Anti-inflammatory effect of *Morinda citrifolia* leaf extract on macrophage RAW 264.7 cells

Aurasorn Saraphanchotiwitthaya\textsuperscript{a,b,*}, Pattana Sripalakit\textsuperscript{b,c}

\textsuperscript{a} Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000 Thailand

\textsuperscript{b} Pharmaceutical Biotechnology Research Unit, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000 Thailand

\textsuperscript{c} Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000 Thailand

\*Corresponding author, e-mail: aurasorns@nu.ac.th

Received 31 Mar 2014

Accepted 2 Dec 2014

\textbf{ABSTRACT:} Leaves of *Morinda citrifolia* (Rubiaceae) have been used in folk medicine to treat pain and inflammation. In this study, the anti-inflammatory activity of *M. citrifolia* leaf extracts was investigated. The extract was prepared by freeze-drying juice squeezed from *M. citrifolia* leaves. HPLC analysis of the crude extract revealed rutin to be a major compound together with small amounts of quercetin and kaempferol. The effect of this extract on TNF-\(\alpha\), IL-1\(\beta\), and NO secretion by lipopolysaccharide-induced macrophage RAW 264.7 cells was investigated and compared to that of rutin, dexamethasone, and indomethacin. The results show that the extract inhibited TNF-\(\alpha\) (98%, 750 \(\mu\)g/ml) secretion four times more than dexamethasone (3.92 \(\mu\)g/ml) and indomethacin (3.58 \(\mu\)g/ml), while rutin had no effect. The extract suppressed IL-1\(\beta\) secretion in a dose-dependent fashion with a maximum inhibition of about 79% (750 \(\mu\)g/ml). This was similar to that achieved by dexamethasone (97%, 3.92 \(\mu\)g/ml), indomethacin (70%, 3.58 \(\mu\)g/ml), and rutin (90%, 48.84 \(\mu\)g/ml). Moreover, the extract inhibited NO secretion (74%, 750 \(\mu\)g/ml) 50% more than rutin (40 \(\mu\)M) and 70% more than dexamethasone and indomethacin (10 \(\mu\)M). This study demonstrates the potential of *M. citrifolia* leaves as an effective therapeutic intervention against inflammatory diseases.

\textbf{KEYWORDS:} noni leaves, TNF-\(\alpha\), IL-1\(\beta\), nitric oxide

\textbf{INTRODUCTION} Inflammation is the first response of the immune system to infection or irritation, and plays important pathological roles in the development of diseases such as cancer, diabetes, atherosclerosis, and arthritis\textsuperscript{1}. In inflammation, macrophages have three major functions: antigen presentation, phagocytosis, and immunomodulation through the production of various cytokines and growth factors. Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation as they are activated and deactivated by the inflammatory process\textsuperscript{2}. Macrophages produce various pro-inflammatory cytokines and inflammatory molecules that play a crucial role during the inflammatory process. However, overproduction of pro-inflammatory cytokines and inflammatory molecules by activated macrophages is critical to the onset of inflammation\textsuperscript{3}. In the presence of stimuli such as lipopolysaccharides (LPS), activated macrophages produce various cytokines such as tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-1\(\beta\) (IL-1\(\beta\)), interleukin-6 (IL-6), and interleukin-10 (IL-10), and inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2)\textsuperscript{4–6}. In our study, LPS was therefore used to activate macrophages to evaluate the anti-inflammatory effects of the leaf extract.

Non-steroidal anti-inflammatory drugs and steroidal drugs are widely used to treat several inflammatory conditions; however, their ability to cause many and severe adverse effects limits their uses. In this regard, traditional medicine continues to use medicinal plants as a substitute for allopathic medicine\textsuperscript{7,8}.

Plant-derived extracts have historically been considered to be effective at maintaining vitality, preventing and treating various diseases, and enhancing overall immune health\textsuperscript{9}. *Morinda citrifolia* Linn. (Rubiaceae family) or noni (‘yo’ in Thai) is a small tropical tree that grows widely in Polynesia. It has been used in folk remedies for over 2000 years. All parts of the plant, including the leaves, fruit, bark, and roots have several pharmacological prop-
Acknowledgment

The authors would like to thank the Office of the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand as well as the local community for their support in the preparation of this manuscript.

1. Medical applications of this plant are based on its anti-inflammatory1, anti-cancer12, and antioxidant properties13. Its fruits have been used as a folk medicine for the treatment of many diseases including diabetes, high blood pressure, inflammation, and cancer14. In 2002, noni juice was accepted in the European Union as a novel food and the noni juice market continues to grow11. Noni fruits contain active components such as phenolic compounds, in particular, coumarin, flavonoid, and iridoid compounds15. The leaves are also the source of a variety of other health-promoting commercial products available in Japan and the US, mainly for use in making infusions. Some manufacturers also produce capsules containing powdered noni leaves16. Previous studies revealed that noni leaves contain a variety of phytochemical constituents, including terpenoid, phytosterol, iridoid, flavonol, and anthraquinone compounds and their glycoside derivatives17–19. Flavonoid compounds have been indicated to possess a variety of biological activities20,21, and may play an important role in noni leaves.

2. The aim of this study was to investigate the therapeutic potential of an aqueous extract of M. citrifolia leaves against inflammation and to determine the possibility of using that extract as a commercial health-promoting product. We studied the effect of the extract on the production of TNF-α, IL-1β, and NO by LPS-induced macrophage RAW 264.7 cells, and compared them with the effects of rutin and two anti-inflammatory drugs (dexamethasone and indomethacin).

MATERIALS AND METHODS

Plant material

M. citrifolia was collected in January 2012 in Lamphun province, Thailand, and authenticated by a botanist from the Office of the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand. A voucher specimen (183 646) was also prepared and deposited at the Office of the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand.

Chemicals

Lipopolysaccharide (LPS), Dulbecco’s modified Eagle medium (DMEM), trypan blue dye, rutin, quercetin, and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), dexamethasone, indomethacin, trypsin-EDTA, N-(1-naphthyl)ethylenediamine, and antibiotic-antimycotic solution (100 U penicillin, 100 µg streptomycin, and 0.25 µg amphotericin B per ml) were purchased from Sigma-Aldrich (Dorset, UK). Foetal bovine serum was purchased from GIBCO/BRL Invitrogen (Paisley, Scotland). TNF-α and IL-1β ELISA kits were from eBioscience Inc. (San Diego, USA). Phosphoric acid, 85% v/v, was from Carlo Erba (Milan, Italy). NaNO3 and sulphanilic acid were from Univar (NSW, Australia). Acetic acid, acetonitrile and methanol were purchased from Labscan (Samut Sakhon, Thailand).

Preparation of aqueous extract of M. citrifolia leaves

Fresh leaves of M. citrifolia were cleaned with tap water and rinsed with distilled water. The leaves (500 g) were chopped into small pieces and homogenized with distilled water (1 l). Leaf juice was filtered and concentrated by freeze-drying. The extract was dissolved in 0.1% (v/v) DMSO in PBS solution. Insoluble material was centrifuged and the extract was sterilized by passing through a 0.2 µm filter. DMSO 0.1% (v/v) in PBS solution was used as a control in all experiments.

Cell culture

Macrophage RAW 264.7 cells were obtained from the American Type Culture Collection. Cells were cultured in phenol red-free Dulbecco’s modified Eagle medium (DMEM) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated foetal bovine serum at 37°C with 5% CO2. Cells were washed with DMEM medium and detached with 0.25% trypsin-EDTA. The cells were resuspended in DMEM medium at a density of 2 x 106 cells/ml. Viability was determined via the trypan blue dye technique. For IFN-γ, IL-1β, and NO production assay, cells were seeded at 2 x 105 cells/100 µl in 96-well plates and incubated for 1 h. After that, the cells were activated with 5 µg/ml LPS with or without test compounds (plant extract, rutin, and anti-inflammatory drugs; dexamethasone and indomethacin) for 48 h. The supernatant was removed and assayed using a commercial enzyme-linked immunosorbet assay (ELISA) kit.

HPLC analysis

The HPLC system consisted of an LC-20AT pump (Shimadzu, Kyoto, Japan), an SPD-20A UV detector (Shimadzu, Kyoto, Japan), equipped with an SPD-20A system controller (Shimadzu, Kyoto, Japan).
Japan), and an SIL-10ADVP sample injector (Shimadzu, Kyoto, Japan) fitted with a 20 µl sample loop. The chromatographic separations were carried out on a Luna C18 column (250 mm × 4.6 mm i.d., 5 µm, 250 Å from Phenomenex, USA). The mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1% (v/v) acetic acid. All separations were performed isocratically at a flow rate of 1 ml/min. Column temperature was maintained at room temperature (27 ± 2°C). The peaks were determined using a UV detector set at a wavelength of 365 nm based on a previous report. Compounds were identified by comparing their retention times and UV-Vis spectral data of the peaks detected with those of original reference standards. Quantification was accomplished using external calibration with pure standards. The calibration curves were linear with \( r^2 = 0.999 \).

Standard solutions of three flavonoid compounds (rutin, quercetin, and kaempferol) were prepared in methanol, at concentrations of 10–330 µg/ml. The dried extract was dissolved in the mobile phase. After filtering through a filter paper and a 0.45-µm membrane filter, the extract was injected directly.

**LPS-induced cytokine production assay**

Production of mouse TNF-α and IL-1β was measured via ELISA according to the manufacturer’s instructions. Briefly, a 96-well microtitre plate was pre-coated overnight with capture antibody. After blocking and several washings, 100 µl of working standards and samples were then added for incubation for 2 h. After washing, 100 µl of working detector solution containing biotinylated anti-mouse cytokine monoclonal antibody was added and incubated for 1 h. After further washing, 100 µl of avidin-horseradish peroxidase conjugate was added to each well and incubated for 30 min. Then, 100 µl of substrate solution was added and the culture was incubated for 15 min, followed by the addition of 50 µl of stop solution, and the absorbance was read within 30 min using a microplate reader at 450 nm. The inhibition of cytokine production compared to the control (LPS) was calculated as: \( \frac{[\text{cytokine}]_c - [\text{cytokine}]_t}{[\text{cytokine}]_c} \), where \([\text{cytokine}]_c\) and \([\text{cytokine}]_t\) are the cytokine concentration in the control and test sample, respectively.

**Nitrite assay**

The presence of nitrite, a stable oxidized product of nitric oxide (NO), was determined in cell culture media using Griess reagent. Briefly, 50 µl of supernatant from the test culture was mixed with 50 µl of 1% (w/v) sulphanilic acid in 5% (v/v) phosphoric acid in a 96-well plate, followed by incubation for 10 min at room temperature. After that 50 µl of 0.1% (w/v) N-1-naphthylethylenediamine HCl in distilled water was added and incubated for 10 min at room temperature. The optical density at 540 nm was measured with a microplate reader. The NO concentration was calculated by comparison with a NaNO\(_2\) (0–100 µM) standard curve. The final concentration of DMSO was adjusted to less than 0.1% for all treatments. Dexamethasone and indomethacin were used as a reference standard. The results were expressed as inhibition of NO production compared to the control (LPS) using: \( \frac{[\text{nitrite}]_c - [\text{nitrite}]_t}{[\text{nitrite}]_c} \), where \([\text{nitrite}]_c\) and \([\text{nitrite}]_t\) are the nitrite concentration in the control and test sample, respectively.

**Statistical analysis**

One-way ANOVA followed by Tukey’s post-hoc test were used to determine the statistical significance of differences between the values for the various experimental and control groups. Data were expressed as mean ± S.D. and the results were taken from three independent experiments performed in triplicate. Values of \( p < 0.05 \) were considered statistically significant.

**RESULTS**

**Plant extraction**

The extract (FMC_CF) was obtained by freeze-drying the aqueous juice obtained from fresh *M. citrifolia* leaves. The physical appearance of the extract was a viscous greenish-brown gum and the yield was 8% (w/w) of fresh leaves.

**Quantitative analysis of flavonoid compounds by HPLC**

Rutin, quercetin, and kaempferol in *M. citrifolia* extract were analysed using HPLC. Methanol-acetonitrile-water (40:15:45 v/v/v) containing 1% (v/v) acetic acid system as the mobile phase demonstrated good separation of rutin, quercetin, and kaempferol at the retention time of 4.1, 9, and 14 min, respectively. Comparison of the retention times of the peaks detected from HPLC chromatograms of 100 mg/ml *M. citrifolia* extract to those of reference standard phytochemicals revealed that rutin was the major compound (0.37% (w/w) of extract) with a small amount of quercetin and kaempferol (less
than 0.01% (w/w) of extract). The HPLC chromatogram of the extract is shown in Fig. 1.

**M. citrifolia** extract inhibited IL-1β, TNF-α, and NO production from macrophage RAW 264.7 cells

The secretion of TNF-α and IL-1β from mouse macrophage RAW 264.7 cell cultures treated with *M. citrifolia* extract, in comparison with rutin, was assayed using ELISA. The commercial inflammatory agents, dexamethasone, and indomethacin, were used as a positive control. Stimulation of RAW 264.7 cells with LPS (5 µg/ml) significantly increased secreted TNF-α and IL-1β levels of approximately 3-folds over basal secretion values and these increases were substantially inhibited by rutin, dexamethasone, and indomethacin, and by *M. citrifolia* extract.

The extract significantly inhibited the secretion of TNF-α from LPS-induced macrophages. The maximum suppression of approximately 98% presented by the extract at 750 µg/ml was observed, and was much higher than that of dexamethasone (23% at 3.92 µg/ml, \( p < 0.05 \)) and indomethacin (25% at 3.58 µg/ml, \( p < 0.05 \)). At the tested concentrations, rutin did not have any effect on TNF-α production. The results are shown in Fig. 2a.

The extract showed maximal inhibition of IL-1β production (79%) at 750 µg/ml; this was not statistically different from the maximal inhibition produced by dexamethasone (97% at 3.92 µg/ml), indomethacin (70% at 3.58 µg/ml), and rutin (90% at 48.84 µg/ml). Comparison of the IC\(_{50}\) values revealed that the potency of inhibition of IL-1β production of the extract (294 µg/ml) was much weaker than that of rutin (30.2 µg/ml), dexamethasone (0.93 µg/ml), and indomethacin (1.50 µg/ml), respectively. These results are shown in Fig. 2b.

NO production increased upon LPS-stimulation, and this increase was suppressed by rutin, dexamethasone, indomethacin and the *M. citrifolia* extract. The inhibitory effect of the latter on NO
production increased with dose, with the maximal inhibition of about 74% (750 μg/ml) and an IC$_{50}$ value of 527 μg/ml. Rutin, dexamethasone, and indomethacin moderately reduced nitrite accumulation with the highest reduction being approximately 40–50%. The results are shown in Fig. 2c.

**DISCUSSION**

The inflammatory response is a dynamic process involving complex interactions among inflammatory molecules that arise in any tissue in response to traumatic, infectious, post-ischaemic, toxic, or auto-immune injury. We have previously studied the immunomodulatory activity of *M. citrifolia* extracts prepared using various techniques on human MOLT-4 cells. We found that the extract prepared by freeze-drying of aqueous juice led to a low IFN-γ/IL-10 ratio, indicating anti-inflammatory activity (unpublished data). The present study was conducted to confirm the anti-inflammatory activity of *M. citrifolia* extract in vitro, by analysing its inhibitory effects on TNF-α, IL-1β, and NO production by RAW 264.7 macrophage cells. We compared its effect with that of rutin, a major component of *M. citrifolia* leaves, and two anti-inflammatory agents (dexamethasone and indomethacin) as reference drugs. LPS was used to stimulate RAW 264.7 macrophages to produce pro-inflammatory mediators (TNF-α, IL-1β, and NO).

TNF-α and IL-1β are pro-inflammatory cytokines that are known to contribute to tissue damage and multiple organ failure. They are significant initiators of the inflammatory response and mediators of the development of various inflammatory diseases. TNF-α is secreted by macrophages and can be induced by LPS. TNF-α plays a key role in the induction and perpetuation of inflammation and up-regulation of other pro-inflammatory cytokines and endothelial adhesion molecules. The production of TNF-α increases the release of IL-1β and is crucially required for the synergistic induction of NO synthesis in LPS-stimulated macrophages.

In our study, macrophages treated with various concentrations of *M. citrifolia* leaf extract showed a concentration-dependent decrease in TNF-α and IL-1β production in LPS-stimulated RAW 264.7 cells. The extract significantly inhibited TNF-α production (four-fold more inhibition than that produced by dexamethasone and indomethacin), while rutin had no effect. The extract inhibited IL-1β production with moderate to high potency. Its potency was slightly lower than that of rutin and dexamethasone but slightly higher than that of indomethacin.

NO is a signalling molecule that plays a key role in the pathogenesis of inflammation, and is considered a pro-inflammatory mediator due to its overexpression in abnormal situations. The transcription of iNOS, and thus NO production, is increased in activated macrophages. At adequate concentrations, NO can generate or modify intracellular signals, thereby affecting the function of immune cells, as well as tumour cells and resident cells of different tissues and organs. However, its uncontrolled release can cause the inflammatory destruction of target tissue during an infection. In this study, NO production was assessed by measuring the accumulation of nitrites, a stable metabolite of NO, in the media via a colorimetric assay based on the Griess reaction. Our study showed that the extract markedly downregulated LPS-induced NO production along with that of pro-inflammatory cytokines (TNF-α and IL-1β) in macrophages. The extract considerably reduced NO production by LPS-induced macrophages, 1.53-fold more than rutin and 1.7-fold more than dexamethasone and indomethacin.

According to the trypan blue exclusion test, the viability of macrophages treated with the extract and test compounds was more than 90%. This indicated that the reduction in TNF-α, IL-1β and nitrite concentration was not due to a cytotoxic effect.

The HPLC analysis revealed that the *M. citrifolia* leaf extract contained the flavonoid compounds rutin, quercetin, and kaempferol, in accordance with a previous report. From this study, rutin (0.37% (w/w) of the extract) was the major compound in *M. citrifolia* leaves together with a small amount of quercetin and kaempferol (less than 0.01% (w/w) of the extract). Phenolic compounds are plant secondary metabolites known for their anti-oxidative and anti-inflammatory properties. Accordingly, flavonoid compounds such as kaempferol and quercetin aglycones, and glycosides have anti-inflammatory effects in vitro, involving inhibition of NO and PGE2 production by activated macrophages. Quercetin has been demonstrated to possess anti-inflammatory activities and could be, at least in part, responsible for the anti-inflammatory effect. Moreover, rutin was no more active against inflammation than the extract, determining by comparison of the equivalent concentrations (25 and 50 μg/ml). One possible explanation could be that the extract contains more chemical constituents that could act in a synergistic or antagonistic manner. Thus the active doses of...
the plant extract were higher than those of the pure compounds or reference drugs. It is well known that flavonoid compounds have a biological effect; the combination of flavonoids in the crude extract may act synergistically, thus amplifying the final effect.

In conclusion, the *M. citrifolia* extract demonstrated anti-inflammatory effects in vitro. The presence of flavonoid compounds would partially explain this activity and the combination of several compounds might boost the pharmacological potential of *M. citrifolia* leaf extract. *M. citrifolia* leaves have therefore a therapeutic potential that supports its use in traditional Thai medicine, and could thus be developed as a commercial anti-inflammatory.

Acknowledgements: This work was financially supported by Research Funds from Yearly Budget, Naresuan University (Grant no. R2556B040), Thailand.

REFERENCES

21. Kampkötter A, Nkwonkam CG, Zurawski RF, Timpel...


