INTRODUCTION

Inflammation is an immediate innate immune response to pathogenic, non-pathogenic challenges, and tissue injuries.\(^1\) It is self-limiting through downregulation of the pro-inflammatory mediators and a reversal in the vascular changes that facilitated the initial immune cell recruitment process, which leads to the restoration of tissue structure and function. During acute inflammation, macrophages and endothelial cells releases inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and related inflammatory mediators such as nitric oxide (NO) and prostaglandinE\(_2\), which are produced by inducible nitric oxide synthase and cyclooxygenase (COX-2), respectively.\(^2\) These inflammatory cytokines and mediators are essential for host survival against infection and tissue repair. Upregulation of these inflammatory mediators leads to tissue damage, in addition; prolonged inflammation causes many inflammatory-related diseases such as multiple sclerosis, rheumatoid arthritis, and atherosclerosis.\(^3\) Moreover, the steroidal and nonsteroidal anti-inflammatory drugs are excellent anti-inflammatory drugs, but non-specific inhibition of inflammatory enzymes and long-term administration causes severe side effects such as gastrointestinal ulceration, obstruction, and bleeding has restricted the therapeutic usage.\(^4\) There is clear evidence addressing that the importance of plant-derived anti-inflammatory agents in the management of inflammatory disorders.

The current strategy focused on the development of novel anti-inflammatory agents with a higher safety profile, particularly medicinal plants of folkloric use as pain releivers and anti-inflammatory agents. A number of phytocompounds with specific activity

---

**ABSTRACT**

**Introduction:** Mesuaferrin-A is a bioactive flavonoid isolated from the bark of ethyl extract of *Mesua ferrea* L. exhibited significant *in vitro* cyclooxygenase -2/5-LOX dual inhibitory activity. **Objective:** The present study was designed to investigate the anti-inflammatory activity of Mesuaferrin-A on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. **Materials and Methods:** To evaluate the effect of isolated bioactive flavonoid Mesuaferrin-A on the production of nitric oxide (NO) and pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in the RAW264.7 macrophages by enzyme-linked immunosorbent assay. **Results:** The Mesuaferrin-A exhibited dose-dependent inhibitory effect on the production of NO and pro-inflammatory cytokines (TNF-α and IL-1β) in LPS-stimulated RAW264 macrophages. **Conclusion:** From this study, it can be concluded that the isolated bioactive flavonoid Mesuaferrin-A act as potent anti-inflammatory agents, it can be used for the treatment of inflammatory related diseases without having any side effects.

**KEY WORDS:** Anti-inflammatory agents, Interleukin-1β, Lipopolysaccharides, Mesuaferrin-A, Nitric oxide, Tumor necrosis factor-α

---

1Department of Chemistry, College of Natural and Computational Sciences, Aksum University, Axum, Ethiopia, 2Department of Biochemistry, Adikavi Nannaya University, Rajahmundry, Andhra Pradesh, India, 3Department of Biochemistry and Molecular Biology, School of Biological Sciences, Central University of Kerala, Kerala, India

*Corresponding author: Dr. Krishna Chaithanya Karri, Department of Chemistry, College of Natural and Computational Sciences, Aksum University, Axum, Tigray Region, Ethiopia. Phone: +251944121156. E-mail: Krishanchaitanyawc@gmail.com

Received on: 16-01-2018; Revised on: 25-02-2018; Accepted on: 27-03-2018
have been identified, screened, and developed to treat the inflammatory associated diseases by targeting pro-inflammatory pathways. Our recent reports showed that the isolated bioactive flavonoid Mesuaferrin-A [Figure 1].

From the Mesua ferrea. L bark ethyl extract exhibited significant COX-2/5-LOX dual inhibitory activities and also revealed that exhibited a potent anti-inflammatory effect on lipopolysaccharide (LPS)-stimulated macrophages by inhibiting NF-B activation and MAPK phosphorylation.54 Hence, the present study has been undertaken to evaluate the anti-inflammatory effects of bioactive flavonoid Mesuaferrin-A from M. ferrea. L by determine the inhibitory effects on LPS-induced NO and pro-inflammatory cytokines TNF-α, IL-1β in RAW 264.7 cells.

MATERIALS AND METHODS

Materials
Mesuaferrin-A from M. ferrea. L, dimethyl sulfoxide (DMSO), and Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin (Invitrogen), DMSO3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Griess reagent, L-nitroarginine methyl ester (L-NAME), LPS, sodium nitrite (NaNO3), TNF-α, and IL-1β ELISA assay kits (eBioscience, USA). All reagents used are of analytical grade.

RAW 264.7 Cell Culture
RAW 264.7 cells, a mouse peritoneal macrophage cell line was obtained from the American type culture collection USA and cultured with DMEM supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen), maintained at 37°C in a 5% CO2 incubator. The Mesuaferrin-A was solubilized with 2% DMSO resulting in concentration 1 mg/ml in DMEM culture medium, and further used for evaluation of anti-inflammatory activities.

Cell Viability by MTT assay
To study the cytotoxicity of Mesuaferrin-A, RAW 264.7 cells were cultured at a density of 1 × 10^5 cells/well in 24 well plates (BD Bioscience) using fresh DMEM medium, and the cell viability was determined using the MTT assay.6 It is a colorimetric assay for measuring the cellular activities of enzymes (NADPH dependent cellular oxidoreductase enzymes). After overnight growth, RAW 264.7 cells were pre-treated with different concentration of (1, 10, 50, 100, and 200 µg/ml) of Mesuaferrin-A for 2 h and then costimulated with 1 µg/ml LPS for 24 h at 37°C, in 5% CO2 incubator for 24 h incubation, the medium containing Mesuaferrin-A was replaced by the MTT solution (0.5 mg/ml) and incubated in the dark for another 4 h at 37°C, in 5% CO2 incubator. The assay is based on the reduction of a soluble yellow tetrazolium salt to insoluble purple formazan crystals by viable cells. After the removing the medium, 100 µl of DMSO was added to the cells for 10 minutes. The absorbance was measured using a microplate reader at 570 nm.

The control group consists of untreated cells were considered as 100% viable cells. Results are expressed as a percentage of viable cells when compared with the control group.

\[
\% \text{ of Cell viability} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}})}{\text{Abs}_{\text{control}}} \times 100
\]

Measurement of Nitric oxide levels in RAW 264.7 cells by Griess assay
The nitrite concentration in the cell supernatant was measured by the Griess method.7,8 RAW264.7 Cells were cultured at a density of 2 × 10^5 cells/well in 24 well culture plate and pre-treated with different concentrations (50 and 100 µg/ml) of Mesuaferrin-A and 100 µM of L-NAME, an inhibitor of NO was used as a positive control. After 2 h of pre-treatment, then stimulation with LPS was carried out at 1 µg/ml for 24 h maintained at 37°C in a 5% CO2 incubator. After 24 h of incubation, 100 µl of the cell culture supernatant was transferred into 96 well plate, and the same quantity (100 µl) of Griess reagent (1% Sulfanilamide and 0.1% N-1-(naphthyl) ethylene}

Table 1: Percentage inhibition at 50 µg/ml and 100 µg/ml and IC50 values of Mesuaferrin-A on NO, TNF-α, and IL-1β

<table>
<thead>
<tr>
<th>Inflammatory mediators</th>
<th>Mesuaferrin-A</th>
<th>% Inhibition at 50µg/ml</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>68.86</td>
<td>74.90</td>
<td>52.17</td>
</tr>
<tr>
<td>TNF-α</td>
<td>40.32</td>
<td>59.40</td>
<td>75.21</td>
</tr>
<tr>
<td>IL-1β</td>
<td>42.65</td>
<td>62.94</td>
<td>68.43</td>
</tr>
</tbody>
</table>

TNF-α: Tumor necrosis factor-α, IL-1β: Interleukin-1β

Figure 1: Mesuaferrin-A
diamine-HCl in 2.5% H_3PO_4 was added. The plate was incubated for 15 min at the room temperature, and then the absorbance was measured at 540 nm with the microplate reader (Bio-Rad). Cells without any treatment served as basal control, but LPS treatment alone acts as a control. The amount of NO was calculated using NaN_3 standard curve (0–100 µM).

% of Inhibition of Nitric Oxide = \frac{(\text{Abs control} - \text{Abs test})}{\text{Abs control}} \times 100

Measurement of Pro-inflammatory Cytokines (TNF-α and IL-1β) in RAW 264.7 cell culture by ELISA

Induction of LPS-stimulated pro-inflammatory cytokine response in RAW 264.7 cell culture

The effect of different concentrations of Mesuaferrin-A on the production of TNF-α and IL-1β was measured by ELISA assay kit. 2 × 10^5 RAW 264.7 macrophages were seeded in 24 well plates for overnight. Cells were pre-treated with different concentrations (50 and 100 µg/ml) of Mesuaferrin-A. After pre-treatment of 2 h, LPS stimulation was performed at 1 µg/ml. Positive controls were only treated with LPS, whereas in negative control well medium containing 0.1% DMSO was added. The cells were maintained at 37°C, in humidified incubation under an atmosphere supplemented with 5% CO_2.

Quantification of Pro-inflammatory Cytokines (TNF-α and IL-1β) by ELISA

The cell supernatants were collected by centrifugation at 2,500 g for 15 min, and the levels of TNF-α and IL-1β in cultured media were quantified using enzyme-linked immunosorbent assay kit, according to manufacturer’s instruction protocol. The cell supernatant (100 µl) was added to appropriate wells of antibody-coated polystyrene plates and incubated for 2 h. After 3–5 washes, the plates were sealed and incubated with biotin-labeled TNF-α and IL-1β anti-cytokine antibody for 2 h; then the plates were washed and incubated for 20 min with streptavidin/horseradish peroxidase conjugate at 37°C. Finally, the plates were washed and incubated with trimethyl benzidine. The reaction was stopped by the addition of 50 µl of 2N sulfuric acid, and the absorbance read at 420 nm with the microplate reader (Bio-Rad). Cells without any treatment served as basal control, but LPS treatment alone acts as a control.

% of Inhibition of TNF-α and IL-1β = \frac{(\text{Abs control} - \text{Abs test})^2}{\text{Abs control}} \times 100

Statistical Analysis

All the results were expressed as the mean ± standard error of the mean of triplicate analysis (n = 3). The statistical analysis was carried out by one-way ANOVA followed by Dunnett’s test using GraphPad Prism software version 6.0. The IC_{50} values were calculated using MS Excel. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 represent a significant difference compared with the control group.

RESULTS AND DISCUSSION

Flavonoids are an important group of polyphenols widely distributed among the plant flora. Structurally, they are made of more than one benzene ring in its structure (a range of C_{15} aromatic compounds), and numerous reports support their use as antioxidants, anti-cancer and anti-inflammatory agents, anti-allergic, and antimicrobial activities.[9] Chaithanya et al. recently reported that Mesuaferrin-A exhibited significant in vitro and in vivo anti-inflammatory activities. The anti-inflammatory activity of bioactive flavonoids Mesuaferrin-A, ascription due to its ability to regulate the production of inflammatory mediators such as inflammatory cytokines and NO by inhibiting NF-kB activation.[10]

Effect of Mesuaferrin-A on Cell Viability of RAW 264.7 Cells

As shown in Figures 2 and 3, Mesuaferrin-A bioactive flavonoid, does not exhibited cytotoxic effect on RAW 264.7 cells having a range of >80 % at the concentration of 1–200 µg/ml; hence, the dose range of Mesuaferrin-A (1–100 µg/ml) was selected for evaluate the inhibitory effects of Mesuaferrin-A on NO, TNF-α, and IL-1β in LPS-stimulated RAW 264.7 macrophages.

Effect of Mesuaferrin-A on Nitric Oxide Production in LPS-induced RAW 264.7 Cells

As shown in the Table 1, Figures 4 and 5, Mesuaferrin-A significantly *P≤0.05 and **P≤0.01 reduced the nitric
oxide production in a dose-dependent manner in LPS treated RAW cells at 50 and 100µg/ml, respectively. The LPS-stimulated RAW 264.7 macrophages significantly increased nitrate level production (652.05 ±0.29µmoles/ml) compared to untreated control RAW 264.7 cells (150.65 ±0.66 µmoles/ml). However, Mesuaferrin-A exhibited a dose-dependent decrease in nitrate levels in the presence of LPS. The nitric oxide inhibitory activities of Mesuaferrin-A at 50 and 100µg/ml were found to be 68.86% and 74.90%, respectively, and L-NAME, an inhibitor of nitric oxide synthase was taken as a positive control for comparing the nitric oxide inhibitory activity of Mesuaferrin-A, and its percentage of inhibition at 100µm was found to be 85.88. The IC\textsubscript{50} values of Mesuaferrin-A were found to be 52.17µg/ml. A number of studies have reported that isolated bioactive flavonoids such as Apigenin-7-O-B-D Glucuronide Methyl Ester, luteolin, and quercetin shown significant inhibitory on LPS induced RAW 264.7 cells.\[11,12]\n
Effect of Mesuaferrin-A on TNF-α production in LPS-induced RAW 264.7 cells

Pro-inflammatory cytokines induced by LPS such as TNF-α, IL-1β, IL-6, and other chemokines such as G-CSF mediates both acute and chronic inflammation, pharmacological inhibition of these inflammatory mediators is an important target in the treatment of inflammatory related disease. As shown in the Table 1, Figures 6 and 7, Mesuaferrin-A significantly \( *P \leq 0.05 \) reduced the TNF-α production in a dose-dependent manner in LPS treated RAW cells at 50 and 100 µg/ml, respectively. LPS-stimulated RAW 264.7 macrophages significantly increased TNF-α production (754.50 ± 2.96 pg/ml) compared to untreated control RAW 264.7 cells (150.70 ± 3.42 pg/ml). The TNF-α inhibitory activity of Mesuaferrin-A at 50 and 100µg/ml was found to be 40.32 % and 59.40%, respectively, with the IC\textsubscript{50} values of 75.21 µg/ml. Our data correlated with the 7-O-methylnaringenin extracted from \textit{Rhododendron speciferum}, belongs to the flavanone class of polyphenols, it inhibited TNF-α, IL-6, and IL-1β production in a dose-dependent manner in LPS-stimulated RAW 264.7 cells.\[11\]
Effect of Mesuaferrin-A on IL-1β Production in LPS-induced RAW 264.7 cells

As shown in the Table 1, Figures 8 and 9, Mesuaferrin-A significantly *$P \leq 0.05$ and **$P \leq 0.01$ reduced the IL-1β production in dose-dependent manner in LPS-treated RAW cells at 50 and 100 µg/ml, respectively. LPS-stimulated RAW 264.7 macrophages significantly increased IL-1β production (710 ± 0.77 pg/ml) compared to untreated control RAW 264.7 cells (252 ± 0.43 pg/ml). The IL-1β inhibitory activity of Mesuaferrin-A at 50 and 100 µg/ml was found to be 42.65% and 62.94%, respectively, with the IC$_{50}$ values of 68.43 µg/ml. Vo et al., 2012, have been reported that Avicularia, quercetin-3-α-L-arabinofuranoside, is a glycoside of quercetin, significantly attenuated LPS-induced extracellular release of IL-1β.[14]

CONCLUSION

The in vitro studies provide scientific evidence; bioactive flavonoid may be the active constituents related to the traditional use of M. ferrea L bark in acute and chronic inflammatory conditions. The results of the present study demonstrated that Mesuaferrin-A belongs to bioactive flavonoid present in ethyl acetate bark extract of M. ferrea L, shown dose-dependent on significant anti-inflammatory activity, by inhibiting the production of pro-inflammatory mediators NO and TNF-α, IL-1β in LPS-induced RAW 264.7 macrophages at 50 and 100 µg/ml without Cytotoxic effect. The in vitro studies provide scientific evidence, bioactive flavonoid may be the active constituents related to the traditional use of M. ferrea L bark in acute and chronic inflammatory conditions.

ACKNOWLEDGMENT

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, and Hyderabad, India, has permitted me to carry out pharmacological studies in his laboratory.

REFERENCES


