50%), 5.alpha.-Cholestan-6-one (2.98%), 4-P-tolyl-5-phenylimino-4,5-dihydro-1,2,4-thiadiazole (2.86%), 3-Deoxy-d-mannoic lactone (2.59%), D-Xylitol (2.12%) Fucoxanthin (1.95%).

Ap extracts reverse the effect of *K. pneumoniae* LPS mediated SAA and inflammasome activation

To understand the role of *K. pneumoniae* LPS further on host innate immune response, THP1 cells were treated with *K. pneumoniae* LPS. A known plant-based inhibitor, *A. paniculata* (Ap) (methanolic extracts) was used to compare the effect of LPS on the cells. Figure 4A shows a dose dependent increase in the levels SAA, NFκBand NLRP3 proteins. A dose dependent increase in the inflammatory cytokines IL-6 and TNFα, pyroptosis cell death marker IL-1β and COX2 enzymes was also observed (Fig 4B). Interestingly the elevated levels of these proteins and their transcripts were reversed by the addition of Ap extracts, although transcript levels of SAA did not show significant change. It is possible that Ap extracts largely affect SAA expression at protein level by either promoting its degradation or by inhibiting its translation, whereas transcription of SAA might be unaffected.

To confirm these findings, THP1 cells were treated with *K. pneumoniae* LPS and Ap extracts. Immunoblotting for apoptosis marker Caspase-8 showed total reversal of cell death in LPS treated cells on co-treatment with Ap. Similarly, pyroptotic cell death markers NLRP3 and IL-1β levels elevated in LPS treatment were reversed drastically on co-treatment with Ap extracts (Figure 5 A and B). It is possible that Ap extracts inhibit the phosphorylation of ERK to inhibit downstream activation of NLRP3 and cleavage of Caspase-1. This maybe resulting in total reversal of inflammatory cytokine production and cell death by apoptosis and pyroptosis. Interestingly, SAA levels were also reversed by the addition of Ap. Hence, Ap extracts might play a significant role in LPS induced infection and inflammatory conditions.

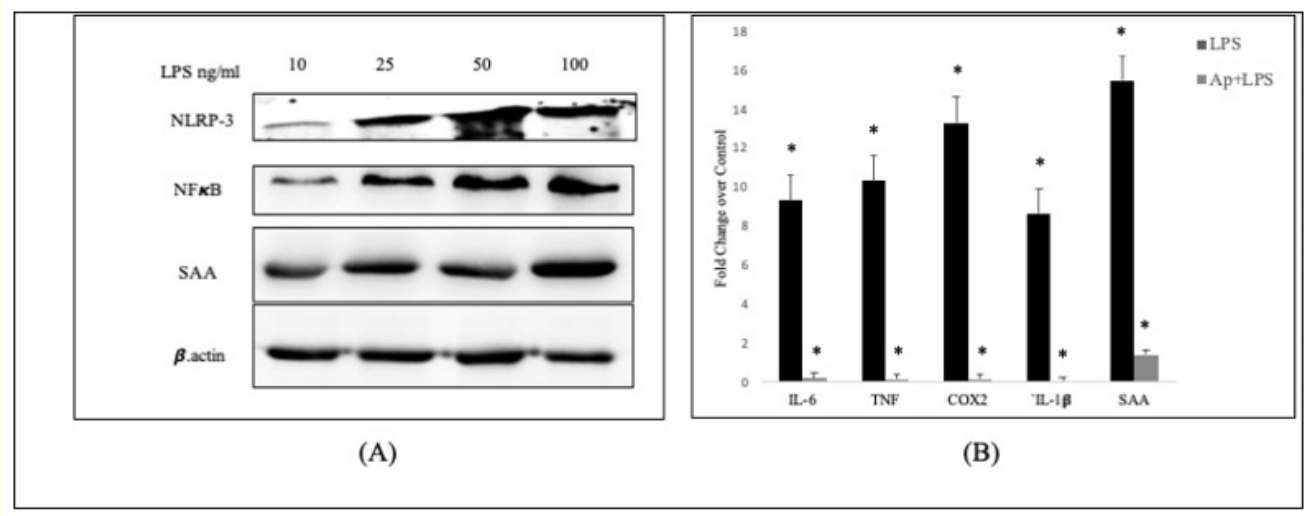

Figure 4: *K. pneumoniae* LPS induced inflammation in human THP-1 cells can be reversed by Ap extract. THP-1 cells were incubated with LPS (100 ng/ml) for 4h followed by Ap for 24 h. The cells were lysed and analyzed for IL-6, TNF-α, COX-2, IL-1β, SAAand GAPDH for changes in protein and mRNA levels. A) Immunoblots showing changes in protein levels; B) Graph showing changes in RNA levels. Values represent the mean ± SD of three independent experiments.

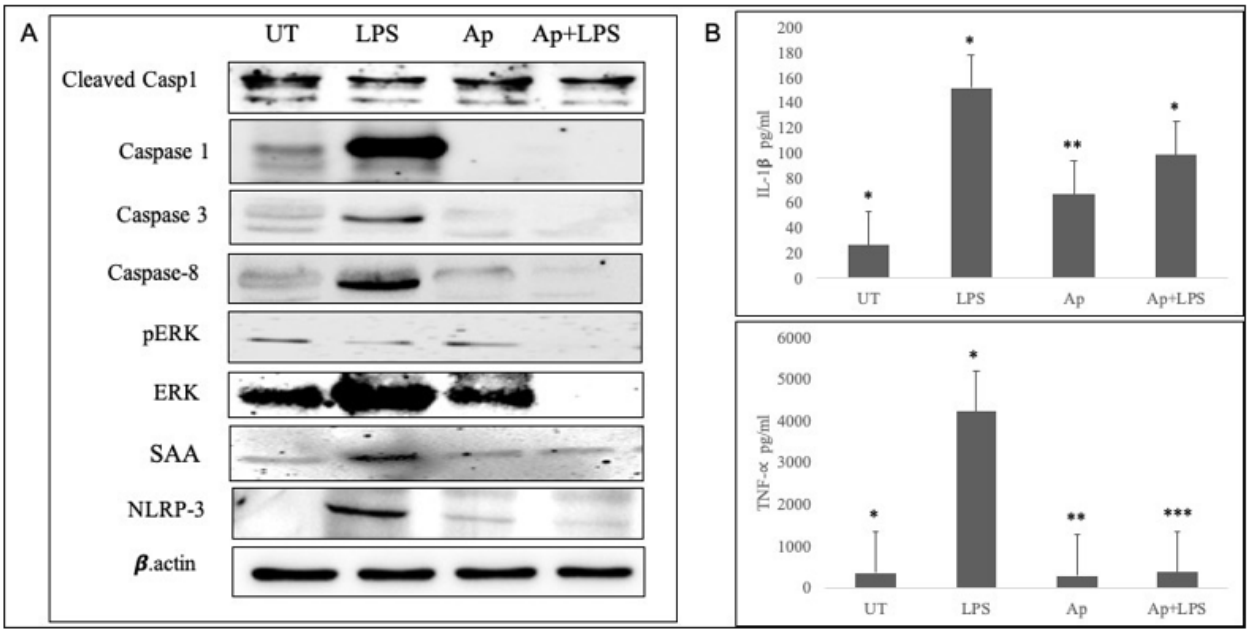

Figure 5: Ap extracts reverse LPS mediated inflammasome activation in THP1 cells. THP-1 cells were stimulated with LPS (100ng/ml) in the presence or absence of Ap (50 µg/ml) for 24 h. After stimulation, supernatants were analyzed for IL-1β and TNF-∝production using ELISA. A) immunoblots of respective proteins; B) Graphs showing changes in TNF-α and IL-1β. Values represent the mean ± SD of two independent experiments.

S. No	Compounds	Formula	Molecular weight (g/mol)	R. Time	Area %	Biological activities	References
1.	Propanamide	C_3H_7NO	73	22.867	0.43	Immune suppressive agent	[31]
2.	Methanesulfonic acid	$C_6H_{14}O_5S$	198	27.408	0.53	Irreversible inhibitors of acetylcholinesterase.	[32]
3.	d-Mannitol, 1-decylsulfonyl	$C_{16}H_{34}O_7S$	370	27.735	0.58	Anti-inflammatory and anticancer effects	[33]
4.	4-P-tolyl-5-phenylimino-4,5-di-hydro-1,2,4-thiadiazole	$C_{15}H_{14}N_4S$	282	28.392	2.86	Anti microbial	[34]
5.	Edulan IV	$C_{13}H_{20}O$	192	28.225	1.66	Antimicrobial, antihistaminic and mast cell stabilizing activities	[35]
6.	2-Methyl-6-(5-methyl-2-thiazolin-2-ylamino)pyridine	$C_{10}H_{13}N_3S$	207	29.121	7.33	Treatment of several types of cancer and as antidiabetics	[36]
7	1-Heptanethiol	$C_7H_{16}S$	132	29.275	4.99		

8.	5.alpha-Cholestan-6-one	$C_{27}H_{45}CLO$	420	29.366	2.98	Anti-inflammatory effects of these compounds were evaluated in LPS-stimulated BV-2 microglia cells.	[37]
9.	2-nonadecanone	$C_{25}H_{42}N_4O_4$	462	29.483	6.56	Anti-inflammatory and antidepressant effect strong cytotoxic activity	[38]
10.	3,7 diazabicyclo[3.3.1]nonane, or bispidinone	$C_9H_{18}N_2$	154	29.800	5.50	Antibacterial and antifungal efficiency anticancer effects	[39]
11.	6-allyl-.alpha.-dimethylamino-o-cresol	$C_{12}H_{17}NO$	191	30.058	2.70		
12.	3-deoxy-d-mannoic lactone	$C_6H_{10}O_5$	162	30.166	2.59	Antibacterial activity	[40]
13.	9,12,15-Octadecatrienoic acid	$C_{27}H_{52}O_4Si_2$	496	30.443	6.78	Antimicrobial, Anticancer, Hepatoprotective, Anti-arthritic, anti-asthama, diuretic Antiinflammatory, Insectifuge Hypocholesterolemic, Cancer preventive, Nematicide, Hepatoprotective	[41]
14.	Fucoxanthin	$C_{42}H_{58}O_6$	658	30.617	1.95	Antiobesity, antitumor, antidiabetes, antioxidant, anti-inflammatory, and hepatoprotective activities cardiovascular and cerebrovascular protective effects. antioxidant, anti-inflammatory, anticancer, anti-obese, antidiabetic, antiangiogenic and antimalarial activities	[42,43]
15.	5-Isopropenyl-1,2-dimethylcyclo-hex-2-enol	$C_{11}H_{18}O$	166	31.025	1.74	Antimicrobial activity	[44]

16.	1,4-Methanophthalazine	$C_{11}H_{16}N_2$	176			Anti-arthritic agent, anti-inflammatory, anti-cancer, anti microbial, anti-bacterial	[45]
17.	Benzenepropanoic acid	$C_{18}H_{18}N_4O_6$	386	31.739	1.35	Anti-neuroinflammatory activities	[46]
18.	3-Trifluoroacetoxydodecane	$C_{14}H_{25}F_3O_2$	282	32.325	0.93	Antimicrobial and anticancer activity.	[47]

Table 1: List of components found in Ap and their pharmacological activities observed in various plants belonging to different species.

Discussion

Medicinal plants are the prime sources of a reliable alternative to mainstream therapeutics.

Serum amyloid A (SAA), is an acute-phase protein produced primarily by hepatocytes and adipose tissues [48] and binds to pattern recognition receptors (PRRs) like toll-like receptors. (TLRs) TLRs are a family of receptors that are expressed on antigen presenting cells, like macrophages, dendritic cells, and neutrophils [49,50] as well as fibroblasts and epithelial cells and play an important role in immune responses against infection [51]. In general, inflammation and immune response is regulated by TNF-α and IL-6 [52]. The mononuclear factor, TNF-α is mainly produced by activated mononuclear macrophages and T lymphocytes [53], while the signaling molecule IL-6 is secreted by various cells of the body which include monocytes/macrophages, lymphocytes, and epithelial cells [54]. Previous studies demonstrated SAA-mediated NLRP3 inflammasome activation in monocytes [55]; Proteolytic processing of the cysteine proteinase caspase-1 is the hallmark of NLRP3 inflammasome activation [56]. Activated caspase-1 processes pro-IL-1β to the bioactive mature form of IL-1β. Our results revealed that SAA stimulation induced pro-IL-1β mRNA expression in neutrophils and indicated that SAA was capable of processing pro-IL-1β. Findings from the present study thus suggest that the endogenous acute phase reactant SAA may trigger the IL-1β-mediated inflammatory response in human neutrophils via two separate signals: SAA first binds to macrophages, activating pro-IL-1β expression and then induces NLRP3 expression resulting in caspase-1-dependent pro-IL-1β processing.

Analysis by qRT-PCR and Western blot further confirmed the effect of Ap extract on the expression of the genes IL-6, TNF-α, and NF-κB. THP-1 macrophages are known to respond to inflammatory stimuli and concurrently, LPS activated THP-1 macrophages can produce numerous inflammatory cytokines commonly used to analyse diseases associated with inflammatory responses or to establish inflammatory models. Results obtained after qRT-PCR and Western blot analysis indicated that the levels of IL-6, TNF-α and NF-κB significantly increased upon LPS treatment, while Ap extract exhibited an anti-inflammatory effect by reducing the mRNA and protein expression levels of these proteins in LPS-activated macrophages.

Ap extracts resulted in reversal of LPS induced expression of several pro-inflammatory cytokines reported to be regulated by NF-κB activation in a wide variety of cells [57,58]. Furthermore, the anti-inflammatory properties of this plant were attributable to its ability to inhibit IL-6 release by blocking NF-κB and TNF-α activation. In this study, we found that components Propanamide, d-Mannitol, 1-decylsulfonyl, 5.alpha.-Cholestan-

6-one, 9,12,15-Octadecatrienoic acid [59], Fucoxanthin [42,60], 1,4-Methanophthalazine, Benzenepropanoic acid present in the extract may be the ones responsible for inhibiting TNF-∝ induced NF-κB activation.

Screening for SAA expression in sepsis patients showed higher SAA protein and mRNA expression in patients with poor outcome (NS) when compared with those who showed better outcome. The effect of Ap extracts in our study demonstrates that inhibiting SAA with plant extracts significantly reduced TNF-α and IL-1β production in LPS-stimulated THP-1 cells. It is unclear whether decreased serum TNF-α can indicate that inhibition of SAA results in decreased TNF-α production in LPS-stimulated THP-1 cells.

Among the identified Phyto compounds from Ap extracts, 2-Methyl-6-(5-methyl-2-thiazolin-2-ylamino)pyridine is used in the treatment of several types of cancer and as antidiabetic; 9,12,15-Octadecatrienoic acid(ODA) is known to possess antimicrobial, anticancer, hepatoprotective, anti-arthritic, anti-asthmatic, diuretic Anti-inflammatory, Insectifuge, cholesterol-lowering, cancer preventive, Nematicide, Antihistaminic, Antieczemic, Antiacne, 5-Alpha reductase inhibitor, Anti-androgenic, Anti-arthritic and Anti-coronary. Since ODA is the predominant component identified by GC-MS, it may be the one responsible for anti-inflammatory effects of the AP extracts. Synergistic effects might also suggest a possible mechanism. Nonadecanone is known to have anti-inflammatory, antidepressant, and strong cytotoxic activity whereas bispidinone is known for its antibacterial and antifungal efficiency as well as anticancer effects. 4-P-Tolyl-5-Phenylimino-4,5-Dihydro-1,2,4-Thiadiazole, 3-Deoxy-d-mannoic lactone, and D-Xylitol are known for their anti-microbial properties [61]. Fucoxanthin could reduce the levels of proinflammatory mediators including NO, PGE2, IL-1β, TNFα, and IL-6 by suppressing the NF-κB activation and the MAPK phosphorylation. Additionally, fucoxanthin reduced the levels of iNOS and COX-2 proteins in a dose-dependent manner [60]. These bioactive molecules must be purified from *A. paniculata* for further evaluation of their biological activity.

Conclusion

To our knowledge, this is the first report on the effect of *Andrographis paniculata* on inflammatory proteins. Ap extracts could significantly inhibit the expression of SAA and also several inflammatory cytokines. The pathophysiological role of SAA in sepsis is previously studied but in this study, we demonstrated that SAA could be one of the indicators of severity of sepsis as it showed a general trend of elevated expression in patients with poor outcome compared to those who recovered. Ap extracts showed reversal of LPS induced cytokine levels in vitro and also SAA expression. Mainly the extracts seemed to be suppress pyroptotic and apoptotic cell death mediated by caspase-1 and caspase-8. Hence, further exploration of the bioactive components identified in the study could help gain a clearer understanding of the mechanism of activity of the extracts for their application in prevention and management of inflammatory conditions.

Acknowledgements

The authors would like to thank OU-DST-PURSE and RUSA 2.0 for providing the fund and logistic support during the entire study.

Conflict of Interest

The authors declare that they have no conflict of interests.

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