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Research Article

# Anti-Inflammatory Activity of Methanol Extract and Fractions from *Alchemilla kiwuensis* Engl. on LPS Activated Macrophages

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**ABSTRACT**

Medicinal plants have been used to treat different diseases over the world and they constitute an inexhaustible source of bioactive metabolites. *Alchemilla kiwuensis* is a Cameroonian medicinal plant used for the treatment of various diseases. The present study investigates the anti-inflammatory properties of the methanol extract and fractions on LPS activated macrophages. The extract was prepared by maceration of dry whole plant powder in methanol, following by fractioning using hexane and ethyl acetate. The activity was evaluated on RAW 246.7 cell and bone marrow differentiated macrophages by measuring their effect on nitric oxide production, phagocytosis, cytokine production and cell surface markers. The crude extract and its fractions significantly ($P<0.05$) inhibited nitric oxide production by LPS activated macrophages. As shown by RT-PCR, iNOS enzyme was inhibited in the presence of extract and its fractions. They also significantly ($P<0.05$) inhibited phagocytosis of *E. coli* and production of TNF-α and IL-6 by LPS activated macrophages. The extracts reduced the expression of the co-stimulatory molecule, CD80. The findings indicate that *Alchemilla kiwuensis* has potent anti-inflammatory properties.

**Keywords:** *Alchemilla kiwuensis*; anti-inflammatory activity; co-stimulatory molecules; nitric oxide; phagocytosis.

## INTRODUCTION

Inflammation is a phenomenon that involves complex biological response of the cellular machinery against foreign particles. Inflammation acts as a main protagonist in the pathogenesis of various diseases including rheumatoid arthritis, arteriosclerosis, infections, and asthma[1]. Plant extracts have gained increased importance as anti-inflammatory drugs and contribute to primary healthcare in a significant world population. Literature studies have conclusively suggested the anti-inflammatory effect of several plant extracts in various model systems[2-4]. Here, the methanolic extract of whole herbaceous perennial plant, *Alchemilla kiwuensis* was used. This plant is used in Cameroon for the treatment of anemia, hemorrhage, diarrhea and diabetes by local populations. The genus *Alchemilla* (Rosaceae family) is reported to comprise of more than one thousand species[5]. Majorly, it is distributed in Western Eurasia, but is also prevalent in mountain slopes of Africa.

Macrophages are the typical immune cells which are critically involved in inflammation. During cytokine surge, bacterial invasion, PAMP recognition or in response to any chemical mediators, macrophages respond and then gradually get deactivated to resolve inflammation. Increasing evidence suggests that classically activated macrophages, also called M1 cells, are the one involved in the inflammation. M1 macrophages are activated by inflammatory cytokines or by microbial products such as lipopolysaccharides[6] and play an essential role in the defense of the organism. They recognize pathogen associated molecular patterns (PAMPs) on bacteria through pattern recognition receptors (PRRs) expressed on immune cells and trigger the production of inflammatory mediators which assist the host in elimination of infectious agents. However, hyper-induction of such mediators by dysregulated innate immune cells leads to the systemic inflammatory response syndrome (SIRS), severe tissue damage and septic shock[7,8]. Macrophages have broad functions in the maintenance of tissue homeostasis through the clearance of microorganisms and repair of tissues after inflammation[9]. Phagocytosis, by macrophages, is one of the important strategy used by innate defense to eliminate the danger from the host system[10]. When activated, macrophages exhibit greater capacity for phagocytosis and kill ingested microbes by producing reactive oxygen intermediates and reactive nitrogen intermediates such as nitric oxide (NO) through the action of inducible nitric oxide synthase (iNOS). NO is known to be one of the major effector molecule in macrophage-mediated cytotoxicity. It can combine with the superoxide anion to yield even more potent antimicrobial molecules[11].Upon stimulation by endotoxins such as LPS, activated

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macrophages secrete cytokines such as TNF-α, IL-1β and IL-6 that induces early inflammatory reactions to infectious agents[12]. Due to their role in causing inflammation and phagocytosis, macrophages are also involved in many chronic inflammatory diseases of immune system, ranging from granuloma formation in tuberculosis to myocardial infarction to cancer progression, all are caused by persistent activation macrophages. Anti-inflammatory drugs such as nonsteroidal anti-inflammatory drug (NSAIDs) are currently used for treatment of inflammation. These drugs are known to cause severe side effects in the body such as heart attacks and strokes. Many plant extracts have been shown to have anti-inflammatory properties without causing any side effects.

In the past, the immunomodulatory activity of *A. kiwuensis* methanol extract was explored on immune system components, both *in-vitro* in tissue culture and *in vivo* in mice[13]; herein, anti-inflammatory properties of this extract and its hexane and ethyl acetate fractions were investigated on LPS-activated macrophages.

## MATERIALS AND METHODS

*Preparation of plant extracts*

*Alchemilla kiwuensis* was collected from the Bamboutous Mountain in the West region of Cameroon in November 2014. It was identified by a botanical expert at the National Herbarium in Yaoundé, Cameroon, by comparing to the voucher specimen registered as 35613/HNC. The whole plant was air-dried, grounded with a laboratory blender and macerated in methanol (500g in 3 l) for 48 hours. The crude extract was obtained after evaporation of the solvent under reduced pressure at 40°C. This extract was further partitioned in hexane and ethyl acetate. The hexane fraction was obtained after dissolution of forty grams of methanol extract in 1.5 l hexane solvent, the mixture was then filtered and concentrated. The residue obtained was soaked in 1.5 l ethyl acetate solvent and 20 ml water was added to obtain two distinct phases. The upper phase was collected and concentrated for ethyl acetate fraction. The crude extract, hexane and ethyl acetate fractions were dissolved in RPMI to obtain concentrations of 128 µg/ml and 512 µg/ml and filtered using 0.2 µm filter.

*Cell line and culture conditions*

RAW 264.7, a murine macrophage cell line was obtained from the American Type Culture Collection. Cells were grown in culture flasks in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% $CO_2$. They were further seeded at $1\times10^6$ cells/well in 24 well tissue culture plates or $1\times10^5$ cells/well in 96 well tissue culture plates, stimulated with LPS (1µg/ml) and incubated with methanol extract, hexane or ethyl acetate fractions (128 and 512 µg/ml) for 24 hours. Untreated cells and LPS treated cells were used as negative and positive controls respectively.

*Animals*

BALB/c mice (6 to 8 weeks old, weight 25 to 30 g) were obtained from National Institute of Immunology, New Delhi. Breeding and maintenance were done at the animal facility of this institute where all animals were maintained in a pathogen-free room.

*Bone marrow cells isolation and culture*

Mice were sacrificed using carbon dioxide ($CO_2$) and pelvic and femoral bones were dissected and all the remaining tissue on the bones was removed. Each bone end was cut off and bone marrow cells were collected from femoral shafts by flushing with 3 ml of cold sterile RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cell suspension was passed through a sieve to remove large clumps and cell suspension was washed 2–3 times with sterile RPMI 1640. Cells from bone marrow were cultured for 7 days with 10 ng/ml M-CSF at 37°C in an incubator containing 5% $CO_2$; culture medium was replaced every three days. At the end of day 7, culture supernatant was discarded and adherent cells were detached using EDTA. These differentiated cells (BMDM) were then seeded at $1\times10^6$ cells/well in 24 well tissue culture plates or $1\times10^5$ cells/well in 96 well tissue culture plates, stimulated with LPS (1µg/ml) and incubated with methanol extract, hexane or ethyl acetate fractions (128 and 512 µg/ml) for 24 hours. Untreated cells and LPS treated cells were used as negative and positive controls respectively.

*Nitric oxide quantification*

Nitric oxide was estimated from the accumulation of nitrite ion ($NO_2^-$) using the Griess reagent. Cell supernatant was incubated with Griess reagent (1% sulphanilamide and 0.1% α-naphthylamine (v/v) in 2.5% phosphoric acid) for 10 min in dark at room temperature. The absorbance values were measured at 540 nm using a microplate reader. The concentrations of nitrites were determined by a standard curve prepared with serial dilutions of sodium nitrite as a standard[14].

*RNA isolation, reverse transcription and quantitative real time (qRT) PCR analysis*

RAW 246.7 macrophages ($2 \times 10^6$ cells/well) were seeded in 6 well plates and were treated as described above for 24 hours. Total RNA was isolated from treated cells using Trizol reagents and RNA concentration and purity were determined by spectrophotometry. One microgram of total RNA from each sample was reverse transcribed using first strand cDNA synthesis kit as permanufacturer's instructions (MBI Fermentas). Real-Time PCR for quantification was done using SYBR green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions (ABIViiA, Applied Biosystems) using gene specific primers iNOS, 5′-ACATGCAGAATGAGTACCGG-3′ and 5′-TCAACATCTCCTGGTGGAAC-3′; L7, 5′-AGCTCATCTATGAGAAGGC-3′ and 5′-AAGACGAAGGAGCTGCAGAAC-3′. Amplification and detection of all genes was performed with ABI ViiA using the following thermal cycling conditions: one cycle of 95 °C for 10 min, 40 cycles of 95 °C for15 s, 60 °C for 1 min. Reactions were performed with cDNAs from three independent experiments and the expression of each transcript was quantified by the comparative ΔΔCT method and normalized to those of L-7 chosen as endogenous control.

Table 1: Effect of *A. kiwuensis* methanol extract and its fractions on some cell surface markers.

| | | Cell surface markers (MFI) | | | |
|---|---|---|---|---|---|
| | | CD11b $\times 10^4$ | CD11c$\times 10^2$ | CD44$\times 10^3$ | CD86$\times 10^3$ |
| RAW 246.7 cells | cells | 1.25 ± 0.02[a] | 5.11 ± 2.37[a] | 2.63 ± 1.29[a] | 6.42 ± 0.00[a] |
| | A1 128 µg/ml | 1.84 ± 0.06[a] | 1.67 ± 4.96[a] | 3.89 ± 0.85[a] | 14.59 ± 3.77[a] |
| | A1 512 µg/ml | 1.07 ± 0.03[a] | 8.81 ± 1.24[a] | 3.25 ± 0.84[a] | 7.89 ± 3.30[a] |
| | A2 128 µg/ml | 1.90 ± 00.00[a] | 18.46 ± 3.91[a] | 4.31 ± 1.80[a] | 13.52 ± 3.78[a] |
| | A2 512 µg/ml | 1.15 ± 0.01[a] | 9.23 ± 1.03[a] | 3.48 ± 1.57[a] | 9.14 ± 1.33[a] |
| | A3 128 µg/ml | 2.28 ± 0.17[a] | 17.21 ± 0.61[a] | 4.19 ± 1.40[a] | 14.62 ± 4.54[a] |
| | A3 512 µg/ml | 1.24 ± 0.03[a] | 6.29 ± 2.85[a] | 3.07 ± 0.91[a] | 10.39 ± 1.92[a] |
| | LPS 1µg/ml | 1.80 ± 0.48[a] | 11.30 ± 1.79[a] | 4.34 ± 1.20[a] | 15.16 ± 0.65[a] |
| BMDM | cells | 0.16 ± 0.01[a] | 1.32 ± 0.23[d] | 3.06 ± 2.26[b] | 2.86 ± 0.43[a] |
| | A1 128 µg/ml | 0.14 ± 0.02[a] | 1.51 ± 0.30[d] | 0.87 ± 0.74[b] | 2.89 ± 0.39[a] |
| | A1 512 µg/ml | 0.11 ± 0.03[a] | 5.16 ± 0.02[a] | 1.20 ± 1.02[b] | 2.62 ± 0.85[a] |
| | A2 128 µg/ml | 0.15 ± 0.05[a] | 1.33 ±0.01[d] | 2.11 ± 1.59[b] | 2.03 ± 0.95[a] |
| | A2 512 µg/ml | 0.14 ± 0.06[a] | 1.45 ± 0.21[d] | 1.47 ± 1.26[b] | 2.10 ± 1.72[a] |
| | A3 128 µg/ml | 0.15 ± 0.04[a] | 1.84 ± 0.17[d] | 4.10 ± 3.34[b] | 1.83 ± 0.51[a] |
| | A3 512 µg/ml | 1,52 ± 0,35[a] | 3.39 ± 0.61[c] | 1.49 ± 1.21[b] | 3.26 ± 0.19[a] |
| | LPS 1µg/ml | 0.21 ± 0.07[a] | 4.48 ± 0.00[b] | 9.41 ± 0.00[a] | 3.41 ± 0.20[a] |

*The cells were stimulated with 1 µg/ml LPS and treated with 128 and 512 µg/ml extract and fractions. Cell surface markers were analysed by FACS. Results are expressed as the mean ± SD (n=3). Values of concentrations bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the LPS treated cultures. A1= Methanol extract, A2= hexane fraction, A3= ethyl acetate fraction.*

Table 2: Effect of *A. kiwuensis* methanol extract and its fractions on macrophages viability.

| | Cell viability (%) | | | |
|---|---|---|---|---|
| | Control | Methanol extract | Hexanec fraction | Ethyl acetate fraction |
| RAW246.7 cells | 100.00 ± 2.50[bc] | 95.40 ± 1.00[c] | 104.60 ± 1.50[b] | 115.2 ± 3.00[a] |
| BMDM | 100.00 ± 1.50[c] | 105.00 ± 2.00[bc] | 107.70 ± 1.00[b] | 113.60 ± 1.50[a] |

*The cells were treated with 128 and 512 µg/ml extract and fractions. Cell exclusion were analysed by FACS. Results are expressed as the mean ± SD (n=3). Values of cell viability bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the LPS treated cultures.*

*Phagocytosis assay*

Phagocytosis of bacteria by macrophages was determined by adding 10 fold of fluorescently labeled *E. coli* to the cells. Overnight bacterial culture was washed and suspended in PBS, followed by labeling with PKH dye for 2 min at room temperature. Excess PKH was removed by washing and the labeled cells were stored at 4°C until used. For phagocytosis, macrophages treated with plant extracts and 1 µg/ml LPS for 24 h were incubated at 37°C with labeled *E. coli* for 1 hour. Cells were harvested and then washed thrice with PBS to remove non ingested bacteria. The amount of bacteria phagocytosed was determined by measuring the fluorescence intensity using a flow cytometer (FACSVerse, BD Biosciences) and data were analyzed using FlowJo software (Treestar, Ashland, OR).

*Quantification of cytokines*

Macrophages were stimulated with 1 µg/ml LPS in the presence of plant extracts. Supernatants were harvested after 24 h and stored at -20°C until analysis. TNF-α, IL-1β, IL-6 and IL-10 levels were measured by ELISA according to the manufacturer's instructions (e-Biosciences, San Diego, CA, USA).

*Flow cytometry analysis for cell surface markers*

Antibodies specific for CD80, CD86, MHC II, CD44, CD14, Gr-1, CD11c, CD11b (BD Biosciences) as direct conjugates to FITC, PE, APC, APC-Cy7 were used. Cells were incubated with staining antibodies on ice for 30 min. Control samples were incubated in staining buffer alone (PBS containing 1% FCS and 0.1% sodium azide). Cells were then washed with PBS. Dead cells were excluded using Sytox dye (Invitrogen) staining. Samples were analyzed on FACSVerse (BD Biosciences) and data analyzed with FlowJo software (Treestar, Ashland, OR).

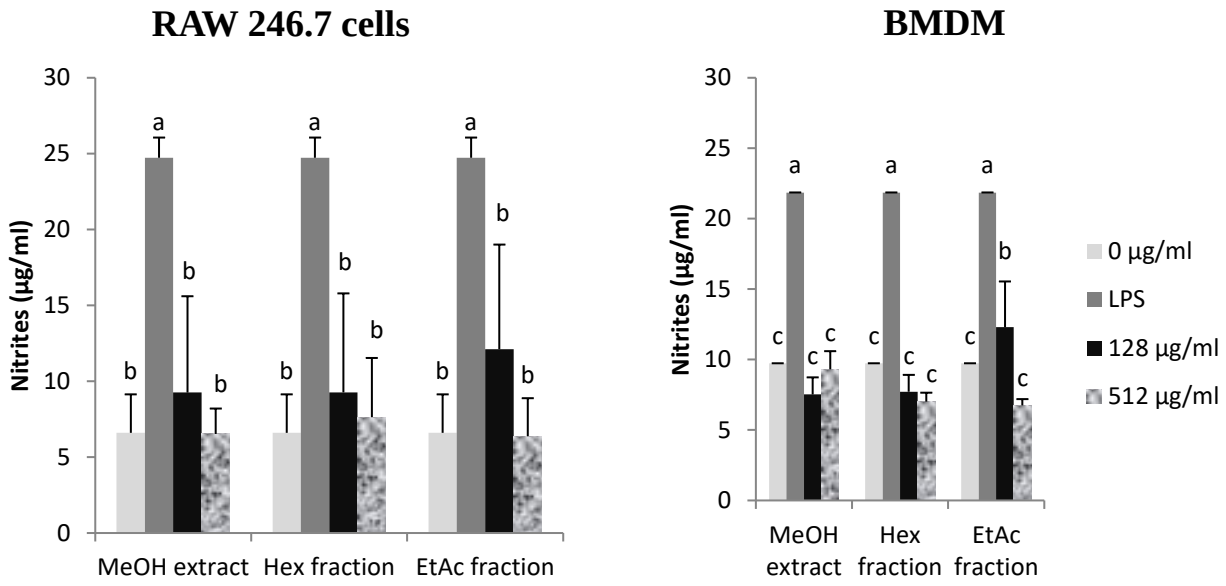
*Statistical analysis*

The results are expressed as mean ± S.E.M. For comparison of means between groups, one way ANOVA was performed. Statistical significance among different concentrations was analyzed by Waller Duncan's multiple range test at 0.05 using SPSS 16.0 software.

## RESULTS

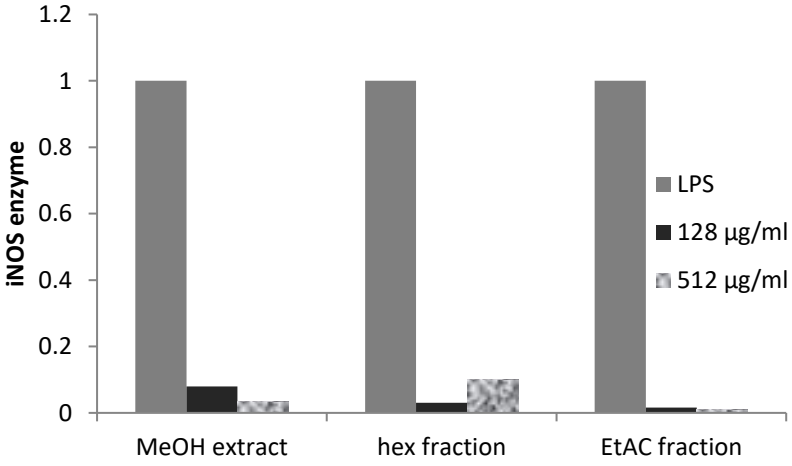
*Methanol extract and fractions of A. kiwuensis inhibit nitric oxide production of LPS activated macrophages*

The formation of NO by macrophages in response to bacterial lipopolysaccharide is a hallmark of inflammation. Herein RAW 264.7 cells and BMDM were stimulated with LPS in the presence or absence of *A. Kiwuensis* extracts. The nitric oxide production of LPS activated macrophages was significantly inhibited ($p<0.05$) in the presence of *A. kiwuensis* methanol extract and its fractions. The inhibition range was of 34.43 to 56.27 % at 128 µg/ml and 25.79 to




*The cells were stimulated with 1 µg/ml LPS and treated with 128 and 512 µg/ml extract and fractions. Nitrite was quantified using Griess reagent. Results are expressed as the mean ± SD (n=3). Values of concentrations bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the LPS treated cultures. MeOH extract: Methanol extract; Hex fraction: Hexanic fraction; EtAc fraction: Ethyl acetate fraction.*

Figure 1: Inhibitory effect of *A. kiwuensis* methanol extract and its fractions on NO production in LPS stimulated macrophages.



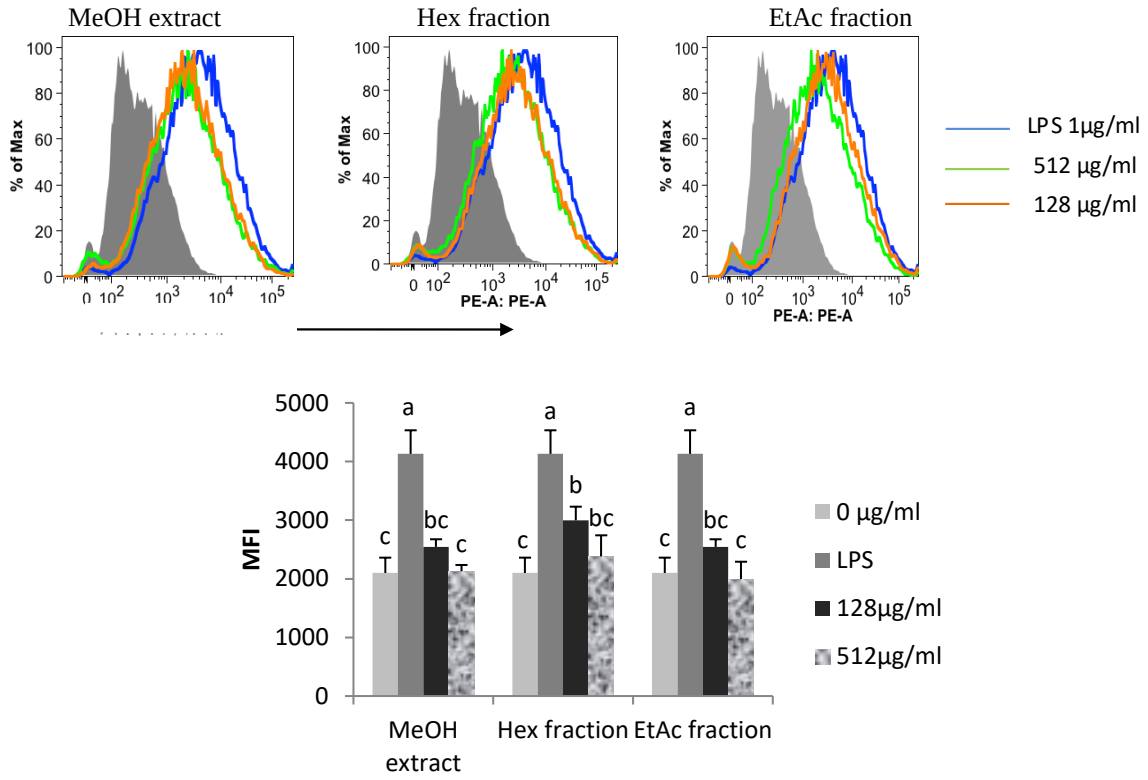

*The cells were stimulated with 1 µg/ml LPS and treated with 128 and 512 µg/ml extract and fractions. The expression of iNOS gene was analysed by RTqPCR. MeOH extract: Methanol extract; Hex fraction: Hexanic fraction; EtAc fraction: Ethyl acetate fraction.*

Figure 2: Inhibitory effect of *A. kiwuensis* methanol extract and its fractions on iNOS gene in LPS stimulated RAW 246.7 cells.

42.62% at 512 µg/ml as compared to positive control, hence indicating towards anti-inflammatory properties of this perennial herb (fig.1). No difference was found between crude extract and its fractions.

*Expression of inducible nitric oxide synthase is down regulated by A. kiwuensis extracts*

Inducible nitric oxide synthase is normally not expressed in cells, but macrophages when exposed to bacterial lipopolysaccharide responds with increased expression of *iNOS* enzyme, leading to generation of NO. The reduction of NO levels in the presence of different extracts of *A. kiwuensis* led to test whether *iNOS* served as a target of this perennial herb. To investigate whether *A. kiwuensis* methanol extract and its fractions affect iNOS gene expression, RT-PCR was done using specific primers for iNOS. *A. kiwuensis* methanol extract and its fractions significantly suppressed LPS-induced mRNA of iNOS expression of RAW 246.7 macrophages when stimulated with LPS (Fig. 2), hence causing regulation at transcriptional level.




*The cells were stimulated with 1µg/ml LPS and treated with 128 and 512 µg/ml extract and fractions. Phagocytosis of treated cells was determined by measuring the fluorescence intensity of engulfment bacteria using a flow cytometer. Representative overlaid histograms show the down-regulation of phagocytosis (**A**). Results are expressed as the mean ± SD (n=3). Values of concentrations bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the LPS treated cultures (**B**). MeOH extract: Methanol extract; Hex fraction: Hexanic fraction; EtAc fraction: Ethyl acetate fraction.*

Figure 3: Inhibitory effect of *A. kiwuensis* methanol extract and its fractions on phagocytosis in LPS stimulated RAW 246.7 cells.

*Phagocytic ability of Raw 264.7 cells are inhibited by different fractions of A. kiwuensis*

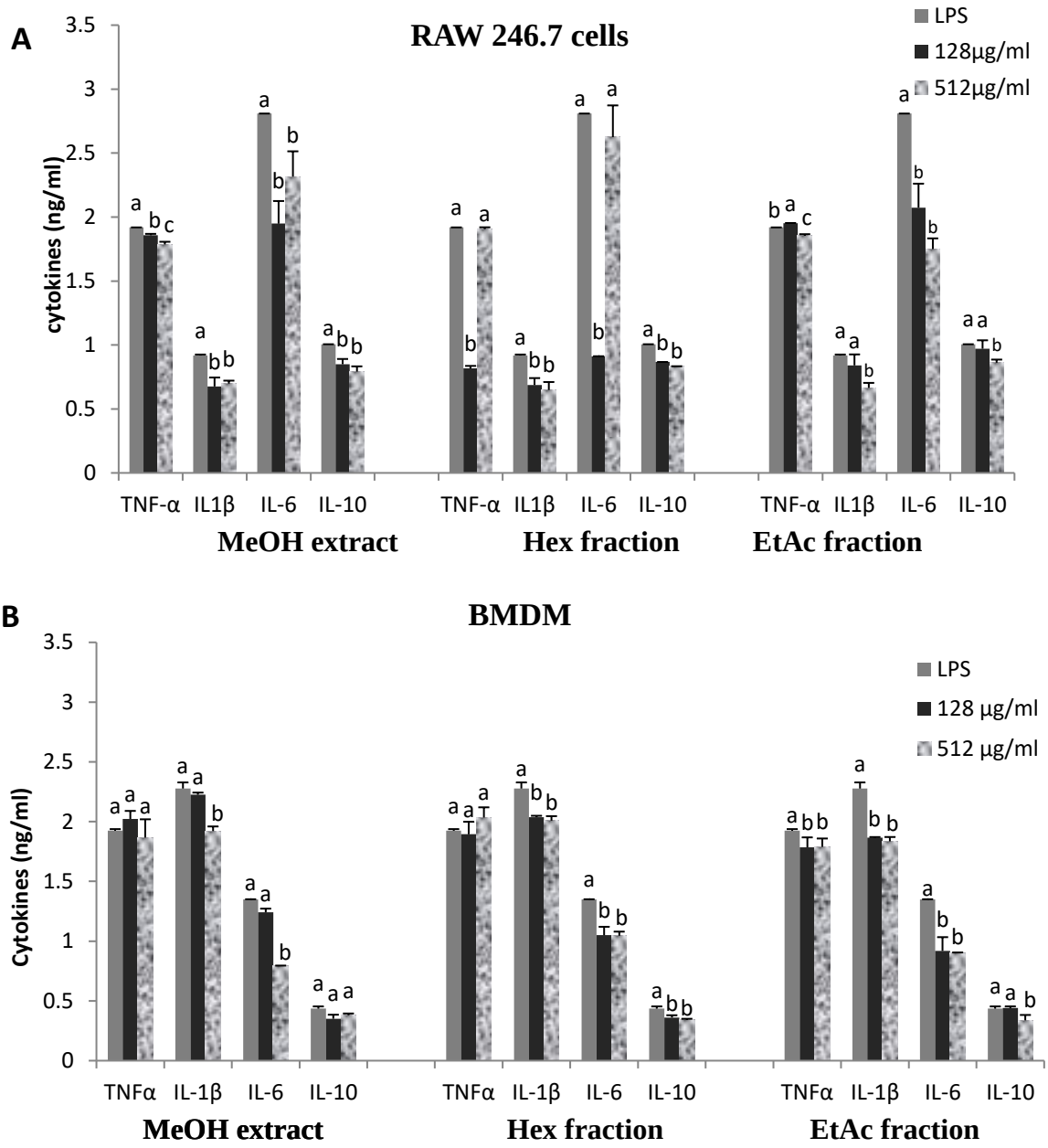
Phagocytosis is one of the important mechanisms by which macrophages initiate innate immune response. Here, the effect of *A. kiwuensis* extracts on the phagocytic capability of Raw 264.7 cells was investigated. It was observed that extract and fractions of *A. kiwuensis* significantly inhibited phagocytic activity of LPS-activated RAW 246.7 cells in a concentration dependent manner (fig. 3). However, there was no difference between crude extract and fractions.

*The levels of inflammatory cytokines in response to LPS are modulated by A. kiwuensis*

The production of pro-inflammatory cytokines following LPS stimulation in macrophages is a well-established mechanism. The inflammatory response of RAW 246.7 cells and BMDM was assessed by measuring the levels of TNF-α, IL1-β and IL-6 after incubation with LPS and various concentrations of extracts. Crude extract of *A. kiwuensis* and its ethyl acetate fraction inhibited TNF-α and IL-6 cytokines production of RAW 246.7 cells while hexane fraction did not have any effect on these cytokines (fig. 4A). All the study extracts significantly inhibited LPS-induced secretion of IL-1β (fig 4A). Crude extract of *A. kiwuensis* and its hexane fraction did not have any effect on TNF-α of BMDM, but they inhibited the secretion of IL-1β and IL-6 (fig 4B). Ethyl acetate fraction of *A. kiwuensis* methanol extract significantly inhibited LPS-induced BMDM secretion of the three cytokines (TNF-α, IL-1β and IL-6) (fig 4B). To evaluate whether the down-regulation of inflammatory cytokines is triggered by an up-regulation of IL-10, an anti-inflammatory cytokine, its level was measured. The crude extract and its fractions significantly inhibited LPS-induced IL-10 of RAW 246.7 and BMDM (fig 4A and B), exception was noted with crude extract on BMDM where no effect was observed (fig 4B). Results therefore confirme the modulatory effect of *A. kiwuensis* on cytokine secretion by macrophages in response to stimulation.

*Extracts of A. kiwuensis up-regulate the expression of LPS receptors on the surface of macrophages without altering the expression of adhesion molecules*

Macrophages express several surface receptors that facilitate the recognition of microbes associated molecular patterns, thus enabling phagocytosis. The expression of two important receptors; TLR4 and CD14 was explored. The flow cytometric analysis revealed the stimulatory effect of methanol extract and their fractions of *A. kiwuensis* on the expression of surface markers of RAW




*The cells were stimulated with 1µg/ml LPS and treated with 128 and 512 µg/ml extract and fractions for 24 hours. Cytokines were quantified in culture supernatants according to the manufacturer's instruction. TNF-α, IL-1β, IL-6 and IL-10 are expressed as the mean ± SD (n=3).Values of concentrations bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the LPS treated cultures. MeOH extract: methanol extract; Hex fraction: Hexane fraction; EtAC fraction: Ethyl acetate fraction. A: RAW 246.7 case; B: BMDM case.*

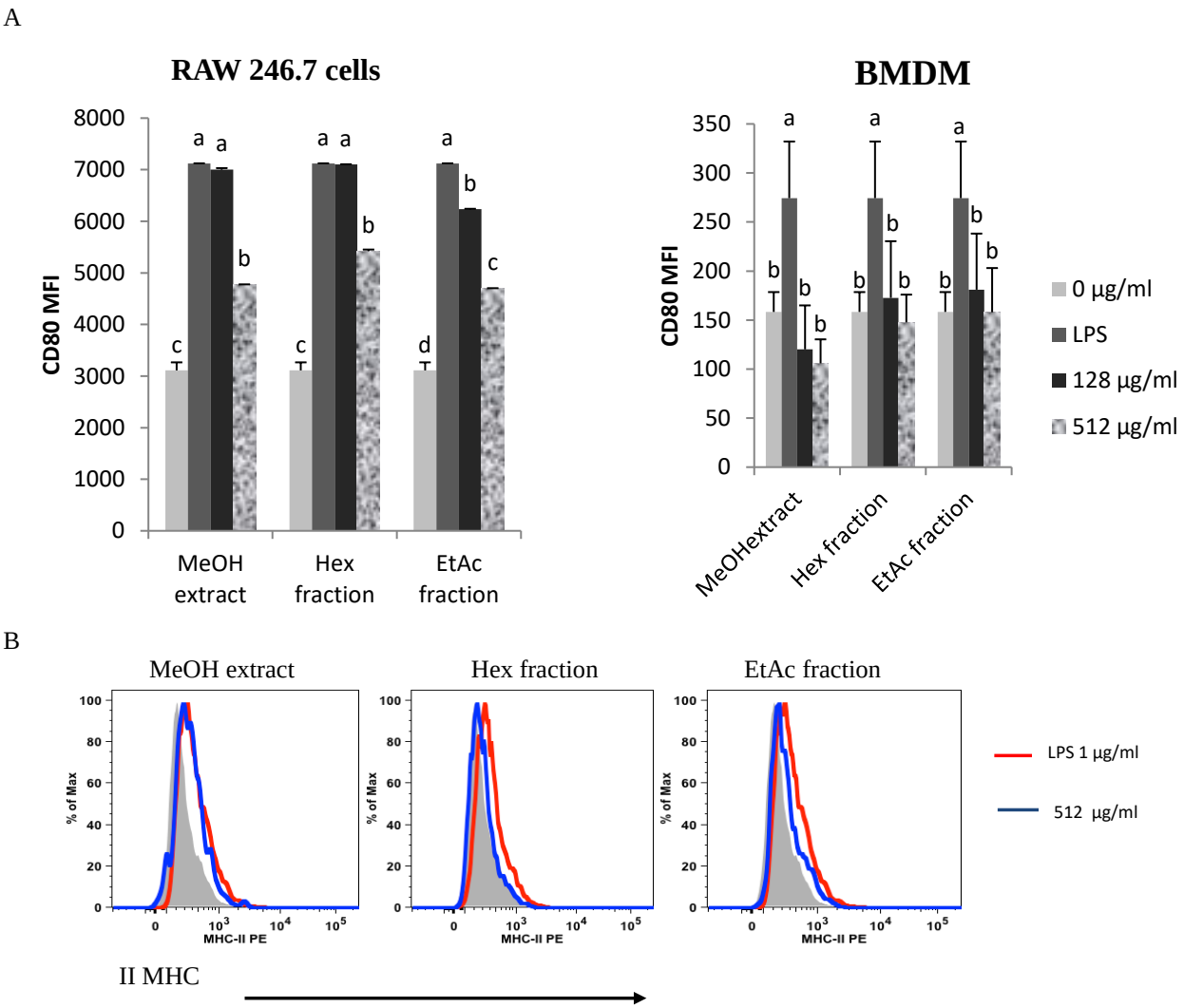
Figure 4: Inhibitory effect of *A. kiwuensis* methanol extract and its fractions on cytokines of LPS stimulated RAW 246.7 cells.

246.7 cells (Fig. 5). The results therefore suggest that these extracts affect the signaling pathway rather than interfering
with the binding of LPS moiety. However, it's not possible to know whether these extracts have any influence on other molecules involved in LPS recognition like LPS Binding Protein.

It was observed that the expression of cell adhesion molecules - CD11b, CD11c and activation marker CD44 on RAW 246.7 cells remain unaltered in the presence of *A. kiwuensis* extracts (Table 1). Though their effect on CD11b expression remains unchanged, they significantly inhibited the expression of CD11c and CD44 on BMDM (Table 1).

*The expression of co-stimulatory molecules and Class II MHC gets influenced by the extracts of A. kiwuensis*

In spite of the fact that methanol extract and its fractions did not showed any effect on CD86 expression of RAW 246.7 cells and BMDM (Table 1), they inhibited CD80 molecules on the surface of these LPS activated




*The cells were stimulated with 1µg/ml LPS and treated with 128 and 512 µg/ml extract and fractions for 24 hours. Cell surface markers were analysed by FACS. **(A)** representative overlaid histograms are shown for CD80 and results are expressed as the mean ± SD (n=3).Values of concentrations bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the LPS treated cultures**. Representative overlaid histograms are shown for MHC II (**B**). MeOH extract: Methanol extract; Hex fraction: Hexanic fraction; EtAc fraction: Ethyl acetate fraction.*

Figure 5: Inhibitory effect of *A. kiwuensis* methanol extract and its fractions on CD80 and II MHC molecules of LPS stimulated macrophages.

macrophages (fig 6). The inhibition on RAW 246.7 cells was significant for crude extract and its hexane fraction at the concentration of 512 µg/ml with percentage of 66.39 and 76.12 respectively. Ethyl acetate fraction showed significant inhibition at both used concentrations with percentage of 87.50 and 66.01 at 128 µg/ml and 512 µg/ml respectively. For BMDM, inhibition was observed at all the tested concentrations for crude extract and its fractions with percentages ranging from 38.60 to 65.97.

Class II MHC molecule was also inhibited by methanol extract and fractions of *A. kiwuensis* (Fig. 6). Results appeared interesting as expression of Class II MHC is required for antigen presentation for initiating adaptive immune response. The extracts have inhibitory effect on phagocytosis, and similarly down-regulation of MHC Class II is an outcome parallel to the earlier observation.

It was noted following dead cell exclusion staining that, there was no significant cytotoxicity due to the plant extract and its fractions on RAW 246.7 macrophages and BMDM in culture at the higher concentration used (512 µg/ml) (Table 2). When macrophages were treated with extracts without stimulation, they didn't have any effect on the different study parameters (data not shown), showing that the extracts by themselves does affect the cell responses of resting cells but, act under inflammatory conditions.

## DISCUSSION

Activated macrophages exhibit myriad functions including the up-regulation of surface molecules such as MHC class II, and the membrane molecules of the B7 family and enhance ability to present antigen and kill intracellular

pathogens[15]. This killing is reached by an increase in the production of toxic oxygen species and an induction of the inducible NO synthase (iNOS) gene to produce NO[16]. These activated cells also produce pro-inflammatory cytokines. However, hyper activation of macrophages can cause extensive damage to the host and leads to sepsis and septic shock[16,17]. The present findings showed that methanol extract of *A. kiwuensis* and its hexane and ethyl acetate fractions inhibited nitric oxide production in LPS-stimulated RAW 264.7 cells and BMDM. NO is a molecule responsible for the anti-tumoricidal and antimicrobial effects of activated macrophages. Nevertheless, its excessive production is associated with cellular and tissue dysfunction and exacerbation of inflammation through the formation of peroxynitrite. The formation of peroxynitrite due to the reaction between NO and superoxide is involved in the pathogenesis of many chronic diseases[18,19]. LPS up-regulates inducible nitric oxide synthase (iNOS) enzyme responsible of the synthesis of NO. Expression of iNOS in activated macrophages is mainly responsible for production of pathological concentration of NO during inflammation[20]. The methanolic extract of *A. kiwuensis* and its fractions decreased the level of this enzyme.

Activated macrophages secrete a number of important cytokines such as TNF-α, IL-1β and IL-6. These cytokines promote inflammatory response to Gram-negative bacteria and some other infectious microorganisms. The methanolic extract of *A. kiwuensis* and its fractions significantly decreased the level of these cytokines in LPS-activated RAW 246.7 macrophages and BMDM. These pro-inflammatory cytokines released at the site of inflammation facilitate both the adherence of immune-system cells to vascular endothelial cells and their migration through the vessel wall into the tissue spaces[21]. The result is an influx of immune system cells to the site of tissue damage, where they participate in clearance of the antigen and healing of the tissue[22]. However, overproduction of pro-inflammatory cytokines can induce systemic reactions that include fever, widespread blood clotting, and shock[23,24]. IL-10 is an anti-inflammatory cytokine produced by macrophages; it inhibits the production of mediators including TNF-α and IL-6[25]. Interestingly, this study revealed that IL-10 induced by LPS was inhibited by crude extract and its fractions; showing that this cytokine is not responsible for the effects of plant extracts on pro-inflammatory cytokine expression. Upon stimulation, macrophages express higher levels of activation markers. Class II MHC molecules are expressed on the cell surface membrane of the antigen-presenting cell (APC). They are implicated in the recognition of antigens by T cells[26]. Indeed, T helper cells recognize antigen only when it is bound to a class II MHC molecule. The membrane molecules of B7 family (CD80 and CD86) bind to their receptors on T cell and promote theirs activation and proliferation. CD80 is not constitutively expressed on the surface of macrophages but it is inducible by cell activation and it is able to deliver a co-stimulatory signal that is necessary for T helper cell activation. However, the level of the expression of these co-stimulatory molecules may play an important role during the course of inflammation[27]. Thus their reduction would be expected to have an effect on inflammation state[27]. In this study, the methanol extract of *A. kiwuensis* and its fractions significantly inhibited the expression of CD80 and class II MHC molecules of LPS stimulated macrophages. On the other hand these extracts up regulated CD14 and TLR4 expression of these cells, showing that *A. kiwuensis* extracts didn't interfere with the binding of LPS. LPS is bound at the cell membrane by a complex of proteins that includes CD14, MD-2, and a TLR[7].

The methanol extract of *A. kiwuensis* and its fractions significantly inhibited phagocytosis of *E. coli* by LPS activated RAW 246.7 macrophages. It is known that LPS increases phagocytosis activity of LPS-activated macrophages. At the area of infection, macrophages recognize the infectious agents, which are ingested and killed intracellularly. During this process, several free radicals use to destroy pathogens may be released into the extracellular space and may injure host tissues leading to closely correlated with the pathophysiology of a variety of diseases and inflammation [29].

In addition to inhibit phagocytosis, methanol extract of *A. kiwuensis* and its fractions significantly inhibited adhesion molecules. Adhesion molecules are implied in the process of leukocyte accumulation at the site of inflammation. As well as being involved in host defense, leukocyte-endothelial interactions can be implicated in chronicity of pathologic inflammation, thus the inhibition of adhesion molecules must be an asset in inflammation condition [30].

The observed differences between RAW 246.7 cells and macrophages differentiated from bone marrow cells may be due to the fact that cell lines are hardy mostly because they have to past a long time in culture, and be capable of surviving multiple rounds of cryopreservation and thawing.

In conclusion, the methanol extract of *A. kiwuensis* and its hexane and ethyl acetate fractions inhibited phagocytosis of *E. coli*, costimulatory molecules, cytokines and nitric oxide production by LPS activated RAW 246.7 macrophages. Thus, this medicinal plant may possibly be useful as anti-inflammatory agent.

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