Inhibition of nitric oxide production and COX-2 protein expression in LPS-stimulated RAW 264.7 cells by the hexane fraction of *Murdannia loriformis*

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ABSTRACT

Background: *Murdannia loriformis* (ML) is a medicinal plant traditionally used for chronic bronchitis, cancers in the initial stage, colds, throat infections, pneumonia, the flu, and wound healing. The crude ethanolic extract of ML has been reported anti-inflammatory, analgesic, antipyretic and gastroprotective activities in various *in vivo* experiments.

Objectives: This study aimed to isolate the active fractions of ML and assess the effect on nitric oxide (NO) inhibition and cyclooxygenase 2 (COX-2) expression.

Materials and methods: The dry powder of ML was extracted with 80% ethanol. The crude ethanolic extract afterward was brought to partition with the various solvents base on polarity difference. Finally, accepted hexane, chloroform, ethyl acetate, and water fractions, respectively. The ML extract and its fractions were screened the cytotoxicity on RAW264.7 cells by sulforhodamine B assay. The non-toxic doses were selected for the next NO inhibition experiment. RAW264.7 cells were treated with the various non-toxic doses of the ML. Lipopolysaccharide (LPS) was added to induce cells inflammation and stimulate NO production. Additionally, the culture supernatants were collected and measured NO levels by Griess reagent. The fraction that revealed potent anti-inflammatory activity by reduced NO accumulation was selected to study COX-2 protein suppression by western blot analysis.

Results: The results showed that crude ethanolic extract and all fractions except for the water fraction significantly inhibited NO production of RAW264.7 cells. The hexane fraction demonstrated a superior on nitric oxide reduction as same as the standard drugs L-NAME and indomethacin. This fraction also reduced COX-2 protein expression in LPS-stimulated RAW264.7 cells.

Conclusions: The hexane fraction possesses an anti-inflammatory activity by reducing nitrite level and COX-2 protein expression. Suggesting that, the active ingredient of ML is the non-polar compound. Further studies should be carried out to isolate the pure compound from the hexane fraction and structure identification.

Introduction

Inflammation is a response process that triggering

by a variety of factors including invading pathogens, toxic compounds, and endogenous signals like damaged cells.[1] Tissue macrophages and dendritic cells represent a key role in danger signals detection. These cells can release pro-inflammatory mediators to promote leukocyte migration and eliminating the cause of infection and contributing to tissue repair.[2] Activated macrophages and other immune system cells inhibit pathogen replication by releasing many kinds of effector molecules, including nitric oxide (NO).[3] Nitric

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oxide is an important mediator of inflammation. It causes vasodilation and tissue injuries in the inflammation process.[4] It has two principal divergent functions in cells: homeostasis and cytotoxicity. For regulatory functions, NO is produced in small amounts under physiological conditions and mediates vasodilation, controls the adhesion and aggregation of platelets and neutrophils, and is involved in neurotransmission.[5] It is considered as a pro-inflammatory mediator that induces inflammation due to overproduction in abnormal situations and involved in the pathogenesis of inflammatory disorders.[4] Therefore, NO inhibitors represent an important therapeutic advance in inflammatory diseases management.[4] The cyclooxygenase (COX) is a key enzyme involved in the inflammation process.[6] The role of NO on the constitutive and induced forms of COX activity were studied in the mouse macrophages cell line RAW264.7. The results showed that NO directly interacts with COX to cause an increase in the enzymatic activity and prostaglandins (PGs) production.[5]

NSAIDs are frequently prescribed drugs for relieving inflammation. In addition to their anti-inflammatory effect, they have antipyretic and analgesic properties.[7, 8] Anti-inflammatory mechanisms of NSAIDs based on cyclooxygenase enzymes (COXs) inhibition. COXs are the key enzymes required in PGs synthesis.[9] COX-1 involves in PGs production that supports platelets and protects the stomach. COX-2 is induced by various inflammatory stimuli, contributed to PGs production that involves pain and swelling.[9, 10] The traditional NSAIDs reduced inflammation, pain, and fever because of both COX-1 and COX-2 inhibition. However, inhibition of COX-1 cause reduction of the PGs that protect the stomach and support platelets and blood clotting then it leads to the gastric ulcer and bleeding.[11] For this point, the NSAIDs drug that selective inhibition on COX-2 was developed. This group increased the selectivity for COX-2 inhibition but also enhanced the risk of cardiovascular diseases.[12] The results from several trials showed that coxib drugs increased the risk of atherothrombotic vascular events.[12, 13] Although, NSAIDs produced an excellent anti-inflammatory activity their adverse effects caused the limitation of NSAIDs using. The critical side effect of NSAIDs including gastrointestinal, renal, and cardiovascular toxicity caused the limitation for NSAIDs user. For these reasons, there is a continuous effort in NSAIDs development with decreasing side effect profiles from the alternative sources.[14]

Murdannia loriformis (ML) is a medicinal plant traditionally used for treating diseases like colds, throat infections, pneumonia, diabetes mellitus, flu, and inflamed wound.[15] In Thailand, the cancer patients used this plant to relieve side effects of chemotherapy and radiotherapy.[16] The crude ethanolic extract of this plant showed anti-inflammatory, analgesic, and antipyretic activities in the various *in vivo* models.[17] In the cotton pellet-induced granuloma formation in the rats, it showed anti-inflammatory activity without gastric ulceration. Suggested that, the anti-inflammatory activity of the extract is the non-steroidal like action since it did not affect the thymus weight of the rats.[17] The ethanolic extract also decreased gastric ulcer in the rats induced by ethanol-hydrochloric acid, indomethacin, and stress.[18] The crude ethanolic extract of this plant revealed both anti-inflammatory and gastro-protection properties. Therefore, it might be the alternative source of the anti-inflammatory agent. This study aimed to isolate active fractions of ML and examine the effect of its fraction on nitric oxide production and COX-2 expression in RAW264.7 cells

Materials and Methods

Preparation of plant crude extract

Dry powders of *M. loriformis* was purchased from Abhaibhubejhr's hospital, Prachinburi Province, Voucher specimen with number 25135 was deposited at the herbarium section of Queen Sirikit Botanical Garden. For preparing of crude ethanol extract, the *M. loriformis* powders (300 gm) was macerated with 80% ethanol at room temperature for 24 hr, evaporated by a rotary evaporator, and lyophilized. The yield of crude EtOH extracted was 14.45%.

Liquid-Liquid partition

Crude ethanolic extract (10 gm) was suspended in double distilled water (100 mL) and partitioned with n-hexane, chloroform ($CHCl_3$), and ethyl acetate (EtOAc), respectively. The partition process was repeated three times. Eluent of each fraction was pooled, evaporated and lyophilized.

Cell lines and cell culture

The RAW264.7 cells were provided by Assoc. Prof. Dr. Siriwan Ongchai, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Thailand. Cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO™, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (GIBCO™, Thermo Fisher Scientific, MA, USA), and 1% antibiotics (penicillin 100 units/mL and streptomycin 100 μg/mL; GIBCO™, Thermo Fisher Scientific, MA, USA). Cells were incubated in a CO_2 incubator at 37 °C, 5% CO_2, and humidified atmosphere and sub-cultured every 2-3 days or when the confluence reaching 70–80%.

Cytotoxicity assay

RAW264.7 cells were seeded into 96 well plates (5×10^4 cells/well) and were incubated in the CO_2 incubator for 24 hr. Crude ethanolic extract and its fractions, in various concentrations (0.5-800 μg/mL), was added to the cells. At 24 hr after the incubation time, cell viability was measured by sulforhodamine B assay (SRB assay).[19] Briefly, 100 μL of 10% cold TCA was added to the cells and incubated at 4 °C. One hour later, the plate was washed with tap water and let air dry. Cells were stained with 0.057% SRB (Sigma-Aldrich) and incubated for 30 min, then added 1 % (v/v) acetic acid to remove the excess dye. Two hundreds microliter of 10 mM Tris base solution (Sigma-Aldrich) were added to dissolve protein-bound dye and measured the OD at 510 nm using a microplate reader.[20]

Nitrite assay

To study the effect of ML fractions on nitric oxide reduction, we stimulated RAW264.7 cells with LPS and measured the nitrite, the stable product of NO, in the culture supernatant. Cells were seeded in 96-well plate (5×10^4 cells/well) and

incubated for 24 hr. Various concentrations of ML fractions were added before cell stimulating with LPS (Sigma- Aldrich). Twenty-four hours after the incubation time, nitrite level in cell culture supernatant was measured by Griess reaction. Briefly, 100 μL of culture medium was mixed with 100 μL of Griess reagent (Sigma-Aldrich). The reaction was performed at room temperature for 15 min and the absorbance was measured at 540 nm. Nitrite level in the sample was determined by comparing with the sodium nitrite standard curve.[21] Non-selective inhibitor of nitric oxide synthase, nitroarginine methyl ester (L-NAME) and non-selective COX inhibitor, indomethacin was used as positive control.[22, 23] The positive controls were kindly provided by Assist. Prof. Dr. Nuttakarn Jiruntanat, Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Thailand.

Western Blot analysis

We chose the potent ML fraction from the earlier nitrite inhibition experiment for COX-2 expression study. RAW264.7 cells (1x10^{6} cells/well) were seeded in the 6-well plate for 24 hr. ML fraction was added for 1 hr before stimulating with LPS and cells were incubated for another 24 hr. RIPA buffer was added to lyse cells. The lysate was centrifuged at 12,000 g 4 °C for 10 min and supernatant was collected for protein determination using a protein assay kit. Total 20 μg of proteins were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membranes were blocked with 5% skim milk overnight and incubated with anti- COX-2 antibody (Merck). After incubation, membranes were washed three times with PBS-Tween 20 and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Merck). Beta actin antibody (Merck) was used as a loading control. The enhanced chemilumi-nescence system was applied to detect the signals. Protein bands intensity from western blot films was quantified by ImageJ software.

Statistical analysis

Data from three independent experiments were presented as the mean±standard error of the mean (S.E.M). Statistical comparisons between the mean of each group were analyzed using the One-way analysis of variance (One-way ANOVA). The statistically significant was considered at $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Results

Liquid-Liquid extraction yield

Dry weight of crude ethanolic extract and its fractions obtained from experiment was shown in the extraction process flowchart (Figure 1). Yields of crude ethanolic extract, hexane, $CHCl_3$, EtOAc, and water fractions were 14.45%, 11.2%, 1.49%, 2.18%, and 87% respectively (Table 1).
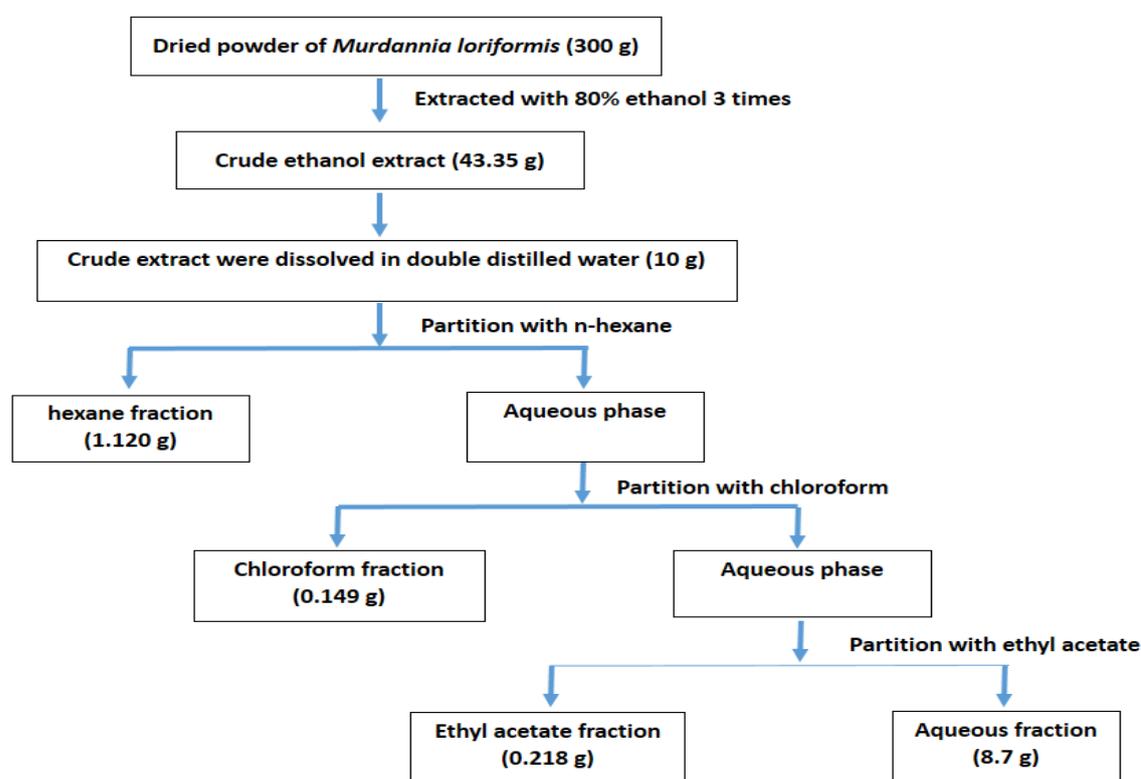

Figure 1. Flow chart of Extraction process.

Table 1 *Extraction yield*.

Extracts	Yield (%)	Weight (g)
1. Crude ethanolic extract	14.45	43.35
2. Hexane fraction	11.2	1.120
3. $CHCl_3$ fraction	1.49	0.149
4. EtOAc fraction	2.18	0.218
5. Water fraction	87	8.7

Cytotoxicity assay

Effect of crude ethanolic extract and its fractions on RAW264.7 cells availability were determined using SRB assay. As shown in Figure 2, no significant difference in cell viability was observed for crude EtOH extract, EtOAc, and water fractions up to concentrations of 100 µg/mL. The difference in cell death was shown in hexane and $CHCl_3$ treated-cell at a concentration of 100 µg/mL. From these results, non-toxic concentrations of crude EtOH extract, EtOAc, and water fractions (up to 100 µg/mL) and hexane and chloroform fractions (up to 50 µg/mL) were selected for subsequent NO inhibition assay.
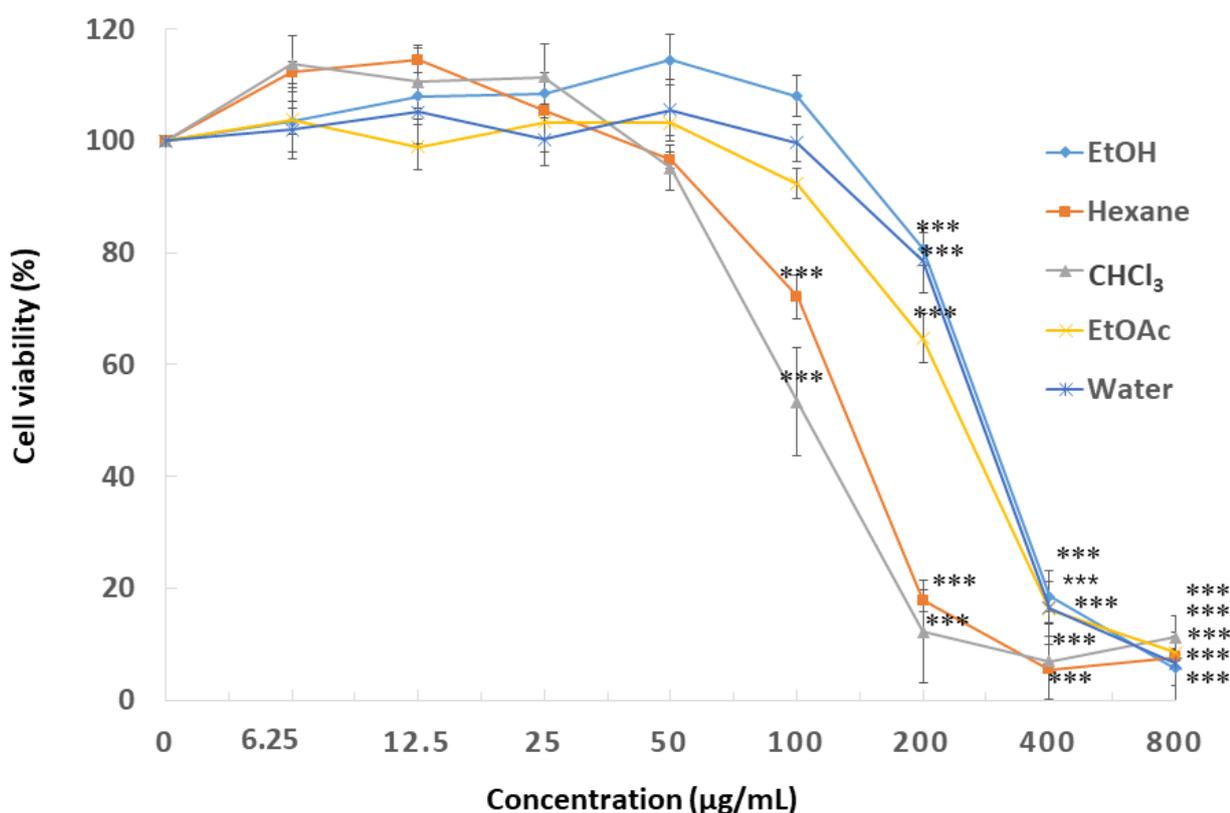

Figure 2. *Effect of crude ethanolic extract of ML and its fractions on RAW264.7 cells viability. Each line represents the mean of three replicates with error bar representing the S.E.M. ***: significant difference compared with media control (concentration 0 µg/mL).*

Inhibition of NO production in LPS-stimulated RAW264.7 cells

Nitrite levels of cells stimulated with LPS were 19.1±5.9 µM. Cells treated with the positive control, L-NAME and indomethacin, caused nitrite reduction to 2.2±0.32 and 2.8±1.19 µM, respectively. Crude ethanolic extract and EtOAc treatment caused nitrite levels reduction comparable with positive control. The potent NO reduction effect was seen in hexane and $CHCl_3$ fractions at a high dose of 50 µg/mL. Nitrite level in the culture supernatant of hexane and $CHCl_3$ –treated cells were 0.83±0.63 µM and 1.1±0.54 µM, respectively. Water fraction tended to reduce nitrite level; however, no significant difference from LPS-induced group (Figure 3). Ethanolic extract and almost ML fractions reduced NO accumulation in culture supernatants without significant reduction of RAW264.7 cells viability as shown in Figure 4.
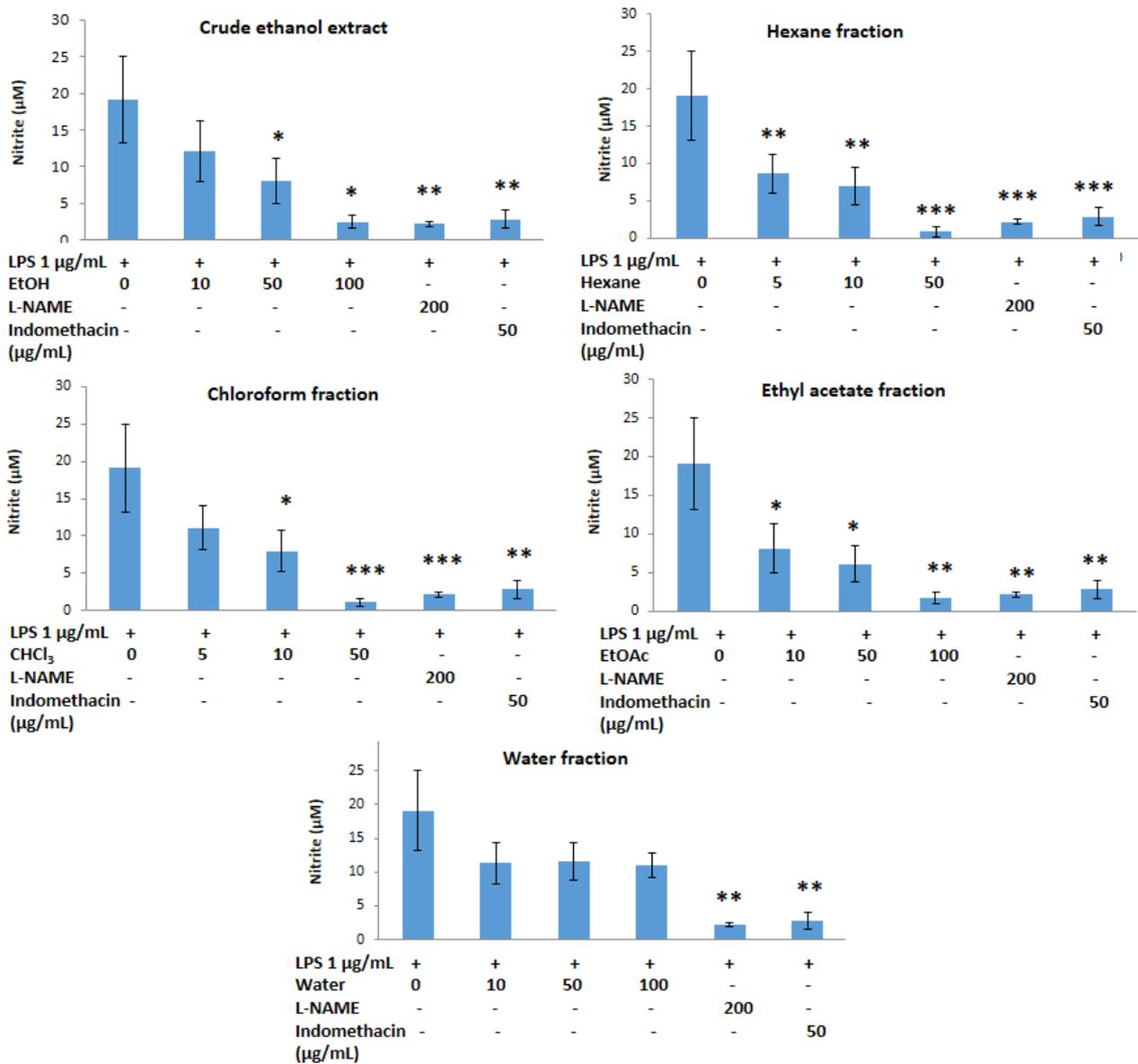
Figure 3. *Effect of crude ethanolic extract and its fractions on the nitrite production in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with various concentrations of ethanol extract and its fractions for 1 hr. LPS (1 µg/mL) was added to the cells and incubated for 24 hr. Nitrite level was determined by using the Griess reagent. *, **, *** : significantly different (p<0.05), (p<0.01), and (p<0.001), respectively, from that of LPS-treated cells.*
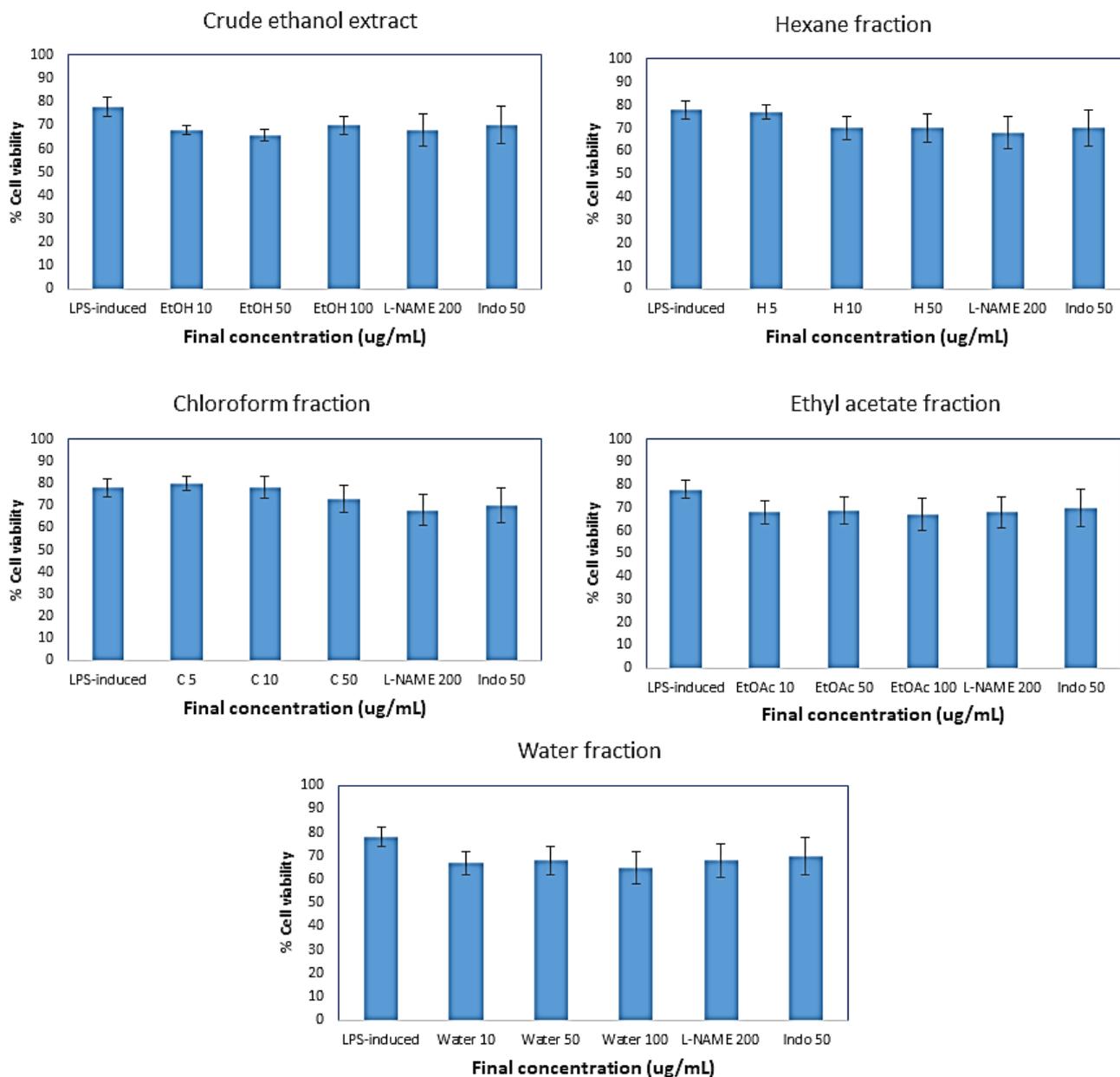
Figure 4. *Cells viability assay after NO experiment. Each bar shows the mean of three replicates experiments with the standard error of the mean (S.E.M.).*

Effect of hexane fraction on COX-2 protein expression

Hexane fraction showed the most potent anti-inflammatory property against LPS-induced inflammation. For this reason, it was selected to study the effect on COX-2 expression by western blot analysis. As showed in Figure 5A, result from western blot analysis demonstrated that COX-2 protein expression was not observed in unstimulated RAW264.7 cells. COX-2 protein expression was presented in LPS treatment cells. Density ratio of COX-2/β-actin was shown in Figure 5B. Indomethacin at a concentration of 50 µg/mL significantly reduced COX-2 expression of LPS-stimulated RAW264.7 cells ($p<0.01$). RAW264.7 cells-treated with hexane (10 and 50 µg/mL) showed a dose-dependent inhibition of COX-2 protein expression when compared with the LPS-treated cells ($p<0.05$ and $p<0.001$, respectively).
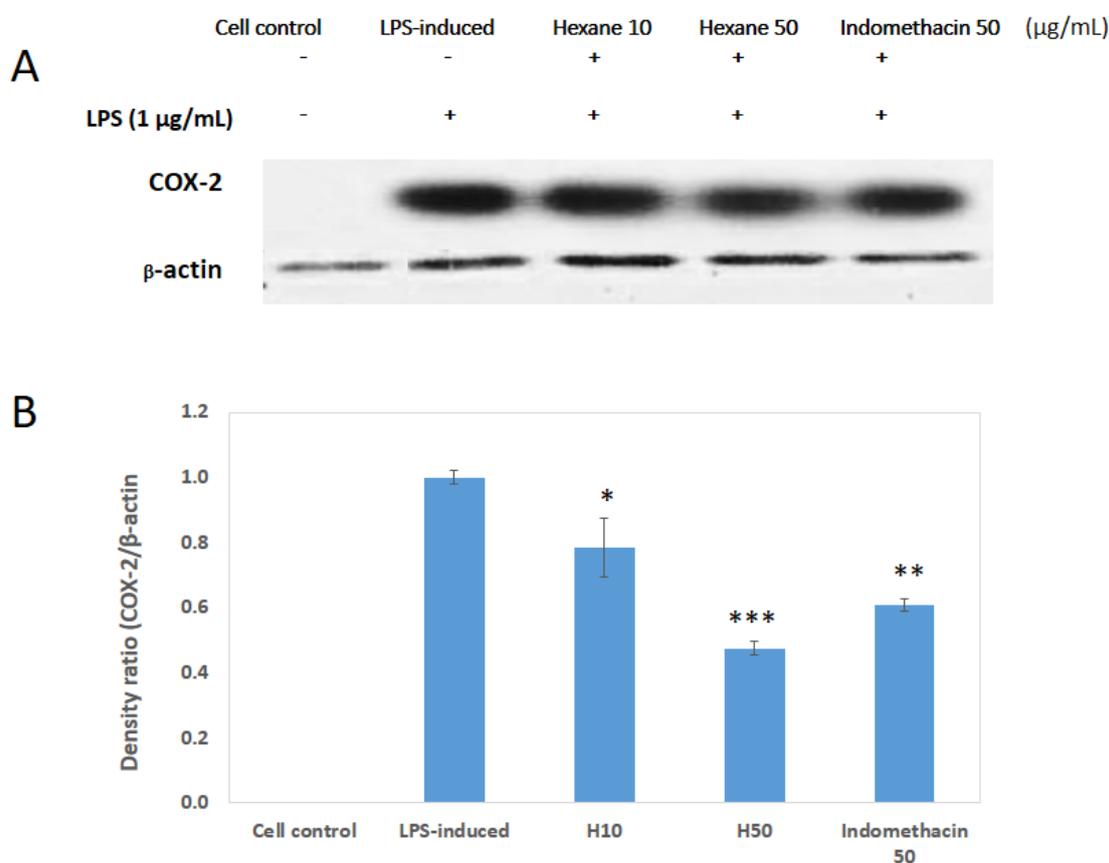
Figure 5. *Effect of hexane fraction of M. loriformis on COX-2 protein expression in LPS-stimulated RAW264.7 cells. A: Expression of the COX-2 protein was assessed by Western blot analysis; β-actin was used as the loading control, B: Density ratio of COX-2/β-actin. Densitometry data represents mean±S.E.M from three independent experiment, *, **, ***: significantly different (p<0.05), (p<0.01), and (p<0.001), respectively, from that of LPS-treated cells.*

Discussion and Conclusion

Macrophages play an essential role in the steps of inflammation including the initiation, maintenance, and resolution.[24] These cells are activated by various activation signals such as cytokines, bacterial lipopolysaccharide, extracellular matrix proteins, and other chemical mediators.[24] RAW264.7 cells are macrophage-like, derived from BALB/c mice. RAW264.7 cells stimulation by LPS causes increasing of NO production and enhance phagocytosis of cells.[25] NO produces from a family of enzymes called nitric oxide synthases (NOSs). The three distinct isoforms of NOS are endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS).[26] Inducible NOS normally is not present in resting cells it can be induced by cytokines, bacterial products or infection in various cells, including smooth muscle cells, endothelium, hepatocytes, monocytes, mast cells, and macrophages.[27] Overproduction of NO from iNOS can result in either protective or damaging effects. Protective effect involves in produces NO against microbial and viral pathogens infection.[28] However, at excessive concentrations, NO can display pathogenic properties. Production of ONOO-, NO•, and other reactive oxidizing compounds in the presence of superoxide radicals or peroxidases finally causing the oxidative damage of tissues.[26, 29]

We investigated effects of ML fractions on NO inhibition in LPS-stimulated RAW264.7 cells. Results showed all fractions except water fraction significantly decreased nitrite accumulation in culture supernatant. The high dose of ML fractions caused reduction of nitrite level similar to the positive controls. Among these, hexane fraction exhibited a potent effect on nitric oxide inhibition compared with other fractions. In NO inhibition assay, we used L-NAME (200 µg/mL) as a positive control. The results indicated its efficacy in reduced nitrite accumulation nearly ninety percent. Mechanism of L-NAME in decreasing of NO level involved iNOS inhibition.[30] We also used indomethacin as the second positive control for nitrite assay. Our results revealed that indomethacin (50 µg/mL) decreased nitrite level close to that of L-NAME. Inducible NOS has been reported correlation with COX-2 pathway.[31, 32] COX-2 an inducible enzyme is stimulated by LPS and several cytokines and involved in pathology of inflammation. COX-2 expression results in inflammatory mediators production including prostaglandins, prostacyclin, and thromboxane.[6] The results

from previous studies, demonstrated that NO activates COX to produce large amounts of prostaglandins and may result in an exacerbated inflammatory response.[32, 33] These studies led to the concept that COX enzymes as an important endogenous "receptor" target for amplifying or modulating the varied roles of NO in physiology and pathology.[31, 33]

Macrophages stimulation by LPS for iNOS expression represents a secondary effect requiring autocrine signaling of endogenously produced PGE_2. It seems to be mediated by up-regulation of COX-2 and followed by produced PGs that stimulate iNOS expression in an autocrine manner.[34] Our results found that indomethacin reduced NO accumulation better than L-NAME. In this study, COX-2 inhibition by indomethacin may be diminished PGE_2 production then decreased iNOS stimulation and afterward reduced NO production.

Since hexane fraction exhibited the most potent effect on NO inhibition, we further evaluated effect of this fraction on COX-2 expression. The results demonstrated that hexane fraction significantly decreased COX-2 expression in LPS-stimulated RAW264.7 cells. Anti-inflammatory mechanisms of the fraction may be via reducing of COX-2 expression and NO inhibition. From this present study, active anti-inflammatory substances of ML fraction are considered non-polar compound.[35] There were many reports about anti-inflammatory activity of non-polar solvent extracts from various plants species. Othman and colleagues have been reported anti-inflammatory activity of hexane partition from root *Jatropha curcas* towards RAW 264.7 cells.[36] In an *in vivo* study, hexane extract of Alchornea and a non-polar extract from *Rhododendron L*. exhibited anti-inflammatory activity over indomethacin.[37, 38] In 2013, Da and colleagues reported that hexane fraction of *Urtica dioica* exhibited anti-inflammatory activity by reduced rat paw edema.[39] These results along well with our findings of present study where hexane fraction demonstrated the most potent anti-inflammatory activity, indicating non-polar compounds were involved.

In conclusion, ML fractions reduced NO production in RAW264.7 cells. Nitric oxide inhibition remains an essential goal in inflammatory management. Therefore, ML fractions may be useful in inflammatory-related conditions. Hexane fraction demonstrated the most effectiveness in reducing NO accumulation and COX-2 expression. These results suggested that hexane fraction of ML might be a potent anti-inflammatory agent for inflammatory-related disease treatment. The active components of hexane fraction should be identified in future study.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

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