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ORIGINAL ARTICLE

# Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells by stem bark of *Ulmus pumila* L.

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**Abstract** This study was designed to isolate and identify a potent inhibitory compound against nitric oxide (NO) production from the stem bark of *Ulmus pumila* L. Ethyl acetate fraction of hot water extract registered a higher level of total phenolics (756.93 mg GAE/g) and also showed strong DPPH ($IC_{50}$ at 5.6 μg/mL) and ABTS (TEAC value 0.9703) radical scavenging activities than other fractions. Crude extract and its fractions significantly decreased nitrite accumulation in LPS-stimulated RAW 264.7 cells indicating that they potentially inhibited the NO production in a concentration dependent manner. Based on higher inhibitory activity, the ethyl acetate fraction was subjected to Sephadex LH-20 column chromatography and yielded seven fractions and all these fractions registered appreciable levels of inhibitory activity on NO production. The most effective fraction F1 was further purified and subjected to $^{1}H$, $^{13}C$-NMR and mass spectrometry analysis and the compound was identified as icariside $E_4$. The results suggest that the *U. pumila* extract and the isolated compound icariside $E_4$ effectively inhibited the NO production and may be useful in preventing inflammatory diseases mediated by excessive production of NO.



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## 1. Introduction

Nitric oxide (NO) is an important molecule for host defense response against various pathogens such as bacteria, viruses, fungi, and parasites (Bogdan et al., 2000). Under normal physiological conditions, NO plays an important role in the regulation of various pathophysiological processes such as neuronal communication, vasodilatation, and neurotoxicity (Moncada et al., 1991; Nakagawa and Yokozawa, 2002). However, overproduction of NO induces tissue damage associated with acute

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and chronic inflammations (Taira et al., 2009). Therefore, more attention is now being paid to the development of new drugs as potent inhibitors of NO production in relation to the treatment of chronic inflammatory diseases (Pacher et al., 2007).

NO is an intercellular mediator produced in various mammalian cells by three forms of nitric oxide synthases (NOS) namely endothelium NO synthase (eNOS), neural NO synthase (nNOS) and inducible NO synthase (iNOS) (Lenon et al., 2008). Macrophages are important components of the mammalian immune system, and they play a key role by providing an immediate defense against foreign agents prior to leukocyte migration and production of various pro-inflammatory mediators including the short-lived free radical NO (Moncada et al., 1991). Lipopolysaccharide (LPS), a component from the cell walls of gram-negative bacteria is one of the most powerful activators of macrophages and involves the production of pro-inflammatory cytokines (Nicholas et al., 2007). Therefore, inhibition of NO production in LPS-stimulated RAW 264.7 cells is one of the possible ways to screen various anti-inflammatory drugs.

Recent studies have stated the mechanisms behind the action of natural antioxidants on the inhibition of NO production (Yen et al., 2008). Plants are considered to be a good source of natural antioxidant molecules especially vitamins, terpenoids, phenolic compounds, tannins, flavonoids, alkaloids, coumarins, and other metabolites. Therefore, considerable attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit NO production (Maksimovic et al., 2005; Mohsen and Ammar, 2009). In this context, the present work was carried out to isolate and identify potent compound from the stem bark of *Ulmus pumila* L. (Ulmaceae) to inhibit the NO production. *U. pumila* is a deciduous tree native to central Asia and widely distributed in Asia, America and southern Europe. In traditional system of medicine, the stem and root parts are used for the treatment of various ailments such as edema, mastitis, gastric cancer and inflammation (College, 1977; Wang et al., 2006). In addition, various bioactive triterpenoids have been identified from the extracts of *U. pumila* (Wang et al., 2006). Free radical scavenging and inhibitory activities on angiotensin converting enzyme and xanthine oxidase of 70% ethanol extracts of *U. pumila* were evaluated (Kim et al., 2012). Ghosh et al. (2012) studied that the methanol extract of *U. pumila* inhibits adipogenesis through regulation of cell cycle progression in 3T3-L1 cells and identified four different triterpenoids such as oleanolic acid, freidelin, maslinic acid and arjunolic acid.

Based on the highly acclaimed properties of *U. pumila*, the present study was aimed to evaluate antioxidant and anti-inflammatory (inhibition of NO production in LPS-stimulated RAW264.7 cells) activities of hot water extract and its sub-fractions of *U. pumila* stem bark. Further, bioassay guided isolation and identification of the compound responsible for the inhibition of NO production from the extract were investigated.

## 2. Materials and methods

### 2.1. Chemicals and plant material

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Folin–Ciocalteu's reagent, Trolox, gallic acid, penicillin–streptomycin solution and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate buffered saline (PBS) were from HyClone Laboratories, Inc (South Logan, UT, USA). The dried stem barks of *Ulmus pumila* L. were purchased from Kyungdong herbal distribution market (Seoul, South Korea). The sample was authenticated and deposited at CodeBio Co., Ltd (Cheonan, South Korea) with batch No. CO-OP-0022.

### 2.2. Isolation and identification of compound

The sample of powdered stem bark (150 g) was refluxed in 3000 mL distilled water for 6 h in a round bottom flask three times. The aqueous extract was then filtered through Whatman No. 2 filter paper. The combined hot water extract (UCE) was evaporated under reduced pressure to get crude extract. The crude extract was re-suspended in distilled water and the aqueous solution was successively partitioned with ethyl acetate (UEF) and *n*-butanol (UBF). The solvents were evaporated using a rotary vacuum-evaporator at 40 °C. The obtained fractions, in addition to the aqueous solution (UWF) remained after the extraction were freeze dried (FD-1000, Eyela, Tokyo, Japan). The crude extract and fractions were used for the assessment of free radical scavenging and inhibition of nitric oxide production assays. The extract yield in different fractions was expressed as percent of dry weight of the hot water extract.

Based on the results from inhibition of NO production, ethyl acetate fraction was selected for further separation and purification studies. The ethyl acetate fraction (1.5 g) was loaded onto an open column (450 × 35 mm) packed with Sephadex LH-20 and eluted with ethanol. The collected seven fractions (F1–F7, each 250 mL) were dried under reduced pressure (Fig. 1). Among the seven fractions collected, F1 was subjected to fractionation with silica gel (Kieselgel 60, Merck, Darmstadt, Germany) column chromatography (350 × 30 mm, 100 g) by eluting with 500 mL of chloroform/methanol (9:1). To analyze the isolated compounds, thin layer chromatography (TLC) was performed on silica gel TLC plate (250 μm thickness, 2–25 μm particle size). The spot was detected by ultraviolet illumination and spraying with 5% (v/v) $H_2SO_4$ in an ethanol solution. The collected fractions were subjected for further purification with RP-18 silica gel column (350 × 30 mm, 100 g) chromatography by eluting with methanol/water (4:6) which afforded compound 1 (15 mg). The structure of the isolated compound was identified through the interpretation of $^{1}H$, $^{13}C$, distortionless enhancement by polarization transfer (DEPT) nuclear magnetic resonance (NMR) and mass spectra.

The content of compound 1 in the *U. pumila* stem bark was determined using HPLC (LC-20A, Shimazu, Tokyo, Japan) analysis. The powdered *U. pumila* stem bark sample was extracted with 50% ethanol for 30 min sonication, and the supernatant was filtered through 0.45 μm syringe filter. HPLC was performed with a Luna $C_{18}$ analytical column (Phenomenex, USA, 10 μm, 250 × 4.6 mm). The column temperature was controlled with a column heater set at 40 °C. The mobile

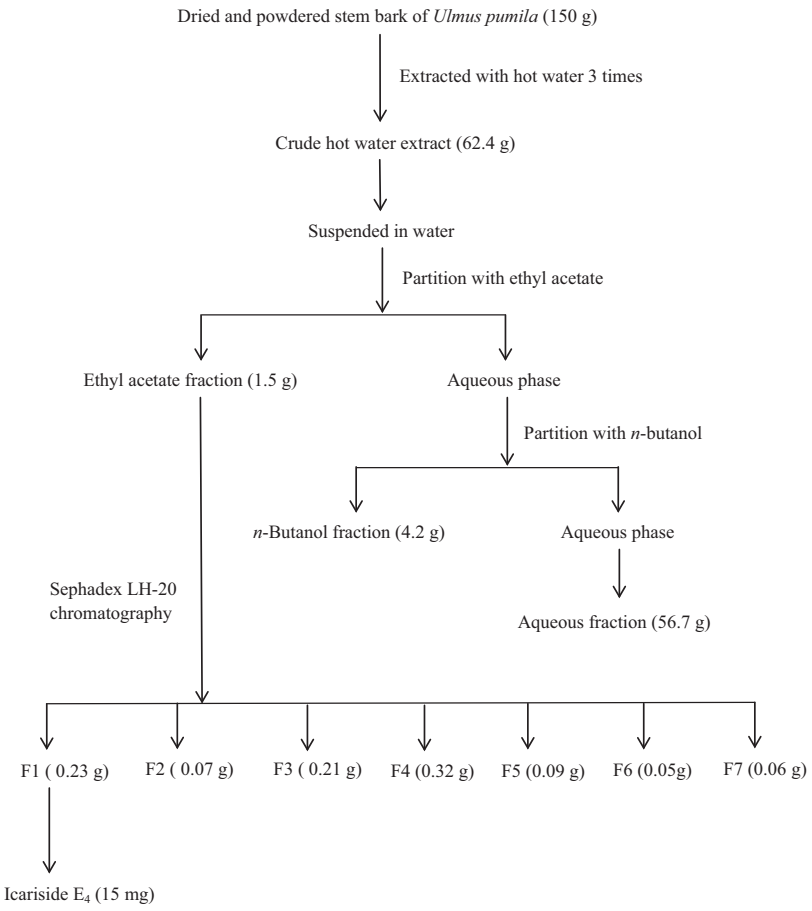


**Figure 1** Extraction and fractions carried out with stem bark of *Ulmus pumila*.

phase, delivered at 1 mL/min, was a 30 min linear gradient from 5% acetonitrile to 50% acetonitrile, and eluent was monitored at 254 nm.

### 2.3. Determination of total phenolic content

The total phenolic content of the crude extract and its sub-fractions of *U. pumila* stem bark was determined spectrophotometrically at 760 nm after reaction with the Folin–Ciocalteu's reagent (Chew et al., 2009). The analysis was performed in triplicate and the results were expressed as milligram gallic acid equivalents (GAE) per gram extract.

### 2.4. Free radical scavenging activity on DPPH

The DPPH radical scavenging activity of the crude extract and different fractions was determined according to the method described by Blois with some modification (Doughari et al., 2012). Sample at various concentrations (50 µL) was added to 2 mL of DPPH (0.4 mM) in ethanol. The mixture was shaken and allowed to stand for 30 min at 27 °C under dark conditions. The absorbance was measured at 517 nm. $IC_{50}$ value of the sample i.e., concentration of sample necessary to decrease the initial concentration of DPPH by 50% was calculated.

### 2.5. $ABTS^{\bullet+}$ scavenging assay

Radical scavenging activity of crude extract and its sub-fractions of *U. pumila* stem bark was assessed spectrophotometrically by $ABTS^{\bullet+}$ cation decolorization assay (Re et al., 1999). $ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol and equilibrated at 30 °C to give an absorbance at 734 nm of 0.7 ± 0.020. Trolox was used as a reference standard. Test sample (10 µL) or Trolox standard at different concentrations were added to 1 mL of diluted ABTS solution and mixed thoroughly. The reaction mixture was kept at room temperature in the dark for 7 min, then the absorbance was measured at 734 nm and the percentage of inhibition was calculated. The Trolox equivalent antioxidant capacity (TEAC) value represents the ratio between the slope of the linear plot for scavenging of $ABTS^{\bullet+}$ radical cation by the different concentrations of samples and the slope of the plot for $ABTS^{\bullet+}$ radical cation scavenging by different concentrations of Trolox.

### 2.6. Cell culture

The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (ATCC, Manassas,

VA, USA) and maintained in DMEM supplemented with 10% FBS, 100 μg/L streptomycin, and 100 IU/mL penicillin at 37 °C in a 5% $CO_2$ atmosphere (HERAcell 150, Thermo Electron Corp. Waltham, MA, USA).

### 2.7. MTT cell viability assay

The mitochondrial-dependent reduction of MTT to formazan was used to measure cell respiration as an indicator of cell viability. Briefly, RAW 264.7 cells were seeded in 96-well plates at the density of $5 \times 10^4$ cells/well. After 24 h of incubation, the adhered cells were treated with various concentrations of the extract or fractions. Twenty-four hours later, after changing the medium, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h at 37 °C and 5% $CO_2$. The medium was then removed and the formazan precipitate was solubilized in DMSO. The absorbance was measured at 550 nm on a microplate reader (Biotek, Winooski, VT, USA).

### 2.8. Inhibition of nitric oxide (NO) production

The RAW 264.7 cells were seeded at a density of $5 \times 10^5$ cells/well in 24 well plates and incubated for 12 h at 37 °C and 5% $CO_2$. Then media of each well were aspirated and fresh FBS-free DMEM media were replaced. Different concentrations of *U. pumila* extract (50–500 μg/mL) or fractions (20–1000 μg/mL) were prepared in FBS-free DMEM to give a total volume of 500 μL in each well of a microtiter plate. After 1 h treatment, cells were stimulated with 1 μg/mL of LPS for 24 h.

The presence of nitrite was determined in cell culture media using commercial NO detection kit (iNtRON, Sungnam, South Korea). Protocols supplied with assay kit were used for the application of assay procedure. Briefly, 100 μL of cell culture medium with an equal volume of Griess reagent in a 96-well plate was incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve.

### 2.9. Statistical analysis

The values expressed are means of three replicate determinations ± standard deviation. The statistical analysis was carried out by analysis of variance (ANOVA) followed by Tukey's test. The data were evaluated with SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Total phenolic content

Total phenolic content of hot water extract and its sub-fractions of *U. pumila* stem bark is shown in Table 1. The total phenolic content of the extract and its fractions was determined through a linear gallic acid standard curve and expressed as milligram of gallic acid equivalents per gram of dry extract. The hot water extract of *U. pumila* stem bark found to contain a moderate level of total phenolics (138.95 mg GAE/g extract). Hot water extract was further separated to ethyl acetate, *n*-butanol and water fractions and the yields obtained are 2.35%, 6.74%, and 90.91%, respectively. The total phenolic content of all fractions from hot water extract varied from 756.93 to 95.35 mg GAE/g. The highest total phenolic content was found in ethyl acetate fraction (756.93 mg GAE/g), whereas the lowest found in water fraction (95.35 mg GAE/g).

### 3.2. DPPH radical scavenging activity

The $IC_{50}$ values of hot water extract and its sub-fractions of *U. pumila* stem bark are given in Table 1. Concentration dependent DPPH radical scavenging activity was observed for the crude hot water extract and its sub-fractions. Crude hot water extract and other fractions showed appreciable levels of DPPH radical scavenging activity with the exception of water fraction. Ascorbic acid is a well-known antioxidant compound used as the reference standard in this study and showed very strong DPPH radical scavenging activity with the $IC_{50}$ of 1.72 μg/mL. Among the different fractions, the most effective DPPH scavenger was ethyl acetate fraction ($IC_{50}$ of 5.6 μg/mL) and *n*-butanol fraction ($IC_{50}$ of 13.76 μg/mL) also seemed to be highly active than crude hot water extract ($IC_{50}$ of 35.87 μg/mL).

### 3.3. ABTS radical scavenging activity

The ability of crude extract and its sub-fractions of *U. pumila* stem bark to scavenge free radicals was measured using the ABTS radical scavenging assay and the results are expressed as TEAC value (Table 1). The results showed that the extract and fractions tested were able to scavenge ABTS radical cation and exhibited similar line to the results of DPPH assay. TEAC value of ethyl acetate (0.9703) was significantly higher ($P < 0.05$) than those of crude extract and other fractions. The ABTS radical scavenging activity of the crude extract and its fractions is in the decreasing order of UEF (0.9703) > UBF (0.5752) > UCE (0.1773) > UWF (0.0958).

### 3.4. Effect of extract and fractions on RAW 264.7 cell viability

To determine the viability of RAW 264.7 cells in the presence of hot water extract or its sub-fractions, cells were incubated for 24 h with the extract/fractions at a wide range of concentrations. The cell viability was evaluated by MTT assay. With this result, concentrations of sample (cell viability >85%) were selected for subsequent NO inhibition experiment.

### 3.5. Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells

In the present study, hot water extract and its fractions of *U. pumila* stem bark were evaluated for the inhibition of NO production in the LPS-stimulated 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 different concentrations of extract or fractions. The RAW 264.7 cells were activated by LPS,

**Table 1** Total phenolic content, DPPH and ABTS radical scavenging activities of crude extract and its sub-fractions of *Ulmus pumila* stem bark.

| Sample | Total phenolics (mg GAE/g extract) | $IC_{50}$ of DPPH (μg/mL) | ABTS (TEAC) |
|---|---|---|---|
| UCE | 138.95 ± 3.270$^{c}$ | 35.87 ± 0.490$^{d}$ | 0.1773 ± 0.002$^{c}$ |
| UEF | 756.93 ± 10.190$^{a}$ | 5.6 ± 0.140$^{b}$ | 0.9703 ± 0.009$^{a}$ |
| UBF | 537.53 ± 10.300$^{b}$ | 13.76 ± 0.260$^{c}$ | 0.5752 ± 0.006$^{b}$ |
| UWF | 95.35 ± 3.780$^{d}$ | 118.81 ± 1.600$^{e}$ | 0.0958 ± 0.001$^{d}$ |
| AA | – | 1.72 ± 0.010$^{a}$ | – |

UCE, crude hot water extract; UEF, ethyl acetate fraction; UBF, *n*-butanol fraction; UWF, water fraction; AA, ascorbic acid.
Values are mean of three replicate determinations ($n = 3$) ± standard deviation. Mean values followed by different superscripts (a–e) in a column are significantly different ($P < 0.05$).

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 as its stable non-volatile breakdown product nitrite. The crude hot water extract and different fractions significantly inhibited ($P < 0.05$) the nitrite accumulation in LPS-stimulated RAW 264.7 cells in a concentration dependent manner (Fig. 2A–D). The $IC_{50}$ values of hot water extract, ethyl acetate fraction, *n*-butanol fraction and water fraction were 279.1, 161.0, 677.5 and 645.9 μg/mL, respectively. Among the different fractions, ethyl acetate fraction exhibited the significant reduction of nitrite level to 32.09 μM at the concentration of 200 μg/mL. The result of MTT cell viability assay revealed that the inhibitory effect of ethyl acetate fraction was not due to cell damage (viability > 90%).

### *3.6. Isolation and identification of inhibitory compound against NO production*

In the present study, ethyl acetate fraction demonstrated that the highest total phenolic content accompanied with highest DPPH and ABTS radical scavenging activities when compared with other fractions. Therefore, the ethyl acetate fraction was selected for further isolation and identification studies. The ethyl acetate fraction was separated using Sephadex LH-20 column chromatography and yielded 7 fractions. All the fractions exhibited appreciable inhibitory activity against NO production and the nitrite levels ranged between 0.86 μM and 26.8 μM at the concentration of 100 μg/mL (Fig. 3). Among these fractions, fraction F1 showed significantly higher reduction of nitrite accumulation in LPS-stimulated RAW 264.7 cells (0.86 μM).

The chemical component was separated on silica gel and RP-18 columns as described in the isolation of compound section (2.2) resulting in the isolation of compound 1. Further, identification and chemical structure of the isolated compound were analyzed using the interpretation of $^{1}H$, $^{13}C$ NMR, DEPT and mass spectral data. Based on the results of spectral data, the isolated compound 1 was identified as icariside $E_4$ ($C_{26}H_{34}O_{10}$) (Fig. 4). The resulting analytical data were as follows:

*Compound 1*. White amorphous solid. ESI-MS *m/z* 528.83 $[M+Na]^{+}$. $^{1}H$-NMR ($CD_3OD$, 600 MHz) $\delta$ : 1.21 (3H, d, $J = 6.4$), 1.81 (2H, m), 2.62 (2H, t, $J = 8.0$), 3.43–3.56 (4H, m), 3.79 (3H, s, OCH3), 3.86 (3H, s, OCH3), 4.05 (1H, d, $J = 2.6$), 5.33 (1H, br s), 5.54 (1H, d, $J = 6.0$), 6.72 (2H, d, $J = 9.5$), 6.90 (1H, dd, $J = 8.5$), 7.02 (1H, br s), 7.07 (1H, d, $J = 8.0$), $^{13}C$-NMR ($CD_3OD$) $\delta$: 16.6 (Rha C-6), 31.5 (C-7′), 34.4 (C-8′), 54.2 (C-8), 55.0 (3-$OCH_3$), 55.4 (3′-$OCH_3$), 60.8 (C-9′), 63.7 (C-9), 69.4 (Rha-C-5), 70.7 (Rha C-3), 70.8 (Rha C-2), 72.4 (Rha C-4), 87.1 (C-7), 100.0 (Rha C-1), 109.9 (C-2), 112.8 (C-2′), 116.5 (C-6′), 117.7 (C-6), 118.2 (C-5), 128.2 (C-5′), 135.7 (C-1′), 137.4 (C-1), 143.9 (C-4′), 145.2 (C-4), 146.1 (C-3′), 150.7 (C-3).

Concentration of icariside $E_4$ in the *U. pumila* stem bark was determined by comparing peak area to external standard calibration curve ($y = 1370.99x - 8120.17$, $r = 0.999$). An HPLC analysis revealed the presence of the isolated compound, icariside $E_4$ in the stem bark extract of *U. pumila* at 378.50 μg/g dry weight.

## 4. Discussion

Macrophages play important roles in inflammation through the production of several pro-inflammatory molecules, including NO. Production of excessive NO has been associated with a range of inflammatory diseases including arteriosclerosis, ischemic reperfusion, hypertension and septic shock (Pacher et al., 2007; Terao, 2009). Recent studies have demonstrated that the 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 (Tsai et al., 2007; Taira et al., 2009). In general, the total phenolic content of the plant extracts is highly correlated with their free radical scavenging activities (Cho et al., 2007). Therefore, the amount of total phenolic content and free radical scavenging capacities are initially assessed for the crude extract and its-sub fractions, which will be a convenient way to identify a potent inhibitory compound against NO production. In this study, ethyl acetate fraction exhibited a higher level of total phenolics when compared with crude extract and other fractions. A number of studies have reported that the phenolic compounds are abundant in ethyl acetate and *n*-butanol fractions of plant extracts (Puangpronpitag et al., 2008; Rao et al., 2008). Their ability as free radical scavengers could be attributed to their redox properties by adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Chang et al., 2001). In addition, phenolic compounds are known to be potent for inhibiting NO and peroxynitrite productions (Conforti and Menichini, 2011).

The antioxidant potential of plant extracts or compounds can be determined using various *in vitro* assays. Among the various assays, DPPH and ABTS radical scavenging assays constitute quick, simple to perform, reproducible and

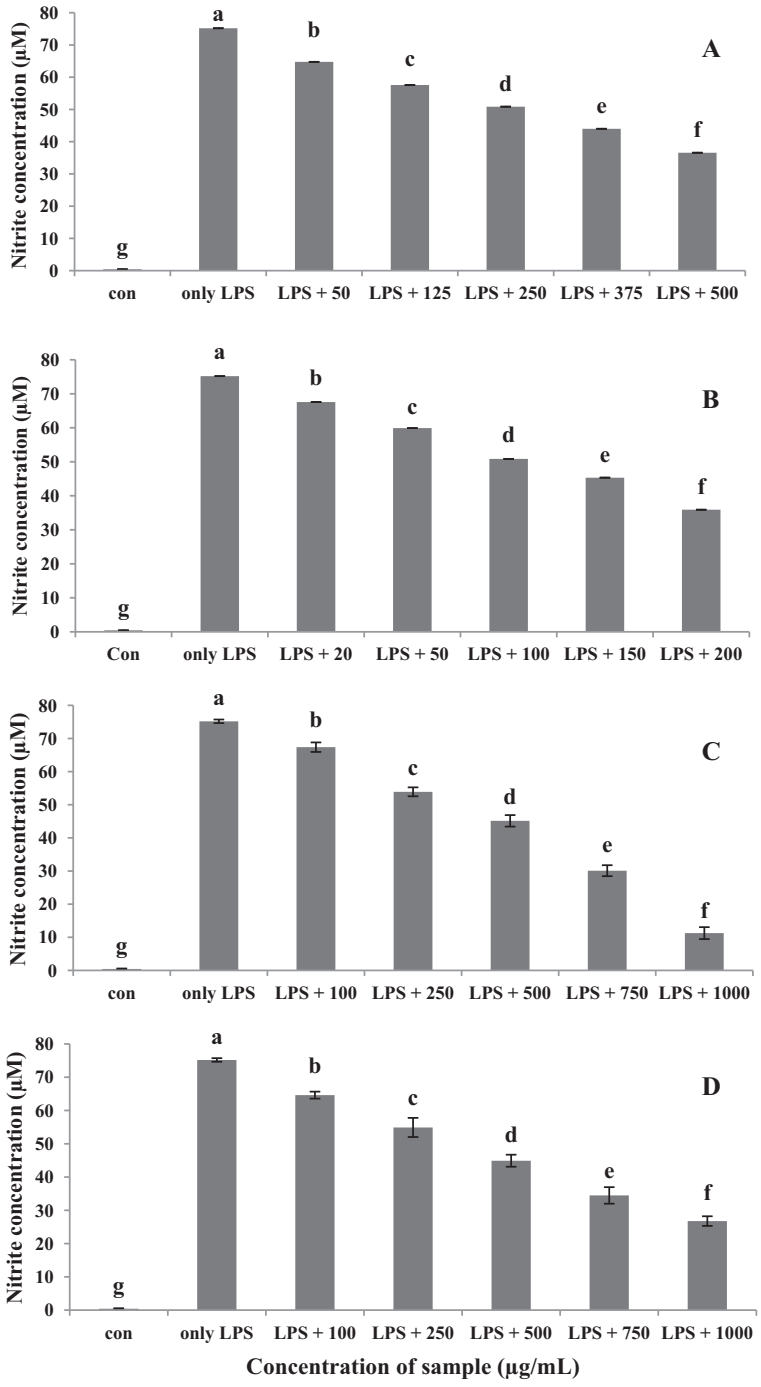


**Figure 2** Inhibitory effects of hot water extract and its sub-fractions of stem bark of *Ulmus pumila* on NO production by LPS-stimulated RAW 264.7 cells (A, hot water extract; B, ethyl acetate fraction; C, *n*-butanol fraction; D, water fraction). RAW 264.7 cells were stimulated with 1 μg/mL of LPS. Values are mean of three replicate determinations ($n = 3$) ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

inexpensive methods and are frequently used for the evaluation of the antioxidant potential of various natural products and functional food materials (Gliszczynska-Swiglo and Tyrakowska, 2003; Sheu et al., 2012). In this study, the DPPH and ABTS scavenging activity of all fractions showed a similar trend with the content of total phenolics in the crude extract and different fractions. Radical scavenging activities by the sample might be due to the presence of the hydroxyl groups in their structure and their electron donating ability. The correlation between antioxidant activity and the content of phenolic compounds has also been reported by many researchers (Sun and Ho, 2006; Liu et al., 2009). Our results also confirmed that total polyphenol contents of plants are regular indices of their antioxidant activity.

Higher level of polyphenol content with strong antioxidant potential of plant samples are a good target for examining the

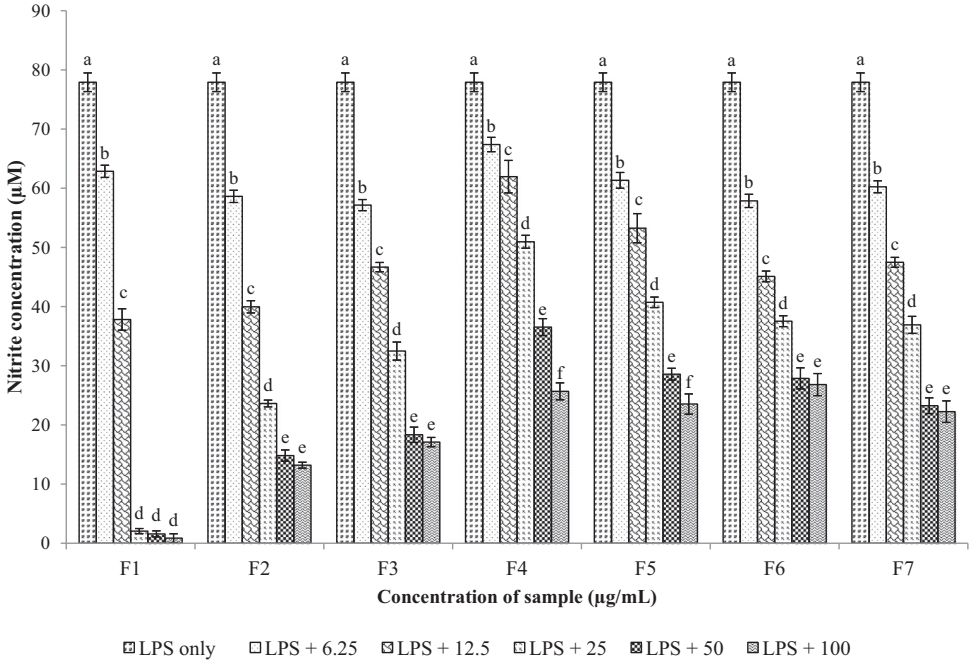


**Figure 3** Effect of different fractions obtained from Sephadex LH-20 column on NO production in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1 μg/mL of LPS. Values are mean of three replicate determinations ($n = 3$) ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

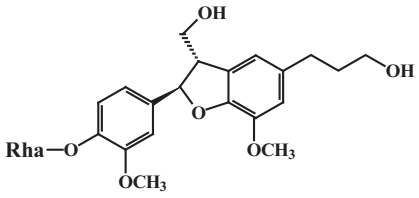


**Figure 4** Structure of the compound icariside $E_4$.

inhibitory activity against NO production. RAW 264.7, a murine macrophage cell line has been frequently used for the screening of anti-inflammatory drugs. The extract and its sub-fractions showed the inhibition of NO production in cells, indicating that presence of antioxidant molecules would be responsible for the inhibitory action. The results of the present study demonstrated that the ethyl acetate fraction significantly decreased the nitrite accumulation in LPS-stimulated RAW 264.7 cells in a concentration dependent manner (Fig. 2). 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 (Lander et al., 1996). Epidemiological studies have shown a positive correlation between consumption of plant foods, which are rich sources of antioxidants, and reduction in risk of diseases mediated by reactive oxygen species (Udenigwe et al., 2009). Previous studies have also suggested that plant secondary metabolites act as excellent anti-inflammatory agents and they play an important role in oxidative stress and inflammation (Sheu et al., 2001; Yen et al., 2008).

In column chromatography study, ethyl acetate fraction was subjected to Sephadex LH-20 column and afforded seven fractions. All the fractions showed inhibitory effect on NO production by decreasing the nitrite accumulation in LPS-stimulated RAW 264.7 cells (Fig. 3). From these seven fractions, fraction F1 registered higher inhibitory activity on NO production. Further purification and characterization studies revealed that the compound F1 was identified as icariside $E_4$. The spectral information of the isolated compound was in agreement with published data for icariside $E_4$ (Miyase et al., 1989; Sadhu et al., 2006). Various authors have reported that the plant extracts or isolated compounds are capable of inhibiting LPS-induced NO production. Taira et al. (2009) isolated five NO inhibitory compounds such as aromadendrin, dihydrokaempferol 3-*O*-*β*-D-glucoside, quercitrin, aglimonolide-6-*O*-*β*-D-glucoside and loliolide from the leaves of *Agrimonia pilosa*. Ten new myrsinol diterpene compounds, named euphorbialoids A–J were isolated from the roots of *Euphorbia prolifera* and all showed inhibitory activity on LPS-stimulated NO production (Xu et al., 2012). In the LPS-stimulated macrophages, NO is generated by the process of single enzymes: an inducible isoform of cyclooxygenase-2 (COX-2), and iNOS (Hempel et al., 1994; Salvemini et al., 2003). Hence, suppression of the iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells are important criteria to inhibit NO production (Ciz et al., 2008; Burk et al., 2009). Further studies are still needed in order to clarify the exact role of the isolated compound icariside $E_4$ on inhibition of NO production.

## 5. Conclusions

The results of the present study indicate that the ethyl acetate fraction from the hot water extract of *U. pumila* stem bark contains a higher amount of total phenolics and provided very strong free radical scavenging activities. Ethyl acetate fraction has a high potential to reduce the level of NO in LPS-stimulated RAW 264.7 cells. Isolation of potent NO inhibitory

compound from the ethyl acetate fraction of *U. pumila* stem bark revealed the identification of icariside $E_4$. The findings of the present study clearly provide evidence that supports the traditional use of stem bark of *U. pumila* in the treatment of inflammatory diseases. Further studies in relation to anti-inflammatory potential of the isolated compound icariside $E_4$ are under progress.

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