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ORIGINAL ARTICLE

Identification of Chemical Constituents and Inhibitory Effect of *Ficus deltoidea* Fraction against Lipopolysaccharide-Induced Nuclear Factor-Kappa B Inflammatory Pathway in Murine Macrophage 264.7 Cells

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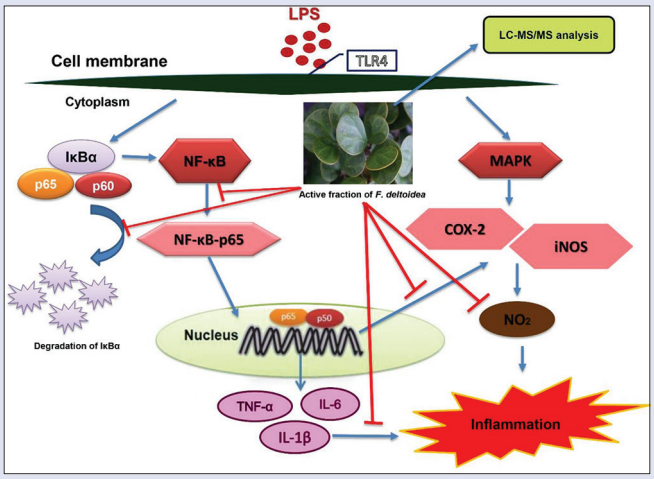
ABSTRACT

Background: *Ficus deltoidea* Jack or locally known as Mas Cotek belongs to the family Moraceae is a conventionally used Malaysian native medicinal plant. **Objectives:** The aim is to determine the anti-inflammatory mechanism of various solvent fractions of *F. deltoidea* methanol extract against Lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) inflammatory pathway in murine macrophage 264.7 cells and to identify the chemical constituents present in the active fraction. **Materials and Methods:** The effect of crude methanolic extract and its fractions (hexane, chloroform, ethyl acetate, and butanol) on murine macrophages against LPS-induced pro-inflammatory cytokines (interleukin [IL]-Iβ, tumor necrosis factor alpha [TNF-α], and IL-6) and biomarkers were tested using enzyme-linked immunosorbent assay and immunoblot analysis. The chemical constituents present in the active fraction were identified using liquid chromatography mass spectrometry and liquid chromatography tandem mass spectrometry analysis. **Results:** The findings indicated that among all the fractions, the ethyl acetate fraction of *F. deltoidea* substantially inhibits the LPS-induced inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokine production including TNF-α, IL-6, and IL-1 β in a dose-dependent manner. The expression of inducible NO synthase, NO synthase, and cyclooxygenase-2 were also effectively downregulated by the treatment of ethyl acetate fraction. Moreover, it also suppressed the expression of LPS-induced NF-κB translocation correlated with the inhibition of NF-κB (inhibitor of kappa B alpha) degradation. The presence of bioactive phenolics, especially flavonoids such as catechin, vitexin, dodecadienyl coumaric acid, (epi)-afzelechin-(epi)-catechin, genistein, and apigenin derivatives were identified in the ethyl acetate fraction of *F. deltoidea*. **Conclusion:** Overall, it has been recommended that the ethyl acetate fraction of *F. deltoidea* could be utilized as a potential natural anti-inflammatory agent.

Key words: Cyclooxygenase-2, *Ficus deltoidea*, inflammatory mediators, inhibitor of kappa B alpha degradation, nuclear factor-kappa B

SUMMARY

- Among the other solvent fractions, the ethyl acetate fraction of *Ficus deltoidea* leaves significantly repressed the lipopolysaccharide-induced damage in murine macrophage 264.7 cells that could be due to the active components such as vitexin, isovitexin, genistein, catechin, coumaric acid, and others present in it.



Abbreviations used: ANOVA: Analysis of variance; ATCC: American Type Culture Collection; BSA: Bovine serum albumin; COX-2: Cyclooxygenase-2; DMEM: Dulbecco's modified minimal essential medium; DMSO: Dimethylsulfoxide; ELISA: Enzyme-linked immunosorbent assay; IL-1 β: Interleukin-1 beta; IL-6: Interleukin-6; iNOS: Inducible nitric oxide synthase; IκBα: Inhibitor of kappa B alpha; LPS: Lipopolysaccharide; LC-MS/MS: Liquid chromatography mass spectrometry and Liquid chromatography tandem mass spectrometry; MAPK: Mitogen-activated protein kinase; MS: Mass spectrometry; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB: Nuclear factor-kappa B; NO: Nitric oxide; PVDF: Polyvinylidene fluoride; SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIC: Total ion chromatography; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-alpha.

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INTRODUCTION

Inflammation is a normal and protective immune response of cell/tissue induced by pathogens, injuries, irritants and microbial infections, etc.[1] Inflammation can be divided into two major type's namely acute and chronic inflammation, acute inflammation is the early stage of inflammation and prompt reaction to various harmful stimuli that activate various types of inflammatory cells to the damaged site. Chronic prolonged inflammation provokes various inflammatory serious chronic diseases such as auto-immune disorders, cancer, inflammatory bowel diseases, sepsis, diabetes, rheumatoid arthritis, multiple sclerosis, asthma, and cardiovascular diseases.[2] Chronic inflammation is a multi-step progression facilitated by activating immune cells, mainly macrophages, and monocytes.[3] It comprises various immune reactions regulated by a flow of pro-inflammatory cytokines, growth factors, inflammatory mediators, and other associated factors released by endotoxin activated macrophages.[4] There are two major signaling pathways triggered (NF-κB and MAPK pathways) by endotoxin, lipopolysaccharide (LPS); LPS facilitated the expression of key inflammatory genes in macrophages and monocytes.[5] Numerous immunological studies have concluded the central role of NF-κB expression in activated macrophages among the other signaling pathways.[6-8] Researchers need to find effective therapeutic targets to inhibit the NF-κB signaling pathway to control the chronic inflammation associated with diseases/disorders. Various steroids and nonsteroidal anti-inflammatory drugs are available in the current market for the treatment of inflammatory-associated diseases. However, prolonged usage of these drugs can induce severe unwanted effects.[9] Thus, biomedical researchers from all over the world explore the effective anti-inflammatory therapeutic molecule from the natural origin without any adverse effects or with minimal side effects.

Ficus deltoidea Jack or locally known as Mas Cotek belongs to the family Moraceae is a traditionally used South East Asian native plant. Various parts of this species are used to treat several chronic ailments such as diabetes and inflammation, cancer. The boiled leaves of this species are conventionally used as a tonic as well as tea for the uterus and vaginal muscle contraction after birth and to treat diabetes.[10] Earlier work has shown the crude extracts enriched with flavonoids exhibited blood-glucose-lowering effect and α-glucosidase inhibition.[11,12] According to Zunoliza *et al.*, the presence of high content of total polyphenols, flavonoids, and tannins in extracts of leaves of *F. deltoidea* have shown good antioxidant activity.[13] The phytochemicals reported from *F. deltoidea* are stigmasterol, catechins and flavones such as apigenin derivatives, vitexin, isovitexin, as well as anthocyanins and proanthocyanins.[14] Furthermore, two known bioactive constituents isolated from this plant, vitexin and isovitexin evaluated to show potent α-glucosidase inhibition and strong antioxidant activities.[10] Abu Bakar *et al*. reported that the leave extract of *F. deltoidea* and its major components such as vitexin and isovitexin had appreciable inhibitory potential toward matrix metalloproteinases (2, 8, and 9) and α-amylase.[15,16] In the present investigation, we studied the anti-inflammatory potential of *Ficus deltoidea* and its active fractions through NF-κB signaling pathway in LPS-stimulated murine RAW 264.7 macrophages.

MATERIALS AND METHODS

Extract and isolation of fraction from *Ficus deltoidea* leaves

The leave material of *F. deltoidea* was collected from Institut of Biosains (IBS), UPM, Malaysia, air dried, ground and the powdered material (40 g) was mixed with 100% methanol and subjected to ultra-sonication for 30 min and left overnight. The sample was authenticated by a botanist, Dr. Shamsul Khamis (IBS, UPM) and the Voucher specimen No: SK2892/15 was deposited at the Herbarium of Institute of Biosains, Biodiversity Unit, Universiti Putra Malaysia. The sample was collected in the month of August 2015 (summer monsoon period in Malaysia). Then, it was filtered, dried under vacuum at 40°C and the crude methanolic extract (6.2 g) was obtained. The extract was then subjected to liquid-liquid partitioning using organic solvents with various polarities. The resulting fractions were dried under vacuum and lyophilized. All the samples such as methanol, hexane (2 g, 32.25%), chloroform (0.89 g, 14.35%), ethyl acetate (1.11 g, 17.90%), and butanol (1.4 g, 22.58%) were stored at −80°C for future analysis.[17]

Cell line and cell culture

The macrophage RAW 264.7 cells were procured from the American Type Culture Collection (ATCC, USA) and it was maintained in Dulbecco's modified minimal essential medium (DMEM, Nacalai Tesque, Japan) supplemented with 1% Pen-Strep and heat-inactivated 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. The cells were sub cultured in a new T75 flask when it reached 70%–80% confluence. Media was changed routinely every alternative day depending on cell growth.

Cell viability assay

The cytotoxicity of *F. deltoidea* extract and its solvent fractions on RAW 264.7 macrophage cells was examined by (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide [MTT], Nacalai Tesque, Japan) assay. Macrophage cells were seeded into 96-well plate at a density of 1×10^4 cells/well and cultured cells were treated with extract/solvent fractions (1000, 500, 250, 125, 62.5, 31.25, and 15.62 μg/ml) and incubated for 24 h. The cells treated with only media served as an untreated control to calculate the cell viability. After the incubation period, the media with drugs were removed and 100 μl of the freshly prepared MTT solution was added. After the incubation period (3 h), MTT solution was discarded followed by centrifugation and further formazan was dissolved in 100 μl of dimethyl-sulfoxide. The viability of treated and untreated macrophages was measured at 570 nm using Microplate reader (Bio-Tek, USA) and the percentage of viable cells was calculated according to the standard method.

Measurement of NO production

RAW 264.7 macrophage cells (1×10^6 cells/well) were seeded and cultured on a 6-well plate for 12 h and cultured cells were pre-treated with extract and fractions (50, 100, and 200 μg/ml) for 2 h. Pre-treated cells were stimulated with LPS (1 μg/ml) for another 24 h and the supernatants were garnered to quantify the nitrite concentration. Equal volumes of cells free culture supernatant were used to measure the nitrite concentration by Griess reagent as previously reported.[18]

Determination of pro-inflammatory mediators

The effect of extract and its fractions on tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1 β production of RAW 264.7 macrophages were estimated using enzyme-linked immunosorbent assay (ELISA). Macrophages were cultured in a 6-well plate for 12 h and preincubated with fractions (50, 100, and 200 μg/ml) for 2 h. Treated cells were challenged with LPS (1 μg/ml) for another 24 h and finally cells free culture media were collected and stored at −20°C until further use. The concentration of TNF-α, IL-6, and IL-1 β production were quantified using ELISA kits according to the manufacturer's guidelines (RandD Systems, USA).

Protein extraction and western blot analysis

RAW 264.7 cells (1×10^6 cells/well) were seeded and cultured in a 6-well plate for 12 h. Cells were pre-treated with extract/fractions (50,

100, and 200 µg/ml) for 2 h and then treated cells were stimulated LPS (1 µg/ml) for 24 h. Finally, cells were washed with ice-cold PBS solution and cold RIPA mammalian protein extraction lysis buffer with protease and phosphatase inhibitor cocktails (Roche) added to washed cells to extract the protein. Protein concentrations of supernatant were quantified using protein assay kit. Bovine serum albumin served as the standard. An equal concentration of protein was separated on a 10% Sodium dodecyl sulfate gel and further transferred to polyvinylidene difluoride membrane. Transferred membrane was subjected to block with blocking buffer for a minimum of 1 h at room temperature on the shaker. Blocked membrane was incubated with specific target primary antibodies overnight in the chiller. The specific dilutions of primary antibodies were diluted with a blocking buffer as mentioned in the datasheet of Source Company. Subsequently, the incubated membrane was washed with a wash buffer and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA) was added to the processed membrane and it was finally detected by Chemidoc™ XRS (Bio-Rad).

Identification of compounds using liquid chromatography mass spectrometry and liquid chromatography tandem mass spectrometry analysis

Preparation of sample solution

1 mg of *F. deltoidea* ethyl acetate fraction was dissolved in 1 ml methanol and then filtered through the membrane filter (pore size 0.2 µm).

Liquid chromatography condition

Chromatographic analysis was performed on an ekspert ultraLC 100-XL System (Sciex Framingham, MA, USA) equipped with an ekspert 100 binary pump, 100-XL auto sampler and ekspert 100 Column Oven. The column was a Phenomenex Aqua C_{18} column (50 mm × 2.0 mm × 5 µM) HPLC column and oven temperature was at room temperature (40°C).

Solvent system conditions

The mobile phase was composed of water (A) and acetonitrile (B), acidified with 5 mM ammonium formate and 0.1% formic acid. The gradient program started from solvent ratio 95: 5 (A: B, v/v) for 10 min followed by 5: 95 (A: B, v/v) for 2 min and further equilibrated with initial conditions 95: 5 (A: B, v/v) for 3 min. The flow rate was set at 0.500 mL/min and the injection volume was 10 µL.

Mass spectral condition

Mass spectrometry was performed on the Linear Ion Trap Quadrupole Liquid chromatography mass spectrometry and Liquid chromatography tandem mass spectrometry (LC-MS/MS) Mass Spectrometer (SCIEX QTRAP 6500, Framingham, MA, USA) The mass spectrum of the samples was attained in negative mode using turbo ion spray by setting the following conditions voltage IS: −4500 V, source temperature: 500°C, desolation gas: 40 psi, source gas: 40 psi, scan range: 100–1200 *m/z* for full scan and 50–1200 *m/z* for MS/MS scan, declustering potential: 40 V, entrance potential: 10 V, collision energy: spread of 35 eV ± 15 eV.

Statistical analysis

All the results are expressed as the mean ± standard deviation. Data were compared using one-way analysis of variance to verify the significant differences between the groups. Differences between LPS treated and untreated groups were considered as statistically significant ($P < 0.05$) and all analyses were done using Graph pad prism Software (version 5) (San Diego, CA). Data acquisition was controlled using Sciex Analyst TF software whereas the analysis and processing of data were performed using Peak View Software V.1.2 (SCIEX, Framingham, MA, USA). All the major active chemical compounds and related metabolites of *F. deltoidea* were identified based on its parent mass and MS/MS spectrum.

RESULTS

Effect of extract and solvent fractions on cell viability and nitric oxide production in lipopolysaccharide -stimulated macrophages

Cell viability in response to extract and solvent fractions were estimated in macrophages after 24 h of treatment. It was found that the lower concentrations of fractions did not affect the cell growth, while the higher concentration (500 and 1000 µg/ml) of samples were seemed to be cytotoxic against RAW 264.7 cells [Figure 1], thus the lower concentration of extract and fractions (50, 100, and 200 µg/ml) have opted for further anti-inflammatory experimental studies.

Excessive production of NO was observed in LPS-stimulated RAW 264.7 cells [Figure 2]. The extract and fractions of *F. deltoidea* treated with LPS-stimulated RAW 264.7 macrophages significantly suppressed the NO production in a concentration dependent manner and these results were comparable with positive control drug, dexamethasone. However, the production of NO in normal cells untreated with LPS was very less.

Extract and solvent fractions inhibits lipopolysaccharide-stimulated pro-inflammatory cytokines productions in macrophages

The anti-inflammatory potential of extract and solvent fractions of *F. deltoidea* on the production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1 β in LPS-stimulated macrophages were determined using ELISA [Figures 3-5]. LPS treated macrophages produced an excessive level of inflammatory cytokines compared to normal control,

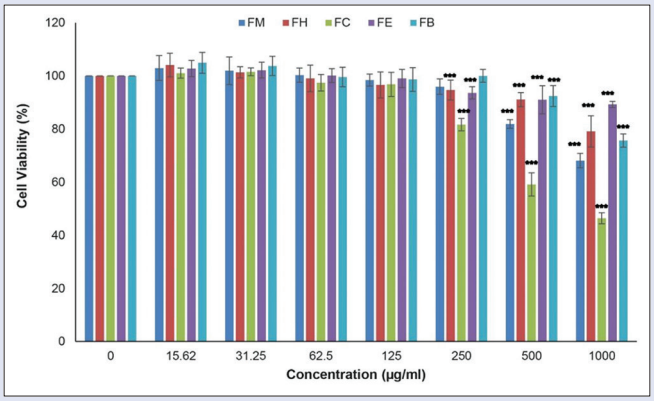

Figure 1: Viability of RAW 264.7 cells treated with various concentration of *Ficus deltoidea* fractions (FM: *Ficus deltoidea* methanol extract; FH: *Ficus deltoidea* hexane fraction; FC: *Ficus deltoidea* chloroform fraction; FE: *Ficus deltoidea* ethyl acetate fraction; FB: *Ficus deltoidea* butanol fraction) for 24 h. Experiments were conducted in triplicate manner and data are expressed as mean ± standard deviation Statistically, $P < 0.05$ were considered to be significant (*$P < 0.05$, ** $P < 0.001$, and ***$P < 0.0001$ control vs. treatment group)

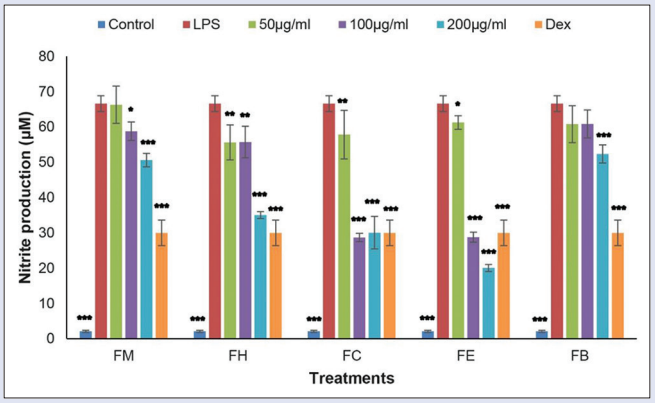

Figure 2: Lipopolysaccharide induced nitric oxide production in RAW 264.7 cells pretreated with various fractions of *Ficus deltoidea* (FM: *Ficus deltoidea* methanol extract, FH: *Ficus deltoidea* hexane fraction, FC: *Ficus deltoidea* chloroform fraction, FE: *Ficus deltoidea* ethyl acetate fraction, FB: *Ficus deltoidea* butanol fraction) and positive control (Dexamethasone) at indicated concentrations (50, 100, and 200 µg/ml) for 24 h studied using Enzyme-linked immunosorbent assay technique. Nitric oxide production values are expressed in µM. Experiments were conducted in triplicate manner and data are expressed as mean ± standard deviation Statistically, $P < 0.05$ were considered to be significant (*$P < 0.05$ **$P < 0.001$, and ***$P < 0.0001$, Lipopolysaccharide -treated group vs. control and samples)

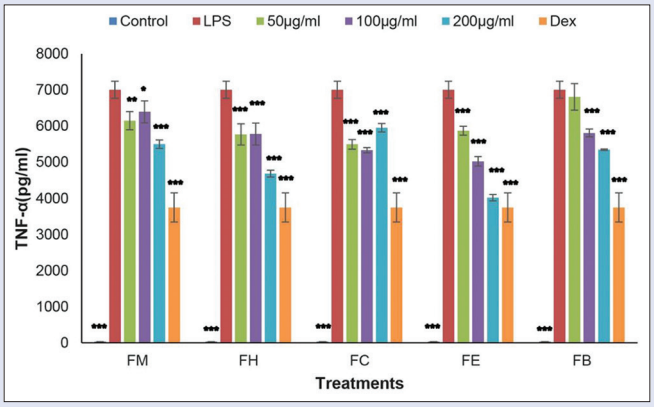

Figure 3: Lipopolysaccharide induced tumor necrosis factor alpha production in RAW 264.7 cells pretreated with various fractions of *Ficus deltoidea* (FM: *Ficus deltoidea* methanol extract, FH: *Ficus deltoidea* hexane fraction, FC: *Ficus deltoidea* chloroform fraction, FE: *Ficus deltoidea* ethyl acetate fraction, FB: *Ficus deltoidea* butanol fraction) and positive control (Dexamethasone) at indicated concentrations (50, 100, and 200 µg/ml) for 24 h studied using Enzyme-linked immunosorbent assay technique. tumor necrosis factor alpha production values are expressed in pg/ml. Experiments were conducted in triplicate manner and data are expressed as mean ± standard deviation Statistically, $P < 0.05$ were considered to be significant (*$P < 0.05$ **$P < 0.001$, and ***$P < 0.0001$, Lipopolysaccharide -treated group vs. control and samples)

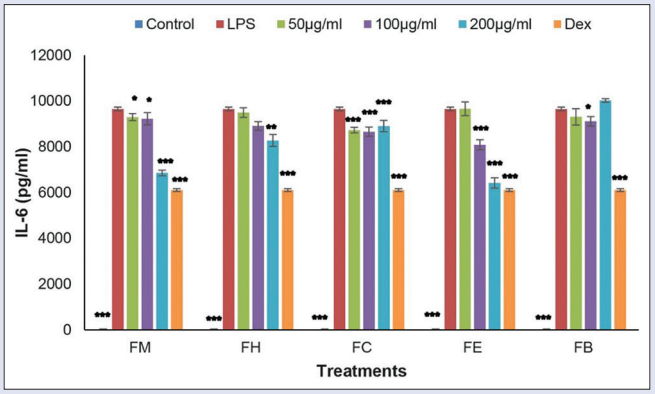

Figure 4: Lipopolysaccharide induced interleukin-6 production in RAW 264.7 cells pretreated with various fractions of *Ficus deltoidea* (FM: *Ficus deltoidea* methanol extract, FH: *Ficus deltoidea* hexane fraction, FC: *Ficus deltoidea* chloroform fraction, FE: *Ficus deltoidea* ethyl acetate fraction, FB: *Ficus deltoidea* butanol fraction) and positive control (Dexamethasone) at indicated concentrations (50, 100, and 200 µg/ml) for 24 h studied using Enzyme-linked immunosorbent assay technique. Interleukin-6 production values are expressed in pg/ml. Experiments were conducted in triplicate manner and data are expressed as mean ± standard deviation Statistically, $P < 0.05$ were considered to be significant (*$P < 0.05$ **$P < 0.001$, and ***$P < 0.0001$, Lipopolysaccharide-treated group vs. control and samples)

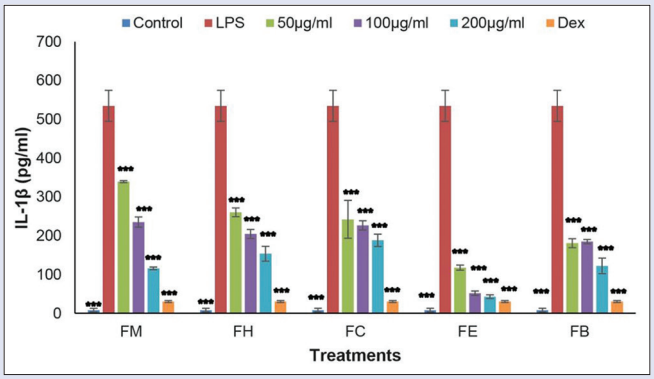

Figure 5: Lipopolysaccharide induced interleukin-1 β production in RAW 264.7 cells pretreated with various fractions of *Ficus deltoidea* (FM: *Ficus deltoidea* methanol extract, FH: *Ficus deltoidea* hexane fraction, FC: *Ficus deltoidea* chloroform fraction, FE: *Ficus deltoidea* ethyl acetate fraction, FB: *Ficus deltoidea* butanol fraction) and positive control (Dexamethasone) at indicated concentrations (50, 100, and 200 µg/ml) for 24 h were studied using Enzyme-linked immunosorbent assay technique. Interleukin-1 β production values are expressed in pg/ml. Experiments were conducted in triplicate manner and data are expressed as mean ± standard deviation Statistically, $P < 0.05$ were considered to be significant (***$P < 0.0001$, Lipopolysaccharide-treated group vs. control and samples)

whereas pre-treatment with extract and solvent fractions resulted in downregulation of those cytokines in a concentration-dependent manner. In this investigation, it was observed that the ethyl acetate fraction offered significant suppressive effects of inflammatory cytokines, therefore, the ethyl acetate fraction was selected to find out further cellular molecular mechanisms to confirm its anti-inflammatory properties. The inhibitory effects were comparable with the positive control drug, dexamethasone.

Ethyl acetate fraction down-regulates the lipopolysaccharide-stimulated inducible nitric oxide synthase and cyclooxygenase-2 expression in macrophages

The expression of inflammatory mediator's inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were observed to

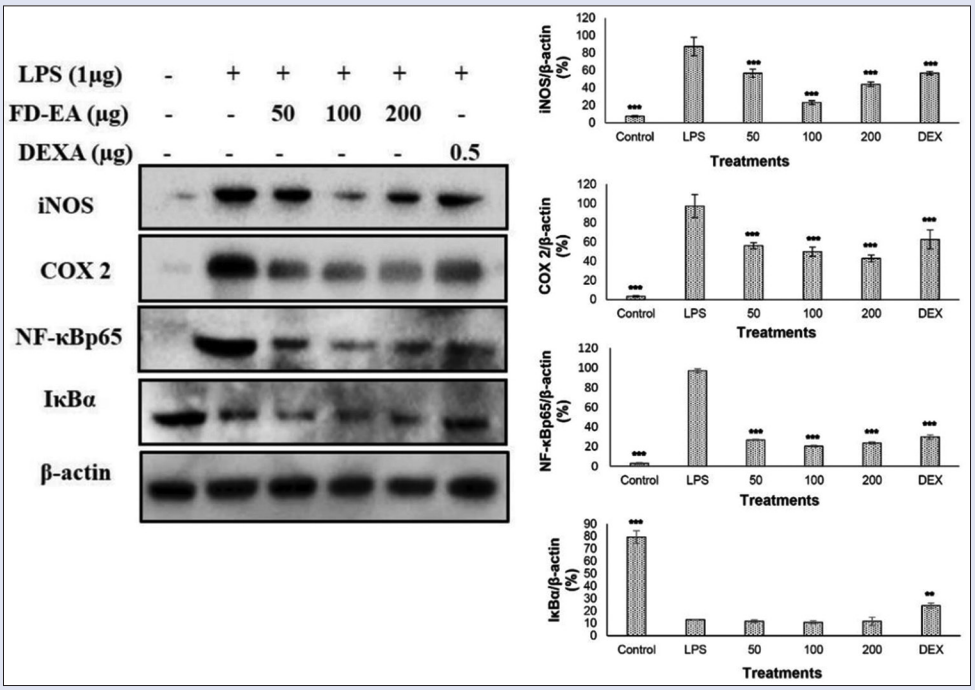

Figure 6: Expression of lipopolysaccharide induced biomarkers such as Inducible nitric oxide synthase, cyclooxygenase-2, nuclear factor-kappa B p65, inhibitor of kappa b alpha, β-acin in RAW 264.7 cells pretreated with ethyl acetate fractions of *Ficus deltoidea* at indicated concentration (50, 100, and 200 µg/ml) for 24 h were studied using Western blotting analysis. Experiments were conducted in triplicate manner and images shown are representatives of triplicate experiments. Statistically, $P < 0.05$ were considered to be significant (**$P < 0.001$, and ***$P < 0.0001$, Lipopolysaccharide-treated group vs. control and samples)

further understand the anti-inflammatory cellular mechanism of ethyl acetate fraction using immunoblot analysis [Figure 6]. Ethyl acetate fraction of *F. deltoidea* treatment effectively down-regulates the expression of iNOS and COX-2 compared to the LPS-stimulated macrophages in a concentration-dependent manner. These findings are concurrent with the inhibitory effects of NO production, since these inflammatory enzymes iNOS and COX-2 stimulates the production of NO.

Effect of ethyl acetate fraction on expression of nuclear factor-kappa B p65 and inhibitor of kappa b alpha degradation in lipopolysaccharide-stimulated macrophages

It is known that NF-κB, the key transcription factor in an inflammatory cascade where it involves the translocation of NF-κB from the cellular cytoplasm to the nucleus to produce various inflammatory mediators headed by the phosphorylation and degradation of inhibitor of kappa b alpha (IκBα). In the present study, we assessed the effects of ethyl acetate fraction on the activation of the NF-kBp65 and IκBα expression in LPS-stimulated RAW 264.7 cells. As shown in Figure 6, IκBα was significantly altered in the LPS-treated macrophages and the expression of nuclear NF-κBp65 indicated the activation of the NF-κB pathway. Treatment with the ethyl acetate fraction of *F. deltoidea* markedly suppressed and inhibited the expression of NF-κBp65 and degradation of IκBα in a dose-dependent manner, respectively. These results suggested that the ethyl acetate fraction of *F. deltoidea* could apparently suppress the LPS-stimulated NF-κB expression in RAW 264.7 macrophages.

Identification of compounds using liquid chromatography mass spectrometry and liquid chromatography tandem mass spectrometry analysis

High resolution and accurate mass LC-MS/MS chromatograms contain comprehensive information of all molecules present in the sample that are amenable to the ionization technique and polarity used. A non-targeted peak finding algorithm, like that integrated into the XIC Manager application of PeakView™ software, allows screening for unexpected compounds (Schreiber, 2011). The total ion chromatogram (TIC) corresponding to the negative signals of the ethyl acetate fraction of *F. deltoidea* were obtained [Figure 7]. The detected characteristic compounds exhibited no difference in all batches. In the full scan mass spectra, most of the authentic compounds exhibited $[M-H]^-$ ions in negative mode. Owing to the presence of formic acid in the mobile phase, ions of $[M-H + HCOOH]^-$ were observed for a part of compounds.

A wide range of phytochemicals including phenolic acids, flavones and their derivatives have been identified. The marker compounds namely isovitexin were identified in the negative ion mode of mass spectrometry at the retention time 7.837 min [Figure 8]. Apart from that, several phenolic acids and the flavonoids such as coumaric acid, genistein, catechin, and apigenin derivatives were also detected in the active fraction of the *F. deltoidea* [Table 1]. These compounds were identified according to their MS data and literature reports.[10,19,20]

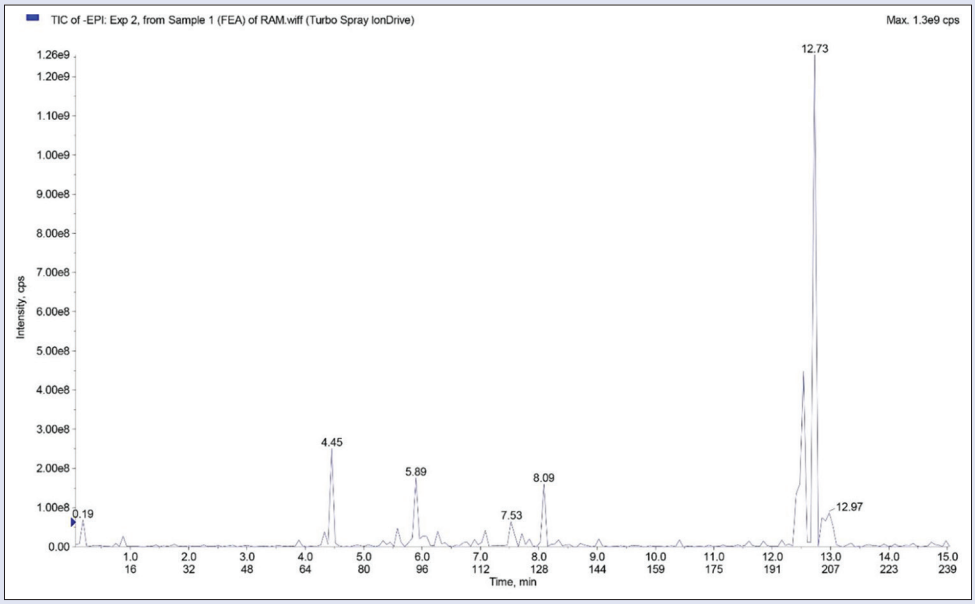

Figure 7: Total ion chromatogram of ethyl acetate fraction of *Ficus deltoidea*

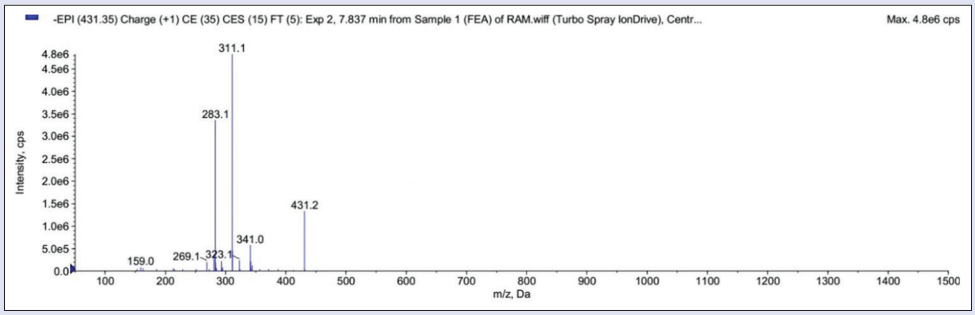

Figure 8: Spectra of ion fragments of isovitexin in negative ion mode

Table 1: Compounds identification in the active fraction of *Ficus deltoidea* using liquid chromatography-mass spectrometry/mass spectrometry analysis

Peak	Tentatively identified compounds	Retention time (min)	$[M-H]^-$	MS/MS fragments
1	Coumaroylquinic acid	0.168	337	293, 173, 163
2	Unknown	0.188	383	337, 295, 277
3	Vanillic acid derivatives	3.888	-	153, 108
5	Quinic acid derivatives	4.327	233	191, 173, 149
6	Catechin	4.452	289	245, 205, 179
7	Coumaric acid (sugar derivatives)	5.455	-	163, 119, 93
8	Luteolin-8-*C*-(6”-*O*-*p*-benzoyl)-glucoside	5.580	567	549, 521,429
9	Dodecadienyl coumaric acid	5.893	327	309, 265, 211, 163, 143, 119
10	Trihydroxyoctadecanoic acid sulphate	6.018	333	315, 229, 151
11	epi-afzelechin-(epi)-catechin	6.896	561	289, 245
12	Amentoflavone (biapigenin)	7.524	537	375, 357
13	Vitexin	7.837	431	341, 311, 283
14	Isovitexin	8.088	431	341, 311, 283
15	Genistein	8.339	269	180, 133
16	Unknown	9.029	419	325, 307, 283, 161
17	Unknown	12.538	365	283, 269
18	Unknown	12.977	487	256, 173

MS: Mass spectrometry

DISCUSSION

In this investigation, the anti-inflammatory properties of *F. deltoidea* extract and its active fractions were determined in LPS-stimulated using macrophages (*in vitro* inflammatory model). Apart from preliminary findings, the extract and fractions on the principal cell signaling pathway which are responsible to produce the inflammatory mediators that trigger the various acute and chronic inflammatory

diseases/disorders were demonstrated. From the investigation, it is clear that the ethyl acetate fraction of *F. deltoidea* extract has appreciable anti-inflammatory potential, with an effective to regulate the production of pro-inflammatory mediators through inhibiting the activation of key signaling pathways; NF-κB in LPS-stimulated macrophages. These findings provide a clear cellular mechanism for the anti-inflammatory potential of fraction and its medicinal value for the prevention of acute and chronic inflammatory associated diseases.

LPS is one of the essential components of the outer membrane of Gram-negative bacteria and is considered as key signal activators of the innate immune system.[21] Many biomedical researchers have described that LPS is recognized by membrane Toll-like receptor 4 on macrophages and activates an acute and early release of pro-inflammatory mediators including NO and pro-inflammatory cytokines through NF-κB key signaling pathways.[22,23] With this information, the LPS-stimulated macrophages were used to investigate the anti-inflammatory potential of extract and active fractions. Thus, the effective suppressors of the production of these pro-inflammatory cytokines and inflammatory mediators have been believed as a potential drug candidate for anti-inflammatory agents without adverse effects.

NO generated by triggered macrophages plays vital roles in the pathology of cells/tissues and inflammatory responses.[24] Several researchers revealed that numerous pro-inflammatory cytokines, NO and inflammatory mediators including endogenous NO production increased during the inflammation. Excessive production of NO is mediated mainly by iNOS, which can be over expressed in LPS-stimulated macrophages[25,26] and its leads to a multiplicity of diseases such as cancer, cardiovascular diseases, rheumatoid arthritis, neurological disorders, and autoimmune diseases.[27] Ethyl acetate fraction treatment effectively reduced the production of NO in LPS-stimulated macrophages in a dose-dependent manner and yet, the dose (50, 100, and 200 μg/ml) which we have chosen for anti-inflammatory studies had no adverse effects on the cell viability. These results indicate that ethyl acetate fraction inhibits the inflammatory response via downregulation of inflammatory gene, iNOS in concentration-dependent manner.

Pro-inflammatory cytokines including TNF-α, IL-1 β, and IL-6 are stimulated by the regulation of NF-κB pathway and play vital roles in the immune reaction to numerous inflammatory mediators.[28,29] Increased production of pro-inflammatory cytokines in macrophages are linked to the progress of numerous inflammatory associated disorders such as diabetes, arthritis, cancer, and other cardiovascular diseases.[30-32] The suppression of these inflammatory cytokines is the suitable option to prevent and treat the inflammatory diseases and it has become a rational target for effective novel anti-inflammatory drugs. In this present investigation, the production of pro-inflammatory cytokines were significantly suppressed by the extract and isolated active fraction in a concentration-dependent manner, these findings suggested that ethyl acetate fraction of extract has effective therapeutic potential compared to other fractions by its suppressive nature of pro-inflammatory cytokines production in endotoxin-induced macrophages.

In addition, to explore the anti-inflammatory potential of active fraction, the targets of NF-κB signaling pathway were examined. NF-κB transcriptional factor is a key mediator of immunologically facilitated transcriptional responses.[33] Endotoxin treatment effectively induced the NF-κB transcriptional pathway through sequential cascade such as IKK-dependent proteolytic degradation, phosphorylation of IκBα in the cytoplasm, and activation of the translocation of cytosolic NF-κB subunits into the nucleus.[34,35] The phosphorylation and degradation of IκBα molecule in the cytoplasm is very important for the initiation and release of NF-κB subunits.[36] Ethyl acetate fraction notably reduced degradation of IκBα in the cytoplasm and suppression of NF-κB nuclear translocation stimulated by LPS in macrophages. In Figure 6, increased expression of IκBα in LPS-stimulated RAW macrophage cells provided a cellular mechanism that ethyl acetate fraction inhibits the translocation and activation of NF-κB signaling molecules in a concentration dependent manner.

Endotoxin, LPS induces inflammatory cells to produce the various cytokines, chemokines and mediators through NF-κB signaling pathway.[37] Increased expression of two important key enzymes iNOS and COX-2, which are accountable to produce the inflammatory mediators NO and PGE2, respectively, through NF-κB signalling pathway in activated macrophages.[5] These enzymes along with the inflammatory cytokines activate the inflammatory cascade to initiate the various acute and chronic inflammatory associated diseases. Various researchers have found that plant extracts, active fractions, and phyto-compounds could effectively inhibits the production of inflammatory cytokines and other biomarkers that mediates the inflammation.[38-40] In Figure 6, we have demonstrated that ethyl acetate fraction significantly downregulated the expression of iNOS and production of NO and also reduced expression of COX-2 in a concentration-dependent manner. These findings have suggested that the ethyl acetate fraction markedly improved the inflammatory response in LPS stimulated macrophages.

Most of the components identified in the active fraction, especially the phenolic compounds such as catechin, coumaric acid, genistein, luteolin glycosides, and apigenin derivatives were reported to possess significant anti-inflammatory properties.[41,42] Zunoliza *et al*. reported the anti-inflammatory properties of the standardized methanol and aqueous extracts through enzymatic assay where isovitexin and vitexin were proclaimed as the major components.[13,16,43] Recently, it was reported that the ethyl acetate fraction of *F. deltoidea* obtained from the crude aqueous extract possess significant antioxidant properties with high phenolic and flavonoid content. Moreover, the presence of vitexin and isovitexin were identified in the crude extract.[41] In this study, bioactive compounds such as vitexin, isovitexin, genistein, and catechin were identified in the active fraction of ethyl acetate obtained from the crude methanolic extract. The presence of more active components in the ethyl acetate fraction could offer synergistic effects to treat various disorders such as antioxidant and anti-inflammation. Overall, in this study, it has proven that the ethyl acetate fraction from *F. deltoidea* as an effective anti-inflammatory natural agent.

CONCLUSION

In this study, the ethyl acetate fraction revealed convincing anti-inflammatory potential *via* suppression of LPS-stimulated pro-inflammatory cytokines, TNF-α, IL-1 β, and IL 6 in macrophages. These suppressive effects are exerted by preventing NF-κB translocation into the nucleus from the cytoplasm. The inhibition of NF-κB signaling pathway and its activated inflammatory mediators such as iNOS, COX-2, and NF-κB p65 may be useful for the prevention and treatment of acute and chronic inflammatory diseases. Overall, this study recommended that the ethyl acetate fraction might be a significant player in the progress of preventive/therapeutic agents or functional foods for treating chronic inflammatory associated diseases.

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Conflicts of interest

There are no conflicts of interest.

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