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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 antiproliferative activities of the methanolic extract and fractions from the stem of *K. gracilis*. TEAC, total phenolic compounds, total flavonoid content, DPPH radical, reducing power method, inhibition on NO productions by LPS-induced RAW264.7 cell, and inhibition on cancer cell proliferation were employed. Among all fractions, Chloroform fraction showed the highest TEAC and DPPH radical scavenging activities, respectively. The Chloroform fraction also had highest contents of polyphenol and flavonoid contents. Chloroform fractions also decreased the 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_{50} of 136.85 ± 2.32 μg/mL. Eupafolin also had good pharmacology activity in the antioxidant, anti-inflammation and antiproliferation. 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 injures (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, enzymes in the body can also maintain a reducing environment to combat and prevent oxidative stress. Additionally, nutrients and active ingredients in foods and herbs may also assist humans to prevent oxidative damage. Recently, extraction via polarity is frequently applied in pharmacology and phytochemistry researches of medicinal plants. Flavonoids are a class of plant secondary metabolites and it 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; Lai et al., 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, cancer, 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 & 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 concentration of NO are found to play important roles in inflammation and carcinogenesis. iNOS can be induced by bacterial endotoxic lipopolysaccahride (LPS), interferon-γ (IFN-γ), and a variety of pro-inflammatory cytokines (Wang, Zhou & Lin, 2011).

Kalanchoe gracilis (L.) is a medicinal plant from the Crassulaceae family. It is called "Da-Huan-Hun" in Chinese, and folklorically, it is also referred to as "Da Hao Ji Zhao Huang" which means chicken claws due to the morphology of its leaves (Huang *et al*, 1998). 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 on the antioxidant, inflammatory and antiproliferative activities of the methanolic extract and fractions from *K. gracilis* root by characterizing their antioxidative potencies, polyphenol contents, anti-inflammatory and cancer growth inhibition activities. Additionally, an HPLC fingerprint of the extract was also established in this study.

MATERIAS AND METHODS

Chemicals

All solvents used were purchased from Merck (Darmstadt, Germany). Folin and Ciocalteu's phenol reagent, sodium carbonate, catechin, 1,1 dipheny l-2-picrylhydrazyl radical (DPPH •), ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), eupaforin, Trolox (6-hydroxy-2,5,7,8-tetramethychroman-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-β-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 of *K. gracilis* stem (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 for three times). Under reduced pressure, fractions were yielded and collected: *n*-hexane fraction（96.1 g, 25.0% 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, 40.2%). All extracts were stored in the refrigerator before the 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 to 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 into concentrations of 125, 62.5, 31.25, 15.63, 7.81 and 3.9 µg/ml. The wells in a 96 well plate were pipitted with 25 µl of sample solutions, 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 micromolar concentration of Trolox equivalent 1 mg/ml of the sample.

Determination of Antioxidant Activity by DPPH Method

The DPPH method, described by Kim (2003), is also a popular way for evaluating antioxidant activities of foods and herbs. The capacities of the extract and fractions in scavenging DPPH radicals were compared with daily prepared standard α-tocopherol (final concentration 9.375-75 µg/ml). The extract and fractions were diluted with

methanol into 0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml and 1.6 mg/ml concentrations of sample solutions. The wells in a 96 well plate were pipetted with 25 µl of the sample solutions and 175 µl of 0.3 mM DPPH solution was then added. The wells were left to 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 (%) = (Ao − As)/Ao × 100 where Ao and As represent the absorbance of the control and 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 into concentrations of 250, 125, 62.5, 31.3 and 15.6 µg/ml. The extract, fractions and eupafolin, on the other hand, were mixed with distilled water into concentrations of 2.5, 1.25, 0.625, 0.313, 0.156 mg/ml. 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℃ for 20 min. Following this, 100 µl of 1% (w/v) trichloroacetic acid solution was added to each solution before centrifugation at 3000 rpm for 10 min, and 175 µl aliquot of the upper layer was combined with 25 µl 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 µg 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 µl, four replicates) were

pipetted into the wells of a 96 well plate before 50 µl of Folin and Ciocalteu's phenol reagent and 125 µl of saturated sodium carbonate solution were added. After shaking the mixtures for 20 s, they were left standing 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 µl aliquots of the extract and fractions were added to equal volumes of 2% $AlCl_3 \cdot 6H_2O$ (2 g in 100 ml methanol) 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 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 CO_2 incubator (5% CO_2 in air) at 37℃ 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 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 µL of 0.5 mg/mL MTT for 2 h at 37℃ testing for cell viability. HepG2 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℃ in a humidified atmosphere of 5% CO_2 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, 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

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 37^0C 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, the absorbance was measured at 540 nm with a Micro-Reader (*Molecular Devices, Orleans Drive, Sunnyvale, CA*). 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 lysates proteins (30 μg of protein) were mixed with an equal volume of electrophoresis sample buffer, and the mixture was then boiled for 10 min. Then, an equal protein content of total cell lysate from control, and different fractions were resolved on 10~12% SDS-PAGE gels. Proteins were then transferred onto nitrocellulose membranes (Millipore, Bedford, MA) 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 three times for 10 min and then incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4 ℃. The membranes were washed with TBST and then incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated, goat antimouse, or antirabbit IgG) for 1 h. After washing the membrane three times for 10 min in TBST, the bands were visualized using ECL reagents (Millipore, Billerica, MA). Band intensity on scanned films was quantified using Kodak Molecular imaging (MI) software and expressed as relative intensity compared with control.

RESULTS AND DISCUSSION

Fingerprint Analysis by HPLC

Eupafolin was used as the maker 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 at 9.28 min at 350 nm (Fig. 1a). Eupafolin was found present in HPLC fingerprints of the methanol extract as shown in Fig. 1b.

Trolox Equivalent Antioxidant Capacity

Table 1 shows TEAC values of the methanolic extract and fractions of *K. gracilis*. TEAC value of the methanolic extract was 126.03 ± 0.93 mM. As for the fractions, the chloroform fraction exhibited the strongest antioxidant activity (1128.7 ± 55.50 mM), followed by ethyl-acetate fraction (1075.1 ± 44.10 mM), *n*-butanol fraction (270.7 ± 0.92 mM), *n*-hexane fraction (157.53 ± 0.93 mM) and water fraction (19.47 ± 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 ethyl-acetate fractions of *Kalanchoe gracilis* exhibited the strongest antioxidant activities in scavenging DPPH radicals, with IC_{50} values of 0.64 ± 0.08 and 0.82 ± 0.03 mg/ml respectively (Table 1).

Reducing Power Assay

The reducing power of the methanolic extract and fractions may be regarded as an indicator of potential antioxidant activity (Li et al., 2009). Previous researches have considered that 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 Fe^{3+}/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 were examined with respect to their concentrations, and the results revealed a direct proportional relationship between the reducing power and concentration of the samples (Table 1). The reducing power of the samples ranged from 5.52 ± 0.01 to 47.96 ± 1.14 µg ascorbate/mg and were in the following decreasing order: ethyl-acetate > 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 power with respect to DPPH radical scavenging activity ($1/IC_{50}$) and Trolox equivalent antioxidant capacity of the fractions 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. The results showed high positive correlations 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 results showed that the chloroform and ethyl acetate fractions had the highest phenolic contents of 169.21 ± 3.82 and 162.64 ± 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 ± 0.09 mg/g, and the total phenolic contents of the fractions are presented in 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 1 g methanolic extract of *Kalanchoe gracilis* was 9.34 ± 0.41 mg of rutin equivalent per gram of dry weight. The results revealed that the total flavonoid contents of the extract and fractions varied from 2.50 ± 0.001 to 151.41 ± 4.30 mg/g. Among all the fractions, the ethyl acetate fraction had the highest total flavonoid content of 151.41 ± 4.30 mg of rutin equivalent per gram of dry weight.

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 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 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 *K. gracilis* were estimated in this study. As shown in

Figure 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 flavonid 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 a MTT assay. Cells cultured with the methanolic extract and fractions at the concentrations (0, 125, 250, 500, and 1000 μg/mL) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability (Fig. 3A). But when RAW264.7 macrophages were incubated for 24 h with 100 ng/mL of LPS in the presence of chloroform fraction and ethyl-acetate fraction at the 1000 μg/mL concentration, chloroform fraction and ethyl-acetate fraction inhibited the cell viability, significantly. And when RAW264.7 macrophages were incubated for 24 h with 100 ng/mL of LPS in the presence of eupafolin at the 50 and 100 μg/mL concentrations, eupafolin inhibited the cell viability, significantly.

In the present study, 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, the the methanolic extract and fractions inhibited nitrite production significantly (Fig. 3B). When RAW264.7 macrophages were treated with different concentrations of chloroform fraction (0, 125, 250, and 500 μg/mL) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of nitrite production was detected. There was either a significant decrease in the nitrite production of group treated with 6.12 μg/mL eupafolin ($p < 0.01$), or highly significant decrease of groups treated respectively with 12.5 and 25 μg/mL of eupafolin when compared with the LPS-alone group ($p < 0.001$).

Inhibition of LPS-induced iNOS and COX-2 Protein by chloroform fraction and eupafolin.

The results showed that incubation with chloroform fraction and eupafolin in the presence of LPS for 24 h inhibited iNOS and COX-2 protein expression in mouse macrophage RAW264.7 cells in a dose-dependent manner (Fig. 3A). 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 μg /mL compared with the LPS-alone (Fig. 3B).

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 & Ho, 2010). 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 *n*-hexane fraction,

chloroform fraction and eupafolin may satisfy the control of the rapid progression of the inflammatory process.

Cell Viability

MTT assay was 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, 1000 μg/mL of the extract and fractions were assayed by MTT. After exposing HepG2 cells to any of the samples with various concentrations, the cell viabilities decreased significantly as compared to the control (100%), indicating cytotoxic effect on HepG2 cells. Among all the fractions, chloroform (IC_{50}= 136.85 ± 2.32) fractions showed excellent inhibitory effects on HepG2 cells, as shown in Table 3. And eupafolin also showed excellent inhibitory effects on HepG2 cells (IC_{50}= 9.48 ± 0.32).

Phenolic and flavonoid 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, methanolic extract of *K. gracilis* have shown cytotoxic activities against human gastric and nasopharyngeal carcinoma cell lines. In addition, one of the compounds isolated from *K. gracilis*, bryophyllin, has shown inhibition activities on 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, inflammation or anticancer agent in the future.

CONCLUSION

This study revealed that chloroform fractions of *K. gracili* exhibited good antioxidant activities, anti-inflammatory activities and inhibited the growth of HepG2 cell. These activities may be attributed to the high polyphenolic contents in these fractions. This study also demonstrated that *Kalanchoe gracilis* had a wide safety dosage range. Furthermore, people are more and more interested in the understanding of antioxidative activities of herbs for the purpose of improving health and preventing of chronic diseases or cancers.

ACKNOWLEDGMENT

This study was supported by grants from China Medical University (CMU 91-CPS-02; CMU 93-CPS-01; CMU 94-156 and CMU 96-085).

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