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Inhibition of cystathionine-γ-lyase-mediated hydrogen sulfide production in LPS-stimulated RAW 264.7 macrophages by polyherbal extract

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The polyherbal formulation chandraprabha vati (CV) comprising 29 herbs is traditionally used as an anti-inflammatory agent for arthritis and urinary ailments in Indian Siddha medicine. Earlier, we reported that lipopolysaccharide (LPS) induced apoptosis in RAW 264.7 macrophages is mediated by hydrogen sulfide (H_2S), a potential target for many inflammatory diseases. Here, we hypothesized that pretreatment with CV decreases H_2S level and thereby alleviate the inflammatory conditions induced by LPS in RAW 264.7 macrophages, and this protective effect occurs through alterations in cystathionine-γ-lyase (CSE), a H_2S synthesizing enzyme. Accordingly, we evaluated the anti-inflammatory effect of CV in LPS-stimulated RAW 264.7 macrophages *in vitro*. The CV pretreatment followed by 12 h of LPS-stimulation showed significantly decreased TNF-α, H_2S production possibly through CSE gene expression and NF-κB activation compared to the non pretreated macrophages. Our results further confirm that the polyherbal extract chandraprabha vati may be a useful therapy for inflammatory disorders by reducing H_2S levels.

Keywords: Anti-inflammatory, Ayurvedic, Chandraprabha vati, Herbal, IκBα, Inflammation, Lipopolysaccharide, NF-κB activation, TNF-α, Siddha

Inflammation is a tightly regulated pathophysiological process, including the production of inflammatory mediators that initiate and maintain inflammatory response[1]. Macrophages play a critical role during inflammation[2] and activated macrophages induce inflammation via the release of numerous mediators including the gaseous mediators such as nitric oxide[3] and hydrogen sulfide[4]. Though not studied in detail, previous studies have shown the involvement of hydrogen sulfide as a crucial player in several inflammatory conditions[6].

Previous studies have shown that H_2S, a gaseous mediator, plays an important role in regulating acute inflammatory processes[6]. Cystathionine-γ-lyase (CSE) is the primary regulator of H_2S production in the normal vasculature and heart[5], however, in inflammation-associated tissue damage there is excessive production of H_2S. Accordingly, literature suggest that H_2S levels increase during the pathogenesis of variety of inflammatory diseases in rodent models[6]. Thus, the level of H_2S may reflect the degree of inflammation and provides an indicator to assess inflammatory processes. Blockage of H_2S production with DL-propargylgycine (CSE inhibitor)[6], and CSE gene silencing (small interfering RNA)[7] has been shown to ameliorate inflammation, suggesting that endogenous H_2S has an important role in the pathophysiology of inflammation[8]. In addition, H_2S activates the transcription factor nuclear factor-κB (NF-κB), required for the activation of several pro-inflammatory genes in inflammation, indicating the important role for H_2S in inflammatory signalling[6].

Currently available nonsteroidal anti-inflammatory drugs have side effects in inflammation associated diseases[9]. Search for safe and effective new drugs to regulate the inflammatory process indicates various herbs to possess significant anti-inflammatory effect, which may be a helpful adjunct in reducing the severity of inflammation.

Chandraprabha vati (CV) is a polyherbal formulation listed in Ayurvedic formulary of India1[10] consisting cumulatively of 29 herbs (*Acorus calamus*, *Cyperus rotundus*, *Phyllanthus niruri*, *Tinospora cordiofolia*, *Curcuma longa*, *Berberis aristata*, *Piper longum*, *Coriandrum sativum*, *Terminalia chebula*, *Terminalia belerica*, *Embelica officinalis*, *Embelia ribes*, *Zingiber officinale*, *Piper nigrum*, *Hordeum vulgare*, *Ipomoea turpethum*, *Cinnamomum zeylanicum*, *Asphaltum punjabianum*, *Commiphora*

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wightii, *Andrographis paniculata*, *Scindapsus officinalis*, *Piper retrofractum*, *Phyllanthus emblica*, *Plumbago zeylanica*, *Baliospermum montanum*, *Perculina turpethum*, *Aconitum heterophyllum*, *Elettaria cardamomum* and *Saraca asoca*)[10]. It has been used commonly in the treatment of inflammation linked disorders like urinary tract infections, urinary calculi, rhinitis, bronchitis, asthma, eczema, dermatitis, etc.[11]. Saralla *et al.*[12] reported the quality, purity and strength of Chandraprabha vati. Previously, carrageenan induced hind-paw edema model treated with CV has demonstrated antiinflammatory response by reducing the paw edema[13]. Though the mechanism behind this anti-inflammatory role is not clear, it has been reported that CV has phenolics, tannins, steroids and alkaloids, which might have played an important role in scavenging the free radicals involved in the inflammatory cascade[13]. Additionally, CV treatment leads to regulation of proinflammatory cytokines, Granulocyte-macrophage colony-stimulating factor and Peroxisome proliferator-activated receptor gamma (PPAR-γ), which are primary regulators during the inflammatory processes[14].

In the present study, we hypothesized that CV pretreatment would decrease the production of H_2S in LPS-stimulated macrophages and that this effect would occur through alterations in cystathionine-γ-lyase (CSE).

Materials and Methods

Cell culture

RAW 264.7 cells (established murine macrophage cell line for inflammation studies were purchased from American Type Tissue Culture Collection (ATCC, Rockville, MD) were suspended in complete medium; DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were plated in 24-well plates at a density of 5×10^5 cells/well. All experiments were performed in a humidified atmosphere under 5% CO_2 at 37°C.

Experimental design

CV powder (20 g) (CV procured from Impcops, Chennai, India) was extracted under reflux with methanol (4X50mL) for 15 min. The extract was filtered and evaporated completely under pressure. Cells were pretreated for 12 hr with or without Chandraprabha vati (40 µg/mL) the concentration of 40 µg/mL of CV corresponds to the therapeutic dose prescribed by traditional medical practitioners[18] and stimulated with LPS (lipopolysaccharide, Sigma Aldrich) (1.0 µg/mL). In positive controls, the cells were pretreated with aspirin (acetyl salicylic acid) (200 µg/mL) (anti-inflammatory agent) for 12 h and stimulated with LPS 1µg/mL for 12 h.

MTT assay

Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to a previously described protocol. RAW 264.7 cells, were harvested by trypsinization and resuspended at a final concentration of 2×10^4 cells/mL in fresh DMEM with 2% FBS. Aliquots of 100 µL cell suspension were plated in 96-well tissue culture plates. In order to detect the cytotoxicity of the cells, cells were treated with CV at a concentration range 20 and 40 µg/mL for12 h. After 12 h, 20 µL of a 5 mg/mL MTT solution was added to each well, and the plate was incubated for 4 h, allowing viable cells to reduce the yellow MTT to dark-blue formazan crystals, which were dissolved in 100 µL of DMSO. The absorbance in individual well was determined at 570 nm using microplate reader [ELx-800 biotek absorbance reader]. The cell viability was calculated as percentage of viable cells and then plotted on a graph.

ELISA for TNF-α

Treated RAW 264.7 cells were collected and immediately frozen at −80°C. The samples were analysed for TNF-α level following the protocol provided by R&D systems.

Assessment of H_2S production:

Effect of Chandraprabha Vati on release of H_2S was determined as described previously[15]. Briefly, cells were homogenised in 50 mM ice-cold potassium phosphate buffer (pH 6.8). Homogenate was then added to microcentrifuge tubes containing 150 µL of zinc acetate (1% w/v) to trap H_2S. The reaction was terminated after 5 min by adding NNDP (dimethyl-p-phenylenediamine dihydrochloride) sulfate (20 nM in 7.2 M HCl) and $FeCl_3$ (30 mM in 1.2M HCl). After the mixture was kept in the dark for 20 min, TCA (10% w/v) was added subsequently and centrifuged at 4000 rpm for 10 min. For quantification, the absorbance of the solution was determined at 670 nm.

Assay of H_2S synthesizing activity

H_2S biosynthesis was measured as described previously[16]. Here, the frozen experimental cells were thawed on ice and homogenized in ice-cold 100 mM potassium phosphate buffer with pH 7.4. The reaction

mixture in the volume 500 µL) consists of homogenate, 10 mM L-cysteine, 2 mM pyridoxal 5'-phosphate and made up using saline. The reaction was performed in tightly sealed eppendorf tubes and the reaction was initiated by transferring the tubes from ice to a water bath at 37°C. In some experiments, the enzymatic reaction was stopped immediately by addition of 10% w/v trichloroacetic acid to denature protein prior to addition of L-cysteine. After incubation for 30 min, 1% w/v zinc acetate was added to trap evolved H_2S, followed by 10% w/v trichloroacetic acid. Subsequently, 20 µM N,N-dimethyl-p-phenylenediamine sulphate in 7.2 M HCl and 30 µM FeCl3 made using 1.2 M HCl were added in the 96-well microplate reader and the absorbance was measured at 670 nm after 15 min incubation. The H_2S concentration of each sample was calculated against a calibration curve of sodium hydrosulphide (NaHS; 3.12–250 µM) and results are expressed as nmol H_2S formed per mg DNA[6].

Semi quantitative PCR

Total RNA was extracted from cells with TRIzol reagent (Sigma Aldrich) according to the manufacturer's protocol and was stored at –80°C until required[15]. The concentration of isolated RNA was quantified spectrophotometrically by measuring the absorbance at 260 nm, and the integrity was verified visually by ethidium bromide staining of 18S and 28S rRNA bands on a denaturing 1% agarose gel for quality. About 1.0 µg of RNA was reverse transcribed into cDNA using verso cDNA Synthesis Kit (Thermo scientific) at 25°C for 5 min and 42°C for 30 min, followed by 85°C for 5 min. The synthesised cDNA was used as a template for PCR amplification by iQ Supermix (Bio-Rad). The PCR primers for detection of CSE were Sense: 5′-GACCTCAATAGTCGGCTT CGTTTC-3′, Antisense: 5′-CAGTTCTGCGTATGCT CCGTAATG-3′, size 618 bp. The PCR protocol consisted of optimized 32 cycles. The reaction mixture was first subjected to 95ºC for 3 min for activation of polymerase. This was followed by amplification cycle consisting of 95ºC for 30 s annealing temperature of 60°C and for amplification 72ºC for 30 s. PCR amplification was performed in MyCycler (Bio-Rad, Laboratories). PCR products were analysed on 1.5% w/v agarose gels containing 0.5 µg/mL ethidium bromide and photographed (Gel Doc-It Imaging System). β-actin was used as an internal control to normalize the signal from genes of interest (sense: 5′-GGGCTGTATTCCCCTCCATC-3′ antisense: 5′-GTCACGCACGATTTCCCTCTC-3′; 22 cycles; Annealing: 61°C).

Real-time PCR

A total of 100 ng of RNA was used for each real-time PCR. It was amplified by Light Cycler real time PCR machine (Bio-Rad) using SYBR green I master mix (Sigma). Gene expression was calculated relative to β-actin levels by the comparative ΔCT values method.

Nuclear extract preparation and NF-κB-DNA binding assay

Nuclear extract was prepared from the cells according to manufacturer's protocol (Cayman's Nuclear Extraction kit NF-κB P65 transcription factor assay kit it, MI, USA). The binding of NF-κB to DNA was measured by ELISA-based. Multiwell plates were coated with un-labelled oligonucleotide containing the consensus binding site for NF-κB (5′-GGGACTTTCC-3′)[16]. To each well extracted cell nuclear proteins (20 µg) were added and incubated for an hr to allow NF-κB to bind DNA. After that, antibody against NF-κB p65 subunit was used and NF-κB complex bound to the oligonucleotide was detected. A secondary antibody conjugated to horseradish peroxidase provides the basis for the colorimetric quantification.

Western blot analysis

Treated cells were homogenized with radio-immunoprecipitation assay lysis buffer, protease inhibitor and phosphatase inhibitor cocktail (Sigma chemicals). The homogenate was centrifuged at 4°C for 15 min at 13500 rpm. Using Bio-Rad protein assay, protein concentration was determined. Protein sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. To block non-specific binding the membranes were incubated with 5% non-fat milk powder for an hour. The membrane was then incubated with primary antibody for IκBα and HPRT (1:1000 dilution) (Cell Signalling Technology). After this, the membrane was washed 3 times with 0.05% Tween 20 in phosphate-buffered solution and incubated for one hour in goat antirabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) (1:2000 dilution). After washing the membranes were then incubated in SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill) and exposed to radiographic films (CL-XPosure, Pierce).

Statistical analysis

All data represent a minimum of 6 experiments and are expressed as the value ± the standard deviation (SD). The significance of changes was evaluated by using one way ANOVA and Tukey′s method was used as a post hoc test for the difference between groups. *P* value <0.05 was taken as the level of significance.

Results

Cytotoxic effect of CV on Raw 264.7 murine macrophages

To study the anti-inflammatory effect of CV, Raw 264.7 murine macrophages were chosen. The cells when treated with 20 and 40 µg/mL of CV did not show any cytotoxic effect (Fig. 1). Since, CV did not show cytotoxic effect, these doses were used for subsequent experiments.

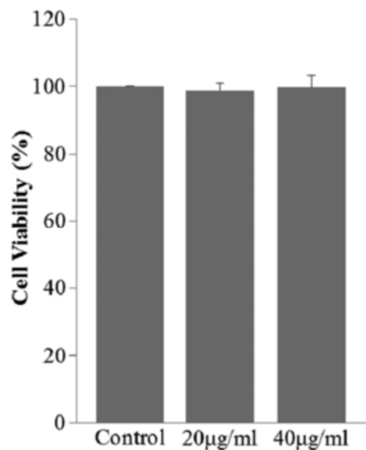

Fig. 1 — Inhibition of Raw 264.7 cell growth by CV (20 and 40 µg/mL, 12 h incubation) as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were exposed for 24 h to the indicated concentrations of CV. [Values are the mean from three experiments. (CV, chandraprabha vati)]

Treatment of murine macrophages with 1.0 µg/mL LPS led to a strong production of the pro-inflammatory cytokines TNF-α (467.74±73.35 pg/mL, *P* <0.001) (Fig. 2). CV (20 and 40 µg/mL) co-treatment significantly reduced LPS-induced TNF-α levels (128.68±30.58 pg/mL, *P* <0.001) in the cells (Fig. 2).

CV inhibits H_2S levels by reducing CSE expression in LPS-stimulated Raw 264.7 macrophages

As shown in Fig. 3C, CSE mRNA expression significantly increased in LPS-induced cells when compared with unstimulated cells whereas LPS-induced CSE mRNA expression was down-regulated after CV treatment in the cells. Compared with the control group (0.05±0.003 nM), H_2S levels significantly (0.12±0.02 nM, *P* <0.001) increased in LPS-induced cells. However, after treatment with CV, H_2S levels significantly (0.08 ± 0.002 nM, *P* <0.01), decreased when compared with LPS treated group (Fig. 3 A & B).

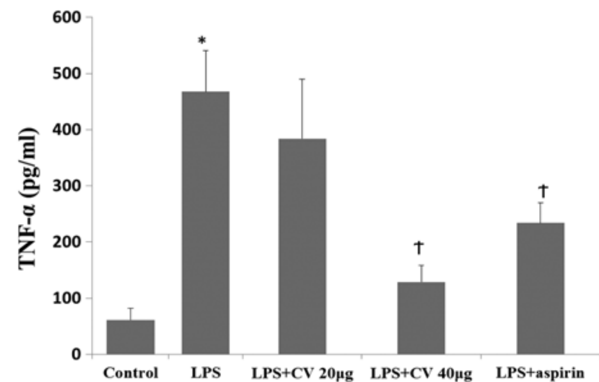

Fig. 2 — Effect of CV (40 µg/mL) on the proinflammatory cytokine TNF-α levels. [Values are expressed as mean ± S.D. of at least six separate determinations. **P* <0.001 when LPS treated Raw 264.7 macrophages were compared with control cells. †*P* <0.001 when LPS treated cells were compared with CV pre-treated cells. (CV, chandraprabha vati)]

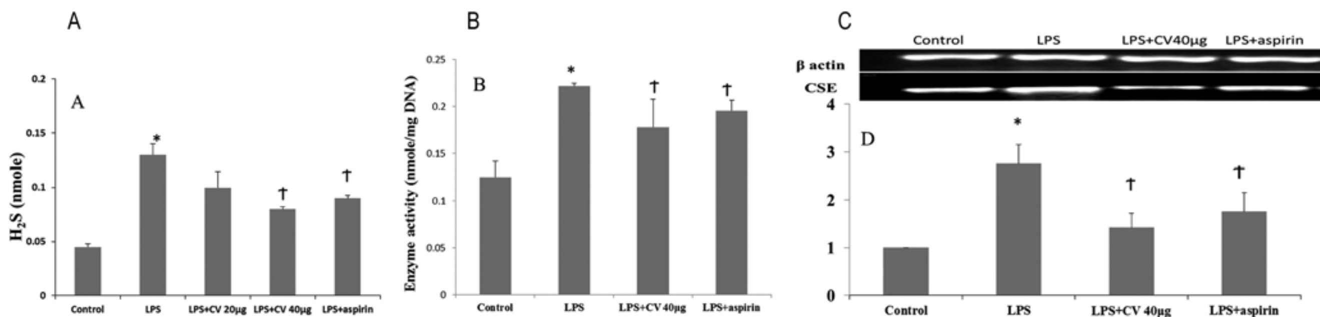

Fig. 3 — CV pretreatment attenuated LPS-induced increase in H_2S concentration in Raw 264.7 macrophages. Raw 264.7 macrophages cells were pre-treated with CV (40 µg/mL) 12 h before LPS (1.0 µg/mL) treatment. (A)H_2S level; (B) H_2S synthesizing enzyme activity, was measured as described in Materials and Methods; (C) Semi quantitative RT-PCR detection of CSE mRNA expression. Sample loading was normalized with β-actin; and (D) A significant decrease in CSE expression in CV pre-treated cells was observed by real-time analysis [Results shown are the mean ± SD of at least six separate determinations. **P* <0.001 when LPS treated Raw 264.7 macrophages were compared with control cells. †*P* <0.01 when LPS treated cells were compared with CV pre-treated cells. (CV, chandraprabha vati)]

Effect of CV on IκBα degradation and NF-κB translocation in LPS-stimulated murine macrophages.

On the basis of anti-inflammatory effect evoked by CV on TNF-α and H_2S responses, we determined whether CV regulates LPS-stimulated inflammation in terms of IκBα degradation and NF-κB DNA binding activity. The result showed that LPS (1.0 μg/mL) induced NF-κB DNA binding activity. CV treatment decreased LPS (1.0 μg/mL) induced NF-κB DNA binding activity in macrophages (Fig. 4B). Western blot analysis revealed that LPS treatment induced IκBα degradation in the cells, whereas CV treatment attenuated LPS-induced IκBα degradation in macrophages (Fig. 4A).

Discussion

In the present study, we evaluated the anti-inflammatory effect of CV and the mechanism using LPS-stimulated RAW264.7 cells. CV at 20 and 40 μg/mL concentrations did not affect the cell viability (Fig. 1). To our knowledge, this is possibly the first report on the anti-inflammatory effects of CV in LPS-induced macrophages. In this well described LPS-stimulated model[4,18], we assessed the inhibition of proinflammatory cytokine, TNF-α by CV (Fig. 2). TNF-α is an early response cytokine in the inflammatory process and an important mediator of inflammation[19]. We evaluated the effects of CV pretreatment (12 h) on TNF-α production in 12 h LPS-stimulated RAW 264.7 macrophages. Confirming our hypothesis, LPS treated cells showed significant increase in proinflammatory cytokine, TNF-α production (467.74±73.35 pg/mL, P <0.001) when compared with LPS untreated cells (61.07±21.06 pg/mL). However, CV (40 μg) pretreatment (128.68±30.58 pg/mL) in LPS stimulated macrophages demonstrated a significant decrease in TNF-α (P <0.001) (Fig. 2). In most inflammatory conditions, the decreased production of TNF-α would imply a reduction in inflammation[20] and the reduction of TNF-α by CV could possibly ameliorate inflammation in the clinical conditions.

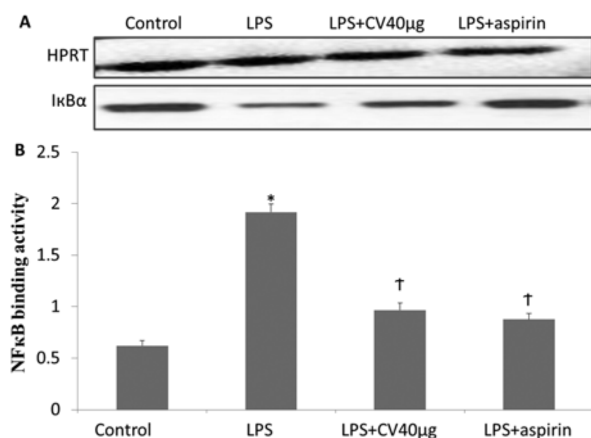

Fig. 4 — (A) IκBα phosphorylation was measured in cytosolic extracts from the same samples by Western blot analysis. Results of 3 to 6 experiments are shown. *P <0.001 when LPS treated Raw 264.7 macrophages were compared with control cells. †P <0.05 when LPS treated cells were compared with CV pre-treated cells. (CV-chandraprabha vati); and (B) NF-κB DNA binding activity was measured in nuclear extracts by ELISA.

Additionally, we have also shown that two potent proinflammatory mediators, TNF-α and H_2S, have two independent pathways in regulating the inflammatory process in RAW macrophages[4]. In this study, we also found that H_2S has a role in mediating the inflammatory effect of LPS, and the herbal formulation CV could effectively quench this inflammatory mediator. For instance, H_2S production by unstimulated macrophages is low (0.05±0.003 nM), and when stimulated with LPS, there was a significant increase in H_2S concentrations at 12 h (0.12±0.02 nM, P <0.001). This effect was significantly decreased by CV pretreatment at a concentration of 40 μg (12 h pretreatment) (Fig. 3A). The H_2S synthesizing enzyme activity was also found to be decreased in CV pretreated cells (40 μg) (Fig. 4B).

A significant portion of H_2S is released after noxious stimuli like pro-inflammatory cytokines, endotoxin, etc., and the level of this response is correlating well with the upregulation of CSE gene transcription[21]. Further, the proinflammatory signalling role played by H_2S is supported by an *in vitro* study, where human monocytes under the influence of H_2S induced cytokine secretion thereby regulating the inflammatory reactions[22].

The decreased H_2S production after treatment with CV suggested the possibility of modulation of CSE gene expression. Hence, we checked the expression level of CSE in CV treated macrophages. As shown in Fig. 3C, the LPS treated RAW 264.7 cells showed distinct increase in CSE expression, and the values were ~1.75 fold higher than the control (Fig. 3D). CV (40 μg) pretreatment decreased LPS-stimulated CSE gene expression at 12 h (~1 fold reduction) (Fig. 3D). In these experiments, we were able to demonstrate that herbal formulation, CV could significantly decrease H_2S production in LPS-stimulated murine macrophages and that this inhibition is associated with altered CSE gene expression. From our findings, it is clear that the flavonoids present in CV possess considerable anti-inflammatory effect[22] especially against the H_2S -associated inflammatory pathways.

On the basis of anti-inflammatory effect evoked by CV on TNF-α and H_2S response, we next determined whether CV regulates LPS-stimulated NF-κB in terms of IκBα degradation and NF-κB DNA binding activity. The result showed that LPS (1.0 µg/mL) induced NF-κB DNA binding activity. CV treatment decreased LPS (1.0 µg/mL) induced NF-κB DNA binding activity in macrophages (Fig. 4B). Western blot analysis revealed that LPS treatment induced IκBα degradation in the cells, whereas CV treatment attenuated LPS-induced IκBα degradation in macrophages (Fig. 4A). It was reported that overexpression of CSE led to enhanced activation of NF-κB and phosphorylation of IκBα in inflammation[6]. Moreover, previous studies have also reported that exogenous H_2S induces NF-κB translocation in inflammation[23]. In this study, we found that CV decreased LPS-induced NF-κB activation and IκBα degradation. Thus, the inhibitory effect of CV on NF-κB translocation and inflammation could be via H_2S signalling mechanism. It has been reported that several plant based compounds[14-16,24] and menadione[25] that inhibited CSE expression and H_2S level lead to the attenuation of inflammatory response.

It is presumed that under pathologic pro-inflammatory conditions, inhibiting macrophage H_2S production could possibly attenuate a variety of proinflammatory mediators. It has been reported that the efficacy of the herbal extract is usually attributed to the multiple phytochemicals present in it. It is believed that the ayurvedic preparation with numerous phytochemical ingredients in it might regulate multiple molecular targets thereby providing with clinically favouring efficacy than a single-molecule drug[26]. The present study supports further the work of Weerasekara *et al*[27] and Rajani *et al*[12] by incorporating the H_2S axis which was not revealed before.

Conclusion

Results of this study demonstrate that the polyherbal extract Chandraprabha vati (CV @40 µg/mL) exhibit promising anti-inflammatory activity against LPS-stimulated Raw 264.7 macrophages, and one prospective machinery is through the suppression of H_2S. Although the precise mechanism of action of CV as an anti-inflammatory agent is yet to be detailed, the presence of numerous phytochemicals may synergistically contribute to its observed activity. The precise role of H_2S and its individual molecular interactions with other inflammatory mediators needs further investigation.

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Conflict of interest

Authors report no conflict of interests.

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