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Original Research Article

Achyranthes Aspera Linn. Crude Extracts Enhance Phagocytic Activity and Attenuate Oxidative Stress-Mediated-TNF-*α* mRNA Expression in Primary Human Monocyte-Derived Macrophage

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ABSTRACT

Achyranthes aspera is used in tropical areas for several purposes based on it being a rich source in polyphenols. However, the direct effect of *A. aspera* on oxidative stress induced-human macrophages remains unclear. Therefore, this study aims to examine the biological activities of ethanol crude extracts of *A. aspera* and their effects on the primary human monocyte-derived macrophage. The individual components of the plant extracts were determined and the phytochemical constituents, antioxidant activity, and cytotoxicity on human white blood cells and red blood cells was examined. The crude extracts subtoxic concentration was used to stimulate the human primary monocyte-derived macrophages for phagocytosis assay and expression of a pro-inflammatory mediator, TNF-*α*, under oxidative stress conditions. The extraction was performed using 70% ethanol which gave a yield of approximately 5-6% for all parts of the plant. The total phenolic and flavonoid contents also reveal a comparable amount. The flower and stem extracts exhibited the highest antioxidant activity. The crude extracts showed relatively low cytotoxicity against the human white blood cells and red blood cells, except for the flower extract, which had a weak hemolytic activity at 75 µg/mL. The crude extracts enhanced the phagocytic activity of the macrophages while suppressing the expression of TNF-*α*, suggesting that *A. aspera* ethanol crude extracts have immunomodulation activity by enhancing phagocytic activity and suppressing a pro-inflammatory cytokine expression of human macrophages. Furthermore, the formulation of a plant-based herbal product should be possible, and it could be a reasonably economical therapeutic anti-inflammatory agent.

Keywords: *Achyranthes Aspera* Linn., Anti-inflammatory, Monocyte-derived macrophage, Oxidative stress, Phagocytic activity, TNF-α

Introduction

Inflammation is an innate defense against foreign antigens, including microbial pathogens, viral infections, allergens, and chemicals. Inflammation caused by activated macrophages contributes to several chronic diseases linked to higher production of reactive oxygen species (ROS) and oxidative stress.[1] The oxidative burst involves macrophage responses to the invading microbial pathogens.[2] Hydrogen peroxide (H_2O_2) is the most stable ROS produced by converting oxygen (O_2) via action of superoxide dismutase. The H_2O_2 is subsequently converted to water by catalase.[3] The H_2O_2 is an amphipathic molecule that is easily diffused through the cell membrane by simple diffusion and aquaporin water channels.[4]

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Therefore, H_2O_2 acts as a second messenger of intracellular redox-sensitive signal transduction pathways via nuclear factor kappa B (NF-κB)-dependent gene expression, including tumor necrosis factor (TNF-α) and inducible nitric oxide synthase (iNOS).[5] The intracellular redox-sensitive signal transduction pathways can be suppressed by various natural compounds that exhibit anti-inflammatory and antioxidant activity, such as α-lipoic acid and polyphenols.[6]

The plant's secondary metabolites, especially the polyphenols are involved in the defense mechanism to protect against pathogens and ultraviolet radiation.[7] Polyphenols are frequently characterized by their bitterness, colour, and odour. Recently, over 8000 phenolic compounds have been identified in plants, such as flavonoids and phenolic acids.[8] These polyphenols have been reported to have protective effect against oxidative processes by activation of Nrf2, a master antioxidant switch. Moreover, polyphenols also exhibit anti-inflammation and antimicrobial properties.[7]

Achyranthes aspera Linn. belonging to the family Amaranthaceae, is a weed found in tropical Asia, Africa, America, Europe, Australia, and Thailand. The seeds, roots, shoots, and leaves of the plant have been used for medicinal purposes in several countries.[9] *A. aspera* is rich in phytochemicals. Their seeds contain saponin A (D-Glucuronic Acid), saponin B (*β*-D-galactopyranosyl ester of D-Glucuronic Acid), and oleanolic acid glycosides present in three forms, namely, α-L-rhamnopyranosyl-(1-4)-(*β*-D-glucopyranosyluronicacid)-(1-3)-oleanolic acid, α-L-rhamnopyranosyl-(1-4)-(*β*-D-glucopyranosyluronicacid)-(1-3)-oleanolic acid-28-O-*β*-D-

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glucopyranoside, and α-L-rhamnopyranosyl-(1-4)-(*β*-D-glucopyranosyluronicacid)-(1-3)-oleanolic acid-28-O-*β*-D-glucopyranosyl-(1-4)-*β*-glucopyranoside).[10] The isolated phytochemical constituents possess broad activities, including laxative, diuretic, anti-periodic, anti-allergic, anti-asthmatic, and hepato-protective properties. *In India, A. aspera* is traditionally used for diarrhea, dysentery, pneumonia, asthma, dropsy, rheumatism, ulcers, cough, piles, snake bite, and skin diseases.[11] In addition, *A. aspera* is also used as a folk antipyretic herb in some parts of Northeastern Thailand. However, the direct effect of *A. aspera* Linn. on oxidative stress induced-human macrophages remains unclear. Therefore, the biological activities of *A. aspera* ethanol crude extracts on the primary human monocyte-derived macrophage were examined. The phytochemical constituents, antioxidant activity, cytotoxicity on human white blood cells and red blood cells were also determined using extracts from individual plant parts.

Materials and Methods

Human peripheral blood collection and ethical statement

Peripheral white blood cells (WBCs) were collected from 5 healthy volunteers who were considered normal by measuring their blood pressures and body temperatures. The volunteers gave written consent for blood collection. The procedure was approved by the Human Ethics Committee of Mahasarakham University (No. 218/63). The WBCs were isolated from whole blood (anti-coagulated by 2 mM EDTA) using red blood cell (RBC) lysis buffer.[12] Briefly, 45 mL of 1× RBC lysis buffer were added to 5 mL of EDTA containing whole blood, and the mixture was placed at room temperature for 5 min, then centrifuged at 2,000 g for 5 min. The WBC pellet was washed twice by adding 10 mL of 1× phosphate buffer saline (PBS) and centrifuged at 2,000 g for 5 min.[12] The WBCs were observed under a microscope by staining with 0.4% trypan blue (Gibco, Thermo Scientific, CA, USA) and counted using a hematocytometer.[13]

Source of plant materials and preparation of crude extracts

The plant materials from *A. aspera* Linn. were collected in August 2019 from an area in Mahasarakham University in northeastern Thailand (16°14'59.2"N, 103°15'04.1"E, A 135 m). The plant was botanically characterized by *Associate Professor* Khwanruan Naksuwankul (Ph.D.), a curator of the Natural Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University, according to the Flora of Thailand manual.[14] Voucher specimens were deposited at the Natural Medicinal Mushroom Museum, under the code number MSUT7390.

The whole plant of *A. aspera* was washed with tap water and separated into four parts; root, stem, leaf, and flower. Each part was dried at room temperature for two weeks. The dried samples were powdered and kept in the dark in a dry container. Fifteen grams of each sample were extracted using 450 mL of 70% ethanol under reflux extraction at 60°C.[15] The extracts were concentrated to dryness in a rotary evaporator. The dried crude extracts were weighed and dissolved in 100% DMSO and filtered using Whatman No. 1, 0.45 µm, and 0.22 µm filters (Whatman, Maidstone, UK). The filtered crude extracts were stored at -20°C until further use.[7]

Phytochemical constituent screening

The total phenolic content of the extract was determined by using the Folin-Ciocalteu's method.[16] First, 100 µL of the 100 µg/mL crude extract was added to 500 µL of 10% Folin-Ciocalteu's reagent and incubated in the dark for 3 min. Next, 400 µL of 7.5% Na_2CO_3 was added to the mixture and incubated in the dark for 30 min. The reaction was determined by a UV-Visible Spectrophotometer at 731 nm against blank without extract. The total phenolic content was expressed as mg gallic acid equivalent (GAE)/g crude extract.[7]

The total flavonoid content of the crude extract was examined by a colorimetric assay.[17] First, 100 µL of the 300 µg/mL crude extract was added to 500 µL of sterilized distilled water. Next, 30 µL of 5% $NaNO_2$ was added to the mixture, then incubated in the dark for 5 min. Then, 60 µL of 10% $AlCl_3$ was subsequently added to the mixture and incubated in the dark for 5 min. Then 200 µL of 1M NaOH was added followed by 110 µL of sterilized distilled water and incubated in the dark for 5 min. The reaction was determined by a UV-Visible Spectrophotometer at 510 nm against a blank without extract. The total flavonoid content was expressed as mg quercetin equivalent (QE)/g crude extract.[7]

Antioxidant activity assays

Antioxidant activity of the crude extracts were determined by two assays, using 2,2-Diphenyl-1-picrylhydrazine (DPPH) or [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) radical scavenging assays.[18] For the DPPH radical scavenging assay, the various crude extracts (0 – 1,000 µg/mL) were mixed with 80 µM of DPPH, and the mixture was then incubated in the dark for 30 min. The reaction was measured by using a UV-visible Spectrophotometer at 515 nm. Ascorbic acid was used as a positive control and for a standard curve. The percentage of antioxidant activity crude extract was calculated by the formula described by Matuszewska *et al*.[19]

$$\text{DPPH scavenging activity (\%)} = \frac{\boldsymbol{A_{control} - A_{sample}}}{\boldsymbol{A_{control}}} \times \mathbf{100}$$

For the [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) radical scavenging assay, 500 µL of 7 mM ABTS solution was mixed with 500 µL of 2.45 mM potassium persulfate and incubated for 12 – 16 h in the dark to produce ABTS radicals. The ABTS radical was diluted with methanol, and the absorbance was adjusted to 0.7 at 734 nm. The ABTS radical solution was mixed with the various concentrations of crude extracts (0 – 500 µg/mL). The mixtures were incubated in the dark for 5 min, and the absorbance at 734 nm was measured using a UV-visible Spectrophotometer. Ascorbic acid was used as a positive control to create a standard curve. The percentage of antioxidant activity crude extract was calculated by the formula described by Re *et al*.[20]

$$\text{ABTS scavenging activity (\%)} = \frac{\boldsymbol{A_{control} - A_{sample}}}{\boldsymbol{A_{control}}} \times \mathbf{100}$$

Cytotoxicity assays

Cytotoxicity of the crude extract was determined by two assays, including a hemolysis assay for RBC and a cell viability assay for WBC.

Firstly, the human AB-type RBC collected from three volunteers were prepared by washing 3 times with 1× PBS and centrifuged at 2000 g for 10 min. 2% RBCs were prepared by diluting them with 1× PBS before use. The assay was performed by mixing 500 µL of 2% RBC and 500 µL of crude extracts in the various concentrations (0 – 300 µg/mL). 1× PBS and 0.5% DMSO in 1× PBS were used as negative controls, while 0.1% Triton-x100 was used as a positive control. The mixture was incubated at 37°C for 30 min and then centrifuged at 3000 g for 6 min. The supernatants were collected, and the absorbance (OD) at 541 nm was measured using a UV-visible Spectrophotometer. The percentage of hemolysis was calculated using the following formula as described by Alencar *et al*.[18]

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

Lastly, the cytotoxic activity of crude extracts on WBCs collected from three volunteers were performed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Gibco, Thermo Scientific, CA, USA). First, 2×10^4 cells/well of WBC were cultured in RPMI-1640 supplemented with antibiotics (Gibco, Thermo Scientific, CA, USA), and 10% fetal bovine serum (FBS) (Gibco, Thermo Scientific, CA, USA), under culture conditions at 37°C, 5% CO_2, and humidity.[21] These cells were treated with various concentrations of crude extracts (0 – 600 µg/mL) and 0.5% DMSO was used as a negative control. The treated cells were incubated for 48 h. Then, 10 µL of 12 mM MTT was added to the culture and incubated for 4 h in the dark. Finally, formazan crystals were dissolved by adding 100 µL DMSO. The WBC viability was determined at 540 nm

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using a UV-Visible spectrophotometer, and the percentage cell viability was calculated by comparison to the control cells.[22]

Primary monocyte-derived macrophage culture

The primary monocyte-derived macrophages were cultured by isolation of $CD14^+$ peripheral monocytes. The isolation of $CD14^+$ monocytes was performed using anti-human CD14 magnetic particles and a BD IMag separator (BD Bioscience, San Jose, CA, USA).[23] In addition, individual monocytes were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Scientific, CA, USA), and antibiotics (Gibco, Thermo Scientific, CA, USA) under culture condition at 37°C, 5% CO_2, and humidity.[21]

Primary macrophage phagocytosis assay

The subtoxic concentration of the crude extract was found from the results of hemolysis and MTT assays. The optimum concentrations of crude extracts were added to cultured macrophage at day three and incubated for 48 h. The gram-positive bacterium, *Staphylococcus aureus* DMST8840 and gram-negative bacterium, *Escherichia coli* ATCC25922, were inoculated into the macrophage culture at MOI of 30 in antibiotic-free conditions. After 1 and 2 h of inoculation, the macrophages were washed twice with 1× PBS and added to 1% SDS for macrophage cell lysis. Tryptic Soy broth was added to lysed macrophage which were then dropped on Tryptic Soy agar plate. The viable engulfed bacteria were calculated and expressed as CFU/mL.[24,25]

Primary macrophage stimulation

$CD14^+$ monocytes were cultured for four days before adding the crude extract at optimum concentrations and controls. The control groups in this study including stimulation groups (LPS, recombinant IFN-γ (Peprotech, NJ, USA), 10 ng/mL LPS + 10 ng/mL IFN-γ, and 2 ng/mL recombinant IL-4 (Peprotech, NJ, USA)) and compound groups (ascorbic acid and quercetin). On day 6 after treatment, the macrophages were washed twice by 1× PBS. The specific MAPK, p38 MAPK (SB203580, 5 μM), ERK (PD98059, 10 μM), and JNK inhibitors (SP600125, 10 μM) were pretreated with the MAPK inhibitor group for 2 h.[23] Then, all the groups were washed and stimulated by 40 μM hydrogen peroxide (H_2O_2) for 6 h.[26] The stimulated macrophages were collected, and RNA extraction was carried out using TRIZOL reagent (Thermo Fisher Scientific, CA, USA) according to the manufacturer's instructions. Briefly, the cell pellet was lysed in TRIZOL reagent and separated in the aqueous phase containing the RNA by adding chloroform and centrifugation. The total RNA was precipitated by adding isopropanol, and centrifugation. The RNA pellets were washed with 75% ethanol and resuspended in RNase free water (Himedia, Mumbai, India). Reverse transcription was performed for cDNA synthesis using the Viva 2-step RT-PCR kit (Vivantis, Malaysia).

The expression of CD14, CD16, TNF-α, and IL-10 mRNA were determined by RT-PCR and gel electrophoresis. In addition, the specific PCR band was semi-quantified by density analysis using ImageJ and normalized by GAPDH internal control.[27]

Statistical analysis

The experiments were performed independently three times. Statistical analysis of this study was performed using GraphPad Prism Software (version 5; GraphPad Soft Inc. La Jolla, CA, USA). The student's t-test and one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test were used. All data were represented as mean ± SD. Differences were considered significant at $p\text{-value} \leq 0.05$.

Results and Discussion

Yield of crude extracts of *A. aspera*

The extraction was performed separately using each part of the plant; root, stem, leaf, and flower (Figure 1A-1D). The yield of crude extracts was measured and expressed as a percentage of dry weight. The highest % yield of the crude extract was found in the stem (6.47%), followed by the leaf, root, and flower, at 5.18, 4.94, and 4.80%, respectively (Figure 1E). *A. aspera* is used over the tropical area for several purposes. The major phytochemical constituents are polyphenols, flavonoids, alkaloids, saponins, proteins, and carbohydrates. The extraction process of this study was performed by using 70% ethanol which showed the yield of crude extracts approximately 5-6% (Figure 1E).

Phytochemical constituents of A. aspera crude extracts

The highest phenolic content was found in the stem and root fractions at 24.561 ± 1.449 and 21.359 ± 3.957 mgGAE/g crude extract, respectively whereas the leaf fraction revealed a low phenolic content at 13.786 ± 0.848 mg GAE/g crude extract (Figure 2A). The flavonoid content was high in the root and leaf fractions at 37.967 ± 0.334 and 35.133 ± 3.167 mg QE/g crude extract. On the other hand, the stem and flower fractions showed less flavonoid content at 28.967 ± 3.334 and 27.133 ± 3.500 mg QE/g crude extract, respectively (Figure 2B).

The seed of this plant contains various saponins including, D-glucuronic acid, *β*-D-galactopyranosyl ester of D-glucuronic acid.[28] The stem also contains other saponins such as pentatriaontane, 6-pentatriacontanone, hexatriacontane, and tritriacontane. The root also contains ecdysterone in methanol extract. These compounds possess vast pharmacological actions, such as that of achyranthine, a water-soluble alkaloid that can decrease blood pressure by dilating blood vessels.[29]

Antioxidant activity of A. aspera crude extracts

Antioxidant activity was measured using DPPH and ABTS free radical scavenging and compared to ascorbic acid as a positive control. The results from both assays revealed the same trend.

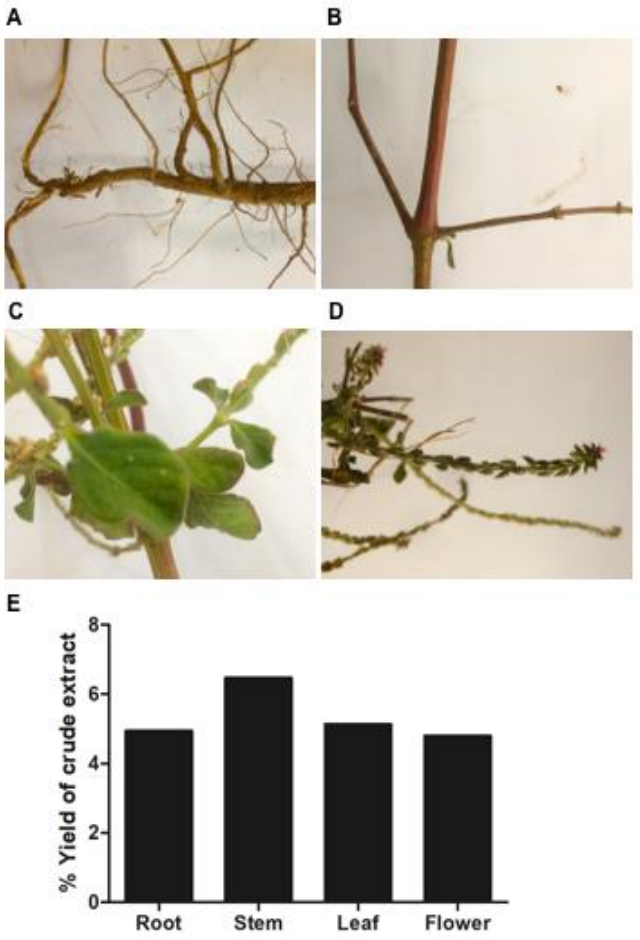

Figure 1: Percentage yield of crude extracts of *Achyranthes aspera* L. *Achyranthes aspera* L. plants were separated into four parts; root **(A)**, stem **(B)**, leaf **(C)**, and flower **(D)**. The yield of crude extracts is shown in black bars **(E)**.

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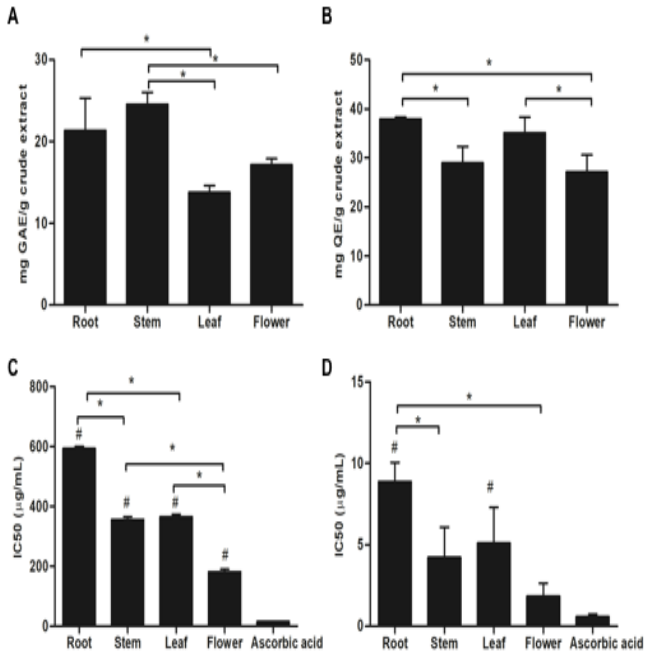

Figure 2: Phytochemical constituents and antioxidant activity of *Achyranthes aspera* L. crude extracts.
The phenolic and flavonoid contents of *Achyranthes aspera* L. were expressed in gallic acid equivalent **(A)** and quercetin equivalent **(B)**, respectively. The antioxidant activity was measured using scavenging of DPPH **(C)** and ABTS **(D)** free radicals and compared to ascorbic acid as the positive control. The experiments were performed in triplicate independent experiments; (#) represents a significant difference at $p \leq 0.05$ compared to the control group; (*) represents a significant difference at $p \leq 0.05$ between groups.

The flower fraction exhibited the highest antioxidant activity with the lowest IC_{50} concentrations of DPPH and ABTS assays at 180.850 ± 9.302 and 1.849 ± 0.790 µg/mL, respectively. The root fraction showed the lowest antioxidant activity with the highest IC_{50} concentration of DPPH and ABTS assays at 593.695 ± 6.548 and 8.872 ± 1.190 µg/mL, respectively. The stem and leaf fractions showed comparable IC_{50} in the DPPH assay at 356.069 ± 10.574 and 365.804 ± 8.808 µg/mL and in the ABTS assay at 4.226 ± 1.852 and 5.106 ± 2.203 µg/mL, respectively (Figure 2C and 2D).
Methanol extracts of the leaves and roots have been reported to exhibit high antioxidant activities in several *in vitro* studies.[30] This study used two methods to determine the antioxidant activity of the crude extracts from different plant parts. The flower and stem showed relatively high antioxidant activities compared to the ascorbic acid control. Second, the correlations of antioxidant activity and total phenolic or flavonoid contents were also analyzed, revealing no correlation between both compound groups [Supplementary figure 1 (not shown)], suggesting that the antioxidant activity of the crude extracts might be derived from the other compound groups.
Tannins, saponins, flavonoids, and alkaloids from *A. aspera* have been shown to possess antibacterial activity against several pathogens such as *Bacillus subtilis*, *Citrobacter species*, *Escherichia coli*, *Micrococcus species*, and *Pseudomonas aeruginosa* using disk diffusion and agar well plate methods.[31] Ethanol crude extract of leaves showed larvacidal activity against the mosquitoes *Aedes aegypti* and *Culex quinquefasciatus.*[32] Antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium was also examined*. *A. aspera* delivers immunomodulatory activity in mice and fish (*Labeo rohita*) models by increasing antigen-specific murine antibody responses in mice and enhancing anti proteases/antigen clearance in fish.[33]

Cytotoxicity of A. aspera crude extracts on white blood cells and hemolysis activity
Cytotoxicity of crude extracts was performed by MTT and hemolysis assays. The percentage viability of white blood cells was not statistically different amongst all the fractions (Figure 3A-3D). There was very low hemolysis activity in the root, stem, and leaf fractions (Figure 3E-3G). However, noticeable hemolysis activity was found in the flower fraction at 75, 150, and 300 µg/mL (Figure 3H). Therefore, the optimum concentrations of crude extracts were considered by both assays and used for further experiments.
The crude extracts showed relatively low cytotoxicity against human white blood cells and human red blood cell except in the flower extract, where a weak hemolysis activity was observed at 75 µg/mL (Figure 3). The subtoxic concentration of the crude extracts was used to stimulate the human primary monocyte-derived macrophages for phagocytosis assay and expression of TNF-α under oxidative stress conditions. Crude extracts enhanced the phagocytic activity of macrophages (Figure 4). On the other hand, crude extracts suppressed the expression of TNF-α (Figure 5D) suggesting that the *A. aspera* extracts had immunomodulation abilities by enhancing phagocytic activity and suppressing a pro-inflammatory cytokine expression of human macrophages. The alcoholic extract of this plant has also been reported to stimulate the phagocytic activity of human neutrophils, which enhance cell-mediated immune responses.[34] The alcohol extracts of roots, leave, and seeds showed anti-inflammatory activity on acute and sub-acute inflammation in several rat models, such as the induced paw edema method, cotton pellet granuloma test, and formalin model.[35] *A. aspera* crude extracts enhance the phagocytic activity of primary human monocyte-derived macrophage.
The phagocytosis assay was performed by using the optimum concentrations of crude extracts, considering 75 µg/mL to be 'low concentration', 150 µg/mL as 'moderate concentration', 300 µg/mL as 'high concentration', while 0 µg/mL was a negative control. The phagocytic activity was represented by increasing intracellular bacteria at 1 h and reducing these bacteria after 2 h. The results showed that the root, stem, leaf, and flower crude extract-treated primary human monocyte-derived macrophages exhibited a high phagocytic activity against *Staphylococcus aureus* at 150 µg/mL (Figure 4A, C, E, and G). The phagocytic activity against *Escherichia coli* showed different patterns for different parts of the plant; the root and stem crude extract-treated primary human monocyte-derived macrophages also had a high phagocytic activity at 150 µg/mL (Figure 4B and F). However, the stem and flower fractions had activities at 75 and 300 µg/mL, respectively (Figure 4D and H).
Macrophages release various cytotoxic substances, including protease, prostaglandins, nitric oxide, cytokines, and reactive oxygen species, such as hydrogen peroxide, superoxide, and hydroxyl radicals. The reactive oxygen species cause a respiratory burst.[5] Many researchers have reported oxidative stress and inflammation, especially a pathogenic role of oxidative stress in chronic inflammatory diseases. Oxidative stress causes the perturbation of several factors such as oxidized proteins, lipid peroxidation, and glycation end products leading to brain disorders by neuron degenerations.[36] Oxidative stress is induced by diabetes which increases pro-inflammatory cytokines such as interleukin-6 (IL-6) and TNF-α, and several adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and NF-κB.[37] Recently, nitric oxide has been recognized as an extracellular signaling molecule with a high permeability by diffusion across the extracellular space and stimulation of downstream signaling pathways.[38] The signaling pathway involves nitric oxide-mediated activation including extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK).[39] Interestingly, hydrogen peroxide is an intercellular messenger of several pro-inflammatory ligands in the murine RAW 264.7 macrophage cell line. Catalase exhibits the complete inhibition of nitric oxide and 40% of TNF-α production at its highest concentration.[40] Exposure to exogenous hydrogen peroxide induces NF-κB activation through phosphorylation at tyrosine residue of I kappa B alpha and serine residue of p65, and oxidations of cysteine residues at a variety of redox-sensitive kinases, upstream of NF-κB.[41]

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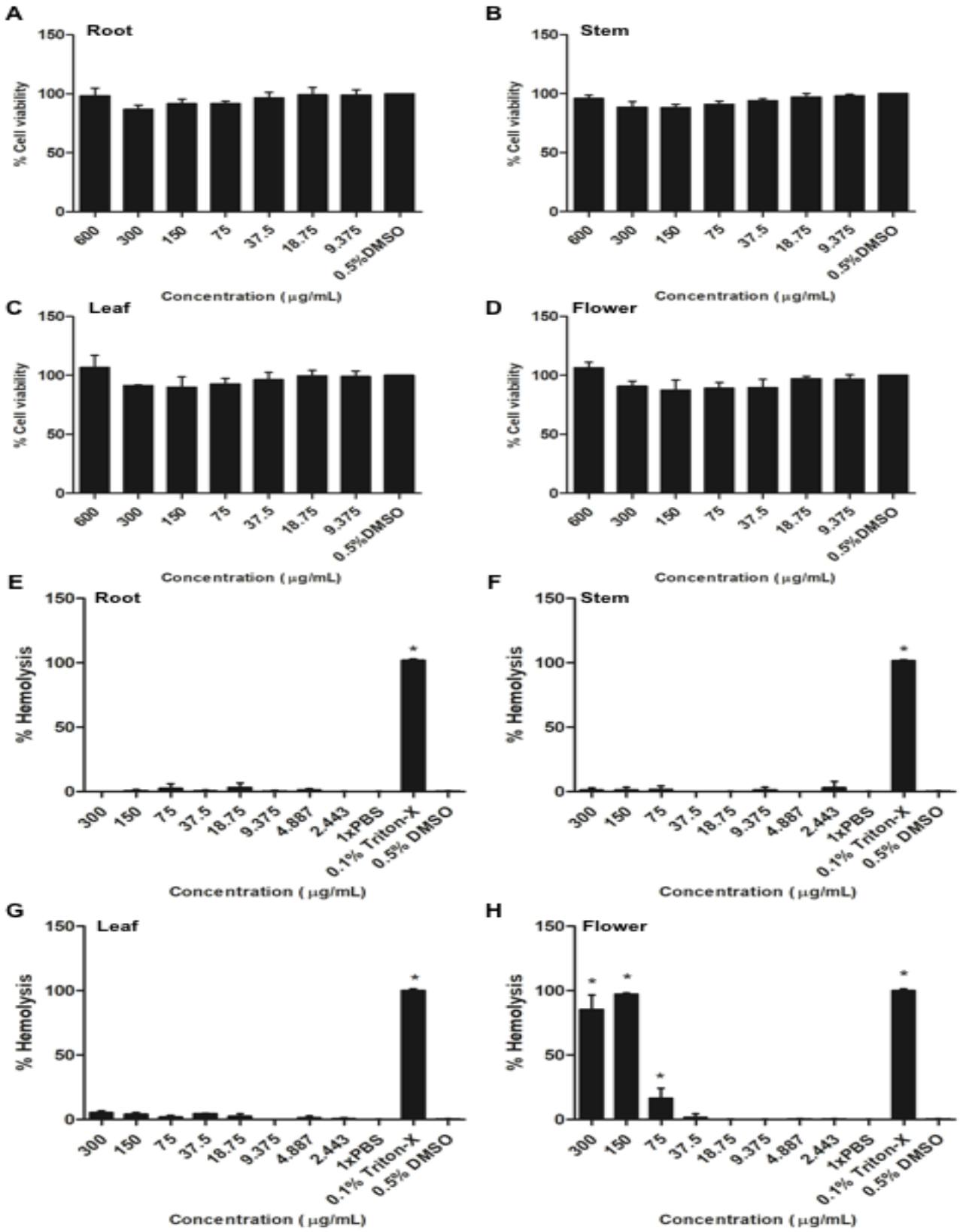

Figure 3: Cytotoxicity of *Achyranthes aspera* L. crude extracts on human white blood cells and red blood cells.
Cytotoxicity of crude extracts was performed by MTT and hemolysis assays. The percentage viability of white blood cells in each part of the plant is represented in the bar graph, for root (A), stem (B), leaf (C), and flower (D). The hemolytic activity also reveals in each part of the plant, including root (E), stem (F), leaf (G), and flower (H), respectively. The experiments were performed in triplicate and three times independent experiments. (*) represented a significant difference at $p \leq 0.05$ compared to the control group.

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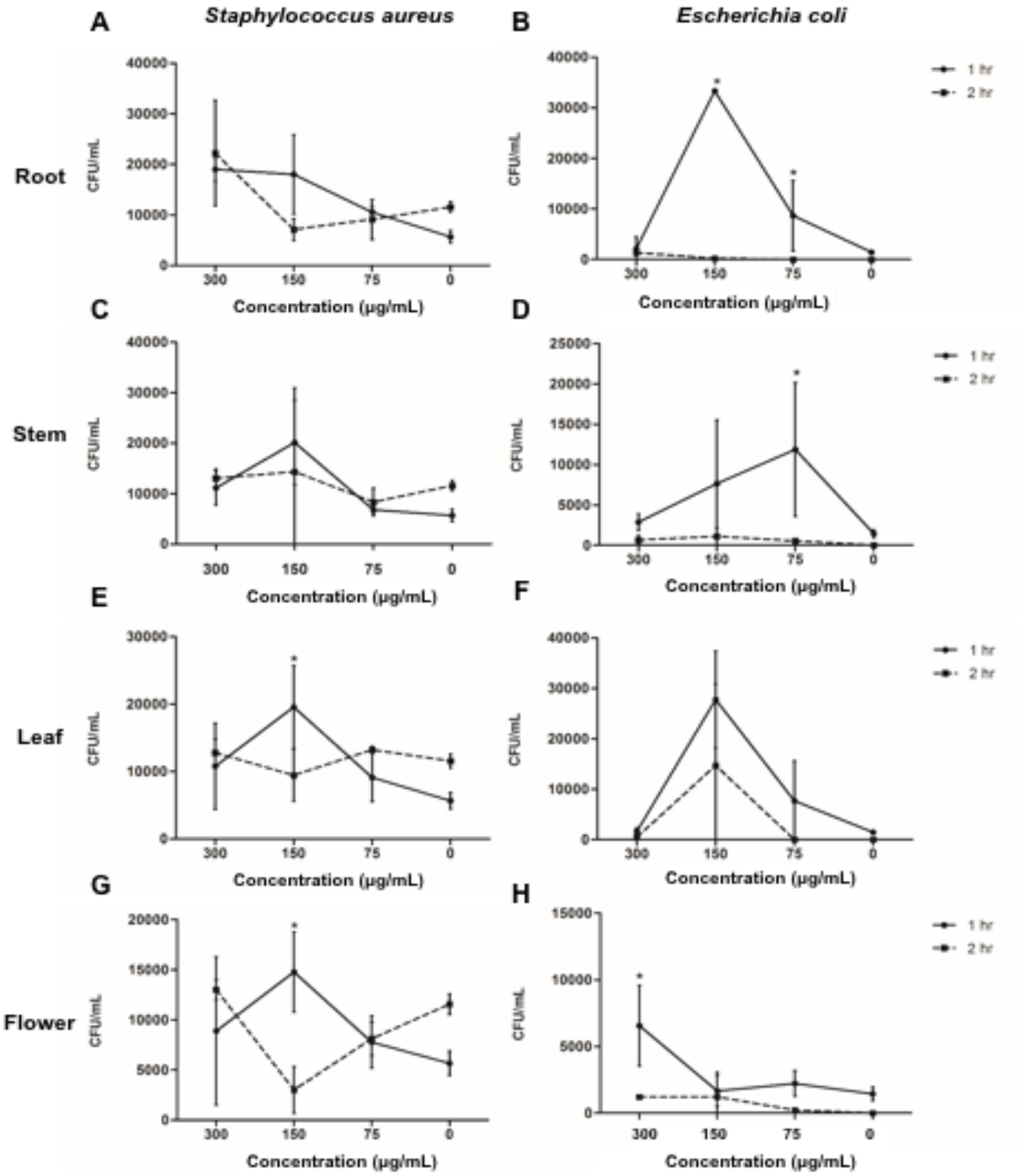

Figure 4: Phagocytic activity of *Achyranthes aspera* L. crude extracts treating primary human monocyte-derived macrophage. The phagocytosis assay was performed by using the optimum concentrations at low (75 µg/mL), moderate (150 µg/mL), high (300 µg/mL), and negative control (0 µg/mL). The phagocytic activity of *Achyranthes aspera* L. crude extracts applied to primary human monocyte-derived macrophage against *Staphylococcus aureus* and *Escherichia coli* are shown for each part of the plant. Monocyte-derived macrophage treated root extract against *Staphylococcus aureus* (A) and *Escherichia coli* (B), stem extract against *Staphylococcus aureus* (C) and *Escherichia coli* (D), leaf extract against *Staphylococcus aureus* (E) and *Escherichia coli* (F), and flower extract against *Staphylococcus aureus* (G) and *Escherichia coli* (H), respectively. The experiments were performed in triplicate using three healthy volunteers. The monocytes from each volunteer were separately cultured and tested. (*) represents a significant difference at $p \leq 0.05$ compare to the control group

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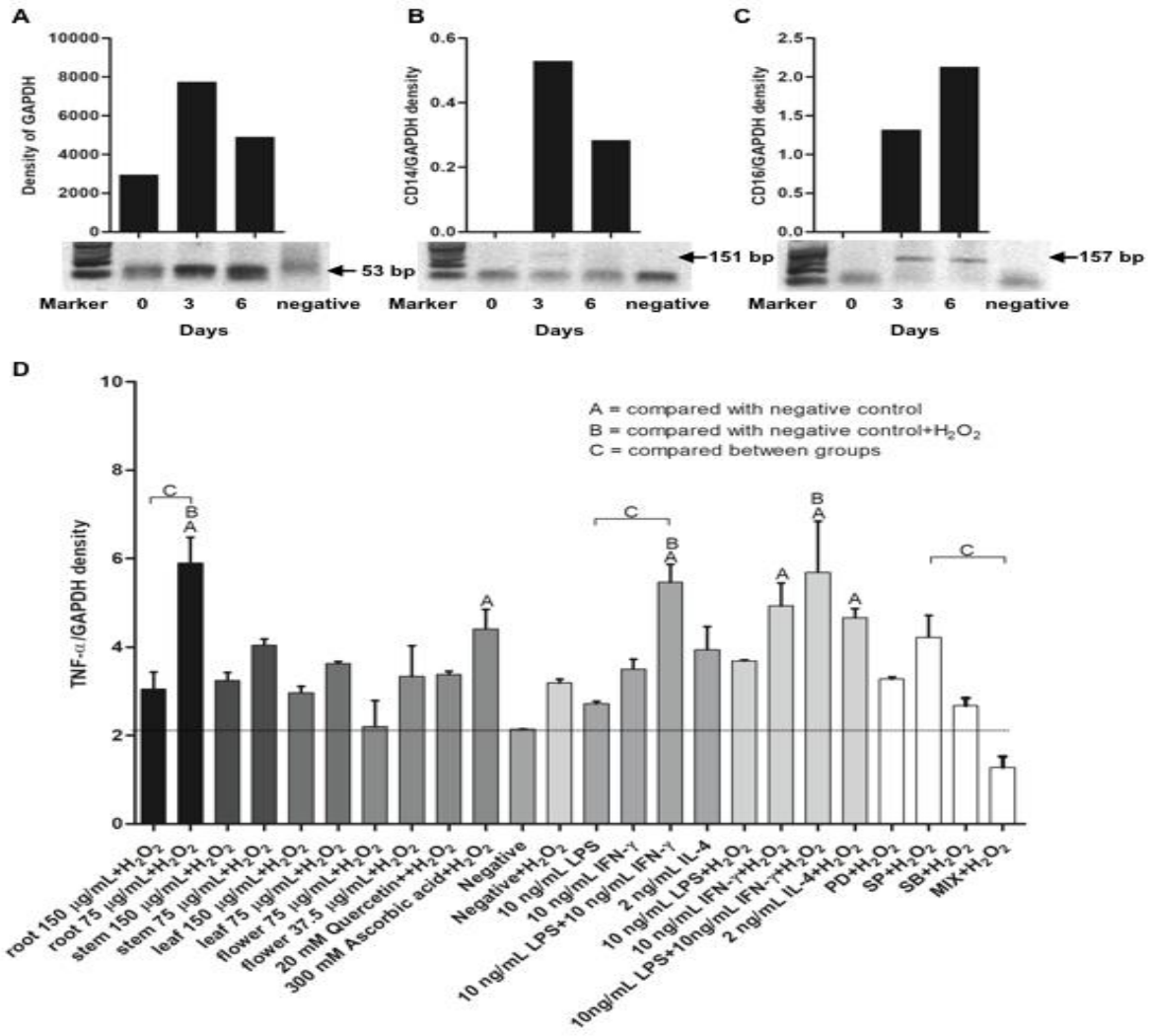

Figure 5: Attenuation of TNF-α mRNA expression in primary human monocyte-derived macrophage by *Achyranthes aspera* L. crude extracts

The production of the pro-inflammatory cytokine TNF-α was performed under H_2O_2-oxidative stress-driven conditions. On day 3 of monocyte-derived macrophage culture, this procedure was done with the CD14-CD16+ expression pattern (A-C). The TNF-α mRNA expression is presented in the bar graph compared to the positive and negative control groups (D). The experiments were performed in triplicate using and three healthy volunteers. The monocytes from each volunteer were separately cultured and tested. In figure 5D, the symbol A represented a significant difference at $p \leq 0.05$ compared to the negative control. The symbol B represented a significant difference at $p \leq 0.05$ compared to negative control + H_2O_2. The symbol C represented a significant difference at $p \leq 0.05$ compare between groups.

A. aspera crude extracts attenuate TNF-α mRNA expression in primary human monocyte-derived macrophage

Production of the pro-inflammatory cytokine TNF-α was performed under H_2O_2-oxidative stress-driven conditions. This procedure was performed on day 3 of monocyte-derived macrophage culture with the $CD14^-CD16^+$ expression pattern (Figures 5A-C). The TNF-α mRNA expression was completely suppressed to the baseline level in 75 µg/mL with the flower extract compared to the negative control. Moreover, the attenuation of TNF-α expression was found in all the fractions of crude extracts in dose-dependent manners. The level of TNF-α was elevated in all the positive control groups after stimulation with H_2O_2. In contrast, the p38 MAPK inhibitor (SB) and a mixture of MAPK inhibitors remarkably decreased the expression (Figure 5D), suggesting that *A. aspera* crude extracts can attenuate TNF-α mRNA expression in primary human monocyte-derived macrophage. This suppression mechanism might involve an inhibition of H_2O_2-activating MAPK pathway or other redox-sensitive signaling pathways.

Various polyphenols have been shown to modulate pro-inflammatory compounds such as prenylated flavonoids and biflavonoids, which inhibit lipopolysaccharide-induced nitric oxide, cyclooxygenase (COX), and lipoxygenase (LOX) production in murine RAW 264.7 macrophage cell line.[42] Black tea and green tea also inhibit LPS-induced iNOS gene expression and NO production in cultured macrophages resulting in decreased oxidative damage.[43] Moreover, several polyphenols such as quercetin, morin, rutin, hesperidin, and hesperetin have been reported to possess anti-inflammatory activities in acute and chronic inflammation.[44] The anti-inflammatory activities of polyphenols may be exerted through radical scavenging activities and modulation of pro-inflammatory mediators, as well as their effects on enzymatic and signaling pathways involved in inflammatory processes or cytokine production by stimulated monocytes.[45] Anti-inflammatory activity of polyphenols is related to the inhibition of several signaling pathways including, the activating protein-1 (AP-1), NF-κB, activation of mitogen activated protein kinase (MAPK), protein kinase-C, nuclear factor erythroid 2-related factor, and activation of phase-II antioxidant detoxifying enzymes.[22] Apigenin, luteolin, kaempferol, and quercetin have been reported as potent inhibitors of lysozyme released from neutrophils.[46] The results suggest that polyphenols play essential roles in anti-inflammatory activities by modulating several parts of the signaling pathways.

Conclusion

Achyranthes aspera Linn. ethanol crude extracts revealed a similar amounts of phenolic and flavonoid contents. The flower extract exhibited the highest antioxidant activity. The crude extracts showed relatively low cytotoxicity against human white blood cells and red blood cells. The flower extract showed weak hemolytic activity. Interestingly, the crude ethanol extracts of *A. aspera* exhibited immunomodulation activity by enhancing phagocytic activity. In addition, the ethanol crude extracts influenced anti-oxidative stress by reducing TNF-α expression specifically in human monocyte-derived macrophages.

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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