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PHYTOCHEMICAL COMPOSITION AND ANTIINFLAMMATORY ACTIVITY OF *NELUMBO NUCIFERA* GAERTN

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ABSTRACT

Objective: Inflammation is a process of injuries caused by physical, chemical, and biological factors. Nitric oxide (NO) plays an important role in the regulation of various pathological and pathophysiological processes. Overproduction of NO induces tissue damage associated with acute and chronic inflammations. This study was conducted to determine the phytochemical composition and the NO inhibitory properties of *Nelumbo nucifera* extracts in lipopolysaccharide(LPS)-stimulated macrophage cell line.

Methods: The dried leaf, stalk, and flower materials of roseum plenum and album plenum (AP) were extracted with 95% ethanol solvents. The phytochemical compounds of the extraction were analysed by gas chromatography-mass spectrometry. The cytotoxic assay of extracts against macrophage cells was conducted using resazurin. The NO was determined using LPS-induced RAW264.7 cells to measure inhibitory activity of extract on the production of NO.

Results: The extracts from Lotus, which exhibited the non-cytotoxic to the RAW264.7 cells. The AP-stalk extracts were capable to reduce the NO level in LPS-activated RAW264.7 cells. GC-MS analysis of AP-stalk extraction revealed pharmacologically active compounds.

Conclusion: The results conduct that the AP-stalk extract effectively inhibited the NO production and may be useful in preventing inflammatory diseases mediated by excessive production of NO. Bio-active phytoconstituents from AP stalk extract could potentially be used for anti-inflammation. These data also suggest that AP-stalk extract may serve as a good indicator of the pharmacological activities of medicinal plants.

Keywords: *Nelumbo nucifera* Gaertn., Nitric oxide, RAW264.7 macrophage cell, Cytotoxicity.

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INTRODUCTION

Inflammation is a protective process of microcirculation in the host, with variety of injuries caused by physical force, irradiation, high temperature, irritation, and most infectious pathogens [1,2]. Inflammation refers to the complex response of the tissue to the injurious agents and the damaged or dead cell or tissue [3]. Major reactions of inflammation include blood vessel changes, leukocyte migration, and changes in body systems.

These reactions occur in the microcirculation are a reaction that protects the tissues and removes the harmful injurious agents, as well as removing damaged or dead tissue [4]. In addition, inflammation plays a role in the repair. Under normal physiological conditions, nitric oxide (NO) plays an important role in controlling and responding to host defenses response against various pathophysiological processes such as neuronal communication, vasodilatation, and neurotoxicity [5,6]. However, overproduction of NO induces tissue damage associated with acute and chronic inflammations [7]. Consequently, more attention is now being paid to the development of new substances as potent inhibitors of NO production in relation to the treatment of chronic inflammatory diseases [8]. Macrophages are important components of the mammalian immune system, and they play a key role by providing an immediate defense against foreign agents before leukocyte migration and production of various pro-inflammatory mediators, including the free-radical NO [9]. Lipopolysaccharide (LPS), a component from the cell walls of Gram-negative bacteria, is one of the most effective activators of macrophages and involves the production of pro-inflammatory cytokines [10]. Therefore, inhibition of NO production in LPS-stimulated RAW264.7 cells is one of the possible ways to screen various anti-inflammatory drugs.

The lotus, also known as *Nelumbo nucifera*, comes under the family Nelumbonaceae, which has various common names as Sacred lotus, Indian lotus, and Chinese water lily. All parts of *N. nucifera* are used as oriental medicine for use as a cure for various diseases [11]. The seeds of *N. nucifera* are used as a diuretic and cooling agent, antiemetic, antidote for the treatment of inflammatory tissue and cancer [12]. The leaves of *N. nucifera* are considered the best for "over-coming body heat," and stop bleeding, and it is used as a drug for hematemesis, epistaxis, hemoptysis, hematuria, and metrorrhagia [13]. It is reported that the extract of lotus rhizome exhibits high antioxidative capacity, and the main antioxidative compositions in lotus rhizome are phenolics including dopa, catechol, gallic acid, catechin, and epicatechin [14]. The antioxidant activities of lotus rhizome extract obtained with a solvent of different polarity are determined. It is found that all extracts obtained with different solvents exhibit higher antioxidant activity than ascorbic acid [15].

Recent studies have stated the mechanisms behind the action of natural antioxidants on the inhibition of NO production [16]. Plants are considered to be a good source of natural antioxidant compounds. Therefore, considerable attention has been focused on the use of natural antioxidants to inhibit NO production [17]. In this study, we conducted to determine the NO inhibitory properties in RAW264.7 cell line stimulated by LPS and phytochemical components of the ethanolic leaf extract of *N. nucifera* extracts.

METHODS

Chemicals and reagents

Dulbecco's modified eagle medium (DMEM), penicillin, and streptomycin were purchased from Gibco (Gibco, USA). DMSO, resazurin, and trypan blue were purchased from Sigma (Sigma-Aldrich, USA).

Plant materials and extraction

The lotus (*N. nucifera)* including of roseum plenum (RP) and Album Plenum (AP) was collected from lotus museum, Rajamangala University of Technology Thanyaburi, Pathum Thani, Thailand. The all parts of *N. nucifera* were dried with oven. Each parts of lotus were extracted by maceration with the ethanolic solvents for 7 days. The extracts were evaporated and dried in a rotary evaporator. The crude powder was suspended in 2% DMSO to a concentration of 10 mg/ml and diluted in distilled water before use.

Cell line and culture conditions

The RAW264.7 murine macrophage cells line were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) at 37ºC in a humidified 5% CO_2 incubator. The RAW264.7 cell was sub-cultured using cell scraper twice a week. The cell viability was determined using 0.4% trypan blue (Sigma-Aldrich, USA) with cell viability >90% was used in all of the experiments [18].

Determination of cell viability of RAW264.7 cell line [19]

The viability of the residual RAW264.7 macrophage cells was seeded at 2×10^5 cells/well in a 96-well plate and incubated for 24 h at 37°C and 5% CO_2 incubator. The cells were fed with fresh media containing 10% FBS. These were treated with extracts at a concentration of 100 µg/ml for 24 h, the residual cells were incubated for 2 h at 37°C in 100 µl fresh DMEM containing 50 µg/ml resazurin. The reaction mixture absorbance at 560 against 600 nm was measured using a microplate reader (Glomax multi detection system, USA). The assays were performed in triplicate cultures, and the mean±SD were calculated.

Measurement of NO production in LPS-stimulated RAW264.7 cell line

The RAW264.7 cells at a density of 2×10^5 cell/ml were seeded in 96-well plate and incubated for 24 h at 37°C and 5% CO_2 incubator. Then media of each well were aspirated and fresh 10% FBS-DMEM media were replaced. The plated-cell was pretreated with various concentrations of 6.25, 12.5, 25, 50, and 100 µg/ml and incubated for 24 h. The treated cells were stimulated with 1 µg/ml LPS for 24 h. An aliquot 100 µl of the culture media was transferred to a 96-well culture plate and incubated with an equal volume of Griess reagent for 30 min. The NO concentrations were determination nitrite production in the induced-cell supernatant using Griess reagent. The optical density of the samples was measured at 560 nm. The assays were carried out in triplicate cultures.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis of these extracts was carried out by following the modify method of Singariya *et al.* [20]. GC-MS analysis was performed on an Agilent 7890 GC system instrument equipped with HP-5MS column (30 m × 0.25 mm × 0.25 µm) and interfaced to a 5975C inert XL MSD with Triple-Axis Detector. An injection volume of 2 µL was employed with spitless mode and an injector temperature 250°C. The column temperature was increased from 60°C to 250°C at a rate 5°C/min. The outlet temperature was 280°C. Mass spectra were taken at 70 eV and fragments from 40 to 550 Da. The MS transfer line temperature was 250°C. Identification of the compounds was conducted using the database of the National Institute of Standards and Technology (NIST) library version 11. The name, molecular weight (MW), molecular formula (MF), and area under the peak of the test material components were ascertained.

Statistical analysis

The values expressed are means of triplicate determinations ± standard deviation. The statistical analysis was carried out by analysis of variance. The data were evaluated with GraphPad Prism 6 version 6.01 (GraphPad Software Inc. La Jolla, CA. USA).

RESULTS AND DISCUSSION

The cell viability of extract on RAW264.7 cells

To determine the viability of RAW264.7 macrophage cell line in the presence of different part of ethanolic extracts, cells were incubated for 24 h with the extract at 100 µg/ml concentrations. The cell viability was evaluated by the resazurin assay. As shown in Fig. 1, the results indicated that all of the extracts were not toxic on RAW264.7 cells (cell viability more than 90%). The RP-flower, AP-stalk, and AP-flower extracts were appeared to stimulate cell proliferation. All of the samples were selected for subsequent NO inhibition experiment.

Inhibition of NO production in LPS-stimulated RAW 264.7 cells

Increasing NO production is a common phenomenon that occurs in LPS-stimulated macrophages and is used as an indicator of an inflammatory response. The results of the cytotoxicity assay showed that the inhibitory effect of all extracts was not caused by cell damage. In this study, the extracts of *N. nucifera* were evaluated to inhibit the NO production in the LPS-induced RAW264.7 cells. The nitrite accumulation in the cells increased due to the LPS treatment. To accomplish this experiment, cells were simultaneously treated with 1 µg/ml LPS and 100 µg/ml final concentrations of extract. The RAW264.7 cells were activated by LPS, and NO production was measured as nitrite concentration in the culture medium. When compared to the untreated control, the pre-treated cells induced with LPS released a lower level of NO in the medium measured. The different part extracts were significantly inhibited ($p<0.001$) the nitrite accumulation in LPS-stimulated RAW264.7 cells in a concentration-dependent manner, as shown in Fig. 2. The NO inhibition values of RP (leaf, stalk, and flower) and AP (leaf, stalk, and flower) extracts were 32.93±3.77, 39.77±2.86, 29.03±2.86, 30.30±4.88,

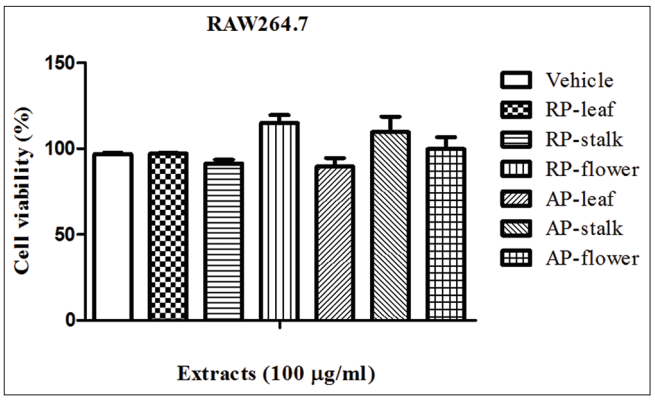

Fig. 1: Cell viability of different parts extracts of *Nelumbo nucifera* on RAW264.7 macrophage cell. Values are expressed as mean±SD

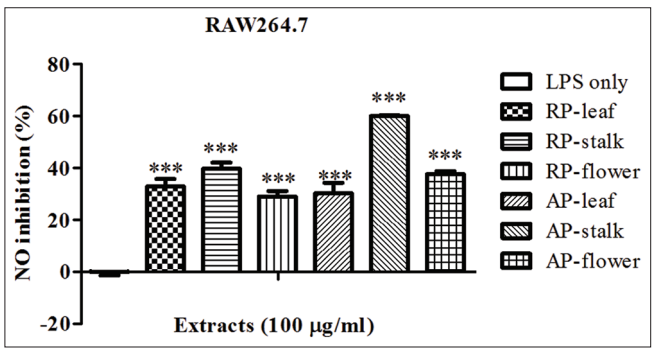

Fig. 2: Inhibitory effects of ethanolic extracts of *Nelumbo nucifera* on NO production by LPS-stimulated RAW264.7 macrophage cells. Values are mean of three replicate determinations (n=3) ± standard deviation. ***p<0.001, compared with LPS only

60.08±0.34, and 37.71±1.29%, respectively. Among the different part extracts, AP-stalk extract exhibited the significant reduction of nitrite level to 32.82±0.90 μM at 100 μg/ml of extract concentration (Table 1). AP-stalk extract was most effective with an IC_{50} of 79.20 μg/ml.

In Table 1, the results showed that the NO concentrations in the LPS-induced macrophage cell were reduced in a concentration-dependent manner. The AP-stalk extract was significantly reduced NO, obtaining the NO inhibition performance with non-cytotoxicity in range of 8.28–67.18% with concentration of 6.25, 12.5, 25, 50, and 100 μg/ml.

In vivo, macrophages play an important role in inflammation through the production of several pro-inflammatory molecules, including NO. Excessive NO production has been associated with a range of inflammatory diseases such as arteriosclerosis, ischemic reperfusion, hypertension, and septic shock [21]. Recent studies have shown that plant foods, including fruits, vegetables, and medicinal herbs are an excellent source of antioxidant molecules that effectively inhibit the inflammatory process by affecting different molecular targets [7]. RAW264.7 cell, a murine macrophage cell line, has been frequently used to screen anti-inflammatory substances. The extractions showed the inhibition of NO production in cells, indicating that the presence of antioxidant molecules would be responsible for the inhibitory action. NO is a multifunctional signaling molecule, thus the impact of the extract or compound on NO production likely has further effects on signaling pathways in many cell types [22].

However, dysregulated inflammation may cause various pathophysiological conditions, including gastritis, esophagitis, hepatitis, atherosclerosis, and cancer [1,2]. Macrophages are involved in chronic inflammation by producing various inflammatory chemical mediators including cytokines, chemokines, interferons, colony-stimulating factors, lysozymes, proteases, growth factors, eicosanoids, and NO [23,24]. Among these, NO is excessively generated by one of the pro-inflammatory enzymes, iNOS, and consequently results in diverse diseases, including asthma, multiple sclerosis, psoriasis, neurodegenerative disorders, tumor development, and transplant rejection of septic shock [25]. Therefore, it is interesting to find new inhibitors of NO production.

Table 1: The NO concentration under variable AP-stem concentrations, compared with vehicle as negative control

AP-stalk extract (μg/ml)	NO concentration (μM)	NO inhibition (%)	IC_{50} (μg/ml)
6.25	91.72±3.32	8.28±3.32	79.20
12.5	87.77±3.96	12.23±3.96	
25	86.48±0.90	13.52±0.90	
50	82.70±0.37	17.30±0.37	
100	32.82±0.90	67.18±0.90	

Table 2: Phytocomponents identified in ethanolic extract of album plenum stalk by gas chromatography-mass spectrometry

Retention time	Compound	Molecular formula	Molecular weight
14.10	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
16.50	9,12-octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280
22.33	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390
26.30	5-Cholene, 3,24-dihydroxy-	$C_{22}H_{36}O_2$	333
26.53	2-Chloroethyl 1-propynyl sulfoxide	C_5H_7ClOS	151
26.72	Naphthalene, decahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1α,4aα,7α,8aβ)]-	$C_{15}H_{28}$	208
26.96	Anthracene, 9-(2-propenyl)-	$C_{17}H_{14}$	218
28.35	Benzo[b]naphtho[2,3-d]furan	$C_{16}H_{10}O$	218
28.77	Benzo[h]quinoline, 2,4-dimethyl-	$C_{15}H_{13}N$	207

Several studies have been demonstrated that various compounds from plants possess rich pharmacological properties that play beneficial roles in various diseases, including diseases related to inflammation [26]. Inflammation is a dynamic process involving pro-inflammatory cytokines and it acts as an important biological response toward injury [27]. NO plays a critical role in the aggravation of chronic inflammatory disease. NO plays an important role in a variety of physiological and pathological processes, including inflammatory reaction. Thus, NO has a potential therapeutic to inhibit the treatment of inflammation [28]. The results of the present study demonstrated that the AP-stalk extraction significantly decreased the nitrite accumulation in LPS-stimulated RAW264.7 cells in a concentration-dependent manner (Table 1).

GC-MS

The phytochemical compounds were characterized and identified. Their retention time, MF, and MW are shown in Table 2. The chromatogram analysis of AP-stalk extract was compared with the mass spectra of the constituents of NIST version 11 library. The GC-MS chromatogram analysis of the ethanolic extract of AP stalk as bioactive compounds showed nine main constituents. Therefore, we assume that bioactivities exhibited by AP stalk in this study are correlated to the occurrence of one or many these phytochemical compounds.

CONCLUSION

This study demonstrated that the ethanolic extract of AP stalk could possibly inhibit inflammation. Bio-active phytoconstituents from extract of AP stalk extract could potentially be used for anti-inflammation. The experimental results of this study indicate that the AP-stalk ethanolic extract contains a high potential to reduce the NO level in RAW264.7 cells stimulated by LPS. The non-cytotoxicity in macrophage cells is presented in all concentrations of AP-stalk extract (6.25–100 μg/ml). The findings of the present study clearly provide evidence the supports the traditional use of the AP-stalk in the treatment of inflammatory diseases. A future study must be conducted to isolate, identify, and mechanism of action of the active phytochemical compounds from the lotus, to establish an effective drug resource for prevention and treatment of disease caused by inflammation.

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CONFLICTS OFINTEREST

The authors declare no potential conflicts of interest.

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