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Studies on the Antimutagenicity and Antioxidant Activity of the Hot Water Extract of *Glechoma hederacea*

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ABSTRACT

Several studies have demonstrated that herbal plants contain various potential antioxidants and these antioxidant components may be potential antimutagens or anticarcinogens. *Glechoma hederacea* belongs to the Labiatae family. According to ancient Chinese prescriptions, *G. hederacea* was used for treating diuresis and stimulating blood circulation. However, there are few reports on its biophysical functions to date. The objective of this study was to evaluate the antioxidant, mutagenic and antimutagenic activities of the hot water extract of *G. hederacea* (HWG). *G. hederacea* was extracted with distilled water at 100°C for 3 h. The antioxidant activities tested included α,α-diphenyl-β-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) and superoxide anion radical-scavenging effects, Fe^{2+}-chelating ability, reducing power and lipid peroxidation inhibition. The *in vitro* mutagenic and antimutagenic properties of the extracts were investigated using the Ames test. The results showed that HWG possessed antioxidant characteristics. Moreover, the antioxidant activities of HWG were significantly higher than those of Vitamin C and Trolox in terms of superoxide anion radical-scavenging activity and Fe^{2+}-chelating ability ($p < 0.05$). The results of Ames test indicated that HWG had no mutagenicity toward all tester strains including *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535 with/without the metabolic activator (S9 mix). The extracts had marked inhibition effects against the mutagenicities of the diagnostic mutagens with the S9 mix system. The HWG showed antioxidant and anti-mutagenic potential. However, the active compounds of HWG need be further investigated.

Key words: *Glechoma hederacea*, antioxidant activity, Ames test, mutagenicity, antimutagenicity

INTRODUCTION

No matter how advanced modern medicine is, cancer is still at the top of the list of fatal diseases and it is continually threatening human health. The prevention of cancer can be achieved by avoiding exposures to mutagens, by fortifying physiological defence mechanisms, or by supplying the intake of protective factors[1]. In recent years, plants have gained much attention due to their antimutagenic activities, which may inactivate or reverse the effects caused by some mutagens. Furthermore, studies have demonstrated that substrates with antioxidant properties are potential antimutagens or anticarcinogens[2].

In the past few years, there is increasing interest in the search for antioxidant phytochemicals, as they can inhibit the propagation of free radical reactions, protect the human body from diseases[3-5], and retard lipid oxidative rancidity in foods[6]. The most effective ones seem to be flavonoids and other phenolic compounds of many raw materials from plants, particularly in herbs, seeds, and fruits. Their metal-chelating capabilities and radical-scavenging properties have enabled phenolic compounds to be thought of as effective free radical scavengers and inhibitors of lipid peroxidation[5,7,8]. *G. hederacea* belongs to the Labiatae family. According to ancient Chinese prescriptions, *G. hederacea* was used in treating abscess, arthritis, asthma, cold, cough, diabetes, flu, gastric diseases, headache, hypochondria, inflammation, jaundice and scurvy[9-12]. Ursolic acid and oleanolic acid from *G. hederacea* showed anti-tumor effects on 12-*O*-tetradecanylphorbol-13-acetate-induced skin tumor[10]. The methanol extract of *G. hederacea* showed antimicrobial effect against several gram positive and negative bacteria with a minimal inhibition concentration of $5.00\text{-}6.25 \times 10^{-2}$ mg/mL[9]. And two unique alkaloids, hederacine A and hederacine B, were isolated from the methanol extract of *G. hederacea*[11]. The hot water extract of *G. hederacea* could inhibit lipopolysaccharide-induced inflammatory mediator release, including nitric oxide, interleukin (IL)-12p70, and tumor necrosis factor-a.

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On the other hand, it increased lipopolysaccharide-induced IL-12p40 production[12]. The *n*-hexane, DCM and methanol extracts of *G. hederacea* exhibited DPPH radical-scavenging ability with IC_{50} values of 1.94×10^{-4}, 2.80×10^{-3} and 1.47×10^{-2}, respectively[9]. However, no further information was documented on the antioxidant properties of *G. hederacea*. Therefore, this work focused on the *in vitro* antioxidant study of *G. hederacea* and proposed that the antioxidant activity of *G. hederacea* endorsed its antimutagenic ability.

Based on the initiation site of antimutagenicity, antimutagens was divided into two classes: desmutagens and bio-antimutagens[13]. Desmutagens include the blocking agents and the scavengers of radicals, and these desmutagens react with the mutagens or free radicals to prevent the genotoxic compounds from reacting with target DNA. Bio-antimutagens can either assist the DNA repairing processes or suppress the evolution of the neoplastic process[2]. In this study, five different tester strains were employed to measure the antimutagenicity of the hot water extract of *G. hederacea* (HWG), and each of the tester stain had its specific site of mutation. TA97 and TA98 are frame-shift-mutants, carrying *hisD6610* and *hisD3052* mutation, respectively, while the others, TA102, TA100 and TA1535, are miss-sense based mutants with TA102 carrying *hisG428* mutation and both TA100 and TA1535 carrying *hisG46* mutation[14]. Prior to applying HWG as antimutagenic agents, the assessments of their safety are essential. Thus, the mutagenic properties of the extracts were determined as well.

MATERIALS AND METHODS

I. *Chemicals*

α-Tocopherol (α-Toc), 2-aminoanthramine (2-AA), 2-aminofluorene (2-AF), 4-nitroquinoline-N-oxide (4-NQO), glucose-6-phosphate (G-6-P), β-nicotinamide-adenine dinucleotide phosphate (β-NADP), α-α-diphenyl-β-pricryl-hydrazyl (DPPH), potassium ferricyanide, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The *Salmonella typhimurium* tester strains including TA97 (hisD6610/rfa/ΔuvrB/pKM101), TA98 (hisD3052/rfa/ΔuvrB/pKM101), TA100 (hisG46/rfa/ΔuvrB/pKM101), TA102 (hisG428/rfa/ΔuvrB/pKM101, pAQ1) and TA1535 (hisG46/rfa/ΔuvrB) were obtained from the Bioresource Collection and Research Centre (Hsinchu, Taiwan). All other chemicals were of reagent grade or higher purity.

II. *Preparation of the Water Extract of G. hederacea*

G. hederacea was planted in pots and the plants were harvested when the leaves grew up to 4-5 cm in diameter, which took about 2-3 weeks of growth during spring time (temperature around 20-25°C). To select an optimal extraction method, the plant samples (whole plant including stems and leaves) were washed and then divided into three groups. Three extracts were obtained by the following extraction methods using 1 : 50 (w/v) of plant (60 g) : water (2940 g). Surface-dried fresh plants extracted with distilled water at room temperature (around 25°C) with stirring for 3 h on a stirring plate was defined as group A, while fresh or oven (50°C)-dried plants extracted with distilled water at 100°C for 3 h using a heating mantle (NEW LAB MN-30000, Sunray Science Co. Ltd., Taipei, Taiwan) were defined as groups B and C, respectively. The decoctions were filtered, and then dried by a vacuum freeze-dryer. The extracts were sealed in plastic bottles and stored at -20°C until use.

III. *Total Phenolic and Anthocyanin Content Assay*

Total phenolic content was analyzed using the Folin-Ciocalteu's reagent method[15]. Different concentration of extract (0.4 mL) was mixed with 0.4 mL of Folin-Ciocalteu's reagent and 0.04 mL of 10% Na_2CO_3, and the absorbance was measured at 735 nm after 1 h of incubation at room temperature. Gallic acid (0.003-0.1 mg/mL) was used as the standard for the calibration curve, and the total phenolic contents were expressed as mg gallic acid equivalents per gram of tested extracts.

The anthocyanin contents of the extracts were analyzed according to the method of Padmavati *et al*.[16] with slight modification. The extracts were mixed with acidified methanol (containing 1% HCl) for 2 h at room temperature in the dark, and then centrifuged at 1000 ×*g* for 15 min. The anthocyanin concentration in the supernatant was measured spectrophotometrically at 530 and 657 nm, respectively. The absorbance values at 530 and 657 nm were indicated as A_{530} and A_{657}, respectively. The extinction coefficient of 31.6 $M^{-1}cm^{-1}$ was used to convert absorbance values into concentrations of anthocyanin. The concentration was calculated using the following equation: Anthocyanin content (μmol/g) = $[(A_{530} - 0.33 \times A_{657}) / 31.6] \times$ [volume (mL) / weight (g)].

IV. *Measurement of the DPPH Radical-Scavenging Activity*

The DPPH radical-scavenging activities of the extracts of *G. hederacea* were measured according to the method of Chung *et al*.[17] An aliquot of the extracts (0.1 mL, 0.08-10.0 mg/mL) or α-Toc (0.02-0.6 mg/mL) was mixed with 100 mM of Tris-HCl buffer (0.4 mL, pH 7.4), and then added to 0.5 mL of 500 μM of DPPH in ethanol (final concentration of 250 μM). The mixture was shaken vigorously and left in the dark at room temperature for 20 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated by the following equation: Scavenging effect (%) = [1 − (absorbance of sample at 517 nm / absorbance of control at 517 nm)] × 100.

V. *ABTS Cation Radical Scavenging Effect*

The $ABTS^{\cdot+}$ radical cation-decolorization assay was

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performed according to the method of Robert *et al*.[18] $ABTS^{\cdot+}$ was prepared by reacting ABTS (7 mM) with potassium persulfate (4.9 mM) in 10 mL of 0.01 M of sodium phosphate buffer (containing 0.15 M of NaCl, pH 7.4) and the solution allowed to stand in the dark at room temperature for 16 h before use. $ABTS^{\cdot+}$ stock solution was diluted with sodium phosphate buffer to obtain an absorbance of 0.85 at 734 nm. A half milliliter of $ABTS^{\cdot+}$ solution was mixed with 0.02 mL of extract, and the mixture was read at 734 nm after being incubated for 10 min. Trolox was used as a standard. ABTS radical scavenging effect (%) = $[(A_{cont} - A_{test}) / A_{cont}] \times 100$, where A_{cont} was the absorbance of the control reaction and A_{test} was the absorbance of the extract or standards.

VI. *Superoxide Anion Scavenging Effect*

Measurement of superoxide anion scavenging effect was based on the method described by Robak and Gryglewski[19]. Superoxide radicals were generated in the PMS-NADP system by the oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the extract (0.3 mL) was mixed with same volume of 120 μM of phenazine methosulfate solution, 936 μM of NADH and 300 μM of NBT. The reaction mixture was kept in the dark at room temperature for 14 min, and the absorbance was measured at 560 nm. Gallic acid was used as a standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following equation: Super oxide anion scavenging activity (%) = $[(A_{cont} - A_{test}) / A_{cont}] \times 100$, where A_{cont} was the absorbance of the control reaction and A_{test} was the absorbance of the extract or standards.

VII. *Measurement of Fe^{2+}-Chelating Ability*

The Fe^{2+}-chelating ability was determined according to the method of Dinis *et al*.[20] The Fe^{2+} ion was monitored by measuring the formation of the ferrous iron-ferrozine complex. The extracts or α-Toc (1.25-20.0 mg/mL) was mixed with 2 mM of $FeCl_2$ and 5 mM of ferrozine in a ratio of 10 : 1 : 2. The mixture was shaken and left at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. A lower absorbance of the reaction mixture indicated a higher Fe^{2+}-chelating ability. The capability to chelate the ferrous iron was calculated by the following equation: Chelating effect (%) = [1 − (absorbance of sample at 562 nm / absorbance of control at 562 nm)] × 100.

VII. *Measurement of Antioxidant Effect on Liposome Peroxidation*

Liposome peroxidation was induced by Fe^{2+}-ascorbate and aquatinted by malondialdehyde-thiobarbituric acid (MDA-TBA) adduct according to the method described by Liao and Yin[21] with slight modification. Liposomes (multi-lamellar vesicles) were prepared from 30 mg of Phosphatidylcholine, 12 mg of cholesterol and 3 mg of dicetyl phosphate at 4°C. The solvents, chloroform and methanol, used for liposome preparation were removed and a lipid film was formed by rotary evaporation under N_2 flush. Then, the liposome was suspended with 10 mL of 50 mM of sodium phosphate buffer (pH 7.2). A mixture containing 1 mL of liposome's suspension, 0.6 mL of sodium phosphate buffer, 0.05 mL of 25 mM of $FeCl_3$, 0.05 mL of 25 mM of ascorbic acid and 0.25 mL of the extracts (0.08-10.0 mg/mL) or α-Toc (0.01-5.0 mg/mL) was incubated at 37°C for 1 h . After incubation, the solution was mixed with TBA (0.4% in 0.2 M of HCl) and BHT (0.2% in 95% ethanol) at a ratio of 1 : 2 : 0.3, and then heated at 100°C for 20 min. After cooling down the mixture, an equal volume of *n*-butanol was added to extract the chromogen in the mixture. The absorbance of the *n*-butanol layer was measured spectrophotometrically at 532 nm. The capability to inhibit MDA formation was calculated by the following equation: Inhibition effect (%) = [1 − (absorbance of sample at 532 nm / absorbance of control at 532 nm)] × 100.

IX. *Preparation of Culture Medium*

Nutrient broth was prepared by dissolving 25 g of Oxoid nutrient broth in 1 L of water. Glucose minimal agar plate (MA plate) contained 1.5% agar, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% citric acid, 1% K_2HPO_4, 0.35% $NaHNH_4PO_4 \cdot 4H_2O$ and 2% glucose. Top agar contained 0.75% agar and 0.5% NaCl.

X. *Salmonella Typhimurium Mutagenicity and Antimutagenicity Assays*

Microbial mutagenicity was assayed by the standard Ames test (standard plate incorporation assay) according to Maron and Ames[22] and Mortelmans and Zeiger[14]. To avoid an erroneous result caused by antimicrobial effect of tested samples toward tester strains, a preliminary toxic dose range experiment was performed to determine an appropriate dose range for the Ames test[22]. Samples were prepared with 0.1 mL of 10 h-cultured test strain (approximately 10^8 cells/mL), 0.1 mL of the extracts (0.02-5 mg/plate), 0.1 mL of phosphate buffer (0.2 M, pH 7.4) and 0.5 mL of S9 mix or phosphate buffer. The serial dilutions were immediately made with phosphate buffer, and then 1 mL of the aliquot was mixed with 12 mL of nutrient agar. After incubation at 37°C for 48 h, the number of colonies was counted. A toxicity effect was confirmed if the standard plate count of the tested compound was lower than that of the control (with no compound added).

A mixture containing 0.1 mL of the extracts (0.02-5 mg/plate), 0.5 mL of S9 mix or phosphate buffer, 0.2 mL of 0.5 mM histidine-biotin and 0.1 mL 10 h-cultured test strain (approximately 10^8 cells/mL) was added to a tube containing 2 mL of top agar. The tube was then gently vortexed and poured onto the MA plate. The extracts were tested with and without S9 mix, and triplicate plates are poured for each dose

of extracts. Diagnostic mutagens, including 2-AF (100 μg/plate), 2-AA (5 μg/plate or 2 μg/plate or 20 μg/plate), and 4-NQO (0.1 μg/plate), AzNa (0.5 μg/plate) and t-BHP (100 μg/plate) were prepared by dissolving in DMSO and served as positive control chemicals. After incubation at 37°C for 48 h, the number of revertants colonies was counted. A compound was considered as a mutagen if there was a two-fold increase in the number of revertants, comparing with the number of spontaneous revertants (negative control) or a dose-related increase in the number of revertants for one or more strains.

The antimutagenic effect of the tested sample was assayed using the Ames test, except for the addition of diagnostic mutagens, 2-AF, 2-AA and 4-NQO, in each tested plate. The antimutagenicity of the diagnostic mutagens in the absence of the extract was defined as 0% inhibition, and the antimutagenicity was calculated according to the formula given by Ong *et al.*[23]: % Inhibition = [1 − T / M] × 100, where T is the number of revertants per plate in the presence of mutagen and the extract and M is the number of revertants per plate in the positive control (without extract). The tests were performed in duplicate with three sub-samples each, and the data was presented as mean ± standard deviation (SD).

XI. *Statistical Analysis*

All data were expressed as mean ± SD Analysis of variance was performed by using ANOVA. Duncan's new multiple-range test was used to determine the difference of means and $p < 0.05$ was considered t statistically significant.

RESULTS AND DISCUSSION

I. *DPPH Radical-Scavenging Activity of the Water Extract of G. hederacea*

Three water extracts (extracts A-C) were prepared by extracting fresh plants with distilled water at 25°C for 3 h, and extracting fresh or oven-dried plants with distilled water at 100°C for 3 h. For a specific antioxidant reaction, the half-inhibition concentration (IC_{50}) can be calculated as the antioxidant concentration required for providing 50% of the antioxidant activity.

The IC_{50} of extracts A-C for scavenging DPPH radicals were 5.48 ± 0.19, 0.34 ± 0.01 and 1.71 ± 0.12 mg/mL, respectively (data not shown). The optimal extraction time was also studied and the results showed that there was no significant difference between 3 h (0.34 ± 0.01 mg/mL) and 6 h (0.35 ± 0.00 mg/mL) of extraction in terms of the IC_{50} of DPPH radical-cavenging activity ($p > 0.05$). Therefore, only extract B, the hot water extract of *G. hederacea* (HWG), was further characterized as follows.

II. *Total Phenolic and Anthocyanin Contents*

The extraction yield of the HWG was 6%. The total phenolic and anthocyanin contents of HWG were 79.70 ± 0.193 mg/g (as gallic acid equivalent) and 1.68 ± 0.02 (μmol/g), respectively. Phenolic compounds and anthocyanins are widely found in food derived from plant sources, and they have been shown to possess significant antioxidant activities[24,25]. The antioxidant mechanisms of phenols include scavenging free radicals, chelating metal ions and inhibiting oxidant-related enzymes[26]. Polyphenols have been shown to be more effective antioxidants *in vitro* than vitamins E and C due to their ideal structural chemistry for free radical-scavenging activities[26,27]. *G. hederacea* belongs to the Labiatae family and the major water-soluble phenols in Labiatae are ursolic acid and rosmarinic acid[28,29]. The antioxidant activities of ursolic acid and rosmarinic acid were documented in previous studies[29-31]. In this study, the total phenolic content of HWG was found to be about 8% of dry matter (79.7 mg/g). This high phenolic content may be related to the antioxidant activities of HWG.

III. *Free Radical-Scavenging Activity of HWG*

Many food materials possess free radical-scavenging ability and their effect on radical scavenging was thought to be due to their hydrogen-donating ability[32]. Figures 1A-C show the dose-response curves of DPPH, superoxide anion and ABTS radical-scavenging activities of the HWG. It was found that the effective concentrations for HWG to scavenge DPPH or superoxide radicals were quite low, and 1.3 mg/mL and 2.0 mg/mL of HWG could reach its maximal-scavenging activities for DPPH (80.6%) and superoxide anion (79.2%), respectively. Compared to the activities toward DPPH and superoxide radicals at low concentration, HWG was not such an efficient scavenger on ABTS. On the other hand, HWG could remove ABTS radicals completely at a concentration as low as 5 mg/mL. The half-scavenging concentrations (IC_{50}) of GH for DPPH, superoxide anion and ABTS radical-scavenging activities were 0.34 ± 0.00, 0.20 ± 0.01 and 1.82 ± 0.02 mg/mL, respectively (Table 1). Based on the IC_{50} results, it was also indicated that HWG had the highest superoxide anion-scavenging activity and the least activity on ABTS. HWG exhibited greater activity toward DPPH than ABTS, which might be due to the less polar condition in the DPPH assay compared to the aqueous system of ABTS. When compared with the commercial antioxidants (including gallic acids, trolox and vitamin C), HWG showed less efficiency towards DPPH and ABTS but was found to be more efficient than trolox and vitamin C towards the superoxide anion. It was evident that HWG displayed hydrogen-donating activity and played an SOD-like role in scavenging the superoxide anion.

It was also found that the Fe^{2+}-chelating ability of HWG also increased with the increase in their concentrations (Figure 1D). The 50%-chelating concentration for Fe^{2+}-chelating ability of HWG and gallic acid were 0.52 and 0.01 mg/mL, respectively (Table 1). Trolex and vitamin C did not exhibit Fe^{2+}-chelating ability. HWG exhibited much lower Fe^{2+}-chelating ability than gallic acid. About 80% of Fe^{2+} ions were eliminated by 3 mg/mL of HWG, indicating that

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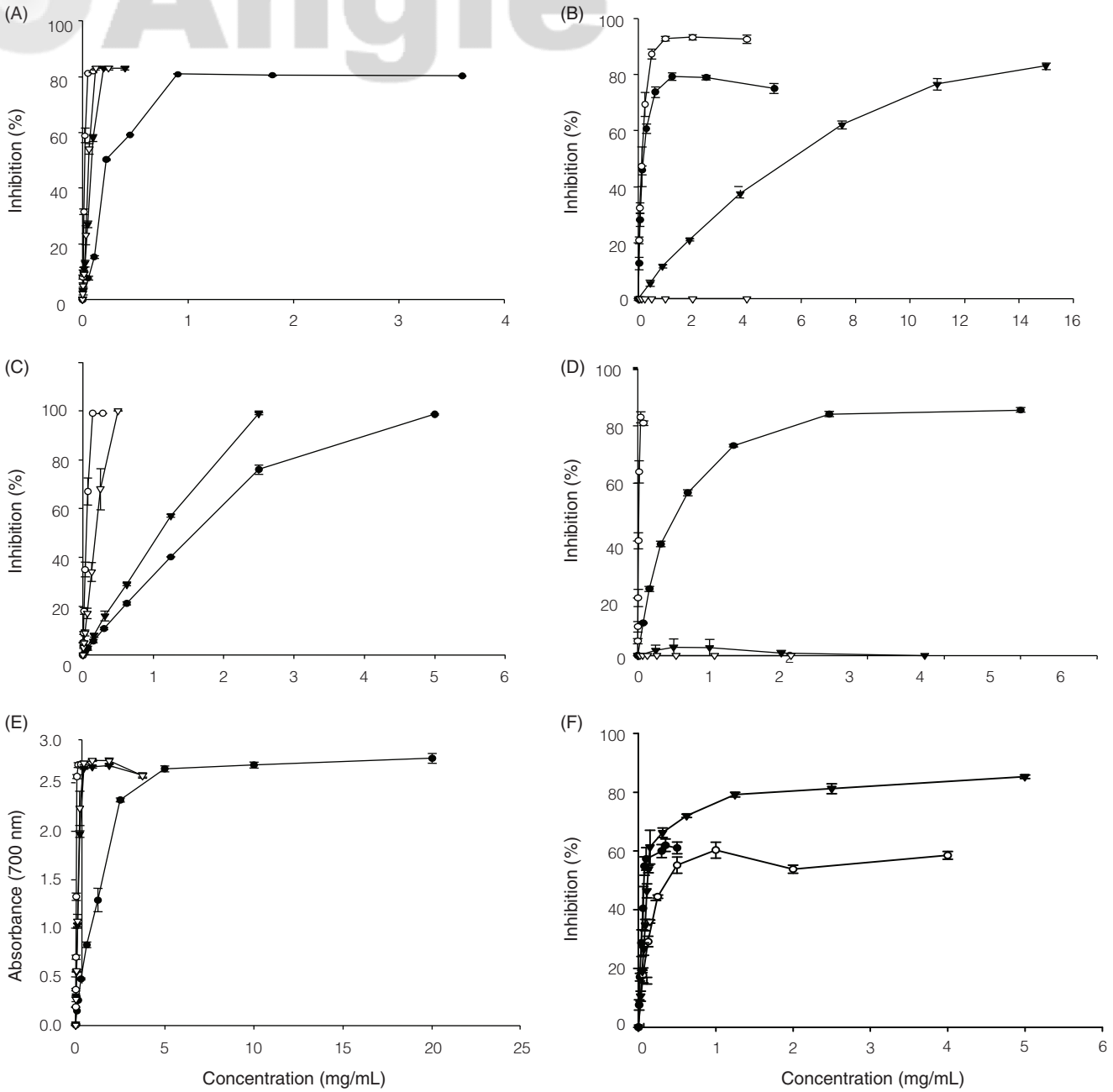

Figure 1. Scavenging effects of the hot water extract of *G. hederacea* extract (HWG) on (A) DPPH, (B) superoxide, (C) ABTS radical, (D) Fe^{2+}-chelating ability, (E) reducing power, and (F) Fe/ascorbate-induced lipid peroxidation. Each value represents mean ± SD (n = 3). -●-, HWG; -○-, Gallic acid; -▼-, Trolox; -▽-, Vitamin C (Figures 1 A, B, C, D and E) or caffeic acid (Figure 1F).

Table 1. The half-inhibitory concentrations (IC_{50}) of the hot water extract of *G. hederacea.*

Extract / Antioxidant	IC_{50} (mg/mL)				
	DPPH-radical	Superoxide - radical	ABTS-radical	Fe^{2+}-chelating	TBARS
HWG	0.34 ± 0.00	0.20 ± 0.01	1.82 ± 0.02	0.52 ± 0.01	0.075 ± 0.01
Gallic acid	0.02 ± 0.00	0.14 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.39 ± 0.02
Trolox	0.08 ± 0.00	5.20 ± 0.21	1.07 ± 0.02	NA	0.12 ± 0.05
Vitamin C	0.08 ± 0.00	NA	0.16 ± 0.03	NA	—
Caffeic acid	—	—	—	—	0.24 ± 0.05

Value are given as mean ± SD (n = 3). N. A. : not applicable. — : not used

HWG could afford good protection from Fe^{2+}-induced oxidation and lipid peroxidation.

The level of thiobarbituric acid reactive substances (TBARS), mainly malonyldialdehyde (MDA), is considered as an indicator of lipid peroxidation. The IC_{50} of GH for TBARS was much lower than that of gallic acid, trolox and caffeic acid. HWG contained 0.005% caffeic acid (data not shown). The results of TBARS indicated that HWG was a potent inhibitor of lipid peroxidation and its caffeic acid composition might provide a partial inhibitory effect.

IV. *Reducing Power of HWG*

In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by the donation of an electron. The amount of Fe^{2+} complex can be then monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicated an increase in reductive ability. Figure 1E shows the dose-response curves for the reducing powers of HWG. It was found that the reducing powers of HWG increased with the increase of concentrations. Though the reductive ability of HWG was significantly less than that of commercial antioxidants, it was evident that HWG displayed reductive potential and could serve as an electron donor and terminate the radical chain reaction[33].

V. *Antioxidant Effect on Liposome Peroxidation*

Lipid peroxidation is a typical free radical oxidation and proceeds via a cyclic chain reaction. Liposomes, artifical biomembanes, have been used extensively as a model system for *in vitro* lipid peroxidation studies[34-36]. The antioxidant activities of HWG, gallic acid, trolox and caffeic acid on MDA formation in Fe^{2+}/ascorbate-mediated lipid peroxidation in the liposome system were shown in Figure 1F. It was also found that the inhibitory effect on the lipid peroxidation of HWG was in a concentration-dependent manner and reached a maximal inhibition of 85% at the concentration of 1 mg/mL. The IC_{50} value of HWG (0.075 ± 0.01 mg/mL) was much lower than those of gallic acid (0.39 ± 0.02 mg/mL), trolox (0.39 ± 0.02 mg/mL) and caffeic acid (0.39 ± 0.02 mg/mL). In other words, HWG exhibited significantly higher lipid peroxidation inhibitory activity than commercial antioxidants. The mechanism of the inhibitory effects by which the HWG protects against lipid peroxidation may involve radical scavenging and reducing capability.

The DPPH radical-scavenging ability of *G. hederacea* was first reported by Kumarasamy *et al*.[9] According to the results of the present study, we further proposed the antioxidant mechanism for the water extract of *G. hederacea* (Figure 2). In addition to preventing cell injury by scavenging various free radicals (provide H^+ proton), HWG exerts its antioxidant power on cycling oxidized materials (Ox'**)** by reducing oxidants and/or avoiding lipid oxidation by chelating metal ions (Fe^{2+}).

VI. *Mutagenicity Test*

Five TA strains containing different types of histidine mutations were used for evaluating the mutagenicity and antimutagenicity of HWG[14,22]. TA1535 can be used to detect mutagens causing base-pair substitutions. Both TA97 and TA98 are used for detecting various kinds of frameshift mutagens. TA97 and TA98 are reverted well by carcinogens of aminoacridine and nitrosofluorene, respectively. TA102 is sensitive to oxidant mutagens, while TA100, which carries a resistance transfer factor, is extremely sensitive to some frameshift mutagens, such as aflatoxins and furylfuramide.

All the tested concentrations (up to 5 mg/plate) of HWG did not show toxicity (antimicrobial effect) to the tester strains, regardless of the presence of the metabolic activator (S9 mix)(data not shown). Therefore, the highest concentration of HWG for mutagenicity assay was selected as 5 mg/plate.

The results of the Ames test conducted to determine the mutagenicity of HWG are shown in Table 2. All tested TA strains were effectively reverted by diagnostic mutagens, including 2-AF, 2-AA, 4-NQO, AzNa (0.5 μg/plate) and t-BHP (100 μg/plate), indicating that the tested strains were sensitive to various kinds of mutagens. The numbers of revertants induced by HWG for all tester strains were close to those of the negative control (spontaneous revertants, without HWG) and were much lower than those of the positive control (with diagnostic mutagens). The results of the Ames test demonstrated that HWG did not have mutagenic effect under the tested dose range.

This study demonstrated that the hot water extract of *G. hederacea* was safe in terms of genotoxicity and exhibited antioxidant activity. The extracts had radical-scavenging effect, reductive capability on reducing Fe^{3+} to Fe^{2+}, and anti-lipid peroxidation activity on a the liposome system.

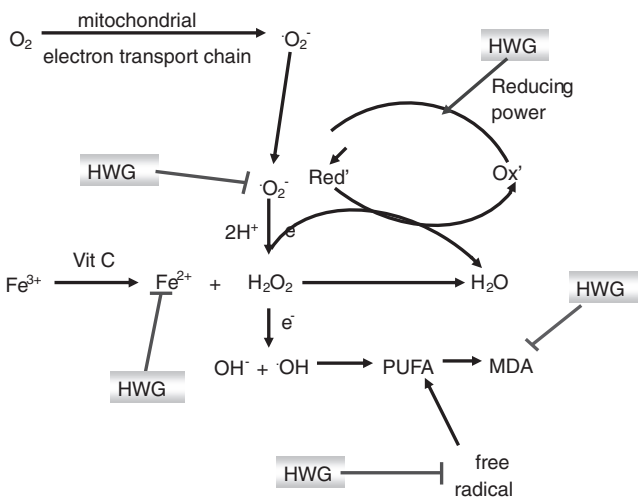

Figure 2. Proposed antioxidative mechanism of the hot water extract of *G. hederacea in vitro*. HWG: hot water extract of *G. hederacea* extract; PUFA: poly-unsaturated fatty acid ; MDA: malonyldialdehyde.

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Table 2. Mutagenicity of the hot water extract of *G. hederacea* towards *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535.

Extracts (mg /plate)	No. of colonies (CFU/plate)[a]									
	TA97		TA98		TA100		TA102		TA1535	
	-S9[b]	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Control[c]	31 ± 1	48 ± 3	49 ± 3	32 ± 1	110 ± 3	124 ± 1	271 ± 18	283 ± 1	12 ± 0	22 ± 1
0.31	22 ± 1	45 ± 5	45 ± 1	29 ± 1	110 ± 1	120 ± 10	228 ± 16	255 ± 2	10 ± 2	21 ± 4
0.63	25 ± 0	52 ± 9	44 ± 2	34 ± 2	112 ± 4	136 ± 13	271 ± 10	265 ± 12	12 ± 1	24 ± 2
1.25	25 ± 1	59 ± 3	50 ± 1	36 ± 1	109 ± 2	132 ± 9	280 ± 20	245 ± 6	12 ± 0	20 ± 2
2.50	29 ± 0	51 ± 4	46 ± 0	32 ± 1	120 ± 4	120 ± 8	254 ± 3	227 ± 5	13 ± 1	19 ± 1
5.00	29 ± 5	53 ± 4	46 ± 2	34 ± 2	113 ± 2	135 ± 4	264 ± 11	266 ± 11	14 ± 1	21 ± 3
4-NQO[d] (0.0001)	124 ± 0	-	233 ± 3	-	660 ± 6	-	-	-	-	-
2-AF[d] (0.1)	-	710 ± 2	-	-	-	-	-	-	-	-
2-AA[d] (0.005)	-	-	-	763 ± 45	-	-	-	-	-	-
2-AA[d] (0.002)	-	-	-	-	-	885 ± 50	-	-	-	-
t-BHP[d] (0.1)	-	-	-	-	-	-	626 ± 6	995 ± 71	-	-
AzNa[d] (0.0005)	-	-	-	-	-	-	-	-	277 ± 21	-
2-AA[d] (0.02)	-	-	-	-	-	-	-	-	-	598 ± 1

[a]Data are mean ± S.D. of triplicates.
[b]S9 is a metabolic activation system consisting of the postmitochondrial fraction of the rats livers.
[c]Negative control: without extract; treated with DMSO; spontaneous revertants/plate.
[d]Positive control: without S9: for strains TA97, TA98 and TA100: 0.1 μg/plate of 4-nitroquinoline-N-oxide (4-NQO) was used; for strain TA102: 100 μg/plate of t-butyl hydroperoxide (t-BHP) was used; for strain TA1535: 0.5 μg/plate of sodium azide (AzNa) was used; with S9: for strain TA97: 100 μg/plate of 2-aminofluorene (2-AF) was used; for strain TA102: 100 μg/plate of t-BHP was used; and for strains TA98, TA100 and TA1535: 5 μg/plate, 2 μg/plate and 20 μg/plate of 2-anthramine (2-AA) were used respectively.

VII. *Antimutagenicity Test*

To evaluate the antimutagenicity of HWG, diagnostic mutagens were incorporated in the sample plates based on the results of mutagenicity (Table 3). Various concentrations of mutagens were chosen to induce noteworthy revertants. Compared to the control, HWG decreased his^+ revertants, induced by mutagens, of TA97, TA98, TA100 and TA1535 in S9 mix up to 73-88%, and the decrease depended on the concentration of HWG at 0.31-5.00 mg/plate. HWG showed very little antimutagenicity toward TA102. On the other hand, HWG without S9 mix did not reveal inhibitory effect on the mutagenicity induced by diagnostic mutagens (data not shown).

In this study, five different tester strains were employed to measure the mutagenicity and antimutagenicity of HWG, and each of the tester strain had its specific site of mutation[14]. Even TA97, TA98, TA100 and TA1535 carried different DNA lesions, their abilities of reversing mutation were all strongly suppressed by HWG in the presence of the metabolic activator (S9 mix). TA102 was the only tested strain that showed no antimutagenicity under the S9 system. TA102 was similar to TA100 and TA1535 in terms of being miss-sense based mutants, but TA102 carried *hisG428* mutation, while TA100 and TA1535 carried *hisG46* mutation[14]. Among the tested strains, TA102 is the most sensitive to free radicals or oxidative agents and its revertants are easily induced by free radicals-generating antibiotics and peroxidants[37,38]. HWG showed high radical-scavenging activity and thus was expected to be a strong antimutagen against the mutation of TA102. However, the inhibitory effect of HWG on induced mutation by *tert*-butylhydroperoxide (t-BHP), a strong hydroxyl peroxidant, was not as notable as expected. Tokuda *et al*.[10] reported that ursolic acid and oleanolic acid, the major phenols in *G. hederacea*, showed anti-tumor effects on 12-O-tetradecanylphorbol-13-acetate-induced skin tumor. However, our results suggested that components other than antioxidant component(s) might play a defensive role in DNA damage.

Furthermore, HWG only exhibited antimutagenicity in the S9 mix system. If the HWG acted as desmutagens, two hypotheses may be proposed based on the need of S9 to facilitate antimutagenic activity. First, since both 2-AF and 2-AA are indirect-acting mutagens with the mutagenicity depending on the activation of the metabolic activator, S9 mix, HWG may conjugate with 2-AF and 2-AA to prevent them from being activated by the S9 mix. Second, both 2-AF and 2-AA are polycyclic aromatic amines and their toxicities are strongly dependent on the acetylation ability of the N-acetyltransferase presented in the complex cytochrome P-450 enzyme system. For example, it was found that rapid acetylators were more susceptible to DNA damage by 2AF[39]. Therefore, it is possible that HWG can interfere with the acetylation reaction of 2-AF or 2-AA in S9 mix system to eliminate the mutagenicity of these components. On the other hand, if HWG was converted into bio-antimutagens by the S9 mix, these bio-converted components might rather contribute to the DNA repairing system than inactivate mutagens before the mutagens attack DNA.

Table 3.The antimutagenicity of the hot water extract of *G. hederacea* toward *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535 with S9 mix.

Treatment group (mg/plate)	Inhibition (%)[a]				
	TA97	TA98	TA100	TA102	TA1535
Control[b]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Mutagen[c] + GH[d] 0.31	0.0 ± 0.0	20.3 ± 7.1	14.0 ± 0.9	32.0 ± 6.3	8.4 ± 1.6
Mutagen + GH 0.63	22.0 ± 5.2	36.1 ± 6.0	20.0 ± 3.3	31.0 ± 4.5	37.0 ± 7.1
Mutagen + GH 1.25	38.1 ± 2.5	56.1 ± 6.8	32.1 ± 7.5	29.0 ± 4.2	63.0 ± 4.4
Mutagen + GH 2.50	61.0 ± 0.5	78.0 ± 3.3	52.1 ± 2.0	29.0 ± 2.5	79.4 ± 2.4
Mutagen + GH 5.00	87.4 ± 1.8	88.0 ± 1.0	73.0 ± 7.2	29.0 ± 4.6	86.0 ± 4.0

[a]Values are two independent experiments done in triplicate. All samples were assayed with S9 mix.
[b]Negative control: without extract; treated with DMSO; spontaneous revertants/plate.
[c]Positive control: for strain TA97: 100 μg/plate of 2-aminofluorene (2-AF) was used; for strain TA102: 100 μg /plate of t-BHP was used; and for strains TA98,TA100 and TA1535: 5 μg/plate, 2 μg/plate and 20 μg/plate of 2-anthramine (2-AA) were used, respectively.
[d]HWG: hot water extract of *Glechoma hederacea* Inhibition (%) = [1 − (His$^+$ revertants in sample of test − Number of His revertant colonies / His$^+$ revertants in control of test − Number of His revertant colonies)]

CONCLUSIONS

HWG exerts antioxidant capabilities on scavenging free radicals, reducing power and chelating metal ions. Components other than the antioxidant component(s) in HWG might play a defensive role in mutagen-induced DNA damage.

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REFERENCES

1. De Flora, S. 1998. Mechanisms of inhibitors of mutagenesis and carcinogenesis. Mutat. Res. 402: 151-158.
2. Bronzetti, G. 1994. Antimutagens in food. Trends Food Sci. Tech. 5: 390-395.
3. Kinsella, J. E., Frankel, E., German, B. and Kanner, J. 1993. Possible mechanisms for the protective role of antioxidants in wine and plant foods. Food Technol. 47: 85-89.
4. Pryor, W. A. 1991. The antioxidant nutrients and disease prevention-what do we know and what do we need to find out? Am. J. Clin. Nutr. 53: 391s-393s.
5. Terao, J. and Piskula, M. K. 1997. Flavonoids as inhibitors of lipid peroxidation in membranes. In "Flavonoids in health and disease". 2nd ed. pp. 277-295. Rice-Evans, C. A. and Packer, L. ed. Taylor & Francis. London, UK.
6. Duthie, G. G. 1993. Lipid peroxidation. Eur. J. Clin. Nutr. 47: 759-764.
7. Bors, W. and Saran, M. 1987. Radical scavenging by flavonoid antioxidants. Free Radic. Res. Commun. 2: 289-294.
8. Miller, N. J. 1997. Flavonoids and phenylpropanoids as contributors to the antioxidant activity of fruit juices. In "Flavonoids in health and disease". 2nd ed. pp. 387-405. Rice-Evans, C. A. and Packer, L. ed. Taylor & Francis. London, UK.
9. Kumarasamy, Y., Cox, P. J., Jaspars, M., Nahar, L. and Sarker, S. D. 2002. Biological activity of *Glechoma hederacea*. Fitoterapia. 73: 721-723.
10. Tokuda, M., Ohigashi, H., Koshimizu, K. and Ito, Y. 1986. Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-O-tetradecanylphorbol-13-acetate. Cancer Lett. 33: 279-285.
11. Kumarasamy, Y., Cox, P. J., Jaspars, M., Nahar, L. and Sarker, S. D. 2003. Isolation, structure elucidation and biological activity of hederacine A and B, two unique alkaloids from *Glechoma hederacea*. Tetrahedron 59: 6403-6407.
12. An, H. J., Jeong, H. J., Um, J. Y., Kim, H. M. and Hong, S. H. 2006. *Glechoma hederacea* inhibits inflammatory mediator release in IFN-*r* and LPS-stimulated mouse peritoneal macrophages. J. Ethnopharmacol. 106: 418-424.
13. Kada, T., Inoue, T. and Namiki, N. 1982. Environmental desmutagens and antimutagens. In "Environmental mutagenesis, carcinogenesis, and plant biology". pp. 127. Klekowski, E. J. ed. Praeger. New York, U.S.A.
14. Mortelmans, K. and Zeiger, E. 2000. The Ames *Salmonella*/microsome mutagenicity assay. Mutat. Res. 455: 29-60.
15. Sato, M., Ramarathnam, N., Suzuki, Y., Ohkubo, T., Takeuchi, M. and Ochi, H. 1996. Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources. J. Agric. Food Chem. 44: 37-41.
16. Padmavati, M., Sakthivel, N., Thara, K. V. and Reddy, A. R. 1997. Differential sensitivity of rice pathogens to growth inhibition by flavonoids. Phytochemistry 46:

499-502.
17. Chung, Y. C., Chang, C. T., Chao, W. W., Lin, C. F. and Chou, S. T. 2002. Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. J. Agric. Food Chem. 50: 2454-2458.
18. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant acitivty appling an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26: 1231-123.
19. Robak, J. and Grygelewski, R. J. 1988. Flavonoids are scavengers of superoxide anions. Biochem. Pharmacol. 37: 837-841.
20. Dinis, T. C. P., Madeira, V. M. C. and Almeida, L. M. 1994. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch. Biochem. Biophys. 315: 161-169.
21. Liao, K. L. and Yin, M. C. 2000. Individual and combined antioxidant effects of seven phenolic agents in human erythrocyte membrance ghosts and phosphatidylcholine liposome systems: importance of the partition coefficient. J. Agric. Food Chem. 48: 2266-2270.
22. Maron, D. M. and Ames, B. N. 1983. Revised methods for the *salmonella* mutagenicity test. Mutat. Res. 113: 173-215.
23. Ong, T. M., Whong, W. Z., Stewart, J. and Brockman, H. E. 1986. Chlorophyllin: a potent antimutagen against environmental and dietary complex mixtures. Mutat. Res. 173: 111-115.
24. Rice-Evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M. and Pridham, J. B. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic. Res. 22: 375-383.
25. van Acker, S. A., van den Berg, D. J., Tromp, M. N., Griffioen, D. H., van Bennekom, W. P., van der Vijgh, W. J. and Bast, A. 1996. Structural aspects of antioxidant activity of flavanoids. Free Radic. Biol. Med. 20: 331-342.
26. Rice-Evans, C. A., Miller, N. J. and Paganga, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20: 933-956.
27. Rice-Evans, C. A., Miller, N. J. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2: 152-159.
28. Kashiwada, Y., Wang, H. K. and Nagao, T. 1998. Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid and structuraly related triterpenoids. J. Nat. Prod. 61: 1090-1095.
29. Kintzios, S., Nikolaou, A. and Skoula, M. 1999. Somatic embryogenesis and in vitro rosmarinic acid accumulation in Salvia officinalis and S. fruticosa leaf callus cultures. Plant Cell Rep. 18: 462-466.
30. Liu, J. 1995. Pharmacology of oleanolic acid and ursolic acid. J. Ethnopharmacol. 49: 57-68.
31. Somova, L. I., Shode, F. O., Ramnanan, P. and Nadar, A. 2003. Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from Olea europaea, subspecies Africana leaves. J. Ethnopharmacol. 84: 299-305.
32. Yamaguchi, T., Takamura, H., Matoba, T. and Terao, J. 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1,-diphenyl-2-picrylhydrazyl. Biosci. Biotechnol. Biochem. 62: 1201-1204.
33. Yen, G. C. and Chen, H. Y. 1995. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem. 43: 27-32.
34. Duh, P. D., Du, P. C. and Yen, G. C. 1999. Action of methanolic extract of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. Food Chem. Toxicol. 37: 1055-1061.
35. Yin, M. C. and Cheng, W. S. 1998. Antioxidant activity of several *Allium* members. J. Agric. Food Chem. 46: 4097-4101.
36. Tsuda, T., Shiga, K., Ohshima, K., Kawakishi, S. and Osawa, T. 1996. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. Biochem. Pharmacol. 52: 1033-1039.
37. Garner, R. C., Miller, E. C. and Miller, J. A. 1972. Liver microsomal metabolism of aflatoxin B1 to a reactive derivative toxic to *Salmonella typhimurium* TA1530. Cancer Res. 32: 2058-2066.
38. Minnunni, M., Wolleb, U., Mueller, O., Pferifer, A. and Aeschbacher, H. U. 1992. Natural antioxidants as inhibitors of oxygen species induced mutagenicity. Mutat. Res. 269: 193-200.
39. Timbrell, J. A. 2000. Chapter 5 Biotransformation of xenobiotics. In "General and applied toxicology". 2nd ed. pp.120. Ballantyne, B., Marrs, T. and Syversen, T. ed. Macmillan Reference Ltd. London, UK.