

## Antioxidant, Anti-inflammatory and Antiproliferative Activities of *Kalanchoe gracilis* (L.) DC Stem

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**Abstract:** Oxidative stress and inflammation are related to several chronic diseases including cancer and atherosclerosis. *Kalanchoe gracilis* (L.) DC is a special folk medicinal plant in Taiwan. The aim of this study was to evaluate the antioxidant, anti-inflammatory and anti-proliferative activities of the methanolic extract and fractions of the stem of *K. gracilis*. TEAC, total phenolic compound content, total flavonoid content, DPPH radical scavenging activity, reducing power, inhibition of NO production in LPS-induced RAW264.7 cells, and inhibition of cancer cell proliferation were analyzed. Among all fractions, the chloroform fraction showed the highest TEAC and DPPH radical scavenging activities. The chloroform fraction also had the highest content of polyphenols and flavonoids. Chloroform fractions also decreased LPS-induced NO production and expressions of iNOS and COX-2 in RAW264.7 cells. The antiproliferative activities of the methanolic extract and fractions were studied *in vitro* using HepG2 cells, and the results were consistent with their antioxidant capacities. Chloroform fractions had the highest antiproliferative activity with an IC<sub>50</sub> of

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$136.85 \pm 2.32 \mu\text{g/ml}$ . Eupafolin also had good pharmacological activity in the antioxidant, anti-inflammation and antiproliferative. Eupafolin might be an important bioactive compound in the stem of *K. gracilis*. The above experimental data indicated that the stem of *K. gracilis* is a potent antioxidant medicinal plant, and such efficacy may be mainly attributed to its polyphenolic compounds.

**Keywords:** *Kalanchoe gracilis*; Antioxidant Activity; Anti-inflammatory Activity; Antiproliferation.

## Introduction

Oxidative stress is caused by reactive oxygen species (ROS). ROS are molecules with unpaired electrons that come from both normal metabolism and external sources. These highly reactive molecules may cause cell membrane injuries (Halliwell, 2007), and are associated with many degenerative diseases, such as aging, cardiovascular diseases and cancer. The body not only has repair mechanisms to restore damaged tissues and enzymes, but can also maintain a reducing environment to combat and prevent oxidative stress. Additionally, nutrients and active ingredients in foods and herbs may also assist in preventing oxidative damage. Recently, extraction via polarity has been frequently applied in pharmacology and phytochemistry research on medicinal plants. Flavonoids are a class of secondary metabolites that can be found in all vascular plants. Plants containing flavonoids have been reported to possess strong antioxidant activities (Raj *et al.*, 1999; Lai *et al.*, 2009, 2010a).

Acute inflammation is part of the defense response, but chronic inflammation has been found to mediate a wide variety of diseases, including cardiovascular diseases, cancers, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, and autoimmune diseases. From the viewpoint of cellular biology, chronic inflammation accompanied by oxidative stress is linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Lin and Tang, 2008). Inflammation leads to up-regulation of a series of enzymes in affected areas. Inducible nitric oxide synthase (iNOS) catalyzes the formation of nitric oxide (NO) from L-arginine. High concentrations of NO have been found to play important roles in inflammation and carcinogenesis. iNOS can be induced by bacterial endotoxic lipopolysaccharide (LPS), interferon- $\gamma$  (IFN- $\gamma$ ), and a variety of pro-inflammatory cytokines (Wang *et al.*, 2011).

*Kalanchoe gracilis* (L.) is a medicinal plant from the Crassulaceae family. It is called "Da-Huan-Hun" in Chinese; folklorically, it is also referred to as "Da Hao Ji Zhao Huang," which means chicken claws, due to the morphology of its leaves (Huang, 1993). In Taiwan, it is a folk medicine commonly used by traditional Chinese medicine (TCM) practitioners for the treatment of pain, fever, inflammation and injuries. Previous phytochemical investigations have isolated one coumarin and eight bufadienolides (Wu *et al.*, 2006), as well as nine flavonoids that were further identified as luteolin, quercetin, quercitrin, kaempferol, eupafolin and four glycosidic derivatives of eupafolin (Karin *et al.*, 1989). In our previous study, we demonstrated that *K. gracilis* increased SOD and GRx activities; its

methanol extract was also shown to have analgesic and anti-inflammatory effects in mice (Lai *et al.*, 2010b).

In this study, we further investigated the antioxidant, anti-inflammatory and anti-proliferative activities of the methanolic extract and fractions of *K. gracilis* root by characterizing their antioxidative potencies, polyphenol contents, and anti-inflammatory and cancer growth inhibition activities. Additionally, an HPLC fingerprint of the extract was established in this study.

## Materials and Methods

### *Chemicals*

All solvents used were purchased from Merck (Darmstadt, Germany). Folin and Ciocalteu's phenol reagent, sodium carbonate, catechin, 1,1 diphenyl 1-2-picrylhydrazyl radical (DPPH·), ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), eupafolin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and LPS (lipopolysaccharide) were purchased from Sigma Aldrich Ltd (Steinheim, Germany). Anti-iNOS, anti-COX-2, and anti- $\beta$ -actin antibodies (Santa Cruz, CA, USA) were obtained as indicated.

### *Plant Materials*

Mature whole plants of *K. gracilis* were collected from farmlands and gardens in Chiayi County, Taiwan, as described by the Flora of Taiwan. The plants were identified by professor Hsin-Fu Yen from the National Museum of Natural Science, Taichung, Taiwan, before being crushed into coarse powder. A plant specimen was deposited in the Institute.

### *Extraction and Fractionation*

The coarse powder from the *K. gracilis* stems (12.8 kg) was extracted with methanol three times. The extract was evaporated under reduced pressure using a rotavapor, and then stored under light protection. A yield equivalent to 3.01% of the original weight was obtained. Next, MKG was dissolved and suspended in 500 ml of water in a separatory funnel prior to being partitioned in sequence with *n*-hexane, chloroform, ethyl acetate and *n*-butanol (300 ml each, three times). Under reduced pressure, fractions were yielded and collected: *n*-hexane fraction (96.1 g, 25.% of total extract), chloroform fraction (8.3 g, 2.2%), ethyl acetate fraction (23.6 g, 6.1%), *n*-butanol fraction (97.2 g, 25.2%) and aqueous fraction (154.8 g, 4.2%). All extracts were stored in the refrigerator before use.

### *Fingerprint Analysis by HPLC*

HPLC fingerprint profiles were established for 0.1 mg/ml of eupafolin and 5 mg/ml of methanol crude extract. HPLC analysis was performed on a Waters HPLC 2695 separation

module. Chromatographic separation was carried out on a LiChroCART RP-18 endcapped column (250 × 4.6 mm, i.d., 5 μm pore size) with an injection of 10 μl by using an elution of 0.2% formic acid: acetonitrile (65:35) solvent at a flow rate of 0.8 ml/min. Peaks were detected at 350 nm with a 2996 PDA detector.

#### *Trolox Equivalent Antioxidant Capacity (TEAC)*

Trolox equivalent antioxidant capacity was performed as reported by Re (1999). Briefly, ABTS was dissolved in water at a concentration of 8 mM. ABTS radical cation was produced by reacting ABTS stock solution with 8.4 mM potassium persulfate in a ratio of 2:1 and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The crude extract, fractions and eupafolin standard were diluted with methanol to prepare sample solutions with concentrations of 0.05, 0.1, 0.2, 0.4 and 0.8 mg/ml. Similarly, the reference standard Trolox was diluted with methanol to concentrations of 125, 62.5, 31.25, 15.63, 7.81 and 3.9 μg/ml. Sample solutions (25 μl) were added to the wells in a 96 well plate, followed by adding 175 μl of ABTS radical cation solution. Absorbance of the resulting solutions was measured at 734 nm using an ELISA reader (power wave X340). The percentage inhibition of absorbance at 734 nm was calculated, and TEAC was expressed as micromoles of Trolox equivalents 1 mg/ml of the sample.

#### *Determination of Antioxidant Activity by DPPH Method*

The DPPH method described by Kim (2003) is a popular way of evaluating antioxidant activities in foods and herbs. The capacities of the extract and fractions to scavenge DPPH radicals were compared to a daily prepared standard of α-tocopherol (final concentration 9.375–75 μg/ml). The sample solutions were diluted with methanol to 0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml and 1.6 mg/ml concentrations, and 25 μl were added to the wells of a 96 well plate. 175 μl of 0.3 mM DPPH solution was then added, and stand at room temperature for 30 min. Absorbance of the resulting solutions was measured at 517 nm using an ELISA reader (power wave X340). The inhibition percentage (%) was calculated using the following equation: inhibition (%) =  $(A_0 - A_s)/A_0 \times 100$ , where  $A_0$  and  $A_s$  represent the absorbance of the control and the sample at 517 nm, respectively.

#### *Reducing Power Assay*

The reducing powers of the extract and fractions were determined according to the method described by Wu (2007). The reference standard ascorbic acid was diluted with methanol to concentrations of 250, 125, 62.5, 31.3 and 15.6 μg/ml. The extract, fractions, and eupafolin, on the other hand, were diluted to concentrations of 2.5, 1.25, 0.625, 0.313, 0.156 mg/ml with distilled water. After 50 μl of 50 μM phosphate buffer (pH 6.6) and 50 μl of 0.1% (w/v) potassium ferricyanide were added, the solutions were incubated in a water bath at 50°C for 20 min. Following this, 100 μl of 1% (w/v) trichloroacetic acid solution

was added and centrifuged at 3000 rpm for 10 min. 175  $\mu$ l aliquots of the upper layer were combined with 25  $\mu$ l of 5 mM ferric chloride prior to measuring the absorbance at 700 nm using an ELISA reader (power wave X340). 3 independent experiments were conducted. The reducing power data were expressed in  $\mu$  of ascorbate equivalents per mg of dry weight.

#### *Determination of Total Phenolic Compounds in the Extract and Fractions of K. gracilis*

Total phenolic compounds in the extract and fractions were estimated by a colorimetric assay (Amarowicz *et al.*, 2004). The extract and fractions (50  $\mu$ l, four replicates) were pipetted into the wells of a 96 well plate before 50  $\mu$ l of Folin and Ciocalteu's phenol reagent and 125  $\mu$ l of saturated sodium carbonate solution were added. After shaking for 20 s, the mixtures remained at room temperature for 30 min. The absorbance was measured at 725 nm using an ELISA reader (power wave X340, Bio-Tek Instrument, Inc), and catechin was used for construction of the standard curve. The total phenolic contents were expressed in mg of catechin equivalents per gram of the samples.

#### *Determination of Total Flavonoid Content*

The flavonoid content was determined according to the method of Lamaison and Carnet (1990). 100  $\mu$ l of the extract and fractions were added to equal volumes of 2%  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  (2 g in 100 ml methanol) solutions. The mixtures were shaken vigorously and incubated for 10 min before the absorbance was read at 430 nm. Rutin was used as the standard for the calibration curve, by which a linear equation was derived to determine the 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 HepG2 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  $\text{CO}_2$  incubator (5%  $\text{CO}_2$  in air) at 37°C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in  $\text{Ca}^{2+}$ -,  $\text{Mg}^{2+}$ -free phosphate-buffered saline (DPBS).

#### *Cell Viability*

RAW264.7 cells ( $2 \times 10^5$ ) were cultured in a 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with the methanolic 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  $\mu$ l of 0.5 mg/ml MTT for 2 h at 37°C, testing for cell viability. HepG2 cells 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<sub>2</sub> and 95% air. The cells were then treated with the methanolic extract and fractions for 72 h. Each concentration was repeated three times. After a period of incubation, the medium was removed, and 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*

NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum, determined by a colorimetric method based on the Griess reaction (Chang *et al.*, 2009). The cells were incubated with the methanolic extract and fractions in the presence of LPS (100 ng/ml) at 37°C for 24 h. 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; absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale, CA, USA). By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

#### *Western Blotting Analysis*

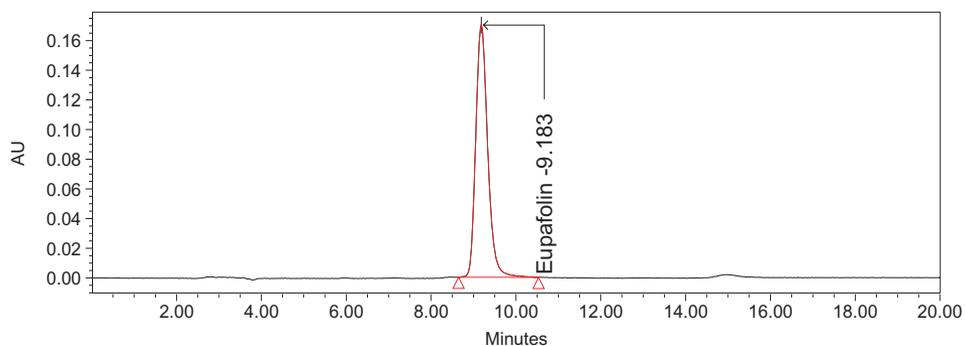
Whole-cell lysate proteins (3 µg) were mixed with an equal volume of electrophoresis sample buffer, and boiled for 10 min. Then, equal protein contents of total cell lysates from the control and different fractions were resolved on 10–12% SDS-PAGE gels. Proteins were then transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) by electroblotting using an electroblotting apparatus (Bio-Rad). Nonspecific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST for 10 min three times and then incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4°C. The membranes were washed with TBST and incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated, goat antimouse, or antirabbit IgG) for 1 h. After washing the membranes in TBST for 10 min three times, the bands were visualized using ECL reagents (Millipore). Band intensity on scanned films was quantified using Kodak Molecular imaging (MI) software and expressed as relative intensity compared to the control.

## **Results and Discussion**

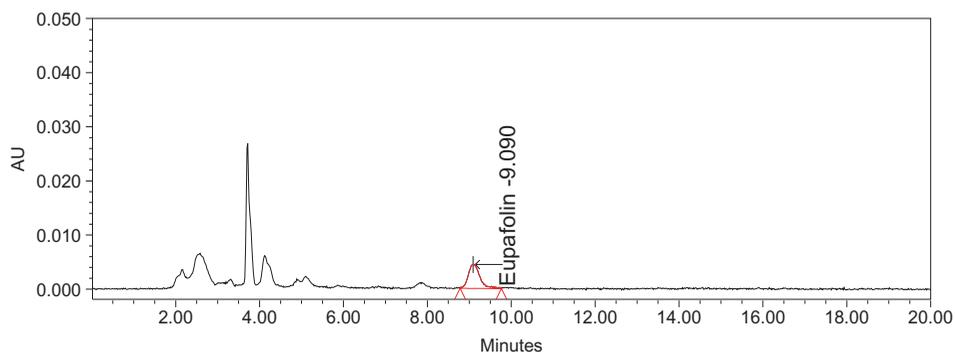
#### *Fingerprint Analysis by HPLC*

Eupafolin was used as the marker component for the standardization of flavonoid ingredients in the methanol extract, ethyl acetate fraction, and *n*-butanol fraction by HPLC. The retention time of eupafolin was found to be 9.28 min at 350 nm (Fig. 1A). According to the

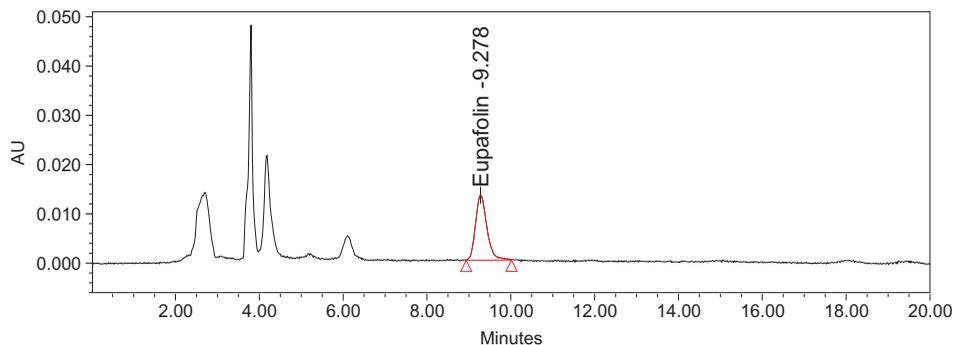
plot of peak-area ratio ( $y$ ) vs. concentration ( $x, \mu\text{g/ml}$ ), the regression equations of the constituents and their correlation coefficients ( $r$ ) were determined:  $y = 16.346 \times +0.0617$  ( $r^2 = 0.9995$ ). The relative amount of eupafolin was  $58.8 \pm 0.32$  and  $16.4 \pm 0.13$  mg/g in the methanolic extract and fractions of *K. gracilis*, respectively (Figs. 1B and 1C).



(A)



(B)



(C)

Figure 1. HPLC fingerprint profiles of eupafolin (A), methanol extract (B) and chloroform fraction (C) at 350 nm.

*Trolox Equivalent Antioxidant Capacity*

Table 1 shows TEAC values of the methanolic extract and fractions of *K. gracilis*. The TEAC value of the methanolic extract was  $126.03 \pm 0.93$  mM. As for the fractions, the chloroform fraction exhibited the strongest antioxidant activity ( $1128.7 \pm 55.50$  mM), followed by the ethyl-acetate fraction ( $1075.1 \pm 44.10$  mM), *n*-butanol fraction ( $270.7 \pm 0.92$  mM), *n*-hexane fraction ( $157.53 \pm 0.93$  mM) and water fraction ( $19.47 \pm 0.16$  mM).

*Scavenging Activity Against 1, 1-Diphenyl-2-Picrylhydrazyl Radical*

A freshly prepared DPPH solution is dark purple in color with a maximum absorption at 517 nm. This color generally fades and disappears when an antioxidant is present in the solution. The reason behind this is that antioxidant molecules can scavenge DPPH free radicals by supplying protons or by donating electrons, converting them to colorless products. The chloroform and ethylacetate fractions of *Kalanchoe gracilis* exhibited the strongest antioxidant activities in scavenging DPPH radicals, with IC<sub>50</sub> values of  $0.64 \pm 0.08$  and  $0.82 \pm 0.03$  mg/ml, respectively (Table 1).

*Reducing Power Assay*

Reducing power may be regarded as an indicator of potential antioxidant activity (Li *et al.*, 2009). Previous researchers have considered that the antioxidant activities of medicinal plants and health foods are related to their reducing powers (Wu *et al.*, 2007). In the reducing power assay, the presence of antioxidants reduces the Fe<sup>3+</sup>/ferricyanide complex to the ferrous form and results in a color change from yellow to greenish blue. Therefore the reducing power can be measured by detecting the formation of Perl's Prussian blue at 700 nm (Jeong *et al.*, 2010). The reducing powers of the methanolic extract and fractions

**Table 1. Antioxidant Activity of the Methanolic Extract and Fractions from the Stem of *K. gracilis*<sup>a</sup>**

Samples	TEAC (mM /mg Extract)	DPPH Scavenging Activity EC <sub>50</sub> (mg/ml)	Reducing Power (Ag E/mg) <sup>b</sup>
Methanol extract	$126.03 \pm 0.93$	$4.90 \pm 0.22$	$11.68 \pm 0.08$
<i>n</i> -Hexane fraction	$157.53 \pm 0.93$	$4.64 \pm 0.34$	$14.24 \pm 0.04$
Chloroform fraction	$1128.76 \pm 55.48$	$0.64 \pm 0.08$	$36.52 \pm 0.47$
Ethyl-acetate fraction	$1075.06 \pm 43.97$	$0.82 \pm 0.03$	$47.96 \pm 1.14$
<i>n</i> -Butanol fraction	$270.70 \pm 0.89$	$2.53 \pm 0.55$	$11.39 \pm 0.03$
Water fraction	$19.47 \pm 0.16$	> 9	$5.52 \pm 0.01$
Eupafolin	$775.36 \pm 14.56$	$1.48 \pm 0.05$	$177.0 \pm 5.94$

<sup>a</sup>Values represented mean  $\pm$  S.D. of three parallel measurements ( $p < 0.05$ ).

<sup>b</sup>Data expressed in  $\mu$ g ascorbate equivalents per gram (mg AE/g).

were examined with respect to their concentrations, and the results revealed a direct proportional relationship between the reducing power and the concentration of the sample (Table 1). The reducing powers of the samples ranged from  $5.52 \pm 0.01$  to  $47.96 \pm 1.14 \mu\text{g}$  ascorbate/mg and were in the following decreasing order: ethylacetate > chloroform > *n*-hexane > *n*-butanol > water. Ethyl acetate and chloroform fractions had stronger abilities to react with free radicals and convert them into more stable, nonreactive forms, thereby terminating radical chain reactions. The relationships of the reducing powers of the fractions with respect to DPPH radical scavenging activity ( $1/\text{IC}_{50}$ ) and Trolox equivalent antioxidant capacity were appraised and expressed as correlation coefficients ( $R^2$ ).  $R^2$  values of reducing power/DPPH scavenging activity and reducing power/TEAC were 0.8116 and 0.8684, respectively. There was a high, positive correlation between these assays. Therefore, the chloroform and ethyl acetate fractions had the best antioxidant activities, in reducing and scavenging free radicals.

#### *Determination of Total Phenolic and Total Flavonoid Contents in the Plant Extract and Fractions*

Plants containing polyphenols have been reported to possess strong antioxidant activities (Raj *et al.*, 1999). The chloroform and ethyl acetate fractions had the highest phenolic contents:  $169.21 \pm 3.82$  and  $162.64 \pm 2.27$  mg catechin equivalents per gram, respectively. However, phenolic contents in the hydrophilic fractions were low. The total phenolic content of *Kalanchoe gracilis* methanolic extract was  $21.02 \pm 0.09$  mg/g, and the total phenolic contents of the fractions are presented in Table 2.

The total flavonoid content was expressed as mg of rutin equivalent per gram of dry weight. As shown in Table 2, the total flavonoid content of 1 g methanolic extract of *Kalanchoe gracilis* was  $9.34 \pm 0.41$  mg of rutin equivalent per gram of dry weight. The total flavonoid contents of the extract and fractions varied from  $2.50 \pm 0.001$  to  $151.41 \pm 4.30$  mg/g. Among all the fractions, the ethyl acetate fraction had the highest total flavonoid content,  $151.41 \pm 4.30$  mg of rutin equivalent per gram of dry weight.

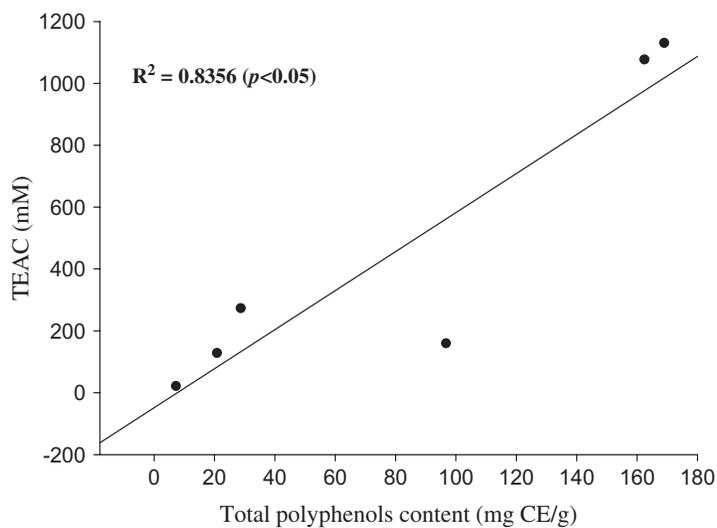
**Table 2. Total Phenolic and Flavonoid Contents of the Methanolic Crude Extract and Fractions from the Stem *K. gracilis*<sup>a</sup>**

Samples	Total Phenolic Content (mg CE/g) <sup>b</sup>	Total Flavonoid Content (mg RE/g) <sup>c</sup>
Methanol extract	$21.02 \pm 0.09$	$9.34 \pm 0.41$
<i>n</i> -Hexane fraction	$96.92 \pm 4.21$	$5.50 \pm 0.01$
Chloroform fraction	$169.21 \pm 3.82$	$60.82 \pm 0.37$
Ethyl-acetate fraction	$162.64 \pm 2.27$	$151.41 \pm 4.30$
<i>n</i> -Butanol fraction	$28.88 \pm 0.17$	$30.91 \pm 2.13$
Water Fraction	$7.44 \pm 0.01$	$2.50 \pm 0.01$

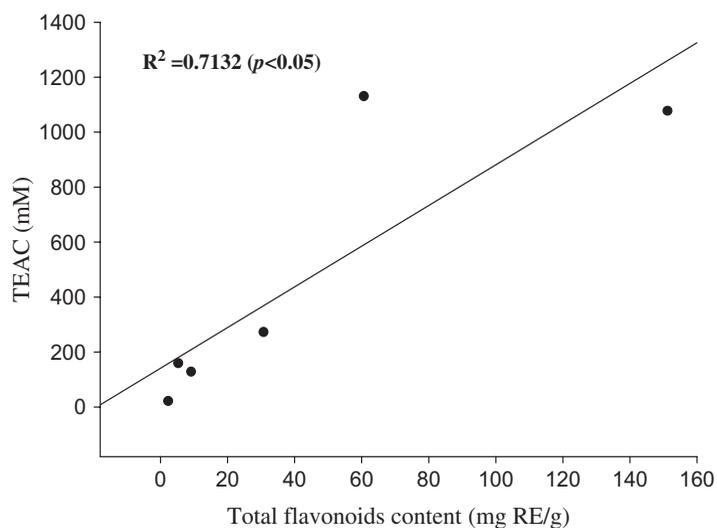
<sup>a</sup>Values represented mean  $\pm$  S.D. of three parallel measurements.

<sup>b</sup>Data expressed in mg catechin equivalents per gram (mg CE/g).

<sup>c</sup>Data expressed in mg rutin equivalents per gram (mg RE/g).



(A)



(B)

Figure 2. Correlation coefficients of TEAC values and total phenolic (A) and total flavonoid (B) contents of the methanolic extract and fractions from the stem of *K. gracilis*.

Phenols and flavonoids are common groups of polyphenolic compounds. Polyphenolic compounds have important roles in stabilizing lipid peroxidation due to their antioxidative activities (Raj *et al.*, 1999). Many studies have indicated that the antioxidant capacities of flavonoids are due to the number and position of hydroxyl groups in their structures (Heim *et al.*, 2002).

*Relationship between Total Antioxidant Power and 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 *K. gracilis* were estimated. As shown in Fig. 2, the correlation coefficient ( $R^2$ ) of TEAC and total phenolic content was 0.8356 (Fig. 2A). The  $R^2$  value of TEAC and total flavonoid content was 0.7132 (Fig. 2B). The results revealed high correlations between TEAC and total phenolic/total flavonoid contents. Therefore, the higher the TEAC activity, the higher the total phenolic and flavonoid contents in the samples.

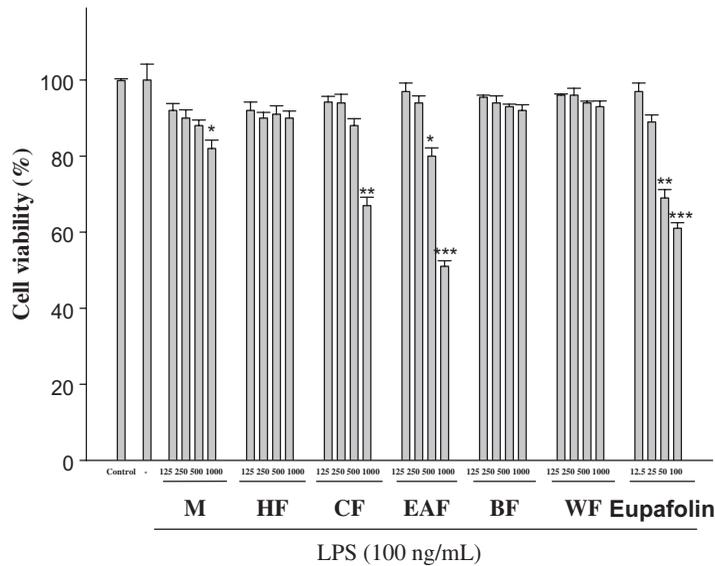
*Effect of the Methanolic Extract and Fractions on LPS-Induced NO Production in Macrophages*

The effect of the methanolic extract and fractions on RAW264.7 cell viability was determined by MTT assays. Cells cultured with the methanolic extract and fractions at the concentrations (0, 125, 250, 500, and 1000  $\mu\text{g/ml}$ ) in the presence of 100 ng/ml LPS for 24 h did not change in viability (Fig. 3A). However, when RAW264.7 macrophages were incubated with 100 ng/ml of LPS and chloroform or ethyl-acetate fraction at the 1000  $\mu\text{g/ml}$  concentration for 24 h, they showed significantly inhibited cell viability. When RAW264.7 macrophages were incubated with 100 ng/ml of LPS and eupafolin at the 50 or 100  $\mu\text{g/ml}$  concentrations for 24 h, cell viability was also significantly inhibited.

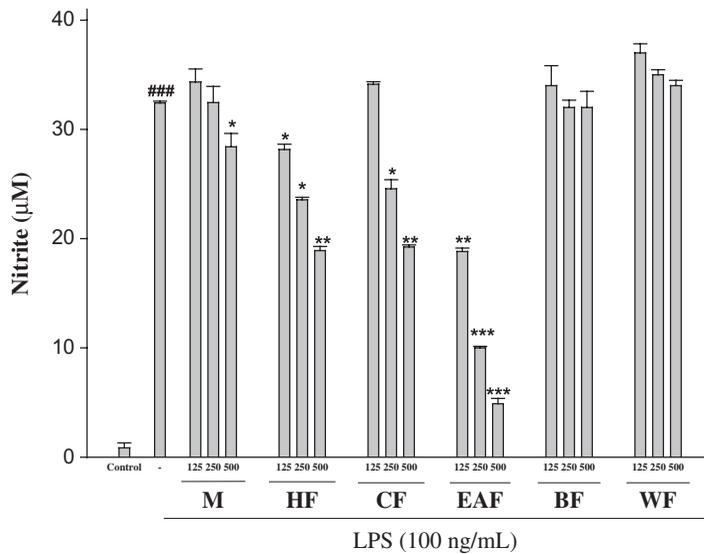
In the present study, the effects of the methanolic extract and fractions on LPS-induced NO production in RAW 264.7 macrophages were investigated. Nitrite accumulated in the culture medium was estimated by the Griess reaction as an index for NO release from the cells. After treatment with LPS (100 ng/ml) for 24 h, the nitrite concentration increased in the medium. When RAW264.7 macrophages were treated with different concentrations of the methanolic extract and fractions together with LPS for 24 h, nitrite production was significantly inhibited (Fig. 3B). When RAW264.7 macrophages were treated with different concentrations of chloroform fraction (0, 125, 250, and 500  $\mu\text{g/ml}$ ) together with LPS (100 ng/ml) for 24 h, a significant dose-dependent inhibition of nitrite production was detected. There was a significant decrease in nitrite production in the group treated with 6.12  $\mu\text{g/ml}$  eupafolin ( $p < 0.1$ ), and a highly significant decrease in the groups treated with 12.5 and 25  $\mu\text{g/ml}$  of eupafolin, when compared to the LPS-alone group ( $p < 0.001$ ).

*Inhibition of LPS-Induced iNOS and COX-2 Protein by Chloroform Fraction and Eupafolin*

In mouse macrophage RAW264.7 cells, the incubation of chloroform fraction or eupafolin with LPS for 24 h inhibited iNOS and COX-2 protein expression in a dose-dependent



(A)



(B)

Figure 3. The effects of the methanolic extract and fractions from the stem of *K. gracilis* on lipopolysaccharide (LPS)-induced cell viability (A) and NO production (B) in RAW 264.7 macrophages. Cells were incubated with 100 ng/ml of LPS for 24 h in the absence or presence of samples (0, 125, 25, 500, or 1000  $\mu\text{g}/\text{ml}$ ). Cell viability assay was performed using MTT assay. Nitrite concentration in the medium was determined by Griess reagent. **M**: methanol extract; **HF**: *n*-hexane fraction; **CF**: chloroform fraction; **EAF**: ethyl-acetate fraction; **BF**: *n*-butanol fraction; **WF**: water fraction. The data were presented as mean  $\pm$  S.D. for three experiments performed in triplicate. ### compared to the control group. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  were compared to LPS-alone group.

manner (Fig. 4). The intensity of protein bands were analyzed and showed an average of 51.2% and 53.6% down-regulation of iNOS and COX-2 proteins, respectively, after treatment with chloroform fraction at 500  $\mu\text{g/ml}$ , compared to LPS alone (Fig. 4).

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). 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 the *n*-hexane fraction, chloroform fraction and eupafolin, may control the rapid progression of the inflammatory process.

### Cell Viability

MTT assays were used to investigate whether the methanolic extract and fractions affected the viability of HepG2 cells. The viabilities of human HepG2 cells treated with 200, 400, 600, 800, and 1000  $\mu\text{g/ml}$  of the extract and fractions were assayed by MTT. After exposing HepG2 cells to any of the samples in various concentrations, the cell viabilities decreased significantly as compared to the control (100%), indicating a cytotoxic effect on HepG2 cells. Among all the fractions, chloroform ( $\text{IC}_{50} = 136.85 \pm 2.32$ ) fractions showed excellent inhibitory effects on HepG2 cells, as shown in Table 3. Eupafolin also showed excellent inhibitory effects on HepG2 cells ( $\text{IC}_{50} = 9.48 \pm 0.32$ ).

Phenolic and flavonoid contents were indicated in this study to be directly proportional to antioxidant activity, as suggested by Huang *et al.* (2008). These phytochemicals may also possess unique or synergic activities with respect to the inhibition of tumor cell proliferation *in vitro*. In previous phytochemical investigations, methanolic extract of *K. gracilis* has shown cytotoxic activities against human gastric and nasopharyngeal

**Table 3. Percent Inhibition of HepG2 Cell Proliferation Induced by the Methanolic and Fractions of *K. gracilis*<sup>a</sup>**

Samples	$\text{IC}_{50}$ ( $\mu\text{g/ml}$ )
Methanol extract	> 1000
<i>n</i> -Hexane fraction	172.41 $\pm$ 3.21
Chloroform fraction	136.85 $\pm$ 2.32
Ethyl-Acetate fraction	164.57 $\pm$ 2.74
<i>n</i> -Butanol fraction	> 1000
Water fraction	> 1000
Eupafolin	9.48 $\pm$ 0.32

<sup>a</sup>Values represented mean  $\pm$  S.D. of three parallel measurements.

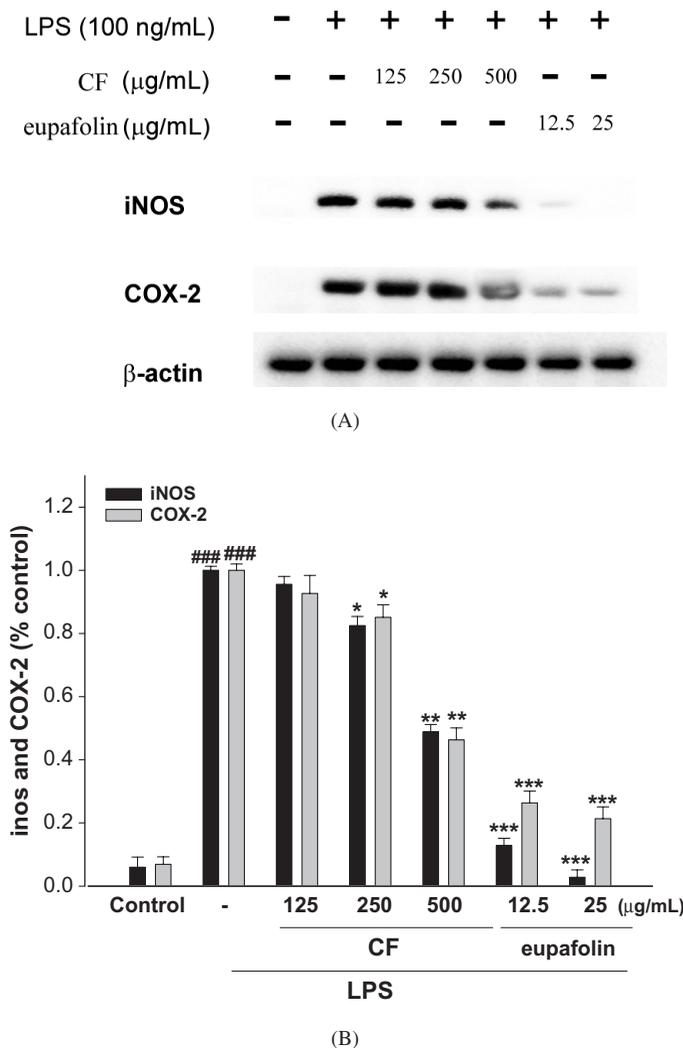


Figure 4. Inhibition of iNOS and COX-2 protein expressions by chloroform fractions and eupafolin of the stem of *K. gracilis* in LPS-stimulated RAW264.7 cells. Cells were incubated with 100 ng/ml of LPS for 24 h in the absence or the presence of chloroform fractions (0, 125, 25, and 500  $\mu\text{g/ml}$ ) and eupafolin (0, 12.5, and 25  $\mu\text{g/ml}$ ). Lysed cells were then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2.  $\beta$ -actin was used as an internal control. (A) Representative Western blot from two separate experiments is shown. (B) Both relative iNOS and COX-2 protein levels were calculated with reference to a LPS-stimulated culture. ### compared to the control group. The data were presented as mean  $\pm$  S.D. for three experiments performed in triplicate. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  were compared to LPS-alone group.

carcinoma cell lines. In addition, one of the compounds isolated from *K. gracilis*, bryophyllin, has been shown to inhibit HIV replication in H9 lymphocyte cells (Wu *et al.*, 2006). Since *K. gracilis* root is rich in polyphenolic compounds, it may be developed into an antioxidant, anti-inflammatory or anticancer agent in the future.

In conclusion, this study revealed that chloroform fractions of *K. gracilis* exhibited good antioxidant and anti-inflammatory activities, and inhibited the growth of HepG2 cells. These activities may be attributed to the high polyphenolic contents in the fractions. This study also demonstrated that *Kalanchoe gracilis* has a wide safety dosage range. Furthermore, it added to our understanding of the antioxidative activities of herbs for the purpose of improving health and preventing chronic diseases and cancers.

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