DOI: https://doi.org/10.5564/mjas.v21i02.903

ANTI-INFLAMMATORY EFFECT OF *PLANTAGO SP* ETHANOLIC EXTRACT IN MURINE RAW264.7 MACROPHAGE CELLS

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ABSTRACT

Besides being recorded as a traditional medicine, nowadays, plantain plants (Plantago sp.) are appreciated in many more aspects. Plantain is a name applied both to a drug and to a vegetable in a number of countries as Vietnam, China, Cambodia, Laos and North American Indians [9, 13]. Plantago sp. traditionally used for treating wound, fever and inflammation in Asia. This study aimed to investigate the anti-inflammatory activity of ethanolic extracts of Plantago sp. including P. major L. and P. depressa Willd. on RAW 264.7 murine macrophage cells. Cells were treated with different concentration of the PAE extract (50, 100, 200, 400 µg/mL) with or without lipopolysaccharide (LPS) stimulation to evaluate its effect on cell viability, using CCK-8 assay. Nitric oxide (NO) production was assessed by Griess reagent on LPS-stimulated cells using preceding PEE treatment. Furthermore, mRNA expression of inflammmatory-related genes were evaluated by RT-PCR analysis. The results revealed that PEE treatment increased cell viability in naive cells whereas inhibited cell profileration in LPS-stimulated cell dose-dependently. In addition, NO emission and mRNA level of IL-1β, IL-6, iNOS, COX-2 and NF-κB decreased by dose dependant manner. As summary, PEE exhibits anti-inflammatory activity through inhibition of pro-inflammatory mediators mRNA expression in macrophages.

KEYWORDS: *Plantago major*L., *Plantago depressa Willd., Plantago* ethanolic extract, anti-inflammatory effect, Murine RAW264.7 macrophage cells, *in vitro* testing.

ABBREVIATIONS

LPS: Lipopolysaccharide
PEE: Plantagoethanolic extract
PME: *Plantago major* ethanolic extract
PDE: Plantago depressa ethanolic extract
DMSO: Dimethylsulfoxide
CCK-8: Cell Counting Kit – 8
N.C: Negative control

INTRODUCTION

The investigation of medicines of plant origin is prospective of the current trend of medical research because of their affordability and accessibility with minimal side effects. Among of medicinal plants, plantain plant (*Plantago* sp.) belongs to Plantaginaceae family, is considered as a potential herb. Recently, this species has attracted much attention and became economically important [5, 13].The plantain plants contain many biologically active compounds such as iridoid glycosides, polysaccharides, flavonoids, lipids, caffeic acid derivatives, terpenoids and some organic acids that involved in the wound healing activity, anti-inflammatory, anti-cancer, anti-oxidant, analgesic, weak antibiotic, immune modulating and anti-ulcerogenic, anti-leukemic and anti-hypertensive activity properties. Indeed, plantain plantwere mentioned as an old medicinal plant that has been used more than a millennium ago for wound healing remedy and in the treatment of a number of diseases, which include diseases related to the skin, respiratory organs, digestive organs, reproduction the circulation, against cancer, pain relief and against infections all around the world [1, 12, 17].Present study was designed to verify the anti-inflamamtory activity of *Plantago* ethanolic extracts (PEE) on murine Raw264.7 macrophage cells.

MATERIALS AND METHODS

Extraction of plant material

Samples of *Plantago depressa* were collected from plant geographical region of Middle Khalkh dry steppe of Mongolia (47.88°N in latitude, 106.90°E in longitude) in July 2015, and samples of *Plantago major* were collected from plant geographical region of Mongolian Altai Mountain steppe of Mongolia (48.39°N in latitude, 89.66°E in longitude) in July 2015. The plant species for sampling was identified according to morphological characters by Grubov, 1982 [21]. The whole plants of *Plantagomajor* L. and *P. depressa* Willd. (200 g of each) were extracted with ethanol (1 Litter) at room temperature. The Plantago ethanol extracts (PEE) were evaporated to obtain powdered samples. The extract was dissolved in dimethylsulfoxide (DMSO) to give 0.1 v/v concentration and used at appropriate concentrations (μg/mL).

Cell line, culture media, cell treatment

The cytotoxicity of *Plantago* ethanolic extracts was determined by a Cell Counting Kit-8 assay (CCK-8) according to the manufacturer's Dojindo Laboratories (Tokyo, Japan). Cytotoxicity was evaluated using HaCaT skin normal cells. Cells were treated with increasing concentrations of the PEE extracts (50, 100, 200, 400 μg/mL) to determine its effect on proliferation.

Nitric oxide (NO) determination

The nitric oxide assay was performed as described previously with slight modification [22]. After pre-incubation of RAW 264.7 cells (5×10^4 cells/mL) with LPS (10μg/mL) for 2hours after treated with increasing concentrations of the PEE extract (25, 50, 100, 200, 400 μg/mL) for 24hours the quantity of nitrite in the culture medium was measured as an indicator of NO production. Amounts of nitrite, a stable metabolite of NO, were measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared by disrupting the RAW264.7 cells in TRIZOL reagent (Life Technologies, USA). Complementary DNA was synthesized from 1 μg of total RNA in a 25 μl reverse transcription reaction mixture. For RT-PCR, aliquots of cDNA were amplified in a 20 μl PCR mixture according to the manufacturer's protocol (Promega, USA) [4]. The primers for each gene were as follows:

Forward primer for β-actin (310 bp): 5′TCATGAAGTGTGACGTTGACATCCGT-3′ and its reverse primer: 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′.

Primers for iNOS were as follows: forward: 5′-GCAGAATGTGACCATCATGG-3′; its reverse primer: 5′-ACA ACC TTGGTGTTGAAGGC-3;

Forward primer for COX-2 (721 bp): 5′GGAGAGACTATCAAGATAGT-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTACA-3′;

Forward primer for IL-1β (387 bp): 5′-TGCAGAGTTCCCCAACTGGTACATC-3′ and its reverse primer:
5′GTGCTGCCTAATGTCCCCTTGAATC-3′;
Forward primer for IL-6 (147 bp): 5′-GAGGAT ACCACTCCCAACAGACC-3′ and its reverse primer:
5′-AAGTGCATCATCGTTGTTCATACA-3′;
Forward primer for NF-kB (194 bp):5′-GAAATTCCTGATCCAGACAAAAAC-3′; and its reverse primer,
5′-ATCACTTCAATGGCCTCTGTGTAG-3′;
Forward primer for TNF-α (351 bp): 5′ATGAGCACAGAAAGCATGATC-3′ and its reverse primer:
5′-TACAGGCTTGTCACTCGAATT-3′;
The thermal cycling conditions were as follows: 24-32 cycles at 94°C for 1 min, 55-60°C for 45 sec and 72°C for 45 sec. PCR products were electrophoresed on 1.5% agarose gels [4].

RESULTS AND DISCUSSION

Effect of Plantago ethanolic extracts on HaCaT skin normal cell viability

In murine macrophage RAW 264.7 cells, LPS stimulation alone has been demonstrated to induce iNOS transcription and its protein synthesis, with a corresponding increase in NO production. Furthermore, LPS stimulation has also been shown to induce IκB proteolysis and NF-κB nuclear translocation [22, 23]. Therefore, this cell system is an excellent model for drug screening and the subsequent evaluation of potential inhibitors against iNOS and NO production. Although a period in which pharmaceutical companies cut back on their use of natural products in drug discovery, there are many promising drug candidates in the current development pipeline that are of herbal origin. After all, traditional cytotoxic chemotherapy despite kills cancer cells by indirectly inducing apoptosis unfortunately, side effects are brutal, and most tumors become resistant [10]. The present study demonstrated the cytotoxicity indices as a measure of percentage cell mortality calculated by CCK assay in HaCaT skin normal cells, in a dose dependent manner at the end of 24 hours incubation with extract. To study the effects of *Plantago* ethanolic extracts on cell proliferation and identify its therapeutic potential we indicated, for the first time, the potent cytotoxic activity of different concentrations of ethanolic extracts of *Plantago* sp. did not significant influenced on normal cell line viability (Fig. 1 and 2). A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells, meaning minimum side effects. This ideal situation is reachable by inducing apoptosis in cancer cells. Cell cycle modulation by various natural and synthetic agents is gaining widespread attention in recent years [2].

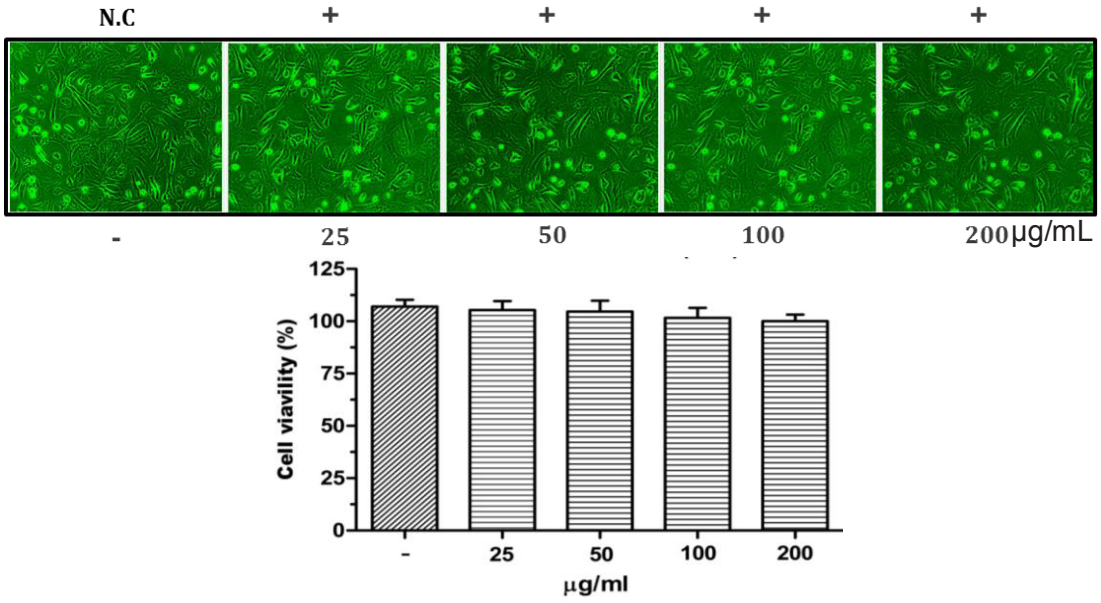

Figure 1. Effects of *P. major* ethanolic extract on HaCaT skin normal cell viability after 24 hour incubation. *Data are means ±SD (n=3) and not significantly different (p<0.05).*

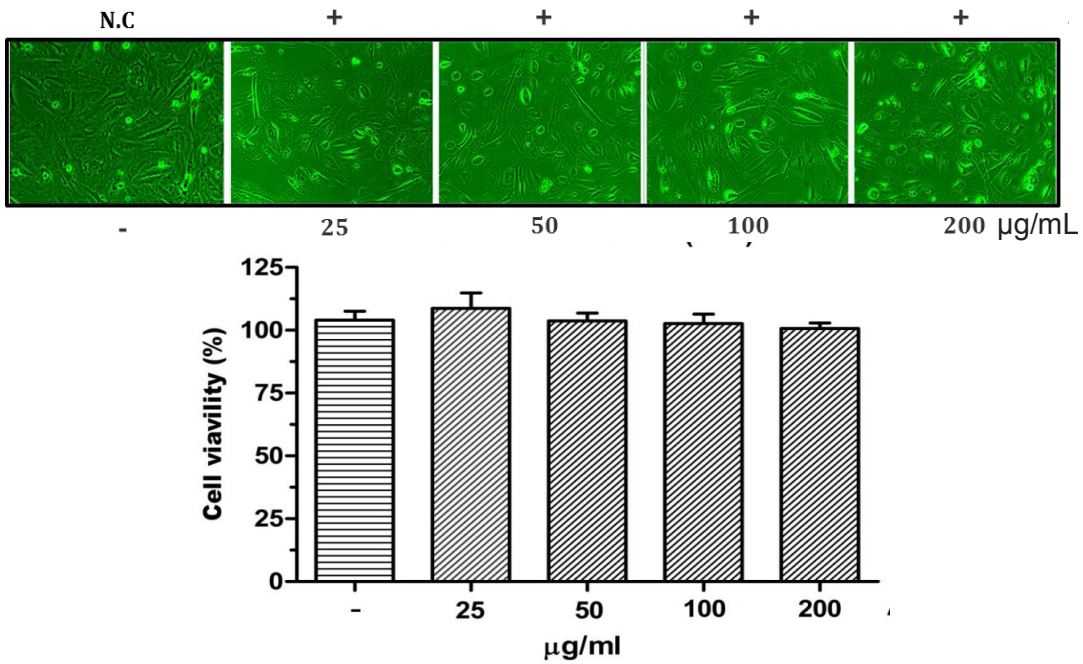

Figure 2. Effects of *P. depressa* ethanolic extract on HaCaT skin normal cell viability after 24 hour incubation.
Data are means ±SD (n=3) and not significantly different (p<0.05).

Anti-inflammatory effect of PEE in RAW 264.7 murine macrophage cells

Plantago sp., particularly *P. major* has been reported containing various bioactive compounds which cure many diseases, such as anti-inflammatory, anti-oxidant, anti-aging properties; cough and wound healing activity [6]. The upper parts of plantain are used not only as crude drug but also as commercial tea for anti-phlogistic purpose [3].*Plantago* sp. has high content of aucubin and flavonoids, which have anti-inflammatory reaction [14]. Present study was designed to verify the anti-inflammatory activity of Plantago ethanolic extracts (PEE) on RAW 264.7 murine macrophage cells. Cells were treated with different concentration of the PEE (50, 100, 200, 400 µg/mL) with or without lipopolysaccharide (LPS) stimulation to evaluate its effect on cell viability, using CCK-8 assay. Nitric oxide (NO) production was assessed by Griess reagent on LPS-stimulated cells using preceding PEE treatment. Furthermore, mRNA expression of inflammmatory-related genes were evaluated by RT-PCR analysis.

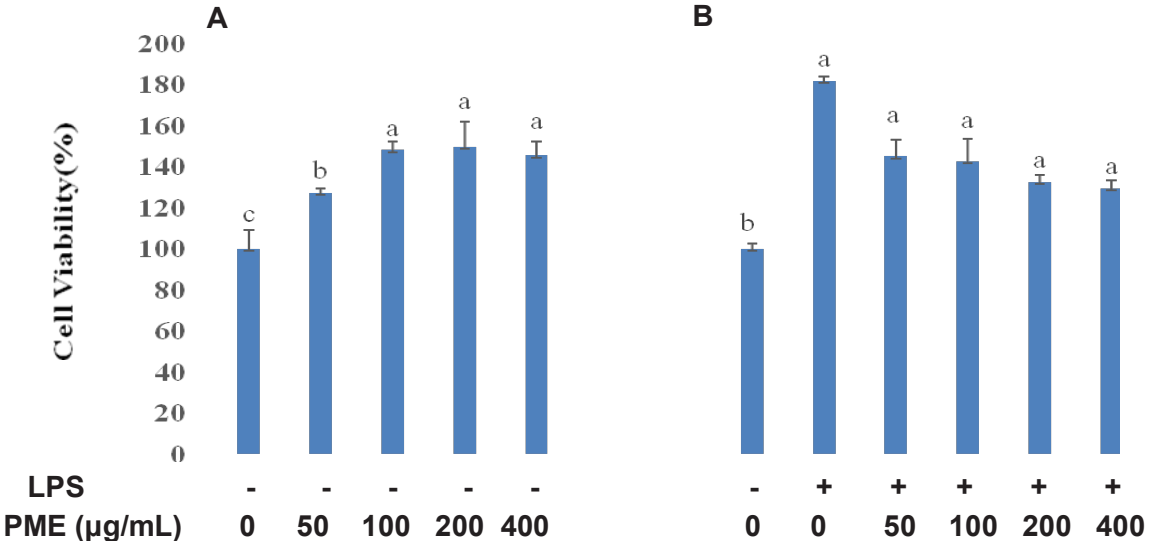

Figure 3. Effect of *P. major* ethanolic extract on RAW 264.7 murine macrophage cell proliferation after 24 hour treatment (A. without LPS treatment, B. with LPS treatment)
Data are means ±SD (n=3). Bars with different superscripts are significantly different (p<0.05).

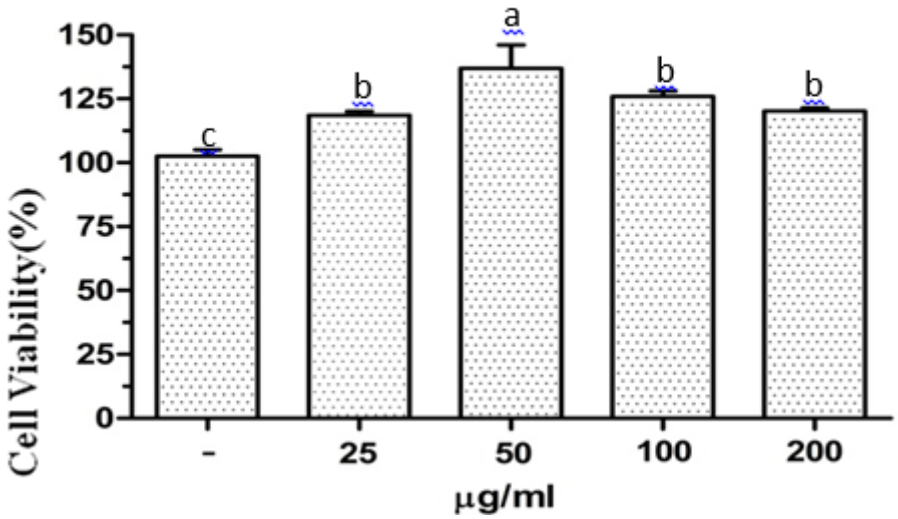

Figure 4. Effect of *P. depressa* ethanolic extract on RAW 264.7 murine macrophage cell proliferation after 24 hour treatment.
Data are means ±SD (n=3). Bars with different superscripts are significantly different ($p<0.05$).

CCK-8 assay was done to evaluate cytotoxic effect of PEE on RAW 264.7 murine macrophage. PEE treatment increased naive RAW 264.7 macrophages, but inhibited cell proliferation of lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages (Fig.3 and 4). Macrophages are important player in innate immune system, thus increasing number of macrophages promotes wound healing [20]. However, increase in activated macrophages may lead to acute or chronic inflammation that will cause dysfunction of tissues. Thus, inhibition of activated macrophage proliferation can prevent prolonged exposure to inflammation and encourage healthy tissue restoration [11]. Inflammation promotes wound healing and tissue repair but prolonged inflammation can cause tissue dysfunction that lead to inflammatory diseases. Macrophages play important role in the initiation, maintenance and resolution of inflammation [16] and can be directly activated by bacterial pathogen such as lipopolysaccharide (LPS) [7]. One of the early chronic inflammation marker is increased level of nitric oxide (NO), produced by many cells involved in immunity and inflammation.

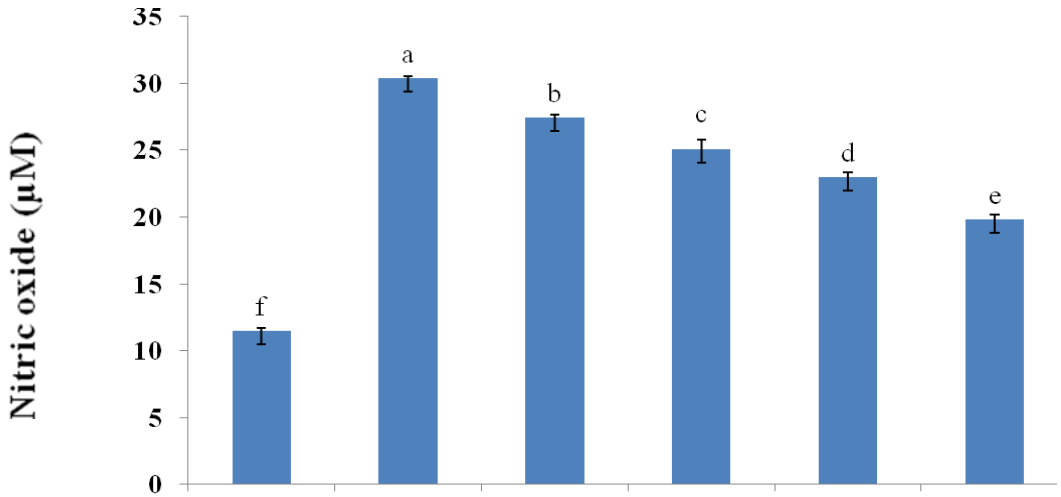

Figure 5. Effect of *P. major* ethanolic extract on RAW 264.7 murine macrophage cell NO production after 24 hour treatment.
Data are means ±SD (n=3). Bars with different superscripts are significantly different ($p<0.05$).

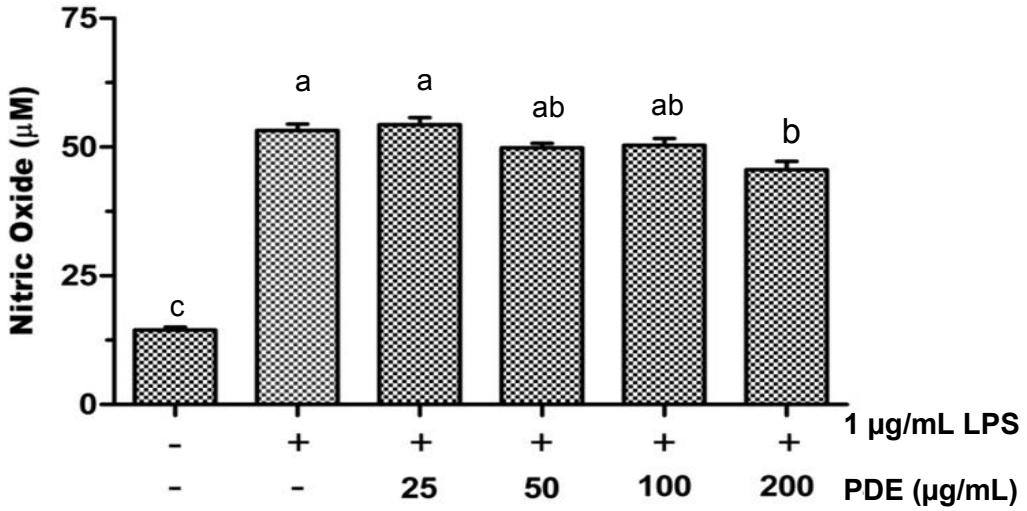

Figure 6. Effect of *P. depressa* ethanolic extract on RAW 264.7 murine macrophage cell NO production after 24 hour treatment.

Data are means ±SD (n=3). Bars with different superscripts are significantly different (p<0.05).

Increase of nitric oxide (NO) production by macrophages is one of the early markers of inflammation. NO protect against infectious agents and influences function, proliferation and apoptosis of immune cells. NO is involved in the regulation of apoptosis and released under expression of inducible nitric oxide synthase (iNOS) and inhibition of iNOS leads to decrease in NO emission in macrophages [19]. The result revealed that the investigation of PEE on NO production in LPS stimulated RAW 264.7 murine macrophage cells showed dose dependent decreasing tendency (Fig. 5 and 6). Moreover, iNOS mRNA expression decreased in dose dependent manner (Fig. 7 and 8), indicating relation with decrease in NO production.

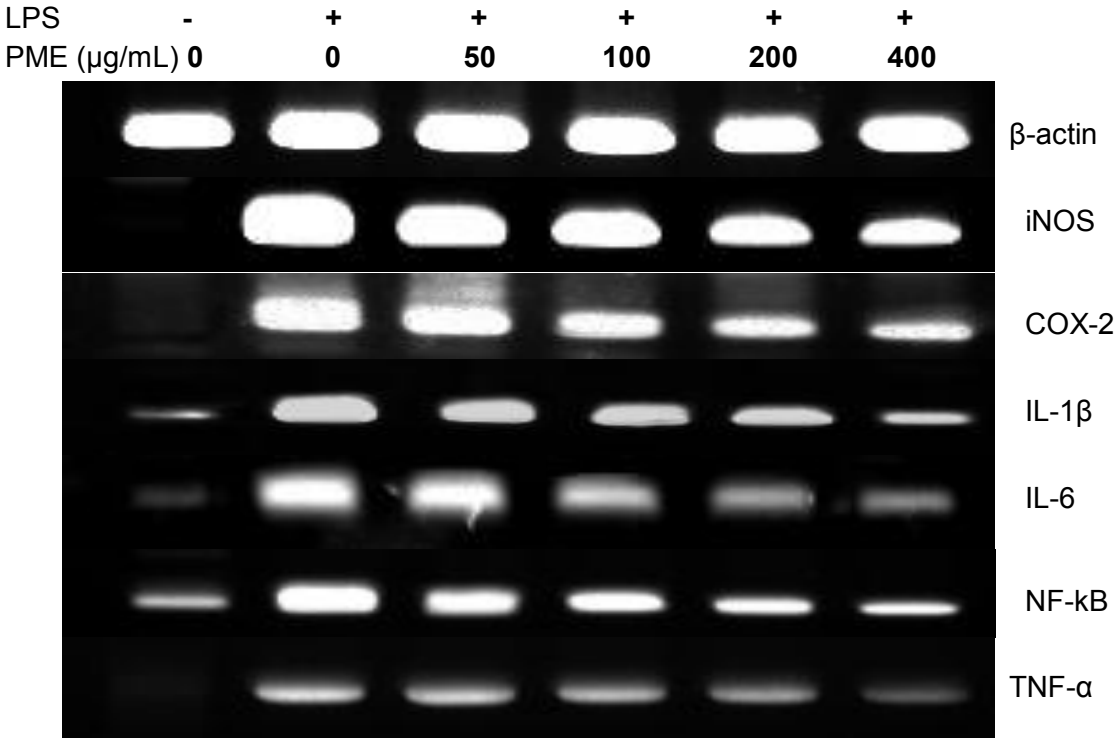

Figure 7. Effect of *P. major* ethanolic extract on RAW 264.7 murine macrophage cell inflammation related mRNA expression after 24 hour treatment.

Beside NO and iNOS, expression of inflammatory cytokines including interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and other inflammatory mediators such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and nuclear factor kappa-B (NF-kB) by activated macrophages are increased at site of inflammation [18]. mRNA expression analysis was done to evaluate preceding gene expression and results showed upon treating LPS stimulated RAW 264.7 macrophages cells with PME, mRNA levels of IL-1β, IL-6, iNOS, COX-2 and NF-κB decreased in dose dependent manner (Fig. 7 and 8), verifying anti-inflammatory activity of PME. Our results demonstrate that Plantago extracts have growth inhibitory and cytotoxic effects on Murine Raw264.7 macrophage cells. These preliminary results could be justified by the cytotoxic activity of the flavone, Luteolin-7-O-β-glucoside, the major flavonoid in all speciesof *Plantago*. Luteolin-7-O-β-glucoside is known to be the responsible agent for the anti-inflammatiory and anti-cancer activities of the plant. Similar results have been established by [8]. [15] who isolated luteolin as an active component of *Terminalia arjuna* in cancer cell lines, which justifies the underlying use of these species in traditional cancer treatment. To sum up, the results showed that PEE treatment increased cell viability in naive cells whereas inhibited cell proliferation in LPS-stimulated cell dose-dependently, especially PME treatment. In addition, NO emission and mRNA level of IL-1β, IL-6, iNOS, COX-2 and NF-κB decreased by dose dependant manner. As summary, PEE exhibits anti-inflammatory activity through inhibition of pro-inflammatory mediators mRNA expression in macrophages.

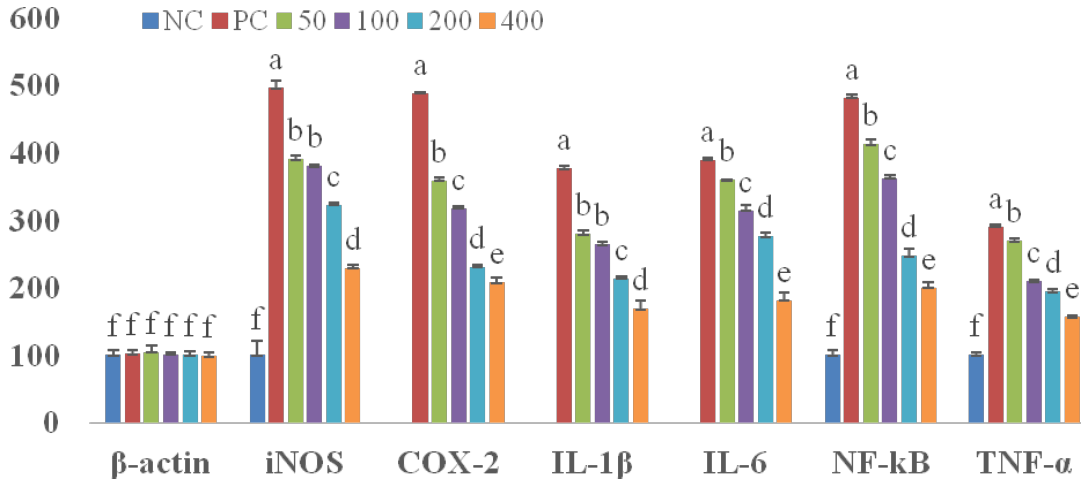

Figure 8. Histogram of *P. major* ethanolic extract effect on RAW 264.7 murine macrophage cell inflammation related mRNA expression after 24 hour treatment.

CONCLUSION

The ethanolic extracts of P. major and P. depressa display their anti-inflammatory activity through suppression of pro-inflammatory mediators mRNA expression in activated macrophages. Thus, plants demonstrating inhibitory activities against NO production will be promising candidates for the activity-guided isolation of active components exhibiting iNOS inhibitory activity, which may have therapeutic potential for the treatment of inflammation accompanying overproduction of NO. Further investigations are underway to characterise the active constituents present in these plant extracts.

ACKNOWLEDGEMENTS

This research was supported by the project named "Biosafety" and biotechnology of agricultural production, KOICA. We thank Prof. Seong Gu Hwang from Hangyong National University, South Korea for assistance with research methodology.

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