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Original Research Article

Phytochemical screening, Antioxidant Activity and *Ex Vivo* Immunological-Associated Properties of a Halophyte Extract, *Maytenus mekongensis* In Sched

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ABSTRACT

Maytenus mekongensis In Sched. is a halophyte found in the saline soil of Northeastern Thailand. This plant has been used in folk medicine for malnutrition in children. However, the medicinal properties and immunological-associated properties are still unclear. This study aims to evaluate the phytochemical, biological and immune-associated activities of crude extracts from *Maytenus mekongensis*. The plant was separated into root, stem, and leaf and extracted using 70% ethanol. Crude extracts were examined for phytochemical constituents and antioxidant activity. The cytotoxicity was determined on human red blood cells (RBC) and white blood cells (WBC). Phagocytic activity and tumour necrosis factor α (TNF-α) mRNA expression of human macrophages were analyzed. All parts of the plant contained alkaloids, phenolic, flavonoids, coumarin, tannins, terpenoids, and glycosides. Saponins were found only in the root extract. Root extract exhibited the highest ($p \leq 0.05$) antioxidant activity with highest phenolic and flavonoid contents. The phenolic/flavonoid contents with antioxidant activity were very strongly (DPPH, $R^2 > 0.9$) and moderately (ABTS, $R^2 > 0.51–0.7$) correlated. Cell viability of WBCs, $CD14^+$ monocytes and $CD14^-$ non-monocytes were increased after treatment with crude extracts. The root, stem, and leaf extracts had no hemolytic activity and enhanced phagocytosis activity of macrophages. Induction of TNF-α mRNA expression in macrophages treated with stem and leaf extracts was higher than when treated with root extracts. The study suggests that *Maytenus mekongensis*, shows promising properties with low cytotoxicity and immune-enhancing activities, and it might be used as an immune-boosting agent in immunodeficiency diseases.

Keywords: *Maytenus mekongensis*, Phytochemical screening, Antioxidant activity, Phagocytosis, Human macrophages, TNF-α

Introduction

Northeastern Thailand is an area of high salinity soils according to its edaphic properties.[1] The distribution of saline soils throughout the region leads to low agricultural productivity.[2] However, some plants have thrived in saline soils which provide stress conditions that might affect their phytochemical content.[3] Medicinal plants that have been found in saline soil areas include species such as *Ocimum basilicum*, which is a low salt-tolerant plant that has anti-inflammatory, anti-microbial, antioxidant, and antiviral activities.[4] The classified halophytes of the Chi River basin of Northeast Thailand include *Azima sarmentosa*, *Maytenus mekongensis*, *Synostema bacciformis*, *Pluchea indica*, and *Gisekia phanaceoides*.[5] These plants might possess significant medicinal properties.

M. mekongensis In Sched. belongs to the Celastraceae family. It is a shrub, has red and purple branches with sharp thorns, and the flowers are white and green.[6] The root of *M. mekongensis* contains 12 sesquiterpene alkaloids. Five of them exhibit anti-plasmodial activity including 7-*epi*-mekongensine, mekongensine (2,9'-di-*O*-acetyl-5-*O*-benzoyl-5-deacetyl wilforidine9), 9'-deacetoxymekongensine, 1-*O*-benzoyl-1-deacetylmekongensine, and 7-*epi*-euojaponine A. Mekongensine and 9'-deacetoxymekongensine have very weak cytotoxicity to human oral epidermal carcinoma (KB) cell line but no cytotoxicity to human breast adenocarcinoma (MCF7) and human small cell lung (NCI-H187) cell lines.[7] This plant is not widely used for medicinal purposes and there have been no reports on immune-associated properties.

Macrophages defend against foreign antigens in the innate immune response.[8] Macrophages recognize microbial molecules by different pattern recognition receptors (PRRs) in response to infection and injury.[9] The mechanisms of macrophage action against microbes involve phagocytosis, stimulating inflammation, and presenting antigens.[8] However, immunomodulation effects of *M. mekongensis* extracts on human macrophages are still unclear. This research aims to study the phytochemical constituents of crude extracts from three parts of *M. mekongensis*, determine antioxidant activity, cytotoxicity on human red blood cells (RBC) and white blood cells (WBC), and immunomodulation activities of crude extracts on human macrophages.

Materials and Methods

Plant sample crude extracts preparation

M. mekongensis In Sched. (Figure 1A) was collected in December 2019 from the saline soil at Ban Phonsim, Hua Na Kham Sub-district, Yang Talat District, Kalasin Province, at 16°24'01.8"N; 103°16'13.1"E. Professor Khwanruan Naksuwankul (Ph.D.) from the Faculty of Science, Mahasarakham University kindly identified the plant according to the Flora of Thailand manual.[10] Voucher specimens were deposited under the code number MSUT7444 at the Natural

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The plant was separated into root (Figure 1B), stem (Figure 1C), and leaf parts (Figure 1D) and then washed with tap water and air-dried in the shade for 14 days.[11] All parts of the plant were ground to powder for extraction. The extraction was performed by reflux in 70% ethanol [12] (ratio of 1:10 g/mL) at 60°C for 24 h, then evaporated to obtain crude extracts.[13] The crude extracts were dissolved with 100% dimethyl sulfoxide (DMSO) by adjusting to a concentration of 100 mg/mL and filtrated with 0.45 µm and 0.22 µm filters and stored at -20°C.

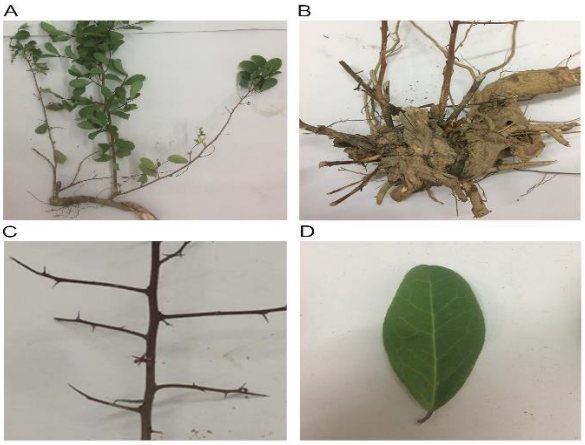

Figure 1: *Maytenus mekongensis*
Maytenus mekongensis (A), root (B), stem (C), and leaf (D).

Phytochemical screening

All crude extracts from *M. mekongensis* were tested for the presence of phytochemicals including alkaloids, phenolic, flavonoids, anthraquinones, coumarin, saponins, tannins, terpenoids, steroids, and glycosides using standardized methods described by Ayoola *et al*.[14]

Total phenolic content (TPC) analysis

TPC was measured by the Folin–Ciocalteu colorimetric method. The method was modified from Hatami *et al*.[15] Briefly, 100 µL of crude extract was mixed with 500 µL of 10% (v/v) Folin & Ciocalteu's phenol reagent (Loba chemie, India), then incubated in dark conditions for 3 min. The mixture was then added with 400 µL of 7.5% (w/v) sodium carbonate (Na_2CO_3) (KemAus, Australia). The reaction was mixed and incubated in dark for 30 min and the absorbance at 731 nm was then measured. Gallic acid (GA) (Sigma Aldrich, USA) was used as a standard reagent. The TPC of crude extract was expressed as GA equivalent (mg GAE/g crude extract).[16]

Total flavonoid content (TFC) analysis

TFC was measured using a colorimetric assay. Briefly, a reaction mixture of 500 µL of deionized water, 100 µL of crude extract, and 30 µL of 5% (w/v) sodium nitrite ($NaNO_2$) was prepared then mixed and incubated in dark conditions for 5 min. Sixty µL of 10% (w/v) aluminium chloride ($AlCl_3$) (Kemaus, Australia) was added, mixed and incubated in the dark for 6 min. Finally, 200 µL of 1M sodium hydroxide (NaOH) (RCI Labscan, Bangkok) and 110 µL of deionized water was added to the reaction mixture, mixed, and incubated in the dark for 5 min. The absorbance was measured at 510 nm. Quercetin (Sigma Aldrich, USA) was used as a standard reagent. TFC was reported as quercetin equivalents (mg QE/g crude extract).[17]

Determination of antioxidant activity

The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was modified from Brand-Williams *et al*.[18] Crude extract (100 µL) was mixed with 900 µL of 80 µM DPPH solution (Sigma Aldrich, Germany), and incubated in dark for 30 min. The absorbance was measured at 515 nm. Ascorbic acid (RCI Labscan, Bangkok) was used as a standard reagent. Antioxidant activity was calculated using the formula.[19]

$$\text{Antioxidant activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

The 2,2'-azinobis-(3- ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging assay was modified from Zheleva-dimitrova *et al*.[20] ABTS (Sigma Aldrich, USA) (7 mM) was mixed with 2.45 mM potassium persulfate (PanReac AppliChem, Spain) and incubated in dark for 12–16 h. Then the $ABTS^{\bullet+}$ solution was diluted with methanol at 1:10 ratio ($ABTS^{\bullet+}$: methanol) to obtain an absorbance value of 0.7 ± 0.01 at 734 nm. The diluted sample was mixed with $ABTS^{\bullet+}$ solution and incubated for 3–5 min and absorbance measured at 734 nm. Ascorbic acid was used as a standard reagent. The results are reported as percentage inhibition using the formula;

$$\text{Antioxidant activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

The vitamin C equivalent antioxidant capacity (VCEAC) was calculated on a weight basis (mg vitamin C equivalent/100 g crude extract) for both the DPPH and ABTS assays.

Correlation analysis of phytochemical components and antioxidant activities

The correlation between phytochemical components with antioxidant activities was analysed at a concentration of 5 µg/mL in crude extracts by using Pearson linear regression.[21] The Pearson correlation coefficient is reported as R^2 (correlation coefficient) and the *p-value* at ≤ 0.05 was considered significant. The correlation was classified into 5 groups: R^2 less than 0.3 was classified as no correlation, R^2 = 0.31–0.5 was considered to be a weak correlation, R^2 = 0.51–0.7 was considered a moderate correlation, R^2 = 0.71–0.9 was classified as a strong correlation and R^2 greater than 0.9 was classified as a very strong correlation.[22]

Isolation of $CD14^+$ monocytes and cultivation of monocyte-derived macrophages (MDMs)

Peripheral blood was collected from three healthy volunteers who had given written consent . The study was approved by The Human Ethics Research Committee of Mahasarakham University (No. 208/2563) on 23 July 2020. The peripheral WBCs were isolated by a centrifugation method with RBC lysis buffer.[23] $CD14^+$ monocytes were separated from WBCs by using anti-human CD14 magnetic particles and BD IMag separator (BD Bioscience, San Jose, CA, USA) according to the method described by Sunthamala *et al*.[24] The purity of $CD14^+$ monocytes was confirmed using flow cytometry to be more than 95%. $CD14^+$ monocytes were cultured in Roswell Park Memorial Institute medium (RPMI)-1640 (Gibco, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, USA), 5% autologous serum, and 1X antibiotic/antimycotic (Gibco, USA). Cultures were conditioned at 37°C with 5% CO_2 overnight to differentiate MDMs adherent cells.[16] $CD14^-$ non-monocyte were cultured in similar conditions.

Analysis of the cytotoxicity of crude extract on human white blood cells and red blood cells

The effect of crude extracts on WBCs, $CD14^+$ monocyte, and $CD14^-$ non-monocyte proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Gibco, USA). Briefly, $2x10^4$ cells/well were seeded into a 96-well culture plate and the crude extract at a concentration of 0–512 µg/mL was then added. DMSO (0.5%) and 0–10%-propolis were used as negative control and positive control, respectively. Cells were incubated for 48 h before adding 12 mM MTT and incubating for 4 h in the dark. The formazan crystals were dissolved with 100 µL of 100% DMSO. The absorbance was measured at 540 nm and the %viability calculated according to the formula;[16]

$$\text{\% Viable cells} = \frac{OD_{sample}}{OD_{control}} \times 100$$

Cytotoxicity of human red blood cells was analysed by hemolysis assay. Peripheral blood was centrifuged at 1,500 x g for 10 min and the bottom layer of RBCs collected. The 2% RBCs were prepared in 1X phosphate-buffered saline (PBS). Hemolysis assay was performed

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by a method modified from Joglekar *et al*.[25] Briefly, 2% RBCs were added to various concentrations of crude extracts in a 1:1 ratio, shaken gently and incubated at 37°C for 30 min. After centrifugation at 2,500 x g for 6 min and the supernatant was collected and the absorbance at 541 nm was measured. 1X PBS and 0.1% DMSO were negative controls and 0.1% Triton-X 100 was the positive control.[26] The % hemolysis was calculated using the following equation:

$$\% \text{ Haemolysis} = \frac{OD_{sample} - OD_{negativecontrol}}{OD_{positivecontrol} - OD_{negativecontrol}} \times 100$$

Phagocytosis assay of monocyte-derived macrophages

$1.5x10^5$ cells/mL of $CD14^+$ monocytes were cultured in 96-well plates. The stimulators of the M1 macrophages (pro-inflammatory macrophages) were 10 ng/mL Lipopolysaccharides (LPS),[27,28] 10 ng/mL interferon γ (IFN-γ),[28,29] and 10 ng/mL LPS+10 ng/mL IFN-γ,[29] the stimulators of the M2 macrophages (anti-inflammatory macrophages) was 20 ng/mL interleukin 4 (IL-4),[28] the stimulators for the control group of compounds were 20 μM quercetin and 300 μM ascorbic acid,[30] and crude extracts were added on day 4. Three bacteria: *Staphylococcus aureus* DMST8840, *S. aureus* (MRSA) DMST20654, and *Escherichia coli* ATCC25922 were added to the cells at multiplicities of infection (MOI) of 30 and incubated for 1 and 2 h. To estimate phagocytosis of bacteria, cells were washed 3 times with 1X PBS to remove non-phagocytosed bacteria. MDM was lysed using 1% sodium dodecyl sulfate (SDS) and examined for phagocytosed bacteria by adding TSB and culturing for 16–18 h. Bacterial growth was determined by measuring the absorbance at 600 nm compared to the bacterial standard. The bacterial standard was cultured at concentrations of $0–10^7$ cells/well in tryptic soy broth (TSB), incubated at 37°C for 16–18 h.[31]

Determination of TNF-α expression in macrophage cells.

$CD14^+$ monocytes were cultured at $1x10^6$ cell/well in 24 well plates for 6 days, changing the media every 3 days.[32] The positive control groups included 10 ng/mL LPS, [27,28] 10 ng/mL IFN-γ,[28,29] 10 ng/mL LPS+10 ng/mL IFN-γ, [29] 20 ng/mL IL-4,[28] 20 μM quercetin and 300 μM ascorbic acid,[30] and crude extracts were added on day 4. On day 6 of cultivation, cells were washed and stimulated with 40 μM hydrogen peroxide (H_2O_2).[27,33] Cells were collected after 6 h of stimulation. Total RNA was extracted using the Trizol method. The expression of TNF-α was investigated by reverse transcription-polymerase chain reaction (RT-PCR) using the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as an internal control. The intensity of PCR products was analyzed by using ImageJ (ImageJ Software, Maryland, USA).

To evaluate whether TNF-α expression involved the microtubule-associated protein kinase (MAPK) pathway, cells were cultured for 6 days, washed, and MAPK inhibitors were added (extracellular signal-regulated kinase (ERK) inhibitor PD98059 (10 μM), Jun N-terminal kinase (JNK) inhibitor SP600125 (10 μM), p38 MAP Kinase (p38 MAPK) inhibitor SB203580 (5 μM), and mixture of PD98059 (10μM) +SP600125 (10 μM) +SB203580 (5 μM)), incubated for 2 h, [23,30] and then stimulated with 40 μM H_2O_2.

Statistical analysis

Experiments were performed three times independently. The data were analyzed by analysis of variance (ANOVA) and Tukey's multiple comparison post-test for One-Way ANOVA and Bonferroni for Two-Way ANOVA by Prism version 5 (GraphPad Soft Inc. La Jolla, CA, USA). The results were expressed as mean ± (SD). Statistical significance was inferred at p-value ≤ 0.05.

Results and Discussion

Extraction yield of *M. mekongensis*

M. mekongensis was harvested from a high salinity area[34] and separated into 3 parts for extraction (Figure 1). The % yield of crude extracts from root, stem, and leaf was 7.11%, 9.70%, and 11.52%, respectively (Figure 2).

Phytochemical screening

The phytochemical screening results for the crude extract are shown in Table 1. Alkaloids, phenolic, flavonoids, coumarin, tannins, terpenoids, and glycoside were present in the crude extract of root, stem, and leaf. Saponins were found only in the root crude extract. These plant secondary metabolites are important sources of bioactive compounds. Some alkaloids are very effective in treating cancer.[35] Phenolics have anti-cancer, anti-microbial, and anti-mutation properties. Flavonoids have potent antioxidant and anti-cancer effects. Coumarin has antioxidant, anti-mutation, anti-inflammatory, and anti-tumour properties.[36] Tannins have antioxidant, wound healing activity, and anti-cancer.[36,37] Terpenoids have antioxidant, anti-inflammatory, anti-tumour activities, and have been used in treatment of heart disease.[38] Cardiac glycosides contain steroid molecule and a sugar that are primarily useful in the treatment of congestive heart failure.[39] Some special saponins have anti-tumour effects.[40] Moreover, natural compound such as alkaloid, phenolic, curcumin, flavonoid, and glycoside had been reported as immunomodulators for T lymphocytes and B lymphocytes.[41,42] Therefore, the presence of these bioactive compounds in *M. mekongensis* extract indicates a medicinal potential and may provide a medicinal source for medical treatment such as immunomodulation.

TPC, TFC analysis and antioxidant properties of crude extracts

TPC was calculated from the standard curve of gallic acid (Y = 0.0112X - 0.0676, R^2 = 0.9883). TFC was calculated from the standard curve of quercetin (Y = 0.001X - 0.001735, R^2 = 0.9733). The result shows that the TPC values of the root, stem, and leaf extract were 408.464 ± 0.000, 158.286 ± 0.536, and 135.964 ± 0.000 mg GAE/g respectively for crude extract (Figure 3A). The TFC values of root, stem, and leaf extract were 557.600 ± 2.000, 271.800 ± 0.000, and 179.800 ± 8.000 mg QE/g respectively for crude extract (Figure 3B). Root extract exhibited the significantly highest TPC and TFC, followed by the stem and leaf, respectively.

The antioxidant activity of *M. mekongensis* crude extracts is reported in Figure 3C and 3D. The 50% inhibitory concentration (IC_{50}) of root, stem, and leaf extract by DPPH assay was 5.388 ± 0.008, 15.266 ± 0.282, and 25.700 ± 0.013 μg/mL, respectively (Figure 3C). The ABTS assay also showed a similar pattern. The IC_{50} of root, stem, and leaf extract by ABTS assay was 0.607 ± 0.022, 0.699 ± 0.029, and 2.875 ± 0.015 μg/mL, respectively (Figure 3D). The root extract showed highest % inhibition in both assays. All crude extracts contained significantly different antioxidant activities. Crude extracts of roots and stem exhibited high antioxidant activity with similar IC_{50} to the positive control, ascorbic acid, suggesting that the root and stem crude extracts might contain powerful antioxidant compounds. However, further studies are still needed to analyse the purified compounds from the extracts and their antioxidant mechanisms.

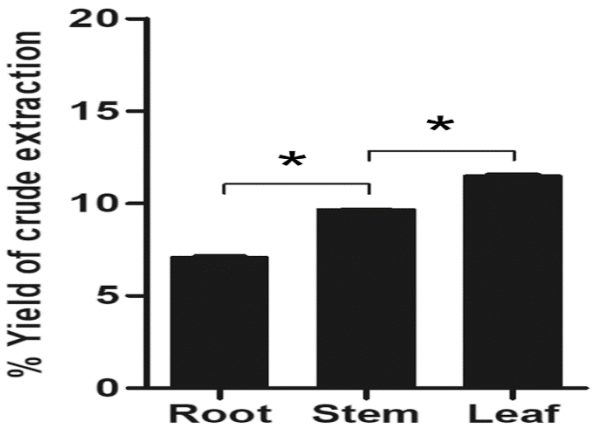

Figure 2: Percentage yield of crude extracts

Yield (%) of crude extracts from *Maytenus mekongensis*. The values are expressed as mean ± SD of data from 3 independent replicates. (*) p-value ≤ 0.05 compared between groups.

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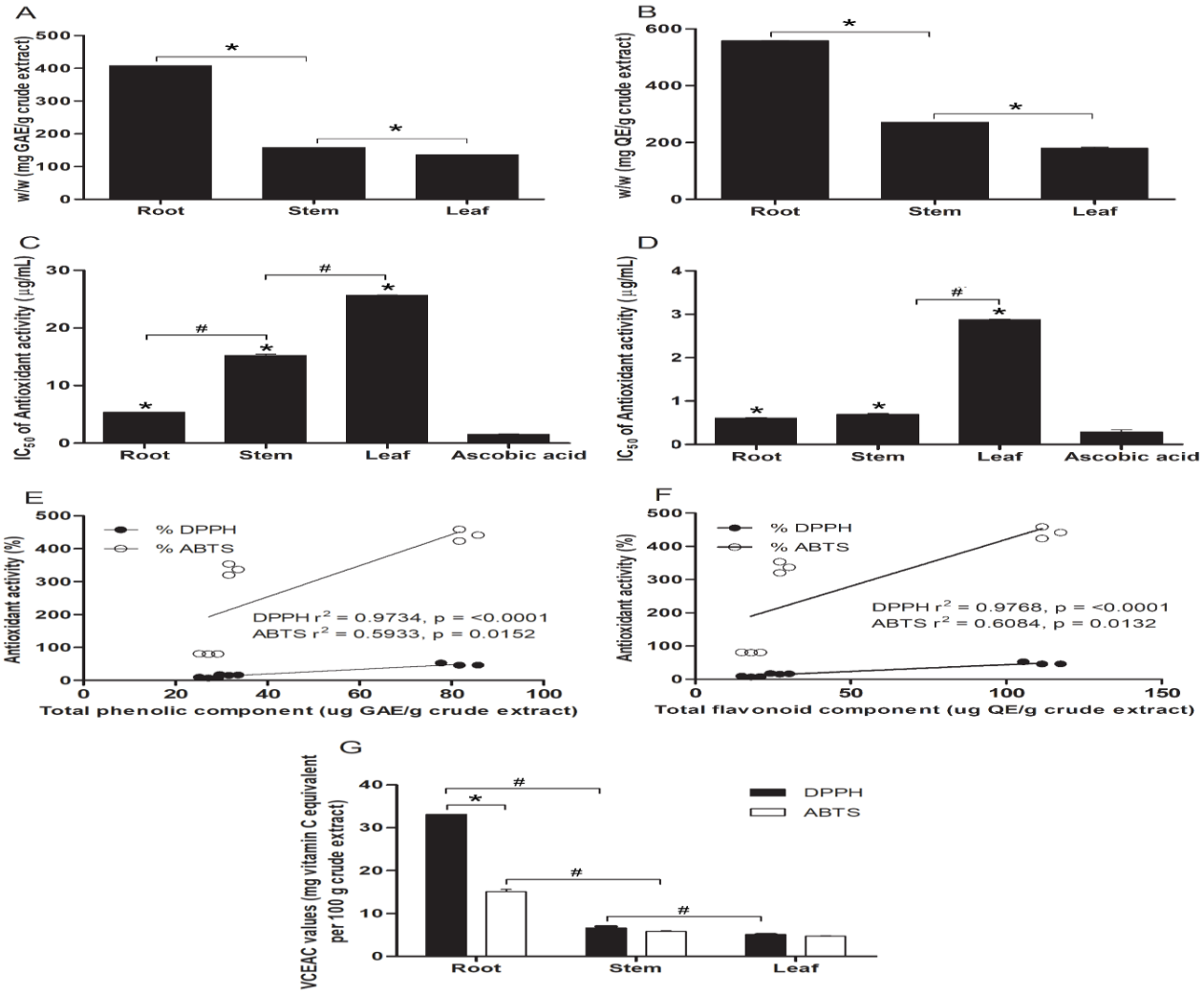

Figure 3: Total phenolic and flavonoid contents and antioxidant activity of the crude extracts

Total phenol content (A) and total flavonoid content (B) of the crude extracts. (*) *p*-value ≤ 0.05 compared between groups. The IC_{50} of the crude extracts by DPPH (C) and ABTS (D) methods. (*) *p*-value ≤ 0.05 compared to ascorbic acid, (#) *p*-value ≤ 0.05 compared between groups. The correlation coefficient of TPC (E) and TFC (F) with antioxidant activity. The vitamin C equivalent antioxidant capacity (mg vitamin C equivalent/100 g crude extract) was calculated by using IC_{50} of DPPH and ABTS methods (G). (*) *p*-value ≤ 0.05 compared between methods, (#) *p*-value ≤ 0.05 compared between groups. The values are expressed as mean ± SD of data in three times independently.

Table 1: Phytochemical constituents of 70% ethanol extract from *Maytenus mekongensis* In Sched.

Phytochemical compounds	Root extract	Stem extract	Leaf extract
Alkaloids	+	+	+
Phenolic	+	+	+
Flavonoids	+	+	+
Anthraquinones	-	-	-
Coumarin	+	+	+
Saponins	+	-	-
Tannins	+	+	+
Terpenoids	+	+	+
Steroids	-	-	-
Glycosides	+	+	+

Note: + = presence (detected); − = absent (Nondetected).

Significant correlations between antioxidant activity and phytochemical content of crude extracts were found with both DPPH and ABTS methods. The TPC and antioxidant activity in the DPPH method had a very strong correlation (R^2 = 0.9734, *p*-value < 0.0001) whereas a moderate correlation was found in the ABTS method (R^2 = 0.5933, *p*-value = 0.0152) (Figure 3E). The TFC and antioxidant activity in the DPPH method were also very strongly correlated (R^2 = 0.9768, *p*-value < 0.0001) while a moderate correlation was found between TFC and antioxidant activity with the ABTS method (R^2 = 0.6084, *p*-value = 0.0132) (Figure 3F). The results suggested that the active antioxidants could be both phenolic and flavonoids. However, further studies are needed to analyze the purified compounds from the extracts and their antioxidant mechanisms. The vitamin C equivalent antioxidant capacity (mg vitamin C equivalent/100 g crude extract, VCEAC) was calculated by using IC_{50} of DPPH and ABTS methods. Antioxidant capacity of the crude extract showed a VCEAC value by DPPH assay of the root, stem, and leaf of 33.115 ± 0.000 6.667 ± 0.546 5.119 ± 0.044, respectively. The VCEAC value by ABTS assay of the root, stem, and leaf was 15.085 ± 0.892 5.822 ± 0.291 4.710 ± 0.127, respectively (Figure 3G).

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Cytotoxicity of crude extract on human white blood cells and red blood cells

The effect of the crude extract on WBCs, $CD14^+$ monocytes that will evolve into macrophages, and $CD14^-$ non-monocytes (lymphocyte and granulocyte) was investigated by using the MTT assay. The survival rate of all three cell types after treatment with root, stem, and leaf extracts demonstrated similar trends. Moreover, the % cell viability of all three cell types were increased in high concentrations of root extract (Figure 4A, 4D, and 4G), stem extract (Figure 4B, 4E, and 4H) and leaf extract (Figure 4C, 4F, and 3I) treated cells, suggesting that the crude extract could increase WBCs, $CD14^+$ monocytes, and $CD14^-$ non-monocytes proliferation. Some natural compounds can increase WBCs proliferation. For example, the propolis, positive control of this experiment, is an effective antibacterial, antiviral, antifungal, and potent immunomodulator[43] that increases cell proliferation in dose-dependent manner for all three cell groups (Supplementary data 1). Honey has also been reported to stimulate the immune response by stimulating the proliferation of monocytes.[44] Oenothein B is reported as a plant phenolic compound from *Epilobium angustifolium* that can stimulate innate lymphocytes that increase CD25 or CD69 expression, as well as promote IFN-γ production in natural killer (NK) cells and T lymphocytes. Genistein increases the number of splenic B cells, macrophages, T-lymphocytes, helper T lymphocytes, and cytotoxic T lymphocytes.[45] Phytohemagglutinin (PHA), a lectin found in plants, can also selectively stimulate human lymphocytes division.[46] 3,3'-diindolylmethane (DIM) extracted from *Brassica* vegetables such as broccoli, cabbage, and kale increases the number of white blood cells or lymphocytes approximately two-fold, which promotes host immunity against infections.[47]

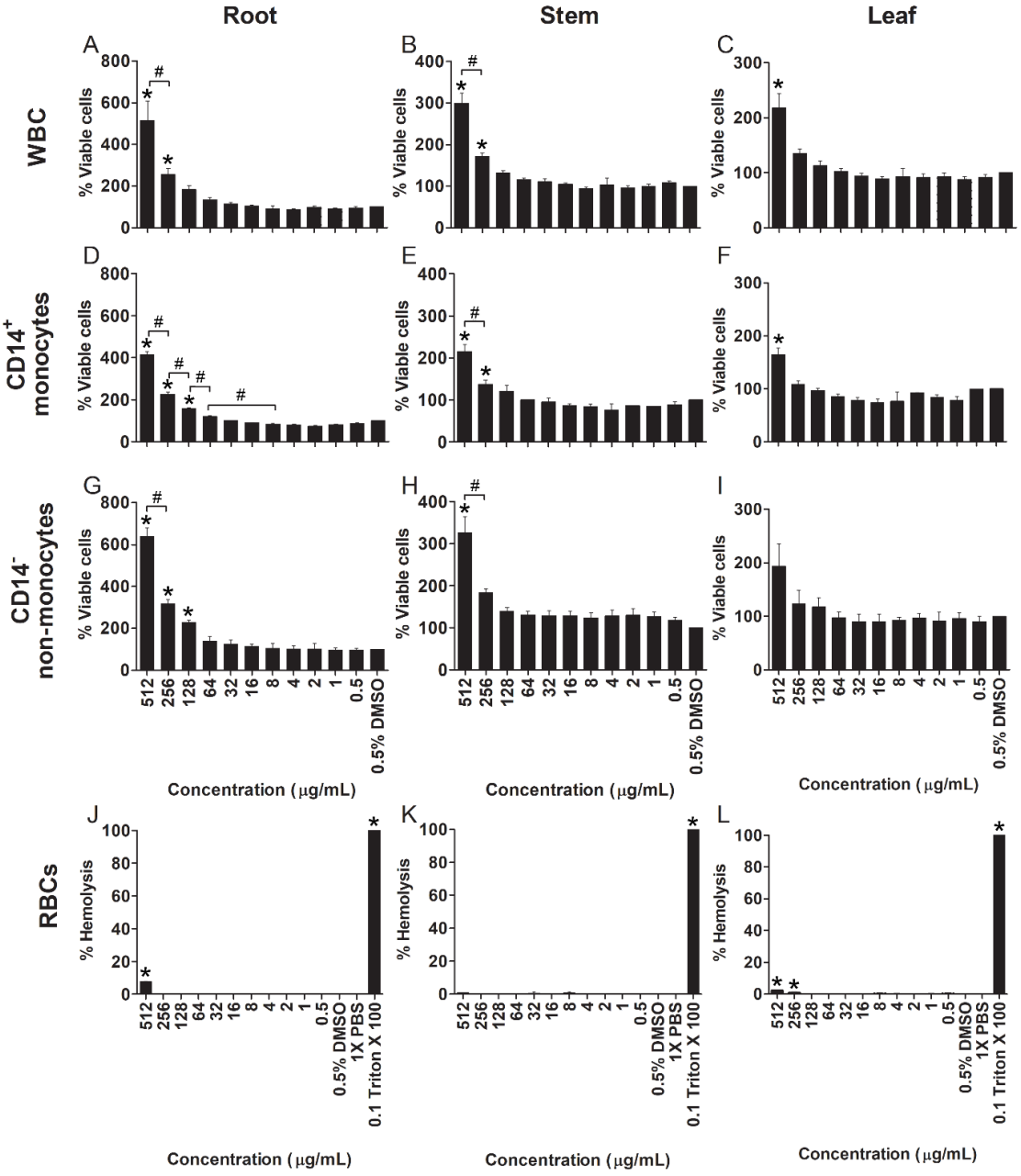

Figure 4: Cytotoxicity of crude extracts on white blood cells and red blood cells

The proliferation profile of WBC (A–C), monocytes (D–F), and non-monocytes (G–I) after treating with crude extracts for 48 h. The toxicity of crude extract on RBC (J–L). The values are expressed as mean ± SD of data from three independent replicates. (*) p-value ≤ 0.05 compared to 0.5% DMSO, (#) p-value ≤ 0.05 compared between groups.

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This research also studied the hemolytic activity of crude extracts against red blood cells. There was no hemolytic activity against red blood cells at a concentration of 0–512 µg/mL in the stem crude extract (Figure 4K) while the root (Figure 4J) and leaf crude extracts (Figure 4L) showed very low hemolytic activity at concentrations of 512 and 256 µg/mL, respectively, compared with 0.5% DMSO as control and 0.1% Triton X-100 as a positive control. The sodium chloride (NaCl) concentration of crude extracts was measured by using a refractometer. The NaCl concentration of these crude extract stock solution (100 mg/mL) was lower than 2%. However, the concentrations of 0-512 µg/mL for the crude extract were chosen for experiments. Therefore, the red blood cell hemolysis was not affected by NaCl concentration from the crude extract in these experiments. The 2,9'-di-*O*-acetyl-5-*O*-benzoyl-5-deacetylwilforidine and 9'-deacetoxymekongensine extracted from *M. mekongensis* have been reported to have low toxicity to human oral epidermal carcinoma (KB) cell line at the IC_{50} concentrations of 28.2 and 46.7 µg/mL, respectively.[7] The cytotoxicity results of this study indicate an excellent prospect that the actions of crude extracts on WBCs and RBCs might be useful in the future. Therefore, considering the cytotoxicity of the crude extracts on the white blood cells and red blood cells, the concentrations at 5, 50, and 500 µg/mL were chosen for further study.

Phagocytosis activity of MDM-treated crude extracts

Conventional macrophages ($CD14^-CD16^+$) are monocytes in tissues that differentiated from $CD14^+CD16^-$ monocytes. Human monocyte-derived macrophages were cultured and monitored for their differentiation by daily examination of CD14 and CD16 mRNA expression (Supplementary data 2). The effect of the crude extract on phagocytosis of macrophages was examined by using three bacteria. The results were shown as CFU (cells) of bacteria/macrophages by comparison with bacterial growth curves. The number of *S. aureus* DMST 8840 cells phagocytosed by MDM at 2 h was higher than at 1 h in three concentrations of all crude extracts. In contrast, 5 µg/mL of root extract-treated with MDM showed a lower number of *S. aureus* DMST 8840 cells phagocytosed at 2 h than at 1 h. This pattern was also found in all groups of positive control conditions compared to the 1X PBS control group (Figure 5A). Another pattern of phagocytosis activity of MDM-treated crude extracts was revealed in *S. aureus* (MRSA) DMST 20654 (Figure 5B) and *E. coli* ATCC 25922 (Figure 5C) models. The number of phagocytosed bacteria at 1 h was higher than at 2 h of three concentrations of all crude extracts. The MDM-treated root crude extract demonstrated an increased number of phagocytosed bacteria at 1 h in a dose-dependent manner and significantly declined at 2 h. The reduction of engulfed bacteria might happen by macrophages releasing reactive oxygen and nitrogen species to destroy the bacteria.[48] Moreover, this pattern of phagocytosis was also shown in positive control and negative control groups. Quercetin has been reported to increase the expression of phagocytosis-related genes and to reduce the expression of inflammatory genes involved in bovine neutrophils.[49] Ascorbic acid can also enhance phagocytosis and microbial killing.[50] Interestingly, all crude extracts of crude extracts exhibited the enhancement of phagocytosis activity of bacteria in different patterns when compared to the negative control.

TNF-α mRNA upregulated by crude extract of M. mekongensis

MDM were treated with crude extract for 48 h and then stimulated with H_2O_2 for 6 h. MDM treated with stem and leaf extracts in all concentrations showed higher TNF-α mRNA expression than root extracts and all control groups. Interestingly, the stem extract enhanced the expression in a dose-dependent manner. The upregulation of TNF-α mRNA in the H_2O_2 -stimulated positive control groups was higher than in non-stimulated groups. Besides, TNF-α mRNA expression was significantly decreased by the p38 MAPK inhibitor SB203580, and 3 MAPK inhibitors suggesting that H_2O_2 stimulated TNF-α expression in macrophages might be regulated via p38 MAPK inhibitor SB203580 (Figure 6). TNF-α is a pro-inflammatory cytokine secreted by macrophages. H_2O_2 induces TNF-α production in macrophages via p38 and a stress-activated protein kinase (SAPK/JNK) (27).

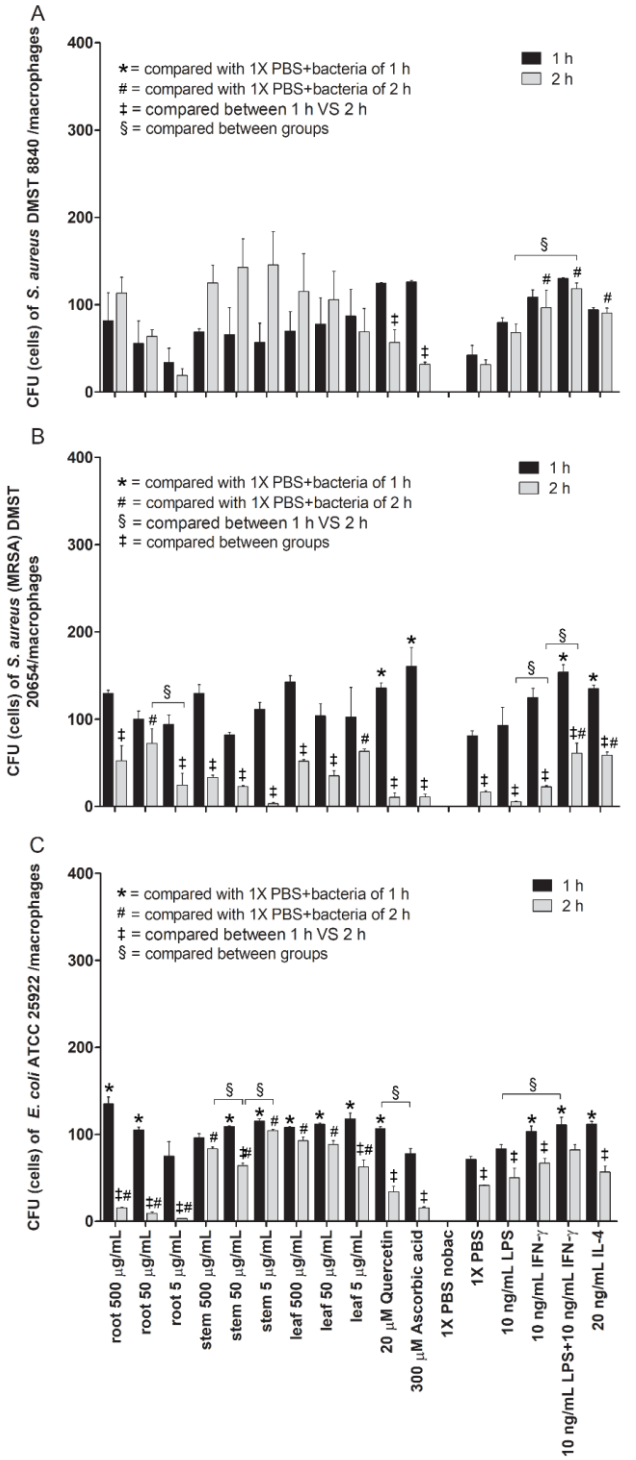

Figure 5: Effect of crude extracts on phagocytosis activity of monocyte-derived macrophages

Engulfed bacteria (CFU (cells) of bacteria/macrophage), *S. aureus* DMST 8840 (A), S. aureus (MRSA) DMST 20654 (B), and E. coli ATCC 25922 (C) by macrophage phagocytosis when stimulated with three crude extracts at various concentration. The values are expressed as mean ± SD of data from three independent replicates. (*) p-value ≤ 0.05 compared to 1X PBS+bacteria at 1 h), (#) p-value ≤ 0.05 compared to 1X PBS+bacteria at 2 h), (‡) p-value ≤ 0.05 compared between 1 h and 2 h, and (§) p-value ≤ 0.05 compared between groups.

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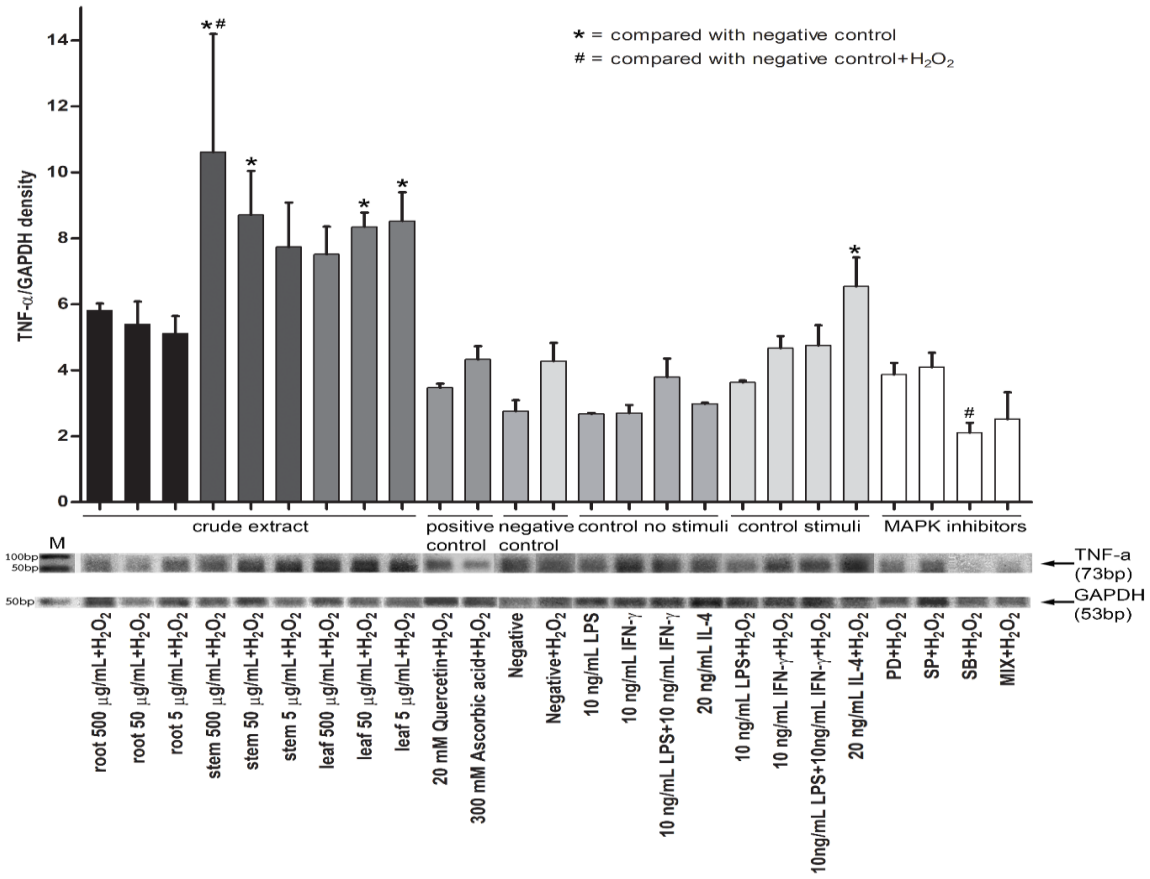

Figure 6: Effect of crude extract on tumour necrosis factor-α
TNF-α mRNA expression by macrophage after treating with crude extracts for 48 h and stimulated by H_2O_2 for 6 h. The values are expressed as mean ± SD of data from three independent replicates. (*) p-value ≤ 0.05 compared to negative control), (#) p-value ≤ 0.05 compared to negative control+H_2O_2).

Also, several natural compounds such as aculeatiside A, naringin, and onionin A, have been reported to stimulate the $CD169^+$ of macrophages, which is involved in the anti-tumour mechanism as well as the stimulation of pro-inflammatory cytokines secretion such as IL-1β, IL-12, and TNF-α in murine peritoneal macrophages.[51] Honey (manuka pasture and jelly bush) stimulates TNF-α, IL-1β, and IL-6 secretion in human monocytes.[45] In addition, the IL-10 mRNA expression was also determined but no expression was not detected (data not shown), possibly due to low IL-10 expression and the absence of IL-10 production in RAW264.7 and Jurkat T cells.[52] In addition, the upregulation of TNF-α expression also involved the stimulation of phagocytosis activity of macrophages and neutrophils, and also directly inhibits bacterial growth.[48,53,54]

Conclusion

This study has revealed effects of *M. mekongensis* crude extract on human monocyte-derived macrophage, which have not been previously reported. We found that the leaf extract gave the highest % yield. Crude extracts of the three parts of the plant contained high phenolic and flavonoid contents. The root extract exhibited high antioxidant activity that was similar to ascorbic acid, whereas the stem and leaf extracts promoted the phagocytic activity and TNF-α mRNA expression higher than the root extract. Taken together, these results indicate that *M. mekongensis*, a neglected halophyte, has promising properties with low cytotoxicity and immune-enhancing activities. The compounds from this plant should be further studied and might be used for immunomodulation in various immunodeficiency diseases.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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