Running title: Pharmcological activities of *Actinidia callosa* var. ephippioides

Chemical compositions, Anti-inflammatory, Antiproliferative and Radical-scavenging Activities of the methanol extracts of *Actinidia callosa* var. ephippioides

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ABSTRACT.

Oxidative stress and inflammation are related to several chronic diseases including cancer. *Actinidia callosa* var. ephippioides (ACE) is a special folk medicinal plant in Taiwan. The aim of this study is to evaluate the antioxidant, anti-inflammatory, and antiproliferative activities of the methanol extract and fractions from the stem of ACE. Trolox Equivalent Antioxidant Capacity (TEAC), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, total phenolic content, flavonoid content, inhibition on nitric oxide (NO) productions by LPS-induced RAW264.7 cell, and on lung cancer cell proliferation were employed. Among all fractions, ethyl-acetate fraction (EA-ACE) showed higher TEAC, DPPH radical scavenging activities, polyphenol and flavonoid contents, respectively. EA-ACE also decreased the LPS-induced NO production and expressions of inducible nitric-oxide synthase (iNOS) in RAW264.7 cells. EA-ACE had the highest antiproliferative activity with an IC_{50} (The concentrations required for inhibition of 50% of cell viability) of 469.17 ± 3.59 μg/mL. Catechin also had good effects in the antioxidant and anti-inflammatory activities. Catechin might be an important bioactive compound in the stem of ACE. The above experimental data indicated that the stem of ACE is a potent antioxidant medicinal plant, and such efficacy may be mainly attributed to its polyphenolic compounds.

Keywords: *Actinidia callosa* var. ephippioides; Antioxidant activity; Anti-inflammatory activity; Antiproliferation;

Introduction

The reactive oxygen species play an important role in the degenerative or pathological processes of various serious diseases, such as cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts, inflammation and aging (Huang *et al*., 2012). The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant activity (Huang *et al*., 2008).

Macrophages play an important role in inflammatory disease through the release of inflammatory factors such as reactive oxygen species (ROS) and cytokines. Production of these macrophage mediators has been determined in many inflammatory tissues, following exposure to immune stimulants including bacterial endotoxin lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). Inappropriate macrophage activation is responsible for the pathology of acute or chronic inflammatory disease (Deng *et al*., 2011). Chronic inflammation leads to the up-regulation of signaling proteins in affected tissues and cells. Among the proinflammatory enzymes, the inducible nitric-oxide synthase (iNOS) and cyclooxygenase (COX-2), are known to be involved in various chronic diseases

including multiple sclerosis and Parkinson's and Alzheimer's diseases, as well as lung cancer (Lai *et al*., 2009). Thus, agents that suppress iNOS and COX-2 overexpression have potential therapeutic value when associated with inflammation and carcinogenic processes.

Actinidia callosa var. ephippioides *(Actinidiaceae*; ACE) is a deciduous tuberous plant, which is distributed in oriental countries. The stems of the plant have been extensively employed to treat various ailments like leprosy, abscess, rheumatism, arthritis inflammation, jaundice, and abnormal leucorrhea and were also useful for the treatment of cancers, especially those of lung, liver, and digestive system (Gan, 1993). *Actinidia species* had showed some pharmacological effects such as anti-inflammatory activity from the fruit of *Actinidia polygama* (Kim *et al*., 2003), antitumor and immunomodulatory activity from the roots of *Actinidia eriantha* (Xu *et al*., 2009). In this study, we examine the inhibitory effect of the antioxidant, anti-inflammatory, and anti-proliferative activities of the methanol extracts of *Actinidia callosa* var. ephippioides (MACE) and fractions. Consequently, the objective of the present study is to determine the effects of ACE against antioxidant, anti-inflammatory, and anti-proliferative activities and its active compounds.

Materials and methods

Materials

Lipopolysaccharide (LPS, *Escherichia coli* O127:B8), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), *N*-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), thiobarbituric acid (TBA), 3-[4,5-dimethyl-thiazol- 2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and other chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-iNOS, anti-COX-2, and anti-β-actin antibodies (Santa Cruz, CA, USA) were obtained as indicated. Plant materials were collected from Taichung country in Taiwan. They were identified and authenticated by Dr. Yuan-Shiun Chang, Professor, School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University. A plant specimen was deposited in the Institute.

Extraction and Fractionation

The coarse powder of the stem of *Actinidia callosa* var. ephippioides (1 kg) was extracted with methanol three times (10 L). The extract was evaporated under reduced pressure using a rotavapor, and then stored under light protection. A yield equivalent to 5.6 % of the original weight was obtained. Next, methanol extract of ACE (46 g) was dissolved and suspended in 100 mL of water in a separatory funnel prior to being

partitioned in sequence with *n*-hexane, chloroform, ethyl acetate and *n*-butanol (800 mL each for three times). Under reduced pressure, fractions were yielded and collected: *n*-hexane fraction (19.4 g, 42.2%), chloroform fraction (4.9 g, 10.7%), ethyl acetate fraction (12.2 g, 26.5%), *n*-butanol fraction (5.2 g, 11.3%) and aqueous fraction (4.3 g, 9.3%). All extracts were stored in the refrigerator before the use.

Fingerprint chromatogram of EA-ACE extracts by HPLC

HPLC was performed with a Hitachi Liquid Chromatography (Hitachi Ltd., Tokyo, Japan), consisting of two model L-5000 pumps, and one model L-7455 photodiode array detector (254 nm). Samples (10 mg/mL) were filtered through a 0.45 μm PVDF-filter and injected into the HPLC column. The injection volume was 10 μL and the separation temperature was 40°C. The column was a Mightysil RP-18 GP (5 μm, 250 mm × 4.6 mm I.D.). The method involved the use of a binary gradient with mobile phases containing: (A) phosphoric acid in water (0.6‰, *v/v*) and (B) MeOH (*v/v*). The solvent gradient elution program was as follows: from 88% A to 78% A in 30 min, from 78% A to 68% A in 15 min. The flow-rate was kept constant at 1.0 mL/min. A precolumn of μ-Bondapak™ C_{18} (Millipore, Milford, MA, USA) was attached to protect the analytical column. For photodiode array detection, the

wavelengths of phenolic compounds at their respective maximum absorbance wavelength can monitored at the same time. Identification is based on retention times and on-line spectral data in comparison with authentic standards. Quantification is performed by establishing calibration curves for each compound determined, using the standards.

Determination of antioxidant activity by $ABTS^{\cdot+}$ scavenging ability

The $ABTS^{\cdot+}$ scavenging ability was determined according to the method of Huang *et al*., (2006). The antioxidative activity assay that uses ABTS is called the TEAC assay. Aqueous solution of ABTS (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 hrs in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). An aliquot (20 μL) of each sample (125 μg/mL) was mixed with 180 μL $ABTS^{\cdot+}$ solution and the absorbance was read at 734 nm after 1 min. Trolox was used as a reference standard. A standard curve was constructed for Trolox at 0, 15.625, 31.25, 62.5, 125, 250, 500 μM concentration.

Determination of antioxidant activity by DPPH radical scavenging ability

The effects of crude extracts and positive controls (BHT) on DPPH radicals were estimated according to the method of Huang *et al*., (2006). Aliquot (20 µL) of crude extracts at various concentrations were each mixed with 100 mM Tris-HCl buffer (80 µL, pH 7.4) and then with 100 µL of DPPH in ethanol to a final concentration of 250 µM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance of the reaction solution was measured spectrophotometrically at 517 nm. The percentages of DPPH decolorization of the samples were calculated according to the equation: % decolorization = [1- (ABS_{sample} /$ABS_{control}$)] ×100. EC_{50} value was the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

Determination of total polyphenol content

The total polyphenol contents of crude extracts were determined according to the method of Huang *et al* (2008). 20 µL of each extract was added to 200 µL distilled water and 40 µL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min and then 40 µL of 20 % sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. Catechin was used as a standard for the calibration curve. The polyphenol content was calibrated

using the linear equation based on the calibration curve. The total polyphenol content was expressed as mg catechin equivalence (CE)/g dry weight.

Determination of Total Flavonoid Content

The flavonoid content was determined according to the method of Lamaison and Carnet (1990). 100 μL aliquots of the extract and fractions were added to equal volumes of 2% $AlCl_3 \cdot 6H_2O$ solutions. The mixtures were shaken vigorously and left incubating for 10 minutes before the absorbance was read at 430 nm. Rutin was used as standard for the calibration curve, by which a linear equation was derived to determine total flavonoid contents of the samples. Total flavonoid data were expressed in mg of rutin equivalents per gram of dry weight.

Cell culture

A murine macrophage cell line RAW264.7 (BCRC No. 60001) and A549 (BCRC No. 60074) were purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in a CO_2 incubator (5% CO_2 in air) at 37°C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (DPBS).

Cell viability

Raw 264.7 cells (2 x 10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with the methanol extract and fractions in the presence of 100 ng/mL LPS for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 h at 37°C testing for cell viability. A549 were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin, 100 mg/mL streptomycin, and 1 mM sodium pyruvate. The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then treated with the methanol extract, fractions, and its active compounds for 24 h. Each concentration was repeated three times. After a period of incubation, the medium was removed, and then the cells were washed with PBS. The medium was then discarded and 100 μL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

Measurement of Nitric oxide/Nitrite

Nitrite levels in the cultured media, which reflect intracellular NO synthase activity, were determined by Griess reaction (Huang *et al*., 2007). The cells were incubated with samples in the presence of LPS (100 ng/mL) at 37°C for 24 hrs. Then, cells were dispensed into 96-well plates, and 100 μL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 10 min. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured form absorbance at 540 nm.

Protein Lysate Preparation and Western blot Analysis of iNOS and COX-2

Total protein was extracted with a RIPA solution (radioimmuno-precipitation assay buffer) at -20°C overnight. We used BSA (bovine serum albumin) as a protein standard to calculate equal total cellular protein amounts. Protein samples (30 μg) were resolved by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using standard methods, and then were transferred to PVDF membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies (iNOS, COX-2, and β-actin) at 4°C overnight, washed three times with PBST, and incubated for 1 h at 37 °C with horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software and represented in the relative intensities.

Statistical analysis

Experimental results were presented as the mean ± standard deviation (S.D.) of

three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests). Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results and Discussion

Fingerprint Analysis by HPLC

To establish the fingerprint chromatogram for the quality control of EA-ACE, protocatechuic acid, catechin, and epicatechin were used as markers. An optimized HPLC-PAD technique was employed. Meanwhile, HPLC chromatograms showed three marker components present in EA-ACE. As shown in Fig. 1, these phenolic components have been identified as protocatechuic acid, catechin, and epicatechin by their retention time and UV absorbance of purified standards. According to the plot of peak-area ratio (y) vs. concentration (x, μg/mL), the regression equations of three constituents and their correlation coefficients (r) were determined as follows: protocatechuic acid, $y = 0.622x + 4.301$ ($r^2 = 0.999$); catechin, $y = 0.443x + 3.104$ ($r^2 = 0.999$); epicatechin, $y = 0.836x + 3.893$ ($r^2 = 0.999$). The relative amounts of three phenolic compounds found in EA-ACE were in the order of epicatechin (3.96 mg/g extract)> catechin (2.52 mg/g extract) > protocatechuic acid (1.64 mg/g

extract), respectively.

The contents of phytochemicals extracted and the antioxidant activities of EA-ACE.

Phenolic compounds occur ubiquitously in plants and are active components in many herbs. Numerous studies have shown that these phenolic compounds possess antioxidant and anti-inflammatory activity (Huang *et al*., 2011). Table 1 shows ABTS and DPPH scavenging activities of MACE and fractions. EA-ACE exhibited the stronger antioxidant activities in scavenging DPPH radicals, with EC_{50} values of 225.29 ± 4.93 μg/mL. And TEAC value of EA-ACE was 159.59 ±1.17 μg /mg extract. The results also showed that EA-ACE had the highest phenolic contents of 627.56 ± 1.15 μg CE/mg (Table 1). Total flavonoid content was expressed as mg of rutin equivalent per gram of dry weight. As shown in Table 1, the total flavonoid content of EA-ACE was 14.31 ± 0.38 μg RE/mg.

As shown in Table 1, reference compounds protocatechuic acid, catechin, and epicatechin in the EA-ACE showed TEAC value of 1252.14 ± 2.87, 1513.36 ± 4.53, and 857.56 ± 3.59 μg/mg extract, respectively. In addition, the reference compounds protocatechuic acid, catechin, and epicatechin in the EA-ACE in the EAAC showed DPPH radical scavenging with an EC_{50} value of 11.55 ± 0.26, 9.38 ± 0.32, and 21.32 ± 0.23 μg/mL.

Polyphenols act as antioxidants via several mechanisms including the scavenging of free radicals, chelation of transition metals, as well as the mediation and inhibition of enzymes (Chang *et al*., 2009). The radical scavenging activity of EA-ACE seems to be correlated with its polyphenolic constituents through active components that could play important roles in its antioxidative effect. In this paper, we demonstrated that EA-ACE inhibited radical scavenging. And the reference compound of protocatechuic acid, catechin, and epicatechin in the EA-ACE also had the antioxidant activities.

Relationship between Total Antioxidant Power with Respect to Total Phenolic and Total Flavonoid Contents

Correlation coefficients (R^2) of the total antioxidant power with respect to total phenols and total flavonoid contents of ACE were estimated in this study. As shown in Figure 2, the correlation coefficient (R^2) of TEAC and total phenolic content was 0.96 (Fig. 2A). The R^2 value of TEAC and total flavonoid content was 0.47 (Fig. 2B). The results revealed high correlations between TEAC and total phenolic contents.

Effect of EA-ACE on LPS-induced NO Production in Macrophages

The effect of MACE and fractions on RAW264.7 cell viability was determined by a MTT assay. Cells cultured with MACE and fractions at the concentrations (0, 62.5, 125, and 250 μg/mL) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability, significantly (Fig. 3A).

In a cellular model of inflammation, the NO inhibitory activity of MACE and fraction were determined by using the LPS activated macrophages to produce NO radicals measured as nitrites in the culture medium by the Griess reaction. As shown in Fig. 3B, EA-ACE reduced the NO production of activated macrophages with an IC_{50} value of 96.29 ± 0.28 μg/mL, respectively. This suggests EA-ACE could be a potential inhibitor of NO related inflammation pathway (Table 2; partial data of table 2 is based on Figure 3A). And the reference compounds of protocatechuic acid, catechin, and epicatechin in the EA-ACE also showed the NO inhibitory activity induced by LPS in RAW264.7 macrophages (Table 2). Protocatechuic acid and epicatechin had weak or no anti-inflammatory activity induced by LPS in RAW264.7 macrophages, respectively. Whatever, we evaluated the reference compounds in the EA-ACE that catechin exhibited good anti-inflammatory activities by LPS-induced NO production in RAW264.7 macrophages with an IC_{50} value of 35.53 ± 0.31 μg/mL (Table.2).

Inhibition of LPS-induced iNOS and COX-2 Protein by EA-ACE.

The results showed that incubation with EA-ACE in the presence of LPS for 24 hrs inhibited iNOS protein expression in mouse macrophage RAW264.7 cells in a dose-dependent manner (Fig. 4A). The intensity of protein bands were analyzed and showed an average of 86.2% and 10.6% down-regulation of iNOS and COX-2 proteins, respectively, after treatment with EA-ACE at 250 μg/mL compared with the LPS-alone (Fig. 4B).

Excessive production of NO plays a critical role in the aggravation of circulatory shock and chronic inflammatory diseases, such as septic shock, inflammatory hepatic dysfunctions, inflammatory lung disease and colitis (Huang and Ho, 2010; Chang *et al*., 2011). As many of these conditions exhibit rapid onset and development, often resulting in the failure of conventional anti-inflammatory therapies and extremely high mortality rates, a simultaneous suppression of NO production pathways, as shown by EA-ACE and catechin may satisfy the control of the rapid progression of the inflammatory process.

Cell Viability

MTT assay was used to investigate whether MACE and fractions affected the viability of A549 cells. The viabilities of human A549 cells were treated with 200,

400, 600, 800, and 1000 μg/mL of the methanol extract and fractions were assayed by MTT. After exposing A549 cells to any of the samples with various concentrations, the cell viabilities decreased significantly as compared to the control (100%), indicating cytotoxic effect on A549 cells. Among all the fractions, ethyl-acetate fraction (IC_{50}= 469.17 ± 3.59) fractions showed excellent inhibitory effects on A549 cells, as shown in Table 3. Protocatechuic acid, catechin, and epicatechin showed little inhibitory effects on A549 cells. The components which are responsible for the antiproliferative activity of the ACE are still unclear. Therefore, further researches must be performed to isolate and identify these components.

Phenolic contents were not only indicated in this experiment to be directly proportional with antioxidant activity, as suggested by Huang *et al*. (2008), these phytochemicals may also possess unique or synergic activities on the inhibition of tumor cell proliferation *in vitro*. In previous phytochemical investigations, water-eluted fraction showed the strongest inhibitory effect on the growth of S180 sarcoma transplanted in mice from *Actinidia eriantha* (Xu *et al*., 2009) and the extracts from roots of *Actinidia indochinensis* possessed the role of antitumors in cell culture (Zhong *et al*., 2005). It is possible that at least part of the antioxidant or inflammation effects observed in the present work may be due to phenolic compounds.

Conclusion

This study revealed that ethyl-acetate fractions of *Actinidia callosa* var. ephippioides exhibited good antioxidant activities, anti-inflammatory activities and inhibited the growth of A549 cell. These activities may be attributed to the high polyphenolic contents in these fractions. This study also demonstrated that *Actinidia callosa* var. ephippioides had a wide safety dosage range and has a potential for local therapeutic applications in chronic diseases. Further studies should be carried out to correlate the pharmacological activities with the chemical constituents

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