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Article

Antioxidant and Glycation Inhibitory Activities of Gold Kiwifruit, *Actinidia chinensis*

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Oxidative stress has been postulated to contribute significantly to the accelerated accumulation of advanced glycoxidation endproducts (AGEs) in collagen, which is implicated in the process of skin aging. Effectiveness of *Actinidia chinensis*, commonly called gold kiwifruit, in counteracting skin aging was investigated. Firstly, primary crude 70% ethanolic extracts of whole *A. chinensis*, pulp, and rind were screened for their *in vitro* antioxidant activities and anti-glycation activity by using 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) assay, and bovine serum albumin-derived glycation model. Result indicated that rind portion exhibited significantly ($p<0.05$) high antioxidant activity as well as high phenolic and flavonoid contents compared to those of pulp and whole *A. chinensis*. Thus, rind was selected for further fractionated with hexane, chloroform, ethyl acetate, and butanol. Among these solvent fractions, *A. chinensis* rind ethyl acetate (ACRE-E) had the greatest radical-scavenging activity and reducing power, comparable to standard antioxidant, vitamin C. Immunofluorescence staining was used to determine AGEs distribution in glycated collagen matrix. ACRE-E inhibited formation of 67% AGEs. High Performance Liquid Chromatography analysis revealed phenolic compound of ACRE-E as quercetin-3-rhamnoside. High antioxidant and anti-glycation activities of ACRE-E in glycated collagen model indicate its contribution to anti-aging process. *A. chinensis* rind, previously considered as a byproduct, may have potential as a low-cost raw material for cosmetic and pharmaceutical industries.

Key words: *Actinidia chinensis,* anti-glycation, antioxidant, collagen, gold kiwi, quercetrin

Aging is a complex and irreversible process which causes damage to all biomolecules, cells, and organs. Skin is the organ, which most easily reflects the result of aging. Vierkotter *et al*. [2009] suggested that there are two distinct types of aging. First type is the aging caused by genetics, the so called intrinsic aging. The other type is extrinsic aging caused by environmental factors, such as exposure to sun's ray, pollution, and unbalanced diet. These environmental factors trigger a biochemical process that generates free radical in our body. When the level of free radicals exceeds the capacity of the body to neutralize them, oxidative stress occurs, leading to damages from the level of cells to tissues and to organs.

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In our skin, these oxidative stress causes damage to skin cell membrane by acceleration of protein glycation and glycoxidation endproduct (AGE) formation [Glenn and Stitt, 2009; Pageon, 2010]. Protein glycation occurs when blood sugar reacts with protein such as collagen, an essential component of skin, to form AGEs, which leads to the degradation of collagen. Oxidative reaction is known to be included in glycation process. Combined glycation and oxidation, "glycoxidation" occurs when Amadori compounds are degraded to form even more reactive dicarbonyl compounds [Lyons, 1993]. Carboxyl-methllysine (CML) and carboxymethyl-hydroxylysine (CMhL) are AGEs formed by oxidative cleavage of Amadori adducts, whereas pentosidine is formed between crosslinking of lysine and arginine. The formation of CML and CMhL from Amadori compounds can be inhibited by free radical scavengers, and formation of pentosidine can be inhibited under anaerobic condition [Baynes, 1991].

Because the formation of AGEs could be dependent on oxidation reaction, the application of antioxidant in cosmetic product is a strategy for the prevention of aging. Human body possesses intracellular antioxidant defense system to battle against free radical and oxidative stress [Steenvoorden and van Henegouwen, 1997]. However, various environmental stresses have resulted in exposure of human body to excessive free radicals and created an imbalance between free radical molecules and endogenous antioxidant. Therefore, our body needs extra exogeneous antioxidants to assist in restraining oxidative stress.

The overwhelming evidence of the importance of oxidative stress in causing skin aging evoked a great interest in the finding of natural antioxidants for cosmetic and food applications. Results showed that plant extracts contain high levels of antioxidant phenolic compounds, and some of them were more effective than synthetic antioxidant, such as butylated hydroxytoluene.

Recently, the recovery, recycling, and upgrading of agricultural wastes as low-cost sources of antioxidants have become active research domains. The external fractions of fruits, such as peels, seeds, and hulls, which usually discarded during processing, are rich in phenolic compounds with good antioxidant activities than their corresponding inner fractions. Mango seed and pomegranate rind (*Punica granatum*) have been reported to possess antioxidant and tyrosinase inhibitory activities [Rout and Banerjee, 2007; Maisuthisakul and Gordon, 2009]. These agricultural wastes are potential raw material in cosmetic and pharmaceutical industries due to their greater concentration of antioxidant phenolic compounds, low cost, and the possibility to reduce environmental problem caused by waste disposal.

Actinidia chinensis, commonly called gold kiwifruit, belongs to the botanical family Actinidiaceae. They were then propagated in New Zealand's HortResearch orchard in Te Puke. The first *A. chinensis* was produced in 1992 in New Zealand. Several studies showed that green kiwi, *Actinidia deliciosa* has strong antioxidant activity [Fiorentino *et al.*, 2009]. However, relatively higher concentration of ascorbic acid was reported in *A. chinensis* compared to *A.deliciosa* [Rassam and Laing, 2005]. Two flavonol glycosides, quercitrin and kaempherol, isolated from *A. chinensis* seed extract, have been reported to have anti-inflammatory and tyrosinase inhibitory activities [Tanaka *et al.*, 2007].

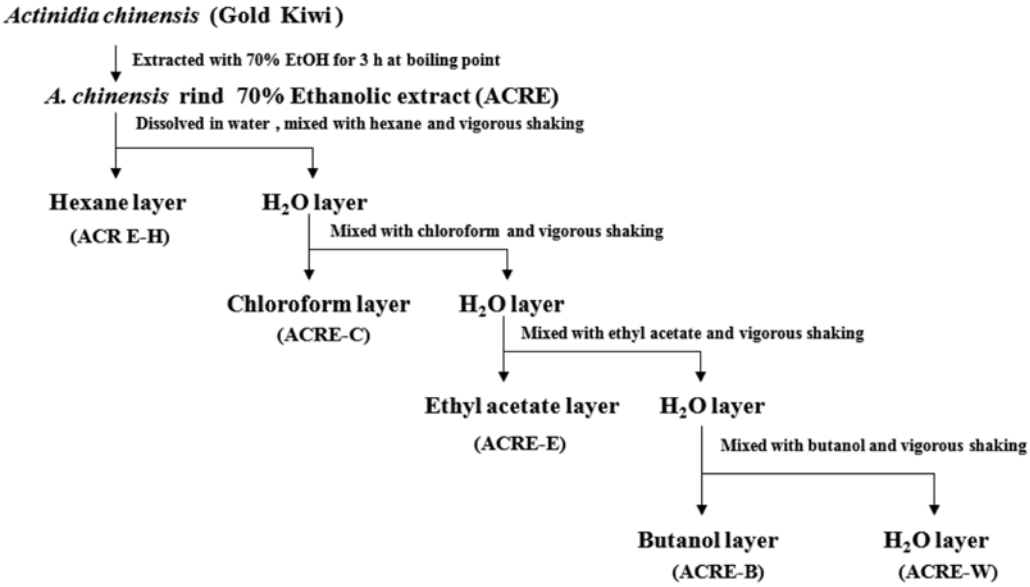
In the present study, antioxidant and glycation-inhibitory activity of solvent extract from different portions of gold kiwifruit *(A. chinensis),* and their flavonoid and polyphenol contents were investigated. Furthermore, effect of *A. chinensis* rind ethyl acetate fraction on the inhibition of AGE formation in a glycated collagen model was also determined.

Materials and Methods

***A. chinensis* sample preparation, crude extraction, and serial fractionation procedure.** *A. chinensis* (New Zealand Kiwifruit Marketing Board, ZESPRI, Auckland) were purchased from local market. The fruits were divided into three groups. First group was used intact as a whole *A. chinensis*, and their main morphological parts of the other groups were separated to rind and pulp. They were freeze-dried and ground into fine powder using a blender.

Solvent extraction was carried out as described by Choi *et al*. [2008]. In order to obtain the comparison result for yield, 100 g from each group were initially extracted with 4 L of 70% ethanol under refluxing for 3 hs. The supernatants were passed through Whatman filter paper No. 2 (Maidstone, England). All filtrates were evaporated under reduced pressure using rotary evaporator at 55°C to remove ethanol. The remaining water was then removed by lyophilization, and dried sample was weighed to determine the production yield. Among the whole *A. chinensis* (ACWE), *A. chinensis* pulp (ACPE), and rind extracts (ACRE), ACRE showed the highest biological activity. Hence, further fractionations of ACRE with different polarity organic solvents were performed. It was dissolved in distilled water and fractionated successively with hexane (ACRE-H), chloroform (ACRE-C), ethyl acetate (ACRE-E), and *n*-butanol (ACRE-B), leaving an ethanol aqueous fraction (ACRE-W) (Scheme 1). They were then concentrated, and lyophilized as described earlier. The yield of these fractions were 0.31, 1.15, 1.51, 24.76, and 16.66 g out of 100 g of crude extract powder and constituted about 0.6, 2.2, 2.9, 47, and 32% of the ACRE, respectively. The above-mentioned extracts and fractions were subjected to biochemical analyses, including 1,1-diphenyl-2-picrylhydrazy (DPPH), Ferric Reducing Antioxidant Power (FRAP) assay, total polyphenol and flavonoid measurements, cellular antioxidant assay, and anti-glycation assay.

Antioxidant assay. Ferric-ion reducing power of the studied material was measured following the method described by Maksimovic *et al*. [2010]. Briefly, the FRAP reagent containing 2.5 mL of 10 mM 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) solution in 40 mM HCl plus 2.5 mL of 20 mM Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and 25 mL of 300 mM acetate buffer, pH 3.6 were prepared. Six microliters of the sample was mixed with 180 μL of FRAP reagent in 96 wells and incubated at 37°C. After the mixture reached equilibrium,



Scheme 1. Extraction and fractionation of bioactive compound from *A. chinensis* rind portion using different organic solvents.

the absorbance at 593 nm was read. $FeSO_4 \cdot 7H_2O$ was used for construction of calibration curve in order to denote the concentration of sample having ferric ion-reducing power equivalent to that of 1 mM $FeSO_4$.

The effects of free radical-scavengers of *A. chinensis* sample on the stable radical DPPH were evaluated by the method described by Wang and Li [2007]. In this assay, sample was mixed with 500 mM DPPH· ethanol solution, and the reaction mixture was incubated at 37°C for 30 min. The absorbance was measured at 519 nm. The scavenging activity was calculated according to following formula: $SC_{50}=[(A_{control}-A_{sample}/A_{control})\times 100]$. The SC_{50} value, obtained through extrapolation from linear regression analysis, denotes the concentration of sample required to scavenge 50% of DPPH radicals.

The total phenolic content was determined using Folin-Ciocalteu's reagent. Sample was dissolved in DMSO (0.1 mL) and added distilled water to a total volume of 0.5 mL. After addition of 0.25 mL Folin-Ciocalteu reagent and 1.25 mL 20% aqueous sodium carbonate solution, mixture were vortexed vigorously and allowed to stand for 40 min. The absorbance was read at 725 nm. A calibration curve of gallic acid was prepared. To measure the flavonoid content, sample and 2% $AlCl_3$ were mixed in 96 wells. Absorbance readings at 413 nm were taken after 10 min incubation. In all experiments, Vitamin C was used as the positive control [Maksimovic *et al.*, 2005].

Cell culture and determination of cellular antioxidant by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human hepatoma HepG2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cell line was grown in a humidified incubator containing 5% CO_2 and 95% air at 37°C. The growth medium was Minimum Essential Medium from GIBCO (Grand Island, NY), supplemented with 10% fetal bovine serum (FBS). The culture medium was changed every other day, and the cells were usually split 1:4 when they reached confluence. The cell viability was measured by colorimetric MTT assay [Lee *et al.*, 2007]. Briefly, 1×10^5 cells were seeded in 24-well plate containing FBS-free medium and were allowed to attach overnight. *A. chinensis* sample were added with FBS-free medium containing 10 μL of 0.3 mM *tert*-butyl-hydroperoxide (*t*-BHP). After 2 h of sample incubation, MTT reagent (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) was added, and treated with 20% sodium dodecyl sulfate (SDS) after 3 h, followed by 16 h incubation at 37°C. In this cell viability assay, positive control was untreated with *t*-BHP, whereas the negative control was treated with *t*-BHP. The optical density was determined by multiplates reader at 564 nm [Kim *et al.*, 2007].

Bovine serum albumin (BSA)-derived glycation model and analysis of AGE formation. In anti-glycation assay, 10 mM BSA was incubated with 10 mM glycolaldehyde in the presence of aminoguanidine or *A. chinensis* primary crude sample at 37°C. The reaction was allowed to proceed for 7 days before extensive dialysis against phosphate buffer three times within 24 h. The fluorescence intensity for the formation of AGEs was measured at an excitation of 370 nm and an emission of 440 nm with a spectrofluorometer [Choi *et al*., 2008].

Quantification of quercetrin by high performance liquid chromatography (HPLC) analysis. HPLC with

Waters Xterra column (3.9×150 mm, Milford, MA) was employed to estimate the content of active compound in the *A. chinensis* rind solvent fractions. HPLC apparatus consisted of a ProStar model 210 binary Pump (Varian, Palo Alto, CA). A flow rate of 1 mL/min, and a gradient elution of 0.05% trifluoroacetic acid anhydride (TFA) in distilled water (solvent A) and methanol (solvent B) from 0 to 100% were used. Sample injection volume was 10 μL. The peak retention time of the purified reference standard of quercetin-3-rhamnoside was used to identify the peak of the sample constituent for HPLC analysis. Compound quantification was performed using a calibration curve of the standard compound.

Immunofluorescence staining for AGE formation on glycated collagen matrix. Pre-glycation was performed before construction of gel lattice. Collagen (2.6 mg/mL) was incubated with 0.001 N HCl buffer and 10 mM glycolaldehyde in the presence of ACRE-E (1 mg/mL) or aminoguanidine (5 and 10 mM). The reaction was allowed to proceed for 9 days. At the end of the incubation, dialysis was carried out for 24 h against 0.001 N HCl buffer. Aliquots were solubilized with 1% pepsin at 37°C for 24 h. After centrifugation for 5 min at 10,000 g, fluorescence intensity of the digested collagen was measured at an excitation of 370 nm and an emission of 440 nm with a spectrofluorometer. Collagen without glycation was used as negative control.

Collagen gel lattice was constructed by using collagen solution and sterile reconstitution buffer at a ratio of 8:1. Collagen solution was prepared by using a 1:1 mixture of untreated collagen and glycated collagen solution while sterile reconstitution buffer is a mixture of 2.2 g $NaHCO_3$ in 100 mL of 0.05 N NaOH and 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The mixture was allowed to form gel in a 48-well plate for 24 h. Subsequently, gel was detached from mold and submerged into liquid nitrogen to ensure gel lattice froze completely. Gel was stored in a deep freezer (−70°C), and the frozen collagen gel lattice was sectioned into 50 μm using cryotome. Finally, the sections were placed on glass slides for immunofluorescence staining [Pageon *et al.*, 2008].

For immunofluorescence staining of AGEs on glycated collagen, nonspecific binding sites were first blocked with a 2% (v/v) dilution of appropriate normal serum in tris-buffered saline (TBS) (pH 7.4) in a humidified chamber. Each sample was subsequently treated with specific antibody against AGEs (TransGenic, Kobe, Japan, 2 μg/mL). Finally, the samples were washed in TBS, incubated with a goat anti-mouse Alexa 488 fluor antibody (Invitrogen, Carlsbad, CA, 1:200), mounted, and cover-slipped. All slides were then examined and evaluated using a confocal laser scanning microscope (LSM 5 Exciter, Carl-zeiss, Hamburg , Germany)

Statistical analysis. For statistical analysis, Sigmastat 3.5 was used. Data were expressed as means±SD of three independent experiments performed in triplicate. Statistical analysis was performed using the Holm-Sidak's test and Dunnett's test. Differences were considered statistically significant with ($p<0.05$).

Results and Discussion

Comparison of the antioxidant potential and glycation inhibitory activities in pulp, rind, and whole *A. chinensis* primary crude 70% ethanolic extract. The biological activities of the three groups were compared. All assays were performed using either ACPE, ACRE or ACWE. Focus was then placed on the activities of ACRE. Arumugam *et al*. [2006] reported that there is a close relationship between the antioxidant activity and the phenolic compounds. Phenolic compounds possess ideal structure chemistry responsible for their reducing activities as a hydrogen or electron-donating agent.

Table 1. Total flavonoid, polyphenol, and antioxidant activities of primary crude 70% ethanolic extracts from gold kiwi, *A. chinensis*

	Total flavonoid (μg QE/1 mg DM)	Total polyphenol (μg GAE/1 mg DM)	FRAP (mM Fe(II)/mg DM)	DPPH· SC_{50} (mg DM/mL)
ACPE	0.81±0.02[a]	37.0±5.7[a]	0.14±0[a]	23.8±7.0[a]
ACRE	1.38±0.20[b]	139.8±5.5[b]	0.82±0.02[b]	8.1±3.4[b]
ACWE	1.05±0.05[ab]	52.3±4.6[a]	0.22±0.01[c]	14.5±4.7[ab]
Vitamin C	-	-	7.85±0.61[d]	(8.77±6.4)×10^{-2c}

Total flavonoid contents expressed as QE, total polyphenol contents expressed as GAE, DPPH-scavenging activity (DPPH SC_{50}), and FRAP of the 70% ethanolic extract of *A. chinensis* are showed at Table 1. Vitamin C was used as positive control.

ACPE: *A. chinensis* Pulp EtOH extract, ACRE: *A. chinensis* rind EtOH extract, ACWE: Whole *A. chinensis* EtOH extract, DM: dry matter. SC_{50}, which means the effective concentration required for 50% radical scavenging activities. Mean±SD, n=3. Means with different letter(s) superscripts along the column are significant at $p<0.05$.

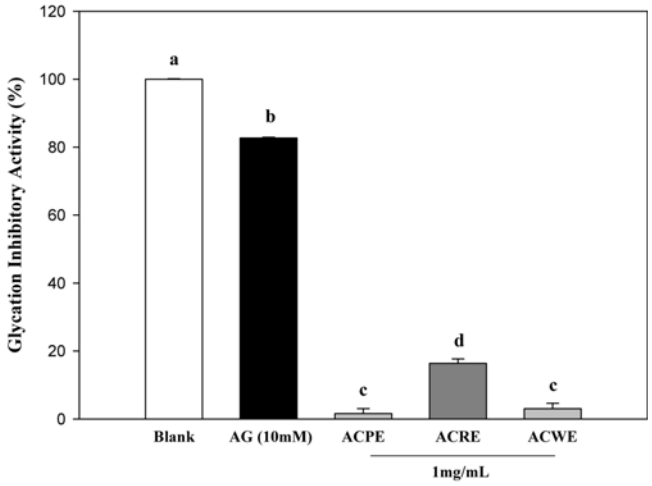


Fig. 1. Comparison of the glycation inhibitory activity in pulp, rind, and whole *A. chinensis* 70% EtOH crude extracts. Glycation inhibitory activity of 70% EtOH extract of *A. chinensis* and aminoguanidine were obtained by BSA assay. The percent of glycation inhibitory activity was calculated by comparison with the blank group (100%). Superscripts indicate statistically significant differences ($p < 0.05$).

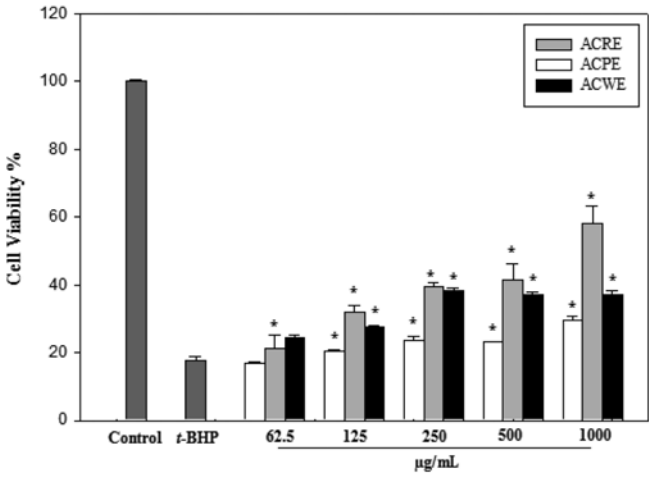


Fig. 2. Protective effect of 70% EtOH crude extract on viability of HepG2 cells against oxidative stress. Each value is mean±SD of triplicate experiments. Asterisks denote significant differences from *t*-BHP-treated group, * $p < 0.05$.

Table 1 shows the total flavonoid and polyphenol contents of ACPE, ACRE, and ACWE. The total flavonoid and polyphenol contents in ACRE were significantly higher, 1.38 μg/mg quercetin equivalents (QE) and 139 μg/mg gallic acid equivalents (GAE), followed by whole *A. chinensis* with 1.05 μg/mg QE and 52 μg/mg GAE, respectively. ACPE had the lowest flavonoid and polyphenol contents, 0.81 μg/mg QE and 37 μg/mg GAE, respectively. In addition, the polyphenol content of ACRE was almost four and three times more abundant than those of ACPE and ACWE, respectively.

The higher contents of flavonoid and polyphenol in ACRE could account for the better antioxidant result found in their free radical-scavenging activities and reducing power. $DPPH^{\cdot}$ was chosen for the radical-scavenging assay due to its advantage of not being affected by metal ion chelation and enzyme inhibition [Sarikurkcu *et al.*, 2010]. ACRE had the highest radical-scavenging ability, inhibiting 50% of $DPPH^{\cdot}$ free radical at 8.1 mg/mL, whereas ACPE and ACWE required 23.8 and 14.5 mg/mL, respectively (Table 1). In other words, it had two- and three-fold higher free radical scavenging capacities in ACWE and ACPE, respectively. These findings correlated well with the suggestion that the total antioxidant activity of many compounds of botanical origin is proportional to the phenolic content [Rice-Evans *et al.*, 1997].

Reducing power of *A. chinensis* might be due to the presence of hydrogen- or electron-donating agent in polyphenol compound. In FRAP assay, the presence of reducers causes the reduction of Fe^{3+}/ferricyanide complex into the ferrous form. Determination of the ferrous ion formation can be used to predict the reducing power of samples. ACRE, which had the highest phenolic content, showed six times higher reducing power than that of ACPE. Both radical scavenging activity and reducing power of vitamin C were remarkably higher when compared to those of *A. chinensis* crude extracts, because it is a highly purified single compound. Further purification of *A. chinensis* crude extract is expected to elevate the antioxidant activity.

Anti-glycation activity of *A. chinensis* crude ethanolic extract was tested by determining its ability to inhibit the formation of glycated BSA. Aminoguanidine at 10 mM inhibited glycation up to 82%. ACRE was able to inhibit the production of AGEs by 16% in 1 mg/mL against the same concentrations of ACWE and ACPE, which resulted in merely 3 and 1.5% inhibition, respectively (Fig. 1). The effectiveness of *A. chinensis* in inhibiting AGE formation shows its potential uses as anti-skin aging agent.

Determination of protective effect against cellular oxidative stress. Cellular antioxidant assay using HepG2 cell culture model is considered as a more biologically representative method to determine antioxidant activity. Fig. 2 shows the protective effect of *A. chinensis* extracts against oxidative stress on the viability of HepG2 cells. A 2-h treatment with 0.3 mM *t*-BHP evoked a great decrease in cell viability to 17%. Result showed that treatment with all portions of *A. chinensis* protected cells from oxidative stress in a concentration-dependent manner.

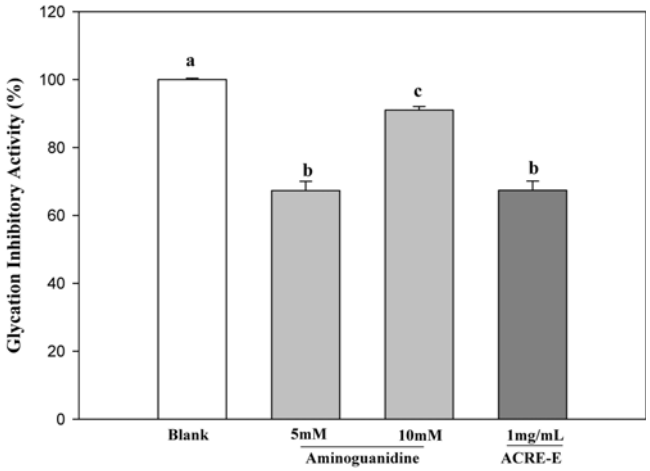


Fig. 3. Glycation inhibitory effect of aethylacetate fraction of *A. chinensis* rind on glycolaldehyde-treated collagen. Glycation inhibitory activity of ACRE and aminoguanidine was measured in a pre-glycated collagen model. The percent of glycation inhibitory activity was calculated and compared with the blank group (100%). Superscripts indicate statistically significant differences ($p<0.05$).

ACRE, at all concentrations examined, was able to reduce cell damage significantly ($p<0.05$). With 1 mg/mL, it protected the cell viability up to 60% compared to the 100% of *t*-BHP untreated cells as the control. It is reasonable to assume that free radical-scavenging activities of *A. chinensis* extract protected HepG2 cells from oxidative stress.

Determination of the free radical-scavenging activities and reducing power in different solvent fractions of 70% ethanolic extract of *A. chinensis* rind. Results obtained from the above experiments showed that, among ACWE, ACPE, and ACRE, ACRE has the highest antioxidant and anti-glycation activities. Therefore, ACRE was further fractionated through solvent-solvent partitioning with hexane, chloroform, ethyl acetate, and butanol to afford ACRE-H, ACRE-C, ACRE-E, and ACRE-B, respectively. The resulting fractions including aqueous fraction (ACRE-W) were tested for their antioxidant potentials.

The DPPH· assay was used first as a screen for antioxidant components. The radical scavenging activity of ACRE solvent fractions decreased in the following order: ACRE-E>ACRE-C>ACRE-H>ACRE-B>ACRE-W.

ACRE-E fraction inhibited 50% of DPPH radical at the concentration of 416 μg/mL, whereas SC_{50} of other fractions were higher than 2 mg/mL (Table 2). The radical scavenging activity was found to be 20-fold elevated in ACRE-E fraction compared to crude extract (ACRE: SC_{50}=8.1 mg/mL). In addition, ACRE-E fraction had significantly higher reducing power (5.7 mM $FeSO_4 \cdot 7H_2O$) compared with other fractions. For reducing power, the same decreasing order was observed, except ACRE-H was slightly more effective than ACRE-C (Table 2). Notably, after fractionation, the antioxidant activity of *A. chinensis* rind extract become comparable to the standard antioxidant, vitamin C.

Glycation inhibitory effect of *A. chinensis* rind ethyl acetate fraction (ACRE-E) on glycolaldehyde treated collagen. Since ACRE-E had the highest antioxidant activities among the other fractions, it was further tested to find out its efficacy of glycation inhibitory activity on collagen. Result indicated that 10 mM of aminoguanidine inhibited formation of AGEs by 91%, whereas ACRE-E resulted in 67% inhibition (Fig. 3). However, ACRE-E exerted slightly higher inhibitory effect when compared to that of 5 mM aminoguanidine.

This result corresponded with that of immuno-fluorescence staining with antibodies specific to AGEs (Fig. 4). The positive control, in the absence of ACRE-E or aminoguanidine, showed high immunoreactivity (Fig. 4B). This glycated collagen matrix theoretically mimics the highly glycated condition on human skin as epitomized by the high levels of AGEs present in human skin. A low level of AGE immunostaining was detected in the group treated with ACRE-E, and similar result was observed in 5 mM of aminoguanidine (Fig. 4E). These results show

Table 2. Determination of the radical scavenging activity and reducing power in different solvent fractions of *A. chinensis* rind 70% EtOH extract

	ACRE-H	ACRE-C	ACRE-E	ACRE-B	ACRE-W	Vit C
FRAP (mM Fe(II)/mg DM)	1.88±0.06^a	1.82±0.05^a	5.72±0.26^b	0.6±0.01^c	0.57±0^d	9.8±0.04^e
DPPH· SC_{50} (μg DM/mL)	2859±180^a	2026±115^b	416±9^c	3434±321^d	18645±568^e	73±6.6^f

FRAP were expressed as the concentration of sample containing ferric reducing ability equivalent to that of mM $FeSO_4$. Data for DPPH was expressed by the scavenging of 50% DPPH radical (SC_{50} of DPPH). Mean±SD, n=3. Means in the same column with different superscripted letters are significantly different ($p<0.05$).

ACRE-H: Hexane fraction of *A. chinensis* rind EtOH extract, ACRE-C: Chloroform fraction of *A. chinensis* rind EtOH extract, ACRE-E: Ethyl Acetate fraction of *A. chinensis* rind EtOH extract, ACRE-B: Butanol fraction of *A. chinensis* rind EtOH extract, ACRE-W: Aqueous fraction of *A. chinensis* rind EtOH extract

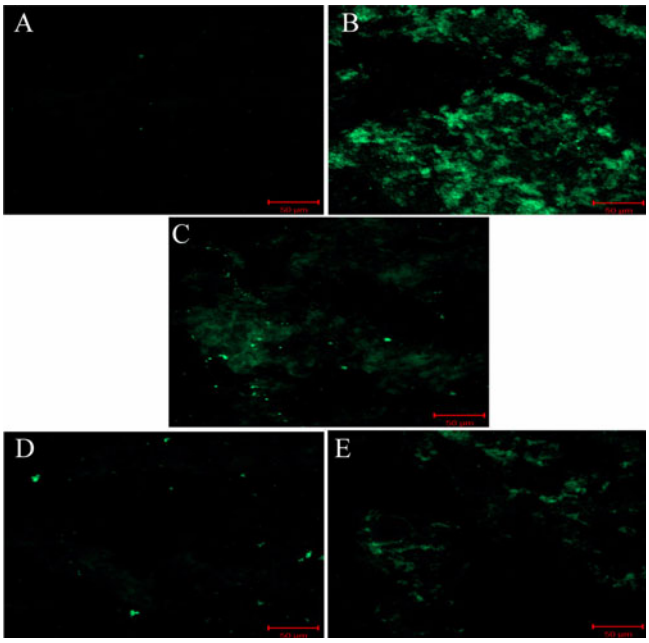


Fig. 4. Immunofluorescence staining for distribution of AGEs in glycated collagen matrix. Collagen section was stained with specific primary antibody against AGEs and incubated with goat anti-mouse Alexa 488 fluor secondary antibody. Highly glycated collagen appearing green. (A) unglycated collagen, (B) glycated collagen, (C) 5 mM Aminoguanidine, (D) 10 mM Aminoguanidine, (E) 1 mg/mL ACRE-E. Images were visualized by confocal laser scanning microscopy.

that ACRE-E fraction significantly inhibited the formation of AGEs on collagen. This inhibitory effect can be attributed to the high content of polyphenol in ACRE-E, which is known to have radical scavenging and reducing activities.

Quantification of quercetin-3-rhamnoside in ACRE-E. Previous study reported the isolation of quercetin-3-rhamnoside (quercetrin) from *A. chinensis* seed extract, showing inhibitory effect on tyrosinase activity [Tanaka *et al.*, 2007]. In the present study, the quercetin-3-rhamnoside compound was identified in ACRE-E by comparing the retention time with a standard compound (Fig. 5), and its content, determined by using the calibration curve of the standard compound was 17.3 μg/10 mg, constituting 0.17% of the total compounds in ACRE-E. Considering the high polyphenol content measured, quercetin-3-rhamnoside may not be the major active compound in *A. chinensis* rind extract. However, it is noteworthy that the quercetin-3-rhamnoside content in *A. chinensis* rind extract was significantly higher than the quercetin-3-rhamnoside isolated from *A. chinensis* seed extract (6.7 μg/10 mg) [Tanaka *et al.*, 2007]. δ-Tocopherol (2.49 mg/100 g) was found in *A. chinensis* peel extract and its antioxidant activity was 0.15±0.01 Caffeic acid

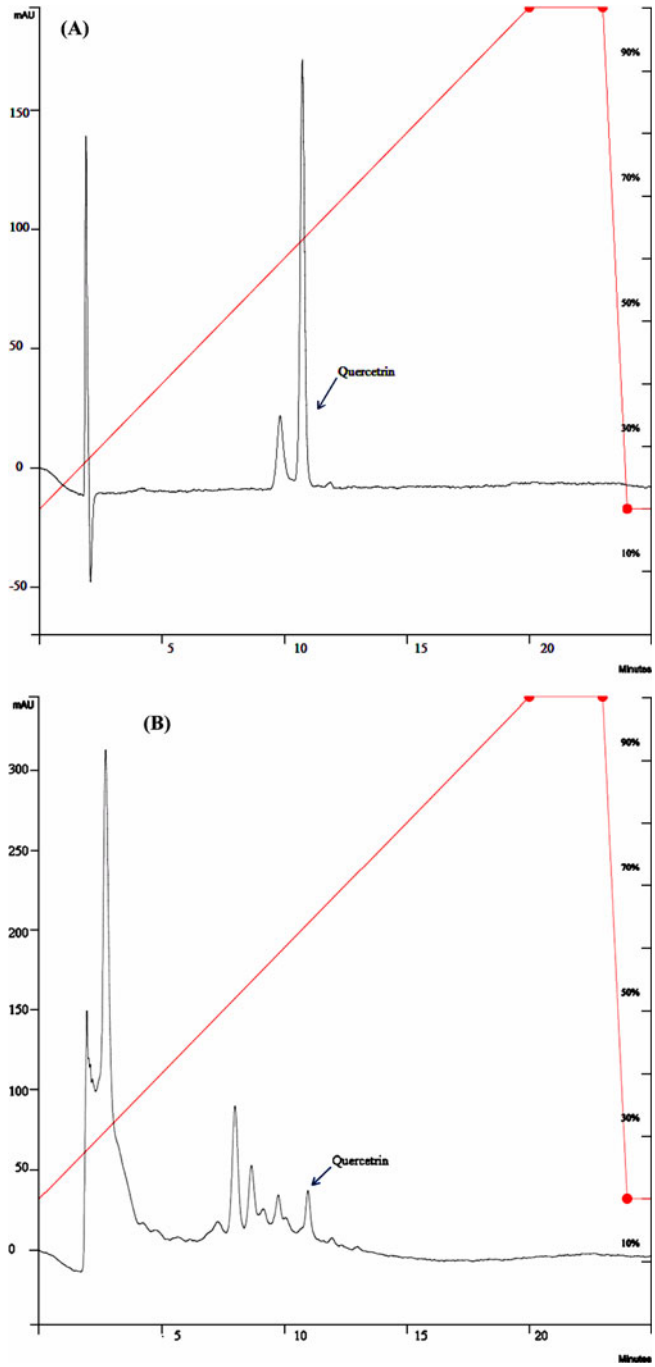


Fig. 5. HPLC characterization of quercetin-3-rhamnoside from ACRE-E. (A) HPLC analysis of purified quercetin-3-rhamnoside as the standard compound. (B) HPLC analysis of ACRE-E. Quercetin-3-rhamnoside compound was identified in ACRE-E by comparing the retention time with that of the standard compound.

equivalent (CAE) [Fiorentino *et al.*, 2009]. Further investigation is required to identify the major active compounds contributing to the antioxidant and anti-glycation activity in ACRE-E, and examine the involvement of quercetin-3-rhamnoside compound in these activities.

In conclusion, the antioxidant and anti-glycation results

obtained were influenced by different portion of *A. chinensis* used. Overall, the rind portion showed better biological activities than the pulp and the whole *A. chinensis*, which is in agreement with the higher content of phenolic compounds found in the rind. The use of the whole *A. chinensis* did not increase the antioxidant effectiveness; possibly because most of the phenol compounds measured in the whole *A. chinensis* were contained in the rind part. Further fractionation of the ACRE resulted in enhanced antioxidant potential in ACRE-E had the highest reducing and scavenging activities. The antioxidant activities in *A. chinensis* rind might account for the inhibition of formation of AGEs on collagen by decelerating the glycation of protein. Being considered as an agricultural waste, *A. chinensis* rind may be an attractive raw material in cosmetic and pharmaceutical industries due to its low-cost as antioxidant source and its contribution in reducing environmental pollution.

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