Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(12):45-52

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Mesuaferrin-A: Bio Active Flavonoid Inhibits LPS Stimulated Inflammatory Response via Down-Regulation of NF-κB and MAPK Signaling Pathways in RAW 264.7 Cell Lines

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ABSTRACT

Background: *Mesuaferrin-A, bio active flavonoid has been isolated from bark of Mesua ferrea L. shown to inhibit NO and TNF –α production in LPS induced RAW 264.7 cells. These flavonoids possess biological properties, such as antioxidant, anti-inflammatory, antimicrobial and anticancer activities.*
Objective: *The main aim of the study is to investigate the mechanisms underlying the anti-inflammatory effects of Mesuaferrin-A on lipopolysaccharide- (LPS) induced inflammation in RAW 264.7 cells.*
Methods: *To evaluate the anti-inflammatory effect of Mesuaferrin-A, on lipopolysaccharide (LPS)-stimulated inflammation in mouse RAW264.7 cells. The inflammatory mediator Cyclooxygenase-2 (COX-2) and regulatory transducing factor like nuclear factor-κB (NF-κB), p38 MAPK (p38) and AKT were analysed by Western blotting.*
Results: *The results showed that Mesuaferrin A (50 μg/mL) significantly inhibited the COX-2 gene and protein expressions through prevent the nuclear translocation of NF-κB by inhibiting the phosphorylation of IkBα and also inhibited activation of the p38 MAPK phosphorylation and Akt in LPS-treated RAW 264.7 cells.*
Conclusion: *Mesuaferrin A, inhibited COX-2 expression through the down regulation of NF-κB-dependent transcriptional factor suggesting that Mesuaferrin A bioactive flavonoids is the important constituent in the bark of Mesua ferrea L and has a potential novel anti-inflammatory agent targeting on the NF-κB signaling pathway, to the treatment of inflammatory diseases.*

Keywords: Lipopolysaccharide; Anti-inflammation; COX-2; Nuclear factor-κB (NF-κB); RAW264.7 macrophages

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INTRODUCTION

Inflammation is an innate immune response that protects host organisms from external injuries and pathogens [1]. However, up regulation of inflammation also contributes to the development metabolic diseases and chronic inflammatory diseases such as arteriosclerosis, rheumatoid arthritis, myocardial infections and cancer [2,3]. Pathogen and host derived molecules such as lipopolysaccharides (LPS) and interferon-γ activates macrophages, which in turn up-regulates synthesis and release of pro-inflammatory mediators such as tumor necrosis factor- α (TNF- α), interleukin-1β (IL-1β), nitric oxide (NO) and Prostaglandin E_2 (PGE_2) by activating their respective enzyme cyclooxygenase-2 and induced nitric oxide synthase [4]. Among these causative factors, lipopolysaccharide (LPS), a major component of the bacterial cell wall and a ligand of Toll-like receptor 4 (TLR4), has the ability to

activate various inflammation- related cellular responses [5]. From *in vitro* and *in vivo* anti-inflammatory studies, the increased pro inflammatory mediators such as IL-1β, TNF- α and NO are associated with activation of nuclear factor-κB (NF-κB), that plays a key role in the up-regulation of inflammatory diseases [6].

NF-κB heterodimer reside in the cytosol bound to an inhibitor known as IκB α kinase. NF-κB heterodimer is activated by a number of pro inflammatory signaling molecules that drive inflammation through degradation and phosphorylation of IκB α kinase. Degradation of IκB α kinase leads to translocation of NF-κB into the nucleus and bind to the DNA at the promoter site that regulates the transcription of inflammatory enzymes and mediators [7]. The mitogen activated protein kinases (MAP kinases) such as extracellular signal receptor activated kinase (ERK), P^{38} are group of signaling molecules that play an important role of controlling inflammatory responses [8]. Phosphorylation of MAPK is responsible for the production of proinflammatory cytokines and enzymes in inflammatory macrophages and PI3K/AKT signaling pathway plays an important role in LPS-induced inflammatory responses. The recent *in vivo* and *in vitro* anti-inflammatory studies have demonstrated that bio active secondary metabolites from medicinal plants such as curcumin, resveratrol and pterostilbene down regulates the NF-κB, MAP kinases and PI3K/AKT inflammatory signaling pathways [9]. Therefore, substances that inhibit LPS-induced signaling pathway could also alleviate the progress of inflammatory diseases.

Mesuaferrin – A (Figure 1) has been isolated from bark of *Mesua ferrea* L, exhibited significant *in vitro* and *in vivo* anti-inflammatory activity.

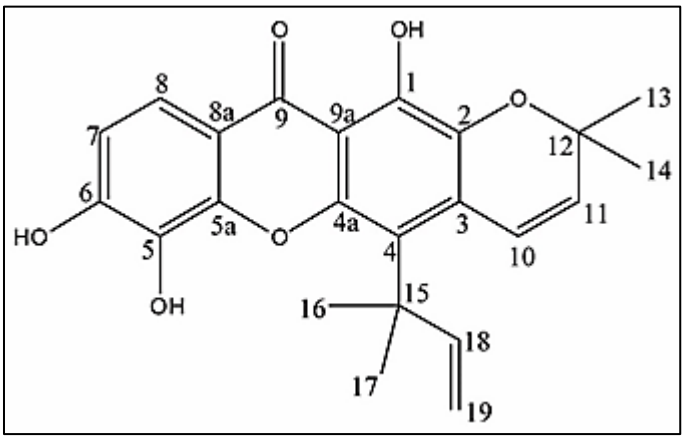


Figure 1: Structure of Mesuaferrin - A

It has been reported that Mesuaferrin - A exhibited a significant dose-dependent COX-2 and 5-LOX inhibitory activities with IC_{50} values of 45.22 µg/ml and 35.74 µg/ml respectively. Based on the above *in vitro* studies Mesuaferrin - A acts as dual inhibitor by inhibiting COX/LOX dual enzymes [10]. However, the molecular mechanism was not elucidated. The objective of present study was aimed to investigate the molecular mechanisms underlying the anti-inflammatory activities of Mesuaferrin - A in LPS-induced RAW 264.7 cells.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium, Fetal Bovine Serum (FBS) and penicillin and antimycotic solution from Invitrogen, Delhi, India. Lipopolysaccharide (LPS) was obtained from sigma Aldrich Company, Shivaji Marg, New Delhi, India. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trizol reagent, dNTP mix, RNAse inhibitor, M-MLV reverse transcriptase, Ethidium bromide, Tris, acrylamide, SDS, APS, Rabbit primary polyclonal antibodies against COX-2, phospho-p38, P^{65} and phospho-Akt, β-actin and secondary antibodies from goat are purchased from Abcam MA, USA, Reagents used in this study were of analytical grade.

RAW 264.7 Cell Culture

The murine peritoneal macrophage RAW 264.7 cell lines were obtained from American type culture collection (ATCC,Manassas, VA, USA) and maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/ml Streptomycin and 100 U/ml penicillin (Invitrogen), maintained at 37°C in a 5% CO_2 incubator.

MTT Assay

To study the cytotoxicity of Mesuaferrin – A, RAW 264.7 cells were seeded in 96 well plates at density of 5×10^4 cells/well (B.D. Bioscience) using fresh DMEM medium. After overnight growth, RAW 264.7 cells were treated with various concentrations of (10, 50, 100 µg/ml) Mesuaferrin – A for 24 hours, the medium was replaced with MTT solution (0.5 mg/ml) and incubated in dark for another 4 hours at 37°C and 5% CO_2. Medium was then removed and the formazan crystals were solubilised in 100 µl of DMSO for 10 minutes. The absorbance was measured at 570 nm on a microplate reader. The control group consists of untreated cells were considered was 100% of viable cells. Results are expressed as percentage of viable cells when compared with the control group.

$$\% \, Cell \, Viablity = \left(\frac{OD \, of \, Control - OD \, of Test}{OD \, of \, Control}\right) \times 100$$

RAW Cell Culture for Gene and Protein Expression Studies

RAW 264.7 macrophages were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% Fetal Bovine Serum, antibiotic and antimycotic solution (Invitrogen). Cells were cultured at 37°C with 5% CO_2 in the incubator. The Mesuaferrin-A compound was prepared with 0.1% DMSO, and cells were then pretreated with 50 µg/ml of compound Mesuaferrin–A, allowed for 12 hrs incubation, followed by stimulated with LPS (1 µg/ml) for another 20 hrs. Positive control wells were only treated with LPS, whereas 0.1% DMSO along with medium was added to the negative control wells. Thereafter, the medium was removed from the wells and the cells were washed twice with ice cold PBS, then trypsinized using 0.025% trypsin-EDTA and incubated at 37°C until cells had lost adherence to the tissue culture dish. After the cells had come off the dish, 5 ml of complete DMEM was added to inactivate trypsin activity. Following inactivation, cells were centrifuged at 2000 rpm at room temperature for 3 minutes, the supernatant was discarded at 100 µl of lysis buffer was added and the pellet was dissolved in it. The pellet was kept on ice and sonicated for three pulses of 30 seconds, each with a break of 30 seconds in between each pulse and then pellet was stored at 70°C till further usage.

RNA isolation and c DNA synthesis for assessment of COX-2 gene expression:

The pellet was resuspended in 1ml of TRIZOL® (Invitrogen) reagent, 20% chloroform and mixed vigorously for 15 minutes. The samples were centrifuged at 14000 rpm for 15 minutes at 4°C to separate the aqueous and organic layers. The upper aqueous phase was transferred to a new tube and mixed with equal volume of isopropanol, incubated at 80°C for 1 hour. RNA pellet was collected by centrifuging at 14000 rpm for 15 minutes at 4°C and washed with 70% ethanol. The RNA pellet was air dried and dissolved in DEPC treated water Quantification of RNA was done by Nanodrop2000 and the integrity of RNA confirmed by 2% agarose gel electrophoresis. 1 µg of RNA was used for cDNA synthesis. The contents of the tube, RNA oligo d(T) and milliQ water were incubated at 65°C for 5minutes and immediately transferred onto ice followed by addition of reaction buffer, dNTP mix, RNase inhibitor (40 Units/µl) and M-MLV reverse transcriptase (200 Units/µl, Invitrogen) and cDNA was prepared.

Semi quantitative polymerase chain reaction (PCR) for amplification of COX-2:

The cDNA was used to amplify GAPDH and COX-2. The amplified products were separated on 2% agarose gel electrophoresis and stained with ethidium bromide. The band intensity was measured using Image gel software. Levels of mRNA expression were normalized with internal control gene GAPDH. The primer sequences, length, annealing temperatures are listed below (Table 1).

Table 1: The sequence of the primers used in RT-PCR analysis

Name of gene	Primers	Sequence	Length (bp)	Tm (°C)	GC%
COX-2	FP	5-CACTACATCCTGACCCACTT-3	20	65	60.4
	RP	5-ATGCTCCTGCTTGAGTATGT-3	20	64.2	60.4
GAPDH	FP	5-GAGGGCTGGGGCTCATTTGC-3	22	60.9	59.1
	RP	5-TGGTGCAGGAGGCATTGCTGAT-3	22	61.1	59.1

FP – Forward primer; RP – Reverse primer

Preparation of whole RAW 264.7 cell lysate for the expression studies of COX-2, NF-κB-P^{65}, P^{38}, P-AKT:

Suspension of RAW 264.7 cells was transferred into a clean 1.5 ml centrifuge tube. Adherent cells were removed by scraping and transferred the detached cells into clean 1.5 ml centrifuge tubes. The cells were collected by centrifugation at 2000 rpm for 3 minutes. The cell pellets are washed with ice-cold PBS. The cells were lysed by

adding 100 µl RIPA (10^8 cells) buffer and 1 µl of 100X protease inhibitor cocktail and incubated for 30 minutes on ice with occasional vortexing. The cell extracts were collected by centrifugation at 14,000 rpm at 4°C for 30 minutes and stored at -20°C. Protein concentration of cell lysates was determined by using Bradford's assay (Sigma) using a BioTek multiplate reader.

Western Blot Analysis

The cell lysate from the above, RAW 264.7 cell culture both in the presence or absence of Mesuaferrin-A, were subjected to Western blotting analysis to study the expression levels of proteins *viz.*, COX-2, P^{65}, P^{38} and P-Akt involved in a signal transduction pathway that needs for the production of proinflammatory mediators. Proteins were separated based upon their molecular size using 10-12% polyacrylamide gels. Separating and stacking solutions were poured into gel chambers and well-forming comb was placed, left to polymerize. After polymerization, the comb was removed and placed in running buffer tank. The sample buffer was added to cell lysates and boiled at 60°C for 5 minutes. Equal amount of protein was loaded along with a marker and run at 70 V for stacking and 120 V to separate on resolving gel. After separation, proteins were transferred onto nitrocellulose membrane at 400 mA for 2 hours. The membrane was stained with ponceau stain to check protein transfer, washed and kept in 5% skimmed dried milk solution to block nonspecific binding sites. The blots were incubated with respective rabbit primary antibody (1:1000) against COX-2 P^{65}, P^{38}, P-Akt and β-actin for overnight at 4°C, followed by washes with TBS, TBS-T and each for 15 minutes. Then the blot was incubated with HRP-tagged goat anti-rabbit IgG secondary antibody (1:10,000), followed by washes with TBS, TBS-T and TBS sequentially for 15 minutes each. Chemiluminescence detection reagents luminol and H_2O_2 (Perkin Elmer) were added to visualize the protein bands using Kodak Image Station.

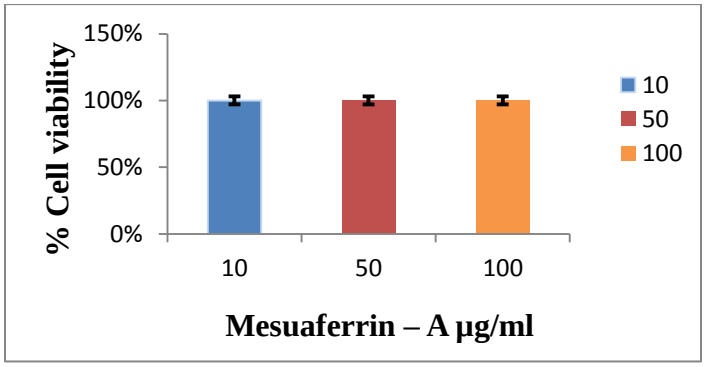
Statistical Analysis

The results were expressed as the mean ± standard error of the mean (SEM). The statistical difference between the test and control groups were evaluated by one way analysis of variance (ANOVA) by Graph pad prism 6.0 software and followed by Dunnets's t test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ represents a significant difference between the control with the test group.

RESULTS AND DISCUSSION

Effects of Mesuaferrin – A on Cell Viability

The cytotoxicity of Mesuaferrin – A in RAW 264.7 cells was measured by MTT assay, as shown Figure 2, Mesuaferrin – A, did not affect cell viability having a range of >90%, at concentration of 10, 50 and 100 µg/ml. Therefore, a concentration range from 10-100 µg/ml of Mesuaferrin – A were used for elucidate its anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages.



Note: Values are mean of three replicates ± SEM. Concentration of Mesuaferrin A µg/ml takenin X-axis % of Cell viability in Y-axis

Figure 2: Effect of Mesuaferrin – A, on cell viability of RAW 264.7 cells

Effect of Mesuaferrin-A on COX-2 mRNA and Protein Expression in LPS-Induced RAW 264.7 Cells

Inflammation underlies in both physiological and pathological processes [11]. LPS activate macrophages through TLR4 and induce a variety of pro-inflammatory mediators and cytokines via distinct signaling pathways. Thus, new anti-inflammatory agents are being discovered based on their suppression of pro-inflammatory cytokine and

mediator production, and related signal transduction [12]. Our study shows that Mesuaferrin-A strongly inhibited the expression of COX-2 as well as their products, PGE2 and NO, in LPS-stimulated RAW264.7 cells [13].

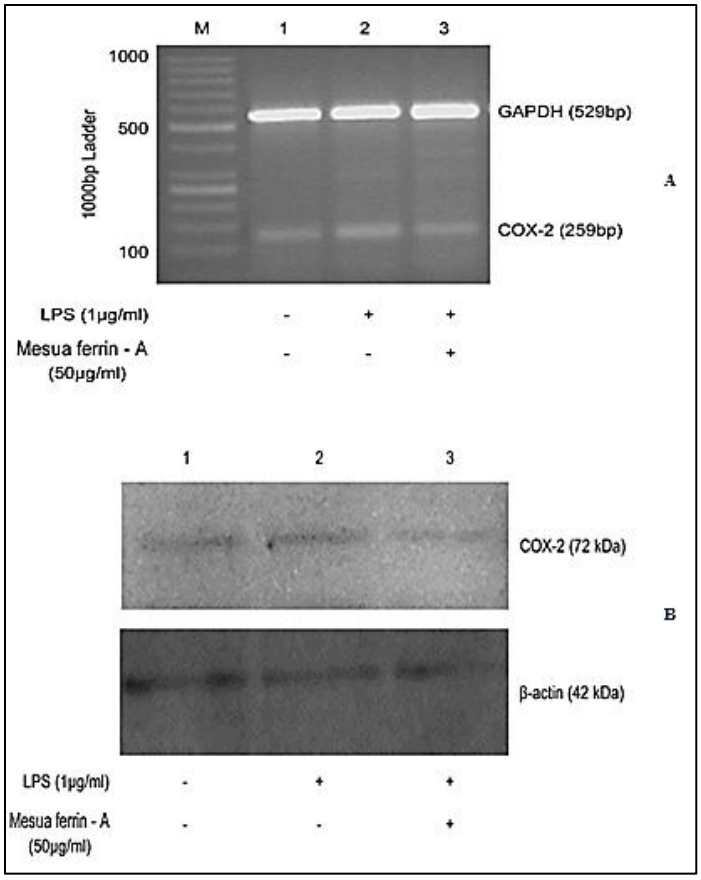


Figure 3: Effect of Mesuaferrin-A on COX^{-2} mRNA and protein expression in LPS-induced RAW 264.7 cells. A) mRNA expression of COX-2 compared with GAPDH . B) Protein expression levels of COX-2 compared with β-actin. Lane 1 – Control; Lane 2 – LPS alone; Lane 3- LPS + Mesuaferrin- A

As shown in the Figure 3, LPS treatment significantly increased the levels of COX-2 mRNA and protein expression in RAW 264.7 cells, and Mesuaferrin-A inhibited the expression of the m-RNA and protein levels of COX-2, which in turn reduce the production of PGE2, the key inflammatory mediators of inflammation. RT-PCR analysis also showed that 50 µg /ml of Mesuaferrin-A attenuated the increased COX-2 mRNA expression at the transcription level induced by LPS in RAW 264.7 cells. It is known that PGE2 plays critical role in the activation of macrophages, and induce acute and chronic inflammation [14]. Therefore, to study the suppression of PGE2 by COX-2 is very important in the development of anti-inflammatory agents [15]. Here, the results shown that Mesuaferrin-A isolated bioactive flavonoid, suppressed LPS-induced expression of COX-2 at the protein levels in RAW 264.7 macrophages, which proposed that Mesuaferrin-A induced reduction of PGE2 may be due to transcriptional suppression of COX-2 genes.

Effects of Mesuaferrin-A on LPS-Induced Nuclear Translocation of NF-κB P^{65} in RAW 264.7 Cells

NF-κB is a key transcription factor for up regulation of proinflammatory enzymes and mediators such as COX-2, 5-LOX, iNOS, and pro-inflammatory cytokines such as TNF-α and interleukin -1β in inflammatory conditions [16,17]. From the *in vitro* anti-inflammatory activities of isolated Mesuaferrin -A from *M. ferrea* bark showed significant inhibitory effects on nitric oxide and tumor necrosis factor–α level in LPS-induced RAW 264.7 cells [18] (data not shown). As a result of inhibitory activity of Mesuaferrin-A on nitric oxide and TNF- α levels, the effect of Mesuaferrin - A has been evaluated on the translocation of NF-κBP^{65}in the nuclear fraction isolated from the RAW 264.7 cells as determined by the western blotting. As shown in the Figure 4, LPS exposure to RAW 264.7 cells significantly increased the level of phosphorylated p65 subunit. Treatment with Mesuaferrin – A, markedly prevented the translocation of NF-κBP^{65} into the nucleus in LPS induced RAW 264.7 cells.

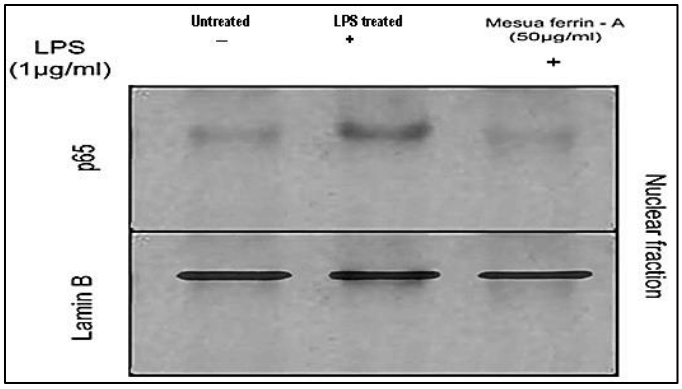


Figure 4: Effects of Mesuaferrin-A on LPS-induced nuclear translocation of NF-κB P^{65} in RAW 264.7 cells

Inhibitory effects of Mesuaferrin - A on NF-κB P^{65}nuclear translocation in LPS stimulated RAW 264.7 cells. These results indicated that the anti-inflammatory effect of Mesuaferrin – A is mediated, through inhibition of NF-κB activation. Recent studies suggest that several plants derived compounds such as curcumin, resveratrol, quercetin inhibits the inflammation by inhibiting the NF-κB Pathway [19]. Our present results were corelated with the anti-inflammatory effect of Wedelolactone (WEL), a major coumestan ingredient in Wedelia chinensis which was reported by Yuan et al. [20].

Effects of Mesuaferrin- A on LPS-induced phosphorylation of MAPK, p38 and phospho-Akt (p-Akt) in RAW 264.7 cells

In order to study whether the inhibition of inflammatory response mediated through MAP Kinase and Akt pathways by Mesuaferrin-A and its effect on the LPS-stimulated phosphorylation of p38 and p-Akt in RAW 264.7 cells has been studied by immunoblotting. MAPKs play an important role in the transcriptional regulation of LPS-induced expression of iNOS and COX-2 [14]. LPS is an effective stimulant of RAW 264.7 cells, LPS treatment results in the increase phosphorylation of p38 in MAPKs and Akt protein in signal transduction pathways [20]. In the present study as shown in the Figure 5 pre-treatment with Mesuaferrin - A for 1 h attenuated the phosphorylation of p38 induced by LPS, whereas the phosphorylated Akt was unaffected. As shown in Figure 6 the ratio of immune intensity between P^{65}, P^{38}, P-Akt, was calculated using image j software. These results suggested that preventing the phosphorylation of p38 MAPK and down regulation of NF-κB by Mesuaferrin – A was attributable for its anti-inflammatory effects. Our reports are correlated with the anti-inflammatory effect of Wedelolactone (WEL), a major coumestan ingredient in *Wedelia chinensis* a potential to be a novel anti-inflammatory agent targeting on the NF-κB signaling pathway [21]. Inhibitory effects of Mesuaferrin - A on MAPKs p^{38}, P-Akt phosphorylation in LPS stimulated RAW 264.7 cells.

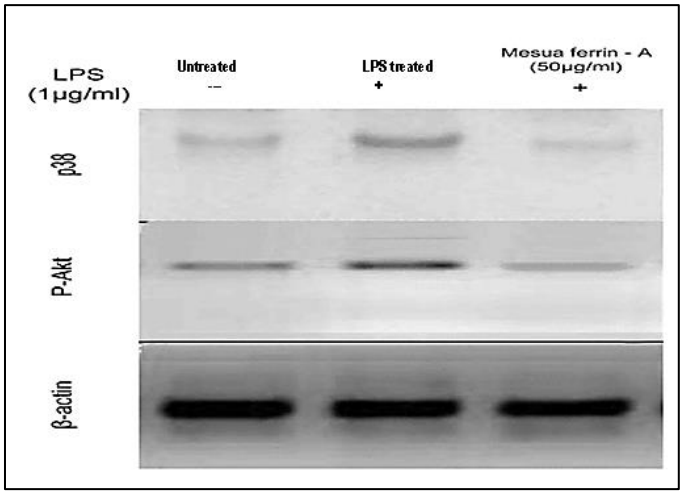
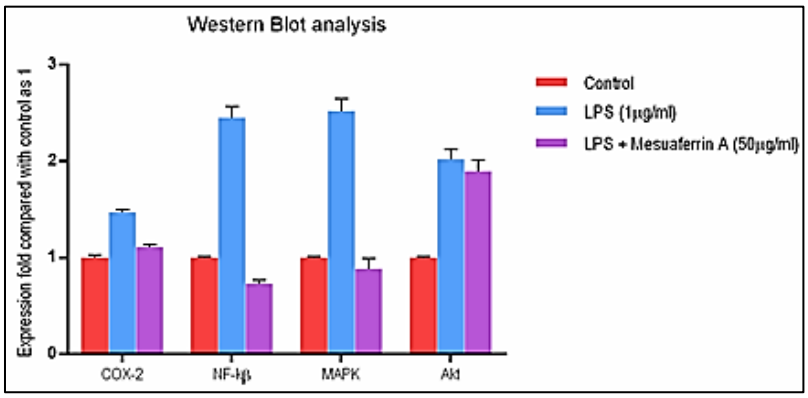


Figure 5: Effects of Mesuaferrin- A on LPS-induced phosphorylation of MAPK, p^{38} and phospho-Akt (p-Akt) in RAW 264.7 cells



Note: Values are mean of three replicates ± SEM

Figure 6: The ratio of immune intensity between P^{65}, P^{38}, P-Akt, β-actin and Lamin-B was calculated using image j software

CONCLUSION

The results of the present study demonstrated that Mesuaferrin – A belongs to flavonoid category present in *Mesua ferrea* bark, exhibited significant anti-inflammatory activity, by down regulating NF-κB, and P^{38} MAPKs activation in LPS-induced RAW 264.7 macrophages. Our findings suggest that Mesuaferrin – A is a functional constituent in the *Mesua ferrea* medicinal and further developed as a novel anti-inflammatory agent for curing inflammatory related disease without causing any side effects.

Conflict of Interest

The author(s) declare(s) that there is no conflict of interest.

ACKNOWLEDGMENTS

I am very happy to convey my sincere thanks to esteemed Professor U.S.N Murthy, Chief Scientist, Head, Department of Biology, Indian Institute of Chemical Technology, Hyderabad, India has permitted me to carry out pharmacological studies in his laboratory.

Financial Support

This work was partially supported by UGC-MRP.F. No. 4N 2-643/2013, New Delhi, India sanctioned to Prof. Duddukuri Govinda Rao, GITAM University, Visakhapatnam.

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