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In vitro Antioxidant and Immunological-Associated Activities of Ethanol Extracts of *Azima sarmentosa* (Blume) Benth. & Hook. F

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ABSTRACT

Azima sarmentosa (Blume) Benth. & Hook. F. is a halophyte, which has traditionally been used to treat various diseases. The *in vitro* antioxidant and immunological-associated activities of *A. sarmentosa* ethanolic extracts were evaluated in this study. The roots, stems, and leaves of *A. sarmentosa* were extracted with 70% ethanol. Phytochemicals were screened, and antioxidant activity was determined. The cytotoxicity of the extracts was examined on white blood cells, red blood cells, and the THP-1 cell line. The anti-inflammatory and antiviral properties of extracts were analyzed for expression of TNF-*α*, IL-6, and IFN-β mRNA by quantitative RT-PCR. The results showed that root, stem, and leaf extracts of *A. sarmentosa* contain alkaloids, flavonoids, coumarins, terpenoids, and stigmasterol. Phenolics and tannins were found in stem and leaf extracts while steroids were found only in the leaf extract. Higher levels of phenolic and flavonoid compounds were found in the leaf extract, in line with their antioxidant activities. All extracts showed no cytotoxicity to immune cells. Root, stem, and leaf extracts exhibited anti-inflammatory properties by down-regulating TNF-α and IL-6 mRNA expression in lipopolysaccharide (LPS) pre-treated THP-1 cells. Moreover, root, stem, and leaf extracts also had antiviral properties by up-regulation of IFN-*β* mRNA expression. The down-regulation of TNF-α and IL-6 and up-regulation of IFN-β mRNA expression were also found in stigmasterol and taraxerone-treated cells. The findings of the present study reveal that crude extracts of *A. sarmentosa* have potential anti-inflammatory and antiviral properties that could be used to develop alternative therapeutic strategies.

***Keywords*:** Anti-inflammatory, Antioxidant activity, Antiviral, *Azima sarmentosa*, Phytochemical screening.

Introduction

Saline soils are a major problem in Northeastern Thailand. The salinity results in low agricultural productivity. The Phon Sim saline soils in Kalasin Province, Thailand, have a salt sediment concentration of more than 50%, which is classified as a very high salinity area. Plants that grow can be classified as salt-tolerant plants, or halophytes, which are mostly weeds and are currently underutilized. Such plants include *Azima sarmentosa* (Blume) Benth. & Hook. F.[1] The plant belongs to the Salvadoraceae family. It is a shrub with pointed or lobe-like thorns on the tips of the leaves and lengthy spines on the axillary leaves. This plant is found in Hainan, Southeast Asia, including New Guinea, the Philippines, and Thailand. A hexane extract from the leaf was found to contain three compounds, namely taraxerol, triterpenoid I, and taraxerone. A 95% ethanol extract of the root contains seven compounds, which include stigmasterol, triterpenoid II, 1-methoxy-indole-3-carboxaldehyde, 1H-indole-3-carboxaldehyde, 5-hydroxymethyl furfuraldehyde, stigmasterol-3-O-Beta-D-glucopyranoside, and 1-methoxy-indole-3-acetonitrile.

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Two compounds, 1-methoxy-indole-3-acetonitrile, and 1-methoxy-indole-3-carboxaldehyde were found to have a toxic effect on *Artemia salina* Linn. at 50% lethal concentration (LC_{50}) levels of 0.09 and 9.24 µg/mL, respectively (Figure 1). In Thailand, *A. sarmentosa* root has traditionally been used to treat fever, inflammation, and mumps viral infection.[2]

Figure 1: *Azima sarmentosa* plant showing the leaf and root.

However, there have been only limited reports of the specific mechanism of *A. sarmentosa* on toll-like receptor (TLR) stimulatory or inhibitory properties. The innate immune response plays an important role in initiating the immune response. The first of the two main responses is the inflammation by lipopolysaccharide (LPS) recognition via TLR4, which leads to the production of inflammatory cytokines such as Interleukin (IL)-1, IL-6, IL-8, IL-12p70, and tumor necrosis factor-α (TNF-α).[3] The second is the antiviral defense, which involves recognition of the nucleic acid of intracellular viruses and producing type I interferons (IFNs) via TLR3, 7, 8, or 9.[4] Human monocyte cells have been observed to have a constant expression of TLR1-10.[5] Moreover, TLR3 and TLR4 ligands can up-regulate TRIM family expression in a human monocyte cell line, THP-1, indicating that THP-1 responds to polyinosinic: polycytidylic acid (poly(I:C)) and LPS, respectively.[6]

The present study was therefore conducted to test *A. sarmentosa* extracts for phytochemicals, antioxidant, and cytotoxicity. The functional modifications of THP-1 cells toward TLR3 and TLR4 signaling pathways were also compared to stigmasterol and taraxerone, compounds identified in *A. sarmentosa* extracts.

Materials and Methods

Source of plant materials

The aerial parts and root of *A. sarmentosa* (Figure 1) were collected in December 2019 from 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 of Mahasarakham University's Faculty of Science identified the plant using the Flora of Thailand guidebook.[7] The specimens were deposited in the Natural Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University, under the code number MSUT7441.

Ethical approval

Ethical approval for this study was obtained from the Human Ethics Research Committee of Mahasarakham University on 23 July 2020 with approval number 208/2563.

Preparation of crude extracts of Azima sarmentosa

The plant was rinsed and dried at 50°C for three days before being crushed and passed through an 80-mesh screen. The plant powders were extracted with 70% ethanol at a ratio of 1:10 g/mL for 5 days. The extract was evaporated to remove the solvent by a pressure-reducing evaporator. The crude extract was then freeze-dried and dissolved in 100% dimethyl sulfoxide (DMSO) to a concentration of 100 mg/mL before being filtered through 0.45 and 0.22 μm filters and kept at -20°C.[8]

Phytochemical screening of Azima sarmentosa extracts

All the crude extracts of *A. sarmentosa* were tested for the presence of phytochemicals including alkaloids, phenolics, flavonoids, anthraquinones, coumarin, saponins, tannins, terpenoids, steroids, and glycosides using standardized methods described by Ayoola *et al*.[9] The total phenolic content (TPC) was measured by the Folin–Ciocalteu colorimetric method as modified by Sankla *et al*.[8] TPC was expressed as gallic acid equivalent (mg GAE/g crude extract). Total flavonoid content (TFC) was measured using a colorimetric assay as modified by Sankla *et al*.[8] TFC was expressed as quercetin equivalents (mg QE/g crude extract).

Determination of the in vitro antioxidant activity of extracts of Azima sarmentosa

The *in vitro* antioxidant activity of *Azima sarmentosa* extracts was analyzed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging assays as modified by Sankla *et al*.[8]

Thin-layer chromatographic analysis of crude extracts of Azima sarmentosa

The crude extracts of *A. sarmentosa* were spotted on thin-layer chromatographic (TLC) aluminum silica gel 60 sheets (Sigma, US). The TLC sheet was developed with a mobile phase of isopropanol: deionized water (95:5). The chromatogram was visualized at 254 nm after being sprayed with 5% sulfuric acid (in absolute ethanol) and heated. The retardation factor (Rf) was measured and compared with stigmasterol and taraxerone. The results were analyzed using the formula:

$$Rf = \frac{distance\ travelled\ by\ compound}{distance\ travelled\ by\ solvent\ front}$$

Isolation of $CD14^+$ monocytes from white blood cells and cell culture

Peripheral blood was collected from six healthy adult volunteers who had given written consent. The peripheral white blood cells (WBCs) and $CD14^+$ monocytes were isolated using the procedure described by Sankla *et al*.[8] $CD14^+$ and $CD14^-$ monocytes were cultured at 37°C with 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher, USA) with 5% fetal bovine serum (FBS) (Gibco, Thermo Fisher, USA) + 5% autologous serum + 1X antibiotic-antimycotic (Gibco, USA).

The effect of crude extracts on the survival rate of white blood cells and THP-1 cell line

The cytotoxicity of the crude extracts was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to the method of Sankla *et al*.[8] Briefly, $2x10^4$ cells/well were seeded into a 96-well culture plate, and the crude extract was added at a concentration of 0–512 μg/mL (for white blood cells) and 0–400 μg/mL (for THP-1 cells). DMSO (0.5%) was used as a negative control. The cells were incubated for 48 h (for white blood cells) and both 24 and 48 h (for THP-1 cells) before being treated for 4 hours in the dark with 12 mM MTT (Gibco, USA). The formazan crystals were dissolved with 100 μL of 100% DMSO. The absorbance was measured at 540 nm and the % viability was determined using the formula:

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

Cytotoxic effect of Azima sarmentosa extracts on red blood cells by haemolysis assay

Peripheral blood from six healthy volunteers was centrifuged at 2,000 rpm for 10 min. RBCs from the bottom layer were collected for processing to 2% RBCs in 1X phosphate-buffered saline (PBS). The hemolysis assay was performed according to the method of Sankla *et al*.[8] The crude extracts were employed at concentrations ranging from 0 to 512 μg/mL. As a negative control, 1X PBS and 0.1% DMSO were utilized, and 0.1% Triton-X 100 was employed as a positive control. The % hemolysis was calculated by the equation:

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

Evaluation of the anti-inflammatory and antiviral effects of Azima sarmentosa extracts on THP-1 cells

In a 6-well culture plate, $1x10^6$ cells were seeded and pre-treated for 2 hours with 100 ng/mL LPS-EK, a TLR4 agonist (Invivogen, US), or 100 ng/mL Poly (I: C) HMW, a TLR3 agonist (Invivogen, US).[10,11] After that, the crude extract was added at concentrations of 12.5 and 5 μg/mL and incubated for 24 hours. Cells were collected for total RNA extraction to examine the mRNA expression of IL-6 and TNF-α for anti-inflammatory, and IFN-β for antiviral activity by the quantitative reverse transcription polymerase chain reaction (RT-qPCR) method.[12] The experimental groups were divided as follows: extract-treated group (1), pre-treated with LPS group (2), pre-treated with Poly(I: C) group (3), LPS-treated positive control group (4), poly(I: C) positive-treated control group (5), and negative control group (6).

Statistical analysis

The experiments were performed three times independently. The data were analyzed using analysis of variance (ANOVA) and Tukey's multiple comparison post-test for one-way ANOVA using Prism

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version 9 (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

Phytochemical and antioxidant properties of Azima sarmentosa crude extracts

Herbal-based alternative therapeutic strategies have recently gained attention around the world.[13] This study has demonstrated the promising potential of *A. sarmentosa* extracts derived from plants collected from saline soils.[14] Due to salinity stress, this plant might contain useful secondary metabolites. *Azima sarmentosa* has been employed in a variety of ways, including as an antipyretic, anti-inflammatory, and anti-mumps viral infection.[2] The results showed that the crude extracts' percentage yields were ranked in the following order: leaf, stem, and root, with percentage yield values of 16.79, 9.93, and 7.68%, respectively. Figure 2A depicts the phytochemical screening results for *A. sarmentosa* extracts. Alkaloids, flavonoids, coumarin, and terpenoids were found in all three portions of the crude extracts. Steroids were only discovered in the leaf extract.

A

Phytochemical components	Root extract	Stem extract	Leaf extract
Alkaloids	■	■	■
Phenolics		■	■
Flavonoids	■	■	■
Anthraquinone			
Coumarins	■	■	■
Saponins			
Tannins		■	■
Terpenoids	■	■	■
Steroids			■
Glycoside			

Presence Absence

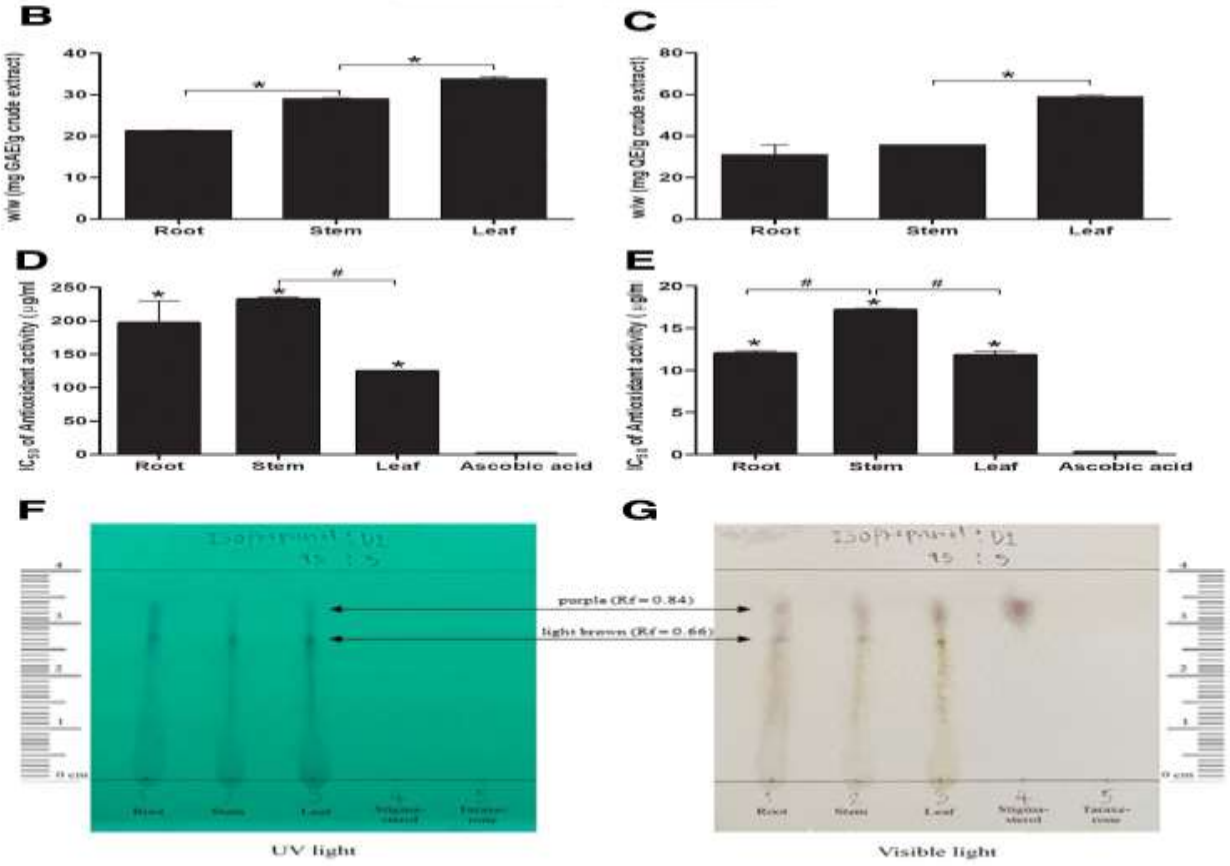

Figure 2: Phytochemical screening, TPC, TFC, antioxidant activities, and TLC screening of ethanolic extracts from *Azima sarmentosa*. A: Phytochemical screening; B: Total phenolic content; C: Total flavonoid content; *: $p \leq 0.05$ compared between the groups; Antioxidant activities of crude extract from D: DPPH assay; E: ABTS assay; *: $p \leq 0.05$ compared to ascorbic acid; #: $p \leq 0.05$ compared between groups; F: TLC separation of crude extract.

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However, phenolic and tannins were present in both the stem and leaf. These results are consistent with a previous finding that detected phenolics and terpenoids in an extract of *A. sarmentosa*.[2] The crude extracts from *A. sarmentosa* contained both phenolics and flavonoids. The standard curves of TPC and TFC were generated by using gallic acid (Y = 0.0112X - 0.0676, R^2 = 0.9883) and quercetin (Y = 0.001X - 0.001735, R^2 = 0.9733), respectively. The leaf extract had the highest phenolic and flavonoid content, with more than 20 mg GAE/g crude extract and more than 25 mg QE/g crude extract, respectively, followed by the stem and root extracts (Figures 2B and 2C).

The antioxidant activities of the extracts were determined by the DPPH and ABTS radical scavenging assays, using ascorbic acid as a standard control. The 50% inhibitory concentration (IC_{50}) values of root, stem, leaf, and ascorbic acid by DPPH assay were 197.133 ± 46.660, 232.805 ± 4.774, 124.856 ± 1.012, and 1.574 ± 0.002 µg/mL, respectively (Figure 2D). The IC_{50} values by ABTS assay were 12.026 ± 0.387, 17.180 ± 0.216, 11.811 ± 0.655, and 0.291 ± 0.068 µg/mL, respectively (Figure 2E). In both assays, the leaf extract demonstrated stronger antioxidant activity than the stem and root extracts. TLC revealed that leaf, stem, and root extracts had a dark purple dot with an Rf of 0.84. The Rf value also coincided with the Rf of stigmasterol. However, Rf values corresponding with taraxerone were not found using the same reading method (Figures 2F-2G). This result is also similar to a previous study of *A. sarmentosa*, which revealed triterpenoids (taraxerol, taraxerone, and triterpenoid I) in a hexane extract of the leaf. In a 95% ethanol extract of the root, triterpenoids (triterpenoid II), phytosterol (stigmasterol), and flavonoids (stigmasteryl-3-O-beta-D-glucopyranoside) were discovered (Figure 1).[2] The crude extracts of *A. sarmentosa* were then confirmed as containing stigmasterol and taraxerone by TLC assay. The results indicated that stigmasterol may be present in all three crude extracts, although taraxerone could be present at very low concentrations (Figures 2F-2G). Two approaches were used to assess antioxidant activity in this investigation. In both assays, the leaf extract demonstrated stronger antioxidant activity than the root and stem extracts (Figures 2D-2E), indicating that the presence of phenolics and flavonoids may contribute to antioxidant characteristics.[15,16]

Cytotoxicity of Azima sarmentosa extracts on white and red blood cells

The cytotoxic effects of *A. sarmentosa* extract on cell viability of total white blood cells, $CD14^+$ monocytes, and $CD14^-$ non-monocyte were expressed as % viability cells, as shown in Figure 3A–3I.

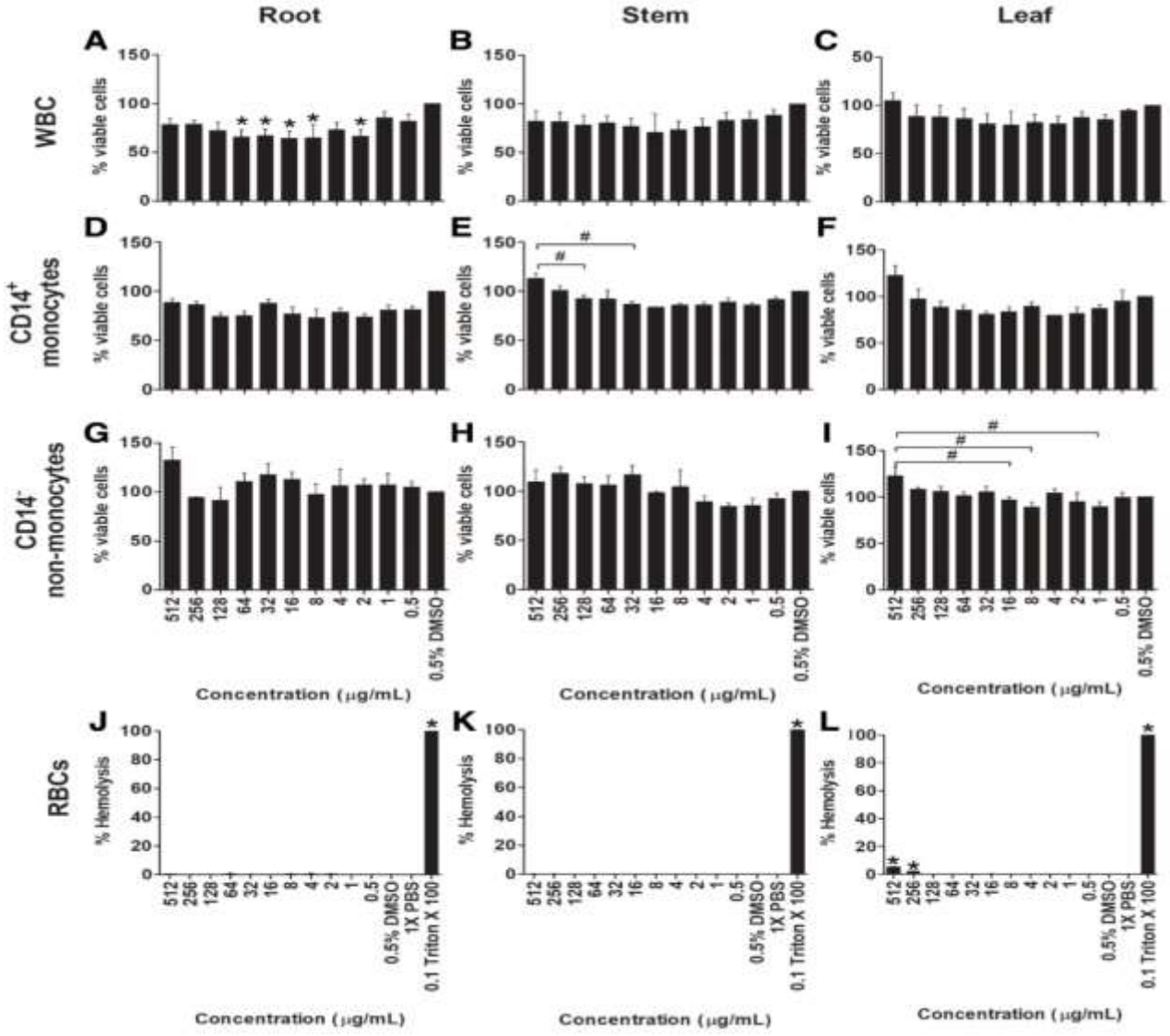

Figure 3: Percentage of viable cells after treatment with crude *Azima sarmentosa* extracts. A–C: Total white blood cells; D–F: $CD14^+$ monocytes; G–I: $CD14^-$ non-monocytes; J–L: Percentage of hemolysis of red blood cells; *: $p \leq 0.05$ compared to 0.5% DMSO; #: $p \leq 0.05$ comparison between groups.

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The crude extract was not cytotoxic to any of the three cell types tested at concentrations ranging from 0 to 512 µg/mL. Only the root extract was subtoxic to total WBCs, with more than 60% viable cells. Therefore, *A. sarmentosa* root extract had subtoxic effects on total WBCs, whereas leaf and stem extracts had no toxicity for WBCs. At high concentrations, the root and stem extracts had no hemolytic activity, whereas the leaf extract had very low hemolytic activity at high concentrations (Figures 3J-3L). The toxicity of the crude extract on THP-1 cells was examined at 24 and 48 h by MTT assay. Root extract was non-toxic after 24 hours but mildly harmful after 48 h (Figure 4A). Stem extract showed no toxicity at 24 h but was moderately toxic at 48 h at a concentration of 25–400 µg/mL (Figure 4B). Leaf extract showed no toxicity at 24 h but was moderately toxic at 48 h at concentrations of 50–400 µg/mL (Figure 4C). Meanwhile, stigmasterol did not show toxicity after 24 and 48 h (Figure 4D), and taraxerone showed moderate toxicity at concentrations of 6.25–50 µg/mL over 24 and 48 h (Figure 4E). Therefore, the concentrations of 12.5 and 25 µg/mL were selected for further analysis of leaf, stem, and root extracts of *A. sarmentosa*. The concentrations selected for stigmasterol and taraxerone were 2.5 and 5 µg/mL, respectively. The 95% ethanolic extract of *A. sarmentosa* root was found to be cytotoxic to *Artemia salina* Linn.[2] However, extracts from the stem and leaves of *A. sarmentosa* demonstrated no cytotoxicity on total WBC, $CD14^+$ monocytes, or $CD14^-$ non-monocytes in the present study, except for the root extract, which had subtoxic effects on total WBCs. Furthermore, the cytotoxicity of monocytes was confirmed by testing the THP-1 cell line, which is a human monocyte cell line derived from individuals with acute leukemia. The results demonstrated that THP-1 cells were not cytotoxic.

Anti-inflammatory and antiviral properties of Azima sarmentosa extracts on THP-1 cells

The effect of the *A. sarmentosa* extracts on the LPS-TLR4-mediated inflammation of THP-1 cells was examined by the expression of pro-inflammatory cytokines, TNF-α and IL-6. The poly (I: C)-TLR3-mediated antiviral properties were determined by the expression of type I IFN and IFN-β. Root and leaf extracts at 25 µg/mL and taraxerone at 2.5 and 5 µg/mL stimulation did not up-regulate the TNF-α expression when compared to the negative control group. Meanwhile, the stem (12.5 µg/mL) and stigmasterol stimulation (12.5 and 25 µg/mL) showed slight expression of TNF-α. However, the LPS-pre-treated THP-1 did elicit expression of TNF-α with any extract when compared to the LPS-stimulated positive control (Figure 5A). IL-6 was shown to be up-regulated in LPS-treated cells, whereas it was found to be down-regulated in the root, stem, leaf, stigmasterol, and taraxerone treatment groups (Figure 5B). This suggests that *A. sarmentosa* extracts, stigmasterol, and taraxerone were anti-inflammatory. IFN-β expression was found in root, stem, leaf extracts, stigmasterol-, and taraxerone-treated groups when compared to the negative control.

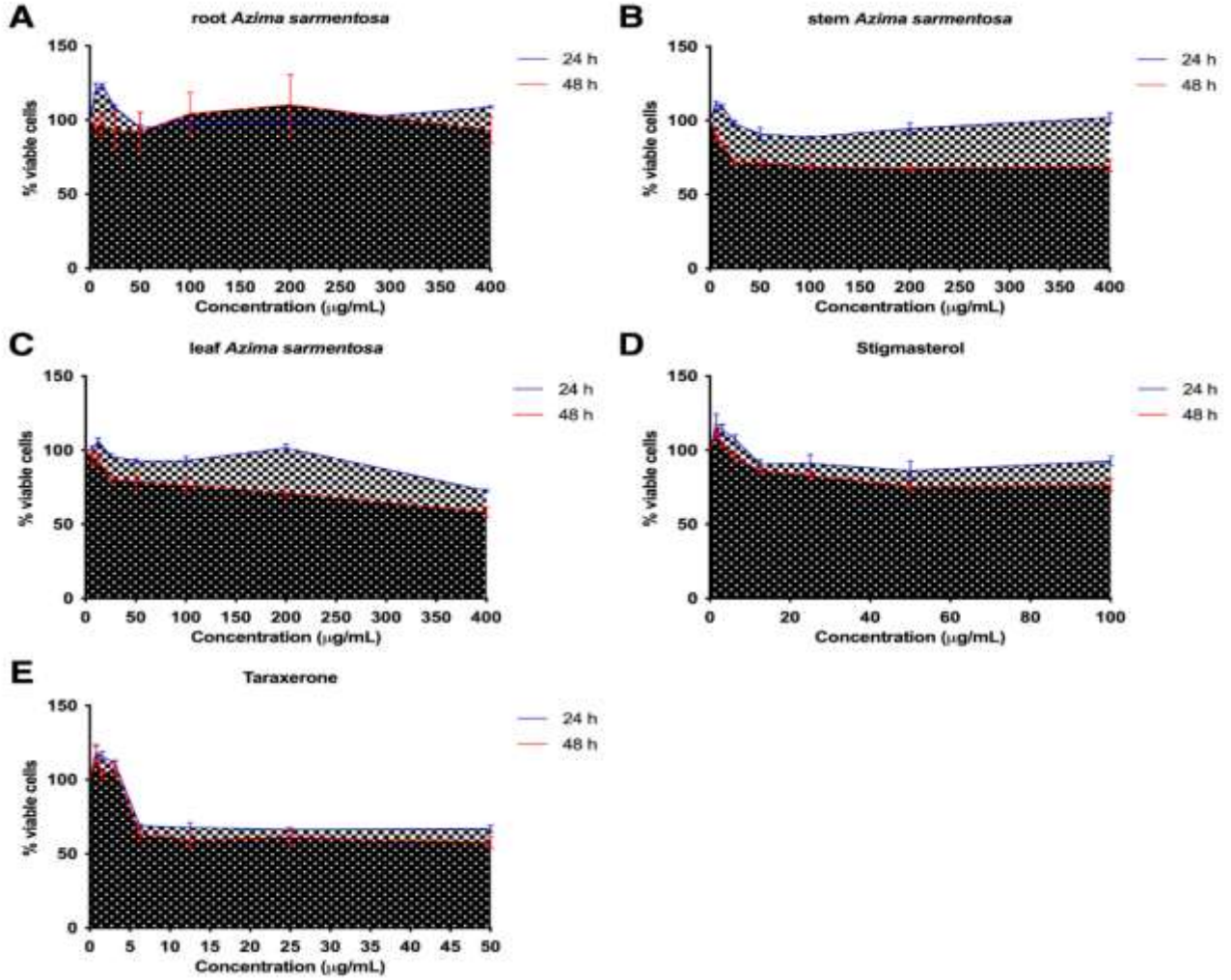

Figure 4: Percentage viability of THP-1 cells treated with crude extracts of *Azima sarmentosa* and compounds. Percentage of viable cells of THP-1 cells were treated with A: Root extract; B: Stem extract; C: Leaf extract; D: Stigmasterol; E: Taraxerone for 24 and 48 h. The values were presented as mean ± SD of data in three times independently.

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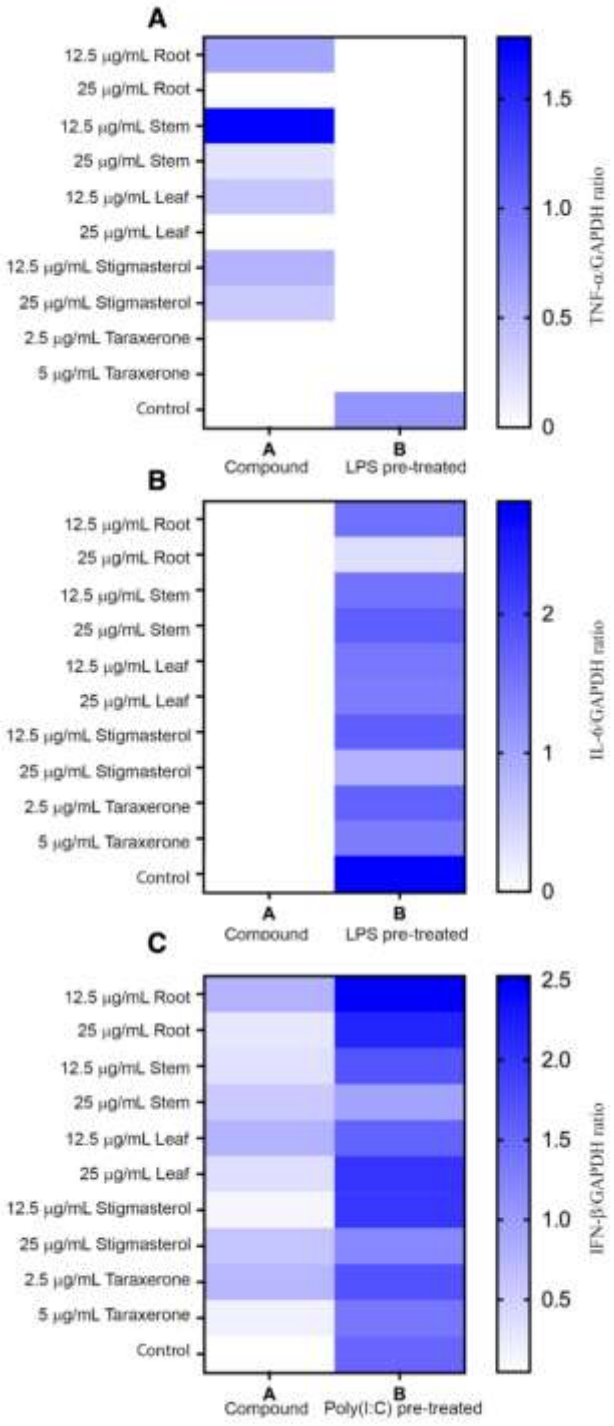

Figure 5: The expression of pro-inflammatory cytokines and antiviral cytokine. The expression of A: TNF-α; B: IL-6; C: IFN-β on THP-1 cells were determined by quantitative RT-PCR.

IFN-β was shown to be up-regulated in poly (I: C)-pretreated THP-1. Higher expression of IFN-β was observed in the root, stem, leaf, stigmasterol-, and taraxerone-treated groups when compared to the poly (I: C)-pre-treated group (Figure 5C), indicating that *A. sarmentosa* extracts, stigmasterol, and taraxerone had antiviral properties by up-regulation of IFN-β expression.

The signal transduction of LPS-TLR4-mediated inflammation via the nuclear factor kappa B (NF-κB) pathway, leads to the production of pro-inflammatory cytokines such as IL-1, IL-6 IL-12p70, and TNF-α.[3]

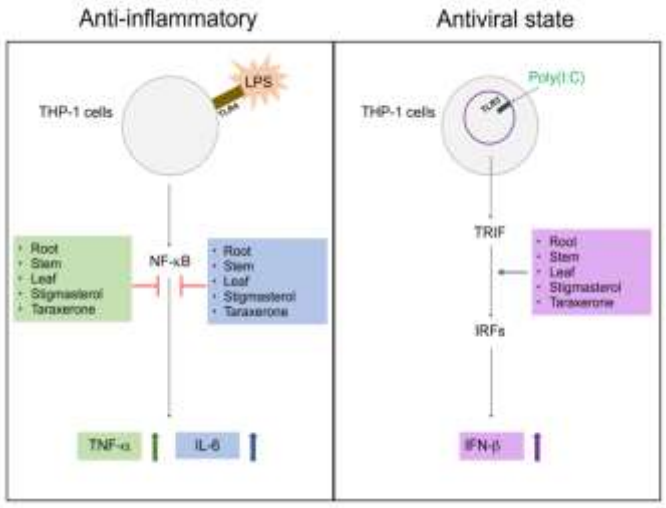

Figure 6: A demonstration of anti-inflammation and antiviral activities of crude extracts from *Azima sarmentosa*.

This signal transduction might be interrupted by root, stem, and leaf extracts, which also showed similar effects with stigmasterol and taraxerone. The antiviral state was induced during the recognition of intracellular viral nucleic acid by endosomal TLR (TLR3, TLR7, TLR8, and TLR9).[4]

The root, stem, and leaf extracts may improve IRF signal transduction and promote the generation of antiviral Type I interferons (IFN-α and IFN-β). In THP-1 cells, both stigmasterol and taraxerone increased IFN-β production (Figure 6). Stigmasterol is an unsaturated plant cholesterol found in various herbs, such as *Aralia cordata, Akebia quinata*, *Croton sublyratus*, *Desmodium styracifolium*, *Eclipta alba* (L.) Hassk, *Eclipta prostrate*, *Eucalyptus globules*, *Emilia sonchifolia*, *Ficus hirta*, *Gypsophila oldhamiana*, *Heracleum rapula* and *Parkia speciosa*.[17] Stigmasterol has pharmacological properties including, antitumor, antihypercholesterolemic, anti-arthritis, antioxidant, anti-inflammatory[17], reduction of oxidative stress and control of DNA damage, anti-angiogenesis of various cancer cells, increased caspase enzyme activities and increased LPS-mediated TNF-α secretion.[18,19] Taraxerone found in *Leucas Lavandulifolia*, which has immune modulation properties such as inhibiting IL-4 and IL-6 production, could be used to treat allergic reactions.[20]

Conclusion

The findings of this study reveal that ethanolic extracts of *A. sarmentosa* have beneficial phytochemicals and antioxidant activity. Furthermore, all crude extracts demonstrated potential anti-inflammatory and antiviral properties in THP-1 cells by down-regulating pro-inflammatory cytokines (TNF-α and IL-6) and up-regulation of IFN-β in THP-1 cells without showing toxicity to white blood cells (total WBCs, $CD14^+$ monocytes, and $CD14^-$ non-monocytes), red blood cells, and THP-1 cells. The anti-inflammatory and antiviral properties could be employed to develop alternative therapeutic strategies.

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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