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Original research article

Free radical scavenging and immunomodulatory activities of the aqueous extract of *Annona muricata* Linn

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ABSTRACT

Annona muricata Linn. has been traditionally used worldwide as an herbal medicine to treat various ailments. All parts of this plant contain several phytochemicals with numerous ethnomedicinal properties, including anti-infective, anti-inflammatory, anticonvulsant, antidiabetic, anticancer, and hepatoprotective activities. This study aimed to assess the antioxidant and immunomodulatory activities of the aqueous extract of *A. muricata* leaves (AM) by using DPPH-radical scavenging and macrophage activation tests. AM was extracted from *A. muricata* dried leaf powder using distilled water at room temperature. The total phenolic and flavonoid contents of AM were assessed, and its antioxidant capacity was determined using the DPPH method. The immunomodulating effect of AM was evaluated on macrophages by determining phagocytic activity using the zymosan-nitroblue tetrazolium (NBT) reduction assay, nitric oxide (NO) production using the Griess reduction assay, and the expression and protein levels of inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) were determined by RT-PCR and Western blot analysis. AM demonstrated its antioxidant potential in correlation with its phenolic and flavonoid contents. AM, at concentrations ranging from 0.312 to 10 µg/ml, significantly increased NO levels, iNOS and TNF-α expression, and induced zymosan phagocytosis in RAW 264.7 macrophages. These results suggest that the aqueous extract of *A. muricata* leaves possesses antioxidant and immunomodulatory potentials. These findings contribute to a better understanding of the medicinal properties of this plant.

Keywords: *Annona muricata*, free radical scavenging, immunomodulatory, macrophages, leave extract

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https://li01.tci-thaijo.org/index.php/JBAP

1. Introduction

For centuries, numerous medicinal plants have been alternatively used for the treatment of various common diseases and many serious diseases such as infections, immunological disorders, and cancer.[1-3] *Annona muricata*, or "thurain-thet" in Thai, is a plant in the *Annonaceae* family and is widely distributed throughout tropical countries, including Thailand. This plant is well-known for its edible fruits and medicinal uses. Several phytochemicals, including acetogenins, alkaloids, phenolic acids, and flavonoid glycosides, have been identified and studied for their activities.[4-8] Several studies have revealed the pharmacological properties of *A.muricata*, such as its antidiabetic, antioxidant, anti-inflammatory, anticancer, and immunomodulatory potentials.[9-15] The immune system is a crucial defense part for protecting the body from any harmful pathogens and foreign antigens. Many herbal medicines have been used as immune modulators to promote health. Several plants containing a wide range of active constituents including terpenoids, flavonoids, glycosides, alkaloids, glucans, and polysaccharides, have been shown to have immunomodulating activities by modulating the immune response, increasing lymphocyte proliferation, and activating macrophages.[16,17] Recent studies have reported that the hot water and the ethanol extracts of *A. muricata* leaves upregulated iNOS and TNF-α and IL-1β expression.[13,14] Although these pharmacological activities of *A. muricata* have been reported, little is known about the immunomodulating activity of *A. muricata* constituents extracted by water at room temperature. So, we intended to examine the immunomodulating effect of aqueous extracts of *A. muricata* leaves on the phagocytosis and the production of NO, and TNF-α production in RAW264.7 macrophages by using distilled water at room temperature. This study also determined antioxidant capacity as well as the phenolic and flavonoid contents of AM.

2. Materials and Methods

2.1 Plant material

The leave part of *Annona muricata* Linn. was collected in Pakpanang district, Nakhon Si Thammarat, Thailand during April 2019. The voucher specimen was identified and deposited at Walailak Botanical Park, Walailak University.

2.2 Preparation of extract

Fifty grams of dried and powdered leaves of *A. muricata* was soaked in distilled water at room temperature for 48 h. The supernatant was filtered through a Whatman paper NO.1 The filtered solution was dried in a rotary evaporator and then lyophilized to become the solid form of *A. muricata* aqueous extract (AM). AM was prepared as AM solutions in either DMEM for determination of cell viability and immunomodulating activity, or in methanol for determination of total phenolic and flavonoid content, and antioxidant capacity.

2.3 Determination of total phenolic content

The total phenolic content of AM was determined using the Folin-Ciocalteu colorimetric method with some modifications.[18] Gallic acid was used as a standard phenolic compound. Briefly, 25 µl of 1 mg/ml AM was mixed with diluted Folin-Ciocalteu reagent (1:1 v/v with distilled water) (Sigma-Aldrich, Switzerland) and 100 µl of 7.5% Na_2CO_3 solution, in a 96-well plate for 2 h in the dark. The absorbance of the mixture was measured at 765 nm. The phenolic content of the mixture was determined from a gallic acid calibration curve. The total polyphenol content of AM was presented as gallic acid equivalent (GAE) in mg/g of the extract.

2.4 Determination of total flavonoid content

Total flavonoid content of AM extract was determined using an aluminum chloride ($AlCl_3$) colorimetric assay.[18] Briefly, 50 µl of 1 mg/ml AM was mixed with 150 µl methanol, 10 µl of 10% $AlCl_3$, 10 µl of 1M potassium acetate, and 30 µl of distilled water. The mixture was kept in the dark for 30 min. Absorbance of the mixture was

measured at 415 nm by a microplate reader (TECAN, Infinite ™ M200 PRO, Männedorf, Switzerland). The total flavonoid content of the mixture was determined from a quercetin calibration curve and presented as quercetin equivalent (QE) in mg/g of the extract.

2.5 Determination of DPPH radical scavenging activity

The antioxidant activity of AM was determined by the 1, 1- diphenyl- 2- picryl hydrazyl (DPPH) radical scavenging activity assay,[19] with slight modification. Briefly, 5 µl of various concentrations (10, 30, 100, 300 µg/ml) of AM was allowed to react with 195 µl of 100 µM DPPH (Sigma- Aldrich, USA) solution in a 96- well plate in the dark for 30 min. The absorbance was measured at 515 nm using a microplate reader. Gallic acid was used as the positive control and methanol was used as the negative control. The scavenging activity was determined as follows: The percentage inhibition = (Absorbance of negative control - Absorbance of sample) X 100/ Absorbance of the negative control. The 50% scavenging concentration (SC_{50}) was calculated by using linear regression with Microsoft Excel.

2.6 Determination of cell viability

Raw 264.7 cells (4 x 10^5 cells/ml) were cultured in a 96- well plate for 24 h at 37°C. The cells were treated with AM at concentrations of 0.312, 0.625, 1.25, 2.50, 5, and 10 µg/ml for 24 h at 37°C. Then, 20 µl of 5 µg/ml of MTT solution was added to each well, and the cells were incubated for an additional 3 hours at 37°C. The supernatant of the treated cells was removed and 100 µl of DMSO (Merck, USA) was added to dissolve the formazan crystals formed by viable cells. The absorbance at 550 nm was measured using a microplate reader. DMEM treated cells were used as the control, and their viability was set as 100%.

2.7 Determination of macrophage phagocytosis

In vitro phagocytic activity of Raw 264.7 cells was determined using the zymosan-nitrobluetetrazolium (NBT) reduction assay.[21] Briefly, Raw 264.7 cells were treated with AM (0.312-10 µg/ml) in a 96-well plate for 24 h at 37 °C. The treated cells were washed twice with DMEM and then incubated with zymosan (800 µg/ml) and NBT (600 µg/ml) in PBS at 37 °C for 1 h. After carefully removing the supernatant, the cells were washed twice with methanol, air dried, and mixed with 2M KOH and DMSO. The absorbance was measured at 570 nm using a microplate reader. The phagocytic stimulation index (SI) was assessed as the OD ratio of the AM- treated condition to the OD of the DMEM-treated condition. LPS at 100 ng/ml was used as the positive control.

2.8 Determination of NO production

Raw 264.7 cells (4x10^5 cells/ml) were treated with an AM at concentrations ranging from 0.312 to 10 µg/ml for 24 h in 96-well plates. Complete DMEM medium and 100 ng/ml LPS were used as the negative and positive control respectively. A hundred µl of the supernatants of the treated cell were aliquoted into a 96- well plate, mixed with Griess reagent, and incubated for 10 min at room temperature in the dark. The nitrite reacts with Griess reagent and forms a purple azo dye which can be detected at 540 nm. The amount of nitrite, which is correlated to the amount of NO, was calculated from nitrite standard curves.

2.9 Detection of iNOS and TNF-α mRNA expression by RT-PCR

Raw 264.7 cells (4x10^5 cells/ml) were treated with AM or LPS for 24 h. The treated cells were collected for RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. From each sample, 1 µg of total RNA was reverse transcribed to single-stranded cDNA by M-MLV reverse transcriptase (Promega, USA). Then polymerase chain reaction (PCR) analyses were performed on the aliquots of the cDNA preparations to detect inducible NO synthase (iNOS), and TNF- α gene expression. *β-Actin* was used as an internal control. The primers used were as follows: iNOS forward, 5′-CCC TTC CAA GTT TCT

GGC AGC AG-3′ and reverse, 5′-GG CTG TCA AGC CTC GTG GCT TTG G-3′ (403 bp); TNF-α forward, 5′-ATG AGC ACA GAA AGC ATG ATC-3′ and reverse, 5′-TAC AGG CTT GTC ACT CGA ATT-3′ (364 bp); and β-actin forward, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and reverse 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′ (603 bp). All primers were synthesized by Eurofins Genomics (Germany). PCR was performed using 1 µl cDNA sample and 24 µl PCR reaction mixtures containing target gene specific primer, mixed with dNTP and Taq polymerase in PCR buffer. Amplification was done for 30 cycles with initial denaturation at 94°C for 3 min, followed by 1 min each for denaturation, annealing, extension at 94°C, 55°C, 72°C and final extension at 72°C for 7 min. The PCR products were then analyzed on a 1.5% (W/V) agarose gel, and stained with FluoroDye Fluorescent DNA Loading Dye for loading and detecting DNA markers (SMOBiO, Taiwan). The findings were captured or visualized using gel documentation equipment (Bio-Rad, USA).

2.10 Capillary Western blot

The treated cells were lysed with RIPA buffer (Abcam, UK) on ice, sonicated, and the supernatant of the lysate was collected for protein determination. Protein concentration was determined using a BCA protein assay (Thermo Fisher Scientific, USA). Protein samples of 1 µg were used for Western blot analysis. The Western blots were performed using the Jess Simple Western System, a ProteinSimple automated Western blot system, following the principles of Western blot analysis and employing a specific capillary vacuum system as instructed by the company. (Protein Simple, San Jose, CA, USA).[22] Protein samples and reagents, including blocking reagent, wash buffer, primary antibodies, secondary antibodies, and chemiluminescent substrate, were prepared and sequentially dispensed into the automated system according to instructions. Protein separation, detection, and quantitation were automatically performed. The primary antibodies were used for determining iNOS (Abcam, UK), TNF-α (Abcam, UK) and *β-actin* (Sigma-Aldrich, USA).

2.11 Statistical analysis

Data are presented as mean± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Turkey's post hoc test. P-values below 0.05 were considered statistically significant.

3. Results

3.1 Antioxidant activities and quantitative phytochemical estimation

The total phenolic and flavonoid content of AM was determined by the Folin-Ciocalteu colorimetric method and the $AlCl_3$ colorimetric assay, respectively. The extract had a phenolic content of 36.7±5.4 mg GAE/g and a flavonoid content of 322.7±11.7 mg QE/g. The antioxidant activity of the extract was evaluated by DPPH radical scavenging. This extract exhibited antioxidant activity with a SC_{50} value of 282.3±8.7 µg/ml (Table1.).

3.2 Immunomodulatory effect of AM on phagocytosis

The effect of AM on the phagocytic activity of RAW 264.7 macrophages was determined from the amount of the formazan product formed in the NBT reduction assay. The extract at all concentrations (0.312-10 µg/ml) significantly increased phagocytic activity of Raw 264.7 cells when compared with the DMEM-treated control (Fig.1). The extract at all concentrations did not have any effect on the viability of RAW 264.7 cells, as determined by MTT assay (Fig. 2).

3.3 Effect of AM on NO production

NO plays a role in phagocytosis by killing microorganism in phagolysosomes of activated macrophages. The effect of AM on NO production from RAW 264.7 cells was determined after the cells were treated with the extract for 24 h. The extract potently elevated NO level, in a concentration-dependent manner, when compared to the

DMEM-treated control. AM increased NO levels to 1.07±0.16, 3.86±0.56, 11.1±3.9, 12.4±2.8, 14.7±1.7 and 15.9±0.90 µM at concentrations of 0.312, 0.625, 1.25, 2.50, 5 and 10 µg/ml, respectively. LPS at 100 ng/ml, as the positive control, also potently increased the NO level (Fig.3).

Table 1. Total phenolic content, total flavonoid content, and antioxidant effect (SC_{50}) of aqueous *Annona muricata* (AM) extract.

Sample	Phenolic content mg GAE/ g of extract	Flavonoid content QE/g of extract	Scavenging ability on DPPH radical (SC_{50} µg/ml)
AM	36.7±5.4	322.7±11.7	282.3±8.7
Gallic acid	-	-	8.5±0.7

The data are expressed as the means ± SD of three independent experiments.

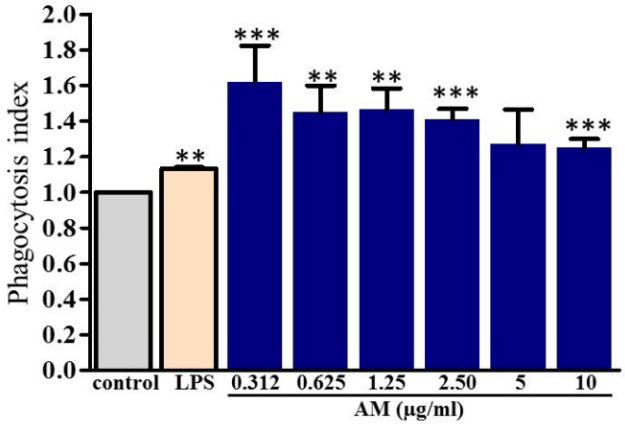

Fig. 1. Effect of AM on phagocytosis. Raw 264.7 cells were treated with various concentrations of AM for 24 h. Data are presented as mean ± S.D. of triplicate measurement. **p-value < 0.01, ***p-value < 0.001 compared to control.

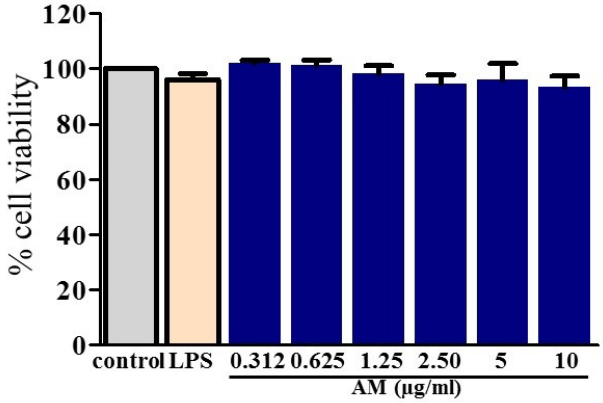

Fig. 2. Effect of AM on cell viability. Raw 264.7 cells were treated with 0.312, 0.625, 1.25, 2.50, 5, and 10 µg/ml of AM or LPS at 100 ng/ml for 24 h. Data are presented as mean ± S.D. of triplicate measurements.

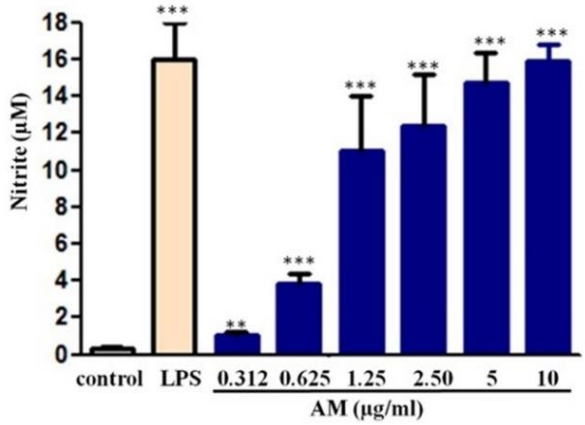

Fig. 3. Effect of AM on NO production. Raw 264.7 cells were treated with 0.312, 0.625, 1.25, 2.50, 5 and 10 μg/ml of AM for 24h. Data are presented as mean ± S.D. of triplicate measurements. **p-value < 0.01, ***p-value < 0.001 compared to control.

3.4 Effect of AM on mRNA and protein expression of iNOS and TNF-α

Large amounts of NO in activated macrophages are generated by iNOS and under regulation by TNF-α. This study investigated the effect of AM on the mRNA expression and on protein levels of iNOS and TNF-α in Raw 264.7 cells by RT-PCR and capillary Western blot, respectively. The results are shown in Fig. 4A and Fig. 4B. The extract remarkably increased both mRNA and protein levels of iNOS and TNF-α after RAW 264.7 cells were treated with the extract for 24 h.

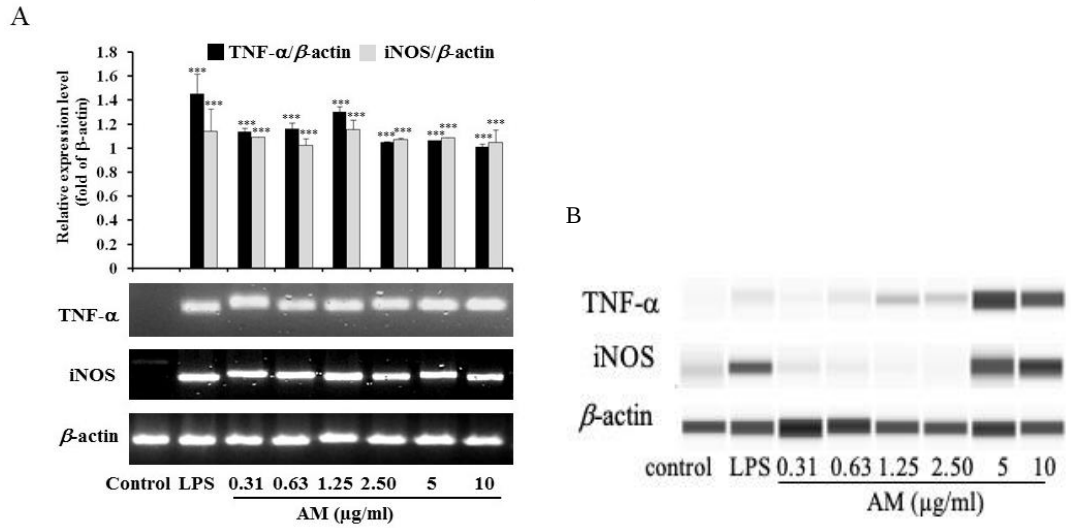

Fig. 4. Effect of AM on the mRNA and protein expression of iNOS and TNF-α. Raw 264.7 cells were treated with 0.312, 0.625, 1.25, 2.50, 5 and 10 μg/ml of AM for 24 h. Total RNA was prepared and mRNA levels encoding iNOS and TNF-α were determined by RT-PCR (A). The protein levels of iNOS and TNF-α were determined by capillary Western blotting (B). β-actin was used as an internal control. The data represent the means ± S.D. of duplicate experiments. ***p-value < 0.001 compared to control.

4. Discussion

Recently, there has been a surge in interest in exploring the pharmacology and clinical potential of herbal extracts and their derivatives, whether employed independently as drugs or as supplementary agents in conjunction with chemotherapy and immunotherapy. Since herbal extracts are often recognized as highly "foreign" by the host immune system, it can have immunostimulatory effect by virtue of local accumulation and an increase in immune cells.[23] Macrophages play a crucial role in defending the host against infection and cancer.[24] Another effect of macrophages on pathogens is the secretion of cytokines, including TNF- α, and inflammatory mediators, such as NO, IL-1β. NO is one of the key mediators of activated macrophages and is involved in the host defense against pathogens[25] and tumor cells.

To the best of our knowledge, this study represents the first exploration of the immunomodulatory activity of an aqueous extract of *A. muricata* leaves prepared at room temperature. AM significantly induced NO production in macrophages. It upregulated the mRNA and protein expression of iNOS and TNF-α.

Previously Kim et al, reported that the aqueous extract using hot water and the ethanol extract of *A. muricata* leaves had immunomodulatory effects on RAW 264.7 macrophages by upregulating TNF- α, interleukin-1β mRNA expression, and only the aqueous extract upregulated iNOS mRNA expression.[13] These effects of both extracts resulted from the activation of the MAP kinase pathways. The effect of AM in our study confirmed the immunomodulatory effects of the hot water extract on TNF-α and iNOS expression, and on NO levels. We have found that AM increases not only mRNA expression but also protein levels of TNF-α and iNOS. We have further demonstrated that AM activated phagocytic activity of macrophages. Phagocytosis is also one of the crucial functions of macrophages for eliminating pathogens, foreign antigens, as well as apoptotic cells. This function of macrophages is an important part for both innate and specific immune response, and very important for keeping homeostasis of cells in the body. This effect of AM further confirmed the potential benefits of aqueous extract of *A. muricata* leaves. In contrast, Saraiva et al. reported that ethyl acetate and butanol extracts of *A. muricata* leaves demonstrated antioxidant and anti-inflammatory properties in activated bone marrow-derived macrophages (BMDMs).[26] In activated BMDMs, both extracts inhibited ROS and IL-6 production, but had no effect on phagocytotic activity and TNF-α levels of these activated cells. The ethyl acetate extract also had *in vivo* antioxidant and anti-inflammatory activities in mice with LPS-induced acute lung injury. It is highly likely that these different effects among the several studied solvent extracts come from the variety of active compounds as well as differing relative amounts of the same active compounds in each solvent used for extraction. By extracting in water of different temperatures, it is also possible to yield different amounts of the same active compounds of *A. muricata* leaves because we have observed a more potent immunomodulating activity on RAW 264.7 cells by AM extracted with cold water compared to warm water. The total phenolic content of AM in our study was lower than that of the aqueous extract in the previous study (36.7±5.4 mg GAE/g (Table 1) versus 683.7 mg GAE/g),[27] while both extracts contained quite similar total flavonoid amounts (322.7±11.7 mg QE/g (Table 1) versus 209.5-372.9 mg QE/g).[29] According to many reports, various phenolic compounds and flavonoids exhibit potent antioxidant capacities.[29] Due to the low content of phenolic compounds in AM, its potential free radical scavenging activity was not high, as determined by a DPPH radical scavenging assay. This activity may instead result mainly from flavonoid compounds identified in the extracts. The antioxidant potential of *A. muricata* leaf extracts were investigated in many in

vitro and in vivo studies. Their antioxidative capacities vary depending on solvents and conditions of extraction, methods and models used for testing.[28] Several phenolic compounds and flavonoids in various plant species have been reported to have both antioxidant and immunomodulating activities.[29,30] Although the immunomodulatory activity of AM has been primarily evaluated in this study, several further studies are needed to investigate other potential activities associated with the use of this medicinal plant, as well as to elucidate the main bioactive compounds in the extract. One of the key limitations of this study is the lack of identification of the main bioactive compounds in the extract. Further research will focus on isolating the active constituents and elucidating the immunomodulatory effects in animal models.

5. Conclusion

In conclusion, results from this study confirm the immunomodulatory activity of *A. muricata* leaves. This study also reveals that different conditions of water extraction can yield different amounts of active compounds. However, only a part of the medicinal activity of *A. muricata* leaves was elucidated by this study.

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Conflicts of Interest

The authors declare that they hold no competing interests.

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