Original Article

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Comparison of antioxidant activity of compounds isolated from guava leaves and a stability study of the most active compound

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ABSTRACT: In the present study, quercetin (QT), morin (MR), and quercetin-3-*O*-glucopyranoside (QG) isolated from guava leaves were comparatively tested for antioxidant activity using DPPH, ABTS, and FRAP methods. QT was the most active among them. The free radical scavenging activity of QT was approximately four times higher than MR and two times higher than QG. The reducing power of QT was eight times higher than MR and two times higher than QG. A mixture of QT with MR or QG showed interesting combination effect. The synergistic antioxidant activity was obtained when QT was mixed with MR whereas the antagonistic effect was found when mixed with QG. The stability study of QT in liquid preparations indicated that the decomposition reaction rate of QT could be explained by a kinetic model assuming a first-order chemical reaction. The aqueous solution of QT was rapidly decomposed with $t_{1/2}$ of approximately five days whereas QT entrapped in chitosan nanoparticles was five times longer. It was concluded that QT was the most active antioxidant from guava leaves. Entrapment of QT in chitosan nanoparticles could significantly enhance its stability.

Keywords: Guava, quercetin, antioxidant, combination effect, stability, chitosan nanoparticles

1. Introduction

Guava (*Psidium guajava* L.), a plant of the Myrtaceae family, is found widespread in hot climate countries including Thailand. Traditional healers of different countries use its leaves to treat diarrhea, one of the leading causes of mortality and morbidity especially in developing countries (*1*). The scientific evaluation of guava with respect to diarrhea has been well studied (*2-5*). The extract of guava leaves has been reported to have narcotic-like activity (*6*), antinociceptive (*7*), antimutagenic (*8*), anticough (*9*), and central nervous system (CNS) depressant activity (*10*). Our previous study on the screening for biological actions of several plants commonly grown in Thailand reported that fruits and leaves of many edible plants possess high antioxidant activity (*11*). Among these, guava leaf extract has shown the highest antioxidant capacity (*12*). It was reported that the antioxidant activity of guava leaves is higher than its fruit (*13*). A phytochemical study of guava leaves has demonstrated many compounds including lipids, carbohydrates, proteins, vitamins, essential oils, tannins, saponins, flavonoids, sterols and triterpenes (*14,15*). Those polyphenolic compounds are believed to be the major principles that provide the plant with high antioxidant properties (*16-19*). However, a literature survey revealed that no data has been made to compare the potency of the active compounds as well as the interaction effects among them. The present study aimed to compare the antioxidant capacity of the active compounds isolated from guava leaves. Moreover, the interaction of the most active compound in combination with others as well as its stability was also our interest.

2. Materials and Methods

2.1. *Plant materials*

Mature leaves of guava (*P. guajava*) were collected during January-February from the botanical garden of Chiang Mai University, Thailand. The leaves were washed with clean water then dried in a hot air oven at 50°C for 48 h. After drying, they were pulverized into fine powder. The guava leaf powder obtained which passed a No. 60 sieve was collected for further study.

2.2. *Chemicals*

Potassium persulfate, ferrous sulfate ($FeSO_4 \cdot 7H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), 2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trolox was from Aldrich Chemical Company (Steinheim, Germany) and 2,4,6-tri (2-pyridyl)-S-triazine (TPTZ) was

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from Fluka Chemicals (Buchs, Switzerland). Column chromatography were carried out on Cosmosil 75 µm C_{18}-OPN (Nacalai Tesque, Kyoto, Japan), Sephadex LH-20 (GE Healthcare Biosciences AB, Uppsala, Sweden), Silica gel 60 (230-400 mesh) (Merck, Darmstadt, Germany), Toyopearl HW-40C (TOSOH, Tokyo, Japan) and MCI-gel (Mitsubishi chemical corporation, Tokyo, Japan). Pre-coated RP-18 F_{254} (0.25 mm) and organic solvents (AR grade) were from Merck (Darmstadt, Germany). Water was deionized and purified using a Milli-Q system (Millipore, Bedford, MA, USA). Sodium alginate was from Sigma-Aldrich (St. Louis, MO, USA), chitosan (95% DD, MW 22 kDa) was a gift from Seafresh Industrial PCL, Thailand. Other chemicals were of the highest available grade and used as obtained.

2.3. *Compound extraction*

Three active compounds, quercetin (QT), morin (MR), and quercetin-3-*O*-glucopyranoside (QG), were isolated according to the description in our previous report (*20*). Briefly, the methanol (MeOH) extract from the dried powder of guava leaves was subjected to column chromatography (Cosmosil C18-OPN, 75 µm, 6.0 cm (i.d.) × 40 cm) and eluted stepwise with MeOH-aqueous mixtures of different polarity (5 to 100% aqueous MeOH). Fractions were collected and subjected to chemical analysis using thin-layer chromatography (TLC) and antioxidant activity was evaluated by the DPPH method. The most active fractions were further isolated by column chromatography using Sephadex LH-20 and silica gel 60 columns to obtain QT, Toyopearl HW-40C and Cosmosil 75 µm C18-OPN columns to obtain MR, and Cosmosil 75 µm C18-OPN and MCI-gel columns to yield QG.

2.4. *DPPH assay*

The DPPH assay is considered to be a good *in vitro* model widely used to assess free radical scavenging efficacy in a relatively short time. In the present study, this method was used to screen the antioxidant power of the extracted fractions and the isolated bioactive pure compounds of guava leaves. In its free radical form, DPPH has an absorbance at 515 nm which disappears upon reduction by an antioxidant compound to become a stable diamagnetic molecule with a result of a color change from purple to yellow (*21*). The test could be taken as an indication of the hydrogen donating ability of the tested compounds. The assay was done using the method described previously by Gamez *et al*. (*22*) with some modification. Briefly, a sample in MeOH (100 µL) was added to a solution of 200 µM DPPH radicals in 100 µL. The reaction mixture was left to stand for 30 min at room temperature in the dark. The scavenging activity of samples was estimated by measuring the absorption of the mixture at 515 nm, which reflected to the amount of DPPH radicals remaining in the solution. The scavenging activity was expressed as the IC_{50}, the concentration of samples required for scavenging 50% of DPPH radicals in the solution.

2.5. *ABTS assay*

The ABTS assay is an excellent method used for determining the antioxidant activity of a broad diversity of substances, such as hydrogen-donating antioxidants or scavengers of aqueous phase radicals and of chain-breaking antioxidants or scavengers of lipid peroxyl radicals (*23*). In the present study, it was used for confirmation on the free radical scavenging potential of the pure and combined compounds isolated from guava leaves. The assay was done in the same manner as previously described by Okonogi *et al*. (*11*). Briefly, the pre-formed radical monocation of ABTS was generated by reacting ABTS solution (7 mM) with 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand for 15 h in the dark at room temperature. The solution was diluted with ethanol to obtain an absorbance of 0.7 ± 0.2 units at 750 nm. The test samples were separately dissolved in ethanol to yield an appropriate concentration. An aliquot of 20 µL of an ethanolic test solution of each sample was added to 180 µL of ABTS free radical cation solution. The absorbance, monitored for 5 min, was measured spectrophotometrically at 750 nm using a microtitre plate reader. The free radical scavenging activity of each sample was expressed as trolox equivalent antioxidant capacity (TEAC), which was obtained by comparing the absorbance change at 750 nm in a reaction mixture containing a test material with that containing trolox. This index is defined as the millimolar concentration of a trolox solution whose antioxidant capacity is equivalent to 1.0 mg of the test sample (*24*).

2.6. *Ferric reducing ability of plasma (FRAP) method*

This method was used to confirm antioxidant activity by mechanism of reducing action of the pure and combined compounds isolated from guava leaves. The reducing power of the test samples was determined by using a FRAP assay described by Benzie & Strain (*25*) with some modification. Briefly, the FRAP reagent containing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6, was freshly prepared. The test samples were dissolved in ethanol to an optimal concentration. An aliquot of 20 µL test solution was mixed with 180 µL of FRAP reagent. The absorption of the reaction mixture was measured at 595 nm by a microtitre plate reader. Ethanolic solutions of known Fe (II) concentration, in the range of 50-500 µM ($FeSO_4$), were used as a calibration curve. The reducing power was expressed as equivalent concentration (EC). This parameter was defined as the concentration of the test

sample having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

2.7. *Interaction effect on antioxidant activity*

The mixture of QT and MR or QT and QG at a weight ratio of 1:1 was subjected to antioxidant test using ABTS and FRAP assays. The experimental antioxidant activity (*E*) of the mixture expressed as TEAC or EC values respectively was determined as described in Sections 2.5 and 2.6, respectively. The theoretical antioxidant activity (*T*) is the sum of the experimental activity of each compound fraction, calculated using individual antioxidant activity in the following equation: $T = [(X_A \times E_A) + (X_B \times E_B)]$, where X_A and X_B represent the mole fraction of compound A and compound B, respectively, and E_A and E_B represent the antioxidant activity of 100% A and B, respectively (*26*).

2.8. *Preparation and characterization of QT loaded chitosan nanoparticles*

QT loaded chitosan nanoparticles were prepared according to the ionic gelation principle described previously by Sarmento *et al*. (*27*) with some modification. Chitosan at 0.5 mg/mL in 1% (v/v) acetic aqueous solution was prepared. A portion of 1 mL of 0.1 mg/mL QT in water-propylene glycol mixture was mixed with 10 mL of 0.03% (w/v) sodium alginate solution. Under magnetic stirring at room temperature, 0.3 mL of 0.18 mM calcium chloride solution was added followed by 1 mL of chitosan solution. Opalescent colloidal dispersion was formed spontaneously. The hydrodynamic diameter and zeta potential of the nanoparticles were measured by dynamic light scattering (DLS) using a Malvern system (Zetasizer, version 5.00, Malvern Instruments Ltd., Malvern, UK) consisting of computerized auto-titrate and DLS software. Measurements were taken at an angle of 173°. For entrapment efficiency determination, the dispersion was subjected to ultra centrifugation at 20,000 g, 4°C for 30 min. The separated nanoparticles obtained were dissolved in DMSO prior to analysis by HPLC using a C-18 column (150 mm × 4.6 mm, 5 µm; Agilent, USA) with a mobile phase of acetonitrile/0.1% (w/v) phosphoric acid solution (30:70, v/v) and detected by UV detector at 373 nm.

2.9. *Stability testing*

The aqueous solution of QT (0.1 mg/mL) and colloidal dispersion of QT loaded nanoparticles of equivalent QT concentration were kept in light protected and non-protected containers for 4 weeks. The amount of QT remaining in each sample during the storage time was determined periodically by HPLC as described in Section 2.8.

3. Results and Discussion

3.1. *Antioxidant activity of the isolated compounds*

The antioxidant activity can be evaluated by different methods depending on the mechanism of antioxidant action. In this study, three compounds, *i.e.* QT, MR, and QG, structures as shown in Figure 1, were isolated from guava leaves and subsequently subjected to the comparative antioxidant test using DPPH and ABTS methods for the free radical scavenging mechanism and the FRAP method for reducing action. The results as shown in Table 1 indicated that QT was the most active antioxidant with an IC_{50} value of 1.20 ± 0.02 µg/mL. The results from ABTS confirmed that QT possessed excellent free radical scavenging activity with a TEAC value of 57.54 ± 0.07 mM/mg. It was noticed from the IC_{50} and the TEAC values that the free radical scavenging potential of QT was approximately four times higher than MR and two times higher than QG. The principle of the FRAP method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. The reducing power property indicates that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process, so that they can act as primary and secondary antioxidants (*28*). The results indicated that QT, MR, and QG possessed reducing activity but not at the same level. These results clearly indicated

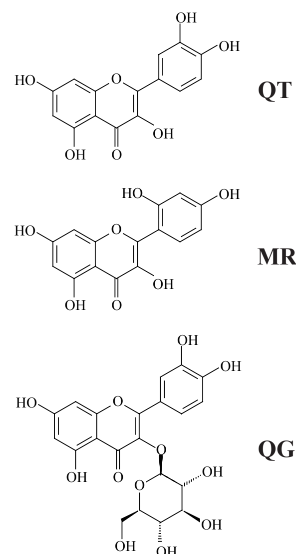

Figure 1. Chemical structure of quercetin (QT), morin (MR), and quercetin-3-*O*-glucopyranoside (QG).

Table 1. Antioxidant activity of the isolated compounds

Isolated compound	IC_{50} (µg/mL)	TEAC (mM/mg)	EC (mM/mg)
QT	1.20 ± 0.02	57.54 ± 0.07	72.69 ± 1.06
MR	5.41 ± 0.20	14.59 ± 0.62	8.56 ± 0.33
QG	3.58 ± 0.05	32.35 ± 0.12	42.52 ± 1.08

that QT was the most potent reducing substance with an EC value of 72.69 ± 1.06 mM/mg. This value was eight and two times higher than MR and QG, respctively. From this point of view, it was confirmed that QT was the most active antioxidant compound among them. According to its obviously high EC value, QT was considered to be a good electron donor and could terminate oxidation chain reactions by reducing the oxidized intermediates into a stable form.

3.2. *Effect of compound combination on antioxidant power*

As QT showed the strongest antioxidant activity among the three active compounds isolated from guava leaves, this compound was combined with each of the other two in order to examine interaction effects. The calculated theoretical antioxidant activities (T) of the mixtures were compared with the experimental activity (E). If E is greater than T ($E/T > 1$), then a positive interaction happened among the compounds, thus displaying synergism. On the other hand, if E is less than T ($E/T < 1$) a negative interaction happened, thus displaying antagonism. An additive effect was obtained when no reaction occurred and the ratio of E/T showed a value of 1. The results of QT in combination with MR or QG are shown in Table 2. It was found that the combination of QT with MR yielded a significant synergistic antioxidant effect, whereas the combination with QG gave an antagonistic antioxidant action. Synergistic effects of complex mixtures are thought to be important in plant defenses against a harmful environment. Plants usually present defenses as a set of compounds, not as individual ones, and it is thought that the minor constituents may act as synergists, enhancing the effect of the major constituents through a variety of mechanisms (*29*). In the present study, our results demonstrated that the antioxidant activity of QT as a major active component was synergized by a minor component, MR.

3.3. *Stability study*

This experiment was done in order to examine the effect of light and aging on the chemical stability of QT extracted from guava leaves. The results revealed that QT decreased rapidly during the first week of storage and further gradually decomposed according to the exponential degradation profiles demonstrated in Figure 2. A high reduction within one week of approximately 70% of QT was found in the samples without light protection as shown in Figure 2A and 30% in the light protection samples as demonstrated in Figure 2B. The result suggested that QT degradation was enhanced by photolysis. This result of light-induced decomposition of QT in our study was in agreement with the report of Smith *et al*. (*30*). The results provided valuable information about the degradation aspect of QT. This revealed the relevance to the real conditions of QT encountered in pharmaceutical or cosmetic products that is limited. To overcome this drawback, in the present investigation, QT was encapsulated in nanoparticles of chitosan. It was found that QT loaded chitosan nanoparticles were formed successfully by the gelation method. The hydrodynamic diameter and zeta potential of the nanoparticles obtained were found to be 310.1 ± 7.1 nm and –35.9 ± 0.5 mV, respectively. This confirmed that QT loaded particles obtained were in the nanometer size range. The resulting zeta potential value, from the viewpoint of the DLVO theory (*31,32*), indicated the high stability of the QT loaded nanoparticle colloidal system obtained. The entrapment efficiency was found to be 96.26 ± 0.1% indicating that QT was quantitatively encapsulated in the nanoparticles. The degradation profile of QT in the nanoparticles was demonstrated to be an exponential curve as shown in Figure 3. It was noted that the degradation of QT entrapped in the nanoparticles was also enhanced by light. However, the amount of QT entrapped in the nanoparticles when compared with that of non-entrapped samples, was found to be significantly higher at the same time of storage. This result indicated that the degradation of QT in the nanoparticles was obviously reduced.

Table 2. Antioxidant activity of the combined isolated compounds

Antioxidant activity	T	E	E/T	Interaction effect
TEAC (mM/mg)				
QT + MR	36.06	57.72	1.60	Synergism
QT + QG	44.95	35.91	0.79	Antagonism
EC (mM/mg)				
QT + MR	40.62	67.54	1.66	Synergism
QT + QG	60.79	40.84	0.67	Antagonism

Mixture of 1:1 weight ratio.

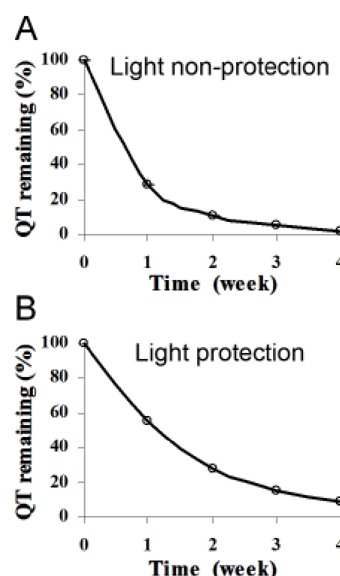

Figure 2. Effect of aging on QT solution kept in light non-protected (A) and light protected (B) containers.

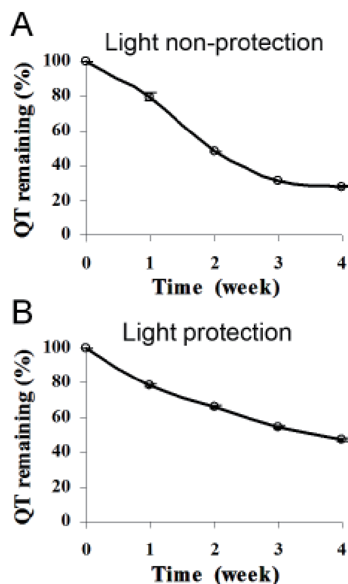

Figure 3. Effect of aging on QT loaded chitosan nanoparticles kept in light non-protected (A) and light protected (B) containers.

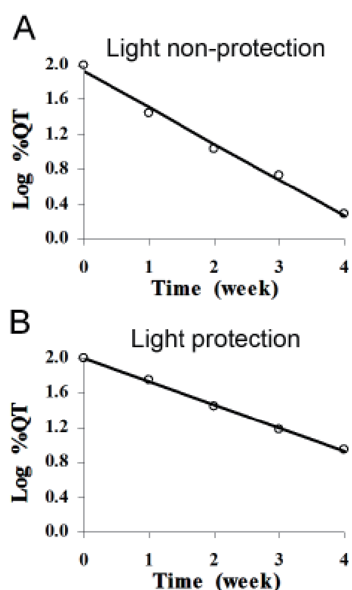

Figure 4. Plot of the logarithm concentration of QT remaining in light non-protected (A) and light protected (B) containers versus time of storage.

The relation of logarithm concentration of the remaining QT and the storage time showed a linear regression of $y = 1.93 - 0.42x$ ($r^2 = 0.991$) and $y = 1.99 - 0.27x$ ($r^2 = 0.998$) for the light non-protected and protected samples, respectively as shown in Figure 4. This result suggested that the decomposition of QT could be explained by a kinetic model assuming a first-order chemical reaction in the following equation: $dC/C = -kdt$ where C is the remaining amount of QT at time t, k is the degradation rate constant, and t is the time point of storage. Similarly with the non-entrapped QT, the linear relationship, of $y = 2.00 - 0.15x$ ($r^2 = 0.967$) and $y = 1.99 - 0.08x$ ($r^2 = 0.993$), was obtained from the light non-protected and protected

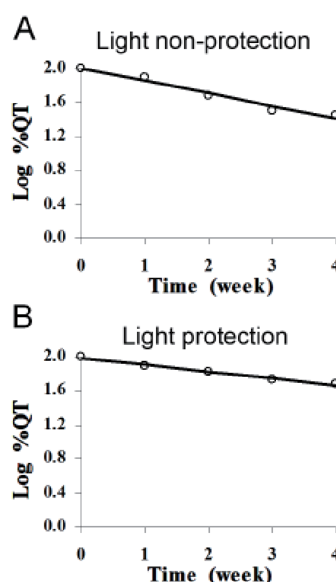

Figure 5. Plot of the logarithm concentration of QT loaded chitosan nanoparticles remaining in light non-protected (A) and light protected (B) containers versus time of storage.

Table 3. Experimental first order kinetic parameters and correlation coefficient of the degradation of QT in different samples

QT samples	r^2	$k_{(OBS)}$(week^{-1})	$t_{1/2}$ (days)
NE-QT-L	0.991	0.961	5.049
NE-QT-NL	0.998	0.612	9.704
NN-QT-L	0.967	0.347	13.966
NN-QT-NL	0.993	0.187	25.995

NE-QT-L: non-entrapped QT solution in light non-protected container; NE-QT-NL: non-entrapped QT solution in light protected container; NN-QT-L: QT entrapped in nanoparticles dispersion in light non-protected container; NN-QT-NL: QT entrapped in nanoparticles dispersion in light protected container.

nanoparticles, respectively as shown in Figure 5. This indicated that the degradation kinetics of QT entrapped in the nanoparticles also followed a first-order kinetic reaction. The degradation rate of QT from each sample could be simply observed from the slope of the straight lines, the higher the slope the higher the decomposition rate. The experimental kinetic parameters of QT degradation are provided in Table 3. The observed first order kinetic rate constant, $k_{(OBS)}$, was obtained from the slope value multiplied by 2.303. The half-life, $t_{1/2}$, of QT from each sample was calculated from the $k_{(OBS)}$. It was found that QT entrapped in chitosan nanoparticles and protected from light was the most stable. The $t_{1/2}$ of QT from this sample was approximately five times longer than that of the non-entrapped sample kept in the light non-protected container.

4. Conclusion

QT, MR, and QG were isolated from guava leaves. Among these three compounds, QT was found to have the most active antioxidant activity through free radical scavenging and reducing mechanisms. The antioxidant activity of QT was synergized by MR and antagonized by QG. The

stability study of QT in liquid preparations revealed that QT was unstable. The degradation of QT was enhanced by light and through a first-order kinetic reaction. Entrapment of QT in chitosan nanoparticles could significantly improve the stability of QT.

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