Thai Journal of Pharmaceutical Sciences (TJPS)

5th International Conference on Pharmaceuticals, Nutraceuticals and Cosmetic Science (IPNaCS 2017)

Determination of Antioxidant Contents and Antioxidant Activity of Gelatin Dessert Containing Guava Leaf Extract

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Keywords: Antioxidant, Gelatin dessert, Guava leaf, Guava leaf extract

Introduction

Free radical is any molecule or atom which has an unpaired electron in its outer orbital. Free radicals are produced from many sources including endogenous source such as metabolism of aerobic respiration, and environmental source such as radiation, pollutant or ultraviolet light.[1] Free radicals are very unstable, and can react with protein, lipid or DNA in human body and cause damage on cells or organs, leading to several human diseases such as cardiovascular diseases, cancer or Alzheimer's disease.[2] Some diseases such as Alzheimer's disease and cancers are difficult to treat and resulted in high economic cost to society.[3] Hence, the prevention could be an alternative method. The antioxidant compounds play an important role in the prevention from oxidative stress. Plants are the rich sources of natural antioxidants. From the study on the antioxidant activity of 24 plant species commonly found in Thailand, the leaves of guava showed the highest antioxidant capacity.[4]

Guava or *Psidium guajava* L. (Family Myrtaceae) is commonly found in Thailand. In southwestern Nigeria, guava leaf has been used to reduce oxidative stress in malaria patient. Many scientific evidences also confirmed this activity.[4-6] The phenolic compounds in guava leaf were responsible for its antioxidant activity.[7] The toxicity study in animal model including acute and subchronic toxicity studies confirmed that guava leaf was safe. In addition, guava leaf was listed in Japan as Food for Specified Health Use (FOSHU). Therefore, guava leaf was considered to be safe for human consumption.[8] Nowadays, most guava leaf products available in the market were in the group of guava leaf infusion tea. It is interesting to develop guava leaf into gelatin dessert which is suitable for all ages as an alternative to existing commercially-available products.

Gelatin dessert is a dessert that contains gelatin as the main ingredient along with other ingredients such as sweetener and flavoring agent. Gelatin dessert is on sale in the market, both as ready-to-eat products and in powder form. This study aims to determine the antioxidant contents and antioxidant activity in the gelatin dessert containing guava leaf extract.

Methods

Chemicals

Ethanol (Merck), hydrochloric acid (Merck), ferric chloride hexahydrate (Merck), sodium nitrite (Merck), 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma-Aldrich), gallic acid (Sigma-Aldrich), quercetin (Sigma-Aldrich), ferrous sulfate heptahydrate (LOBA Chemie), aluminium chloride hexahydrate (LOBA Chemie), sodium carbonate anhydrous (Univer), sodium hydroxide (Carlo Erba), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Alfa aesar), methanol (Burdick & Jackson), acetic acid (BDH), citric acid (UCS) and sterile water (GHP) were used in this study. Ethanol and methanol were analytical grade.

Extraction

Guava leaves were collected from Namatoom, Panutnikhom, Chonburi province, Thailand. The extraction was carried out according to the method of Witayapan et al.[9] with some modifications. First, guava leaves were washed with tap water, then blanched in boiling water for 30 seconds and soaked in cold water for 15 minutes. The blanched leaves were dried in a hot air oven at 50°C for 20 hours. Dried leaves were ground into coarse powder using a blender, then extracted with deionized water using ultrasonication for 10 minutes. The extract was filtered through a Whatman No.4 filter paper, under reduced pressure. The filtrate was evaporated *in vacuo* at 45°C. The concentrated extract was subsequently lyophilized (Labconco FreeZone 2.5plus, Labconco, USA) into dry powder. The guava leaf extract powder was collected and stored in a dark and dry place.

Gelatin dessert preparation

The dessert formula consisted of guava leaf extract (0.09%w/w), sucrose (7.27%w/w), gelatin (1.82%w/w) and water (90.83%w/w) Gelatin leaf was soaked in cold water at 4-10°C for 5 minutes before use. Water was heated to 70-80°C. Soaked gelatin leaf, and sucrose were then added to the water and stirred until dissolved. The gelatin mixture was filtered through a muslin cloth and allowed to set at 4°C for 12 hours.

Sample preparation

Due to the possible interactions of phenolic compounds with other food constituents such as carbohydrates and proteins in gelatin dessert[10], the suitable sample preparation method was determined. The five sample preparation methods were performed according to the methods that was previously described with slight modification.[11-13] (**Figure 1**)

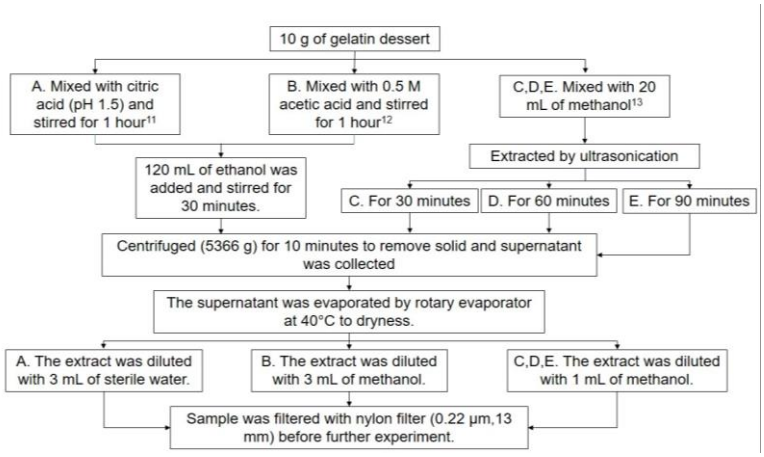

Figure 1 Schematic overview over the five sample preparation methods

The extracts obtained from all sample preparation methods were subjected to TLC analysis using silica gel 60 F 254 as an adsorbent and toluene-ethyl acetate-formic acid (10:10:4) as developing solvent. Determination of total phenolics content was also performed in order to clarify the result from TLC analysis.

Determination of total phenolics content

Total phenolics content was determined using Folin–Ciocalteu method described by Vongsak et al.[14] with some modifications. Sample solution was prepared by dissolving sample in 50% methanol to the concentration of 100 µg/mL. Twenty µL of sample solution and 50 µL of 10% Folin–Ciocalteu reagent were mixed in a 96-well microtiter plate. After 3 minutes, the mixture was neutralized by adding 80 µL of 7.5% Na_2CO_3. Then, the mixture was incubated in the dark at room temperature for 2 hours. The absorbance was measured using a UV spectrophotometer microplate reader (Tecan Infinite M200, Tecan, Austria) at 765 nm; gallic acid at the concentrations of 25-200 µg/mL were used to construct a calibration curve. The result was expressed as milligram of gallic acid equivalent per gram sample and all determinations were performed in triplicates.

Determination of total flavonoids content

This method was performed as described by Herald et al.[15] with some modifications. One hundred microliters of sterile water was added to a test tube and mixed with 25 µL of sample solution at the concentration of 500 µg/mL and 10 µL of 50 g/L $NaNO_2$. The reaction mixture was allowed to react for 5 minutes. Fifteen µL of 100 g/L $AlCl_3$ was then added. After 6 minutes, 50 µL of 1M of NaOH and 50 µL of distilled water was added to the mixture. Vortex mixer was used to mix the mixture for 30 seconds. The mixture was added to 96-well microtiter plate and the absorbance was measured with a UV spectrophotometer microplate reader at 510 nm. Quercetin at the concentrations of 4-1500 µg/mL was used as a standard for calibration curve. The result was expressed as milligram of quercetin equivalent per gram sample and all determinations were performed in triplicates.

Determination of antioxidant activity

DPPH free radical scavenging assay

DPPH radical scavenging activity assay was carried out according to the method introduced by Brand-William et al.[16] with minor modification. The 0.4 mM DPPH stock solution was prepared by dissolving

16 mg DPPH in 100 mL of methanol, and stored in amber container until use. Then 100 µL of DPPH solution was mixed with 100 µL of sample solution at the concentration of 10 µg/mL in 96-well microtiter plate. The mixture was incubated in the dark for 30 minutes, then the absorbance was measured at 517 nm using UV spectrophotometer microplate reader at the wavelength of 517 nm. All determinations were performed in triplicates, and ascorbic acid was used as a positive control. The standard curve is constructed from the plot between the concentrations and the absorbances of Trolox ranged from 5 to 80 µM. The radical scavenging capacities of samples were expressed as mM TE/g sample.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out according to the method described by Benzie et al.[17] with some modifications. The FRAP reagent was freshly prepared by mixing 200 mL of 300 mM of acetate buffer (pH 3.6), 20 mL of 10 mM TPTZ in 40 mM HCl, 20 mL of 20 mM $FeCl_3$ solution and 24 mL of distilled water. The FRAP reagent was stored in a plastic bottle and placed in a water bath at 37°C during use. The samples were dissolved in sterile water to give a concentration of 1 mg/mL. Five hundred and twenty five µL of FRAP reagent and 40µL of sample solution were added to a micro cuvette and mixed thoroughly. After 4 minutes, the absorbance was measured using a UV spectrophotometer (UV-2600, Shimadzu, Japan) at 593 nm. Aqueous solutions of known ferrous (Fe^{2+}) concentration, in the range of 0.1-1 mM $FeSO_4$, was used for calibration curve. The ferric reducing antioxidant power was expressed as mM of ferrous sulfate equivalent per milligram sample. Ascorbic acid was used as a positive control and all determinations were performed in triplicates.

Results and Discussion

Guava leaf extract was used as the active ingredient in gelatin dessert. The suitable method for extraction of bioactive compounds from gelatin dessert was determined. TLC chromatograms of the extracts obtained by method C, D and E showed the chemical composition similar to guava leaf extract and was found to contain the band having the same Rf value and color as gallic acid. By varying the ultrasonication time in method C, D and E, the quantity of bioactive compounds in each extract could not be differentiated (**Figure 2**). Thus, the determination of total phenolics content of the extracts obtained by those methods was performed. It was found that the extract obtained by method C had highest total phenolic contents (21.01 mg gallic acid/g sample) when compared with those obtained by method D and E (16.52 and 16.15 mg gallic acid/g sample, respectively). This was probably because the longer duration of ultrasonication (60 and 90 minutes) generated more heat than shorter duration. This led to the decomposition of phenolic compounds. Therefore, extraction with methanol by ultrasonication for 30 minutes (method C) was the most suitable method to obtain the highest content of bioactive compounds from gelatin dessert containing guava leaf extract. Because gelatin did not dissolve in methanol during extraction, the supernatant obtained from method C, D and E were free of gelatin. On the other hand, in method A and B, acid was used in the extraction process, resulting in the dissolution of gelatin; elimination of gelatin could not be carried out. Moreover, acid may cause the changes in the original form of phenolic compounds contained in guava leaf extract.[18] Extraction by ultrasonication was better than extraction by maceration since the ultrasonic vibration disrupted the molecules in the sample and the ultrasonic jet passed through the solid surface of sample leading to a greater contact area between liquid and solid phase of sample, enhancing the dissolution of the bioactive compounds into the solvent.[19]

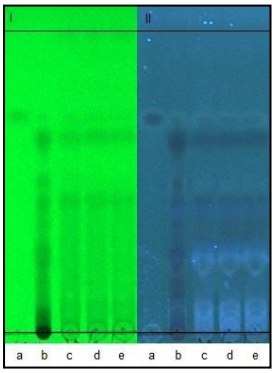

Figure 2 TLC chromatograms of a) gallic acid, b) guava leaf extract, c, d, e) the extracts obtained from method c, d, e, respectively. Plate I and II were observed under UV light at 254 and 366 nm, respectively.

The antioxidant contents and antioxidant activity of gelatin dessert containing guava leaf extract (GTGV) were totally contributed by phenolic and flavonoid compounds from guava leaf extract since the gelatin dessert base formula, GTB, showed negative results in both determinations (**Table 1**).

Table 1 Antioxidant contents and antioxidant activity of tested samples

Experiment \ Samples		GTGV	GTB	GV	Ascorbic acid
Antioxidant contents	Total phenolics (mg GAE/g sample)	14.47±0.00	nd	272.70±1.91	-
	Total flavonoids (mg QTE/g sample)	100.56±1.59	nd	961.56±8.7	-
Antioxidant activity	DPPH assay (mg Trolox/g sample)	61.29±0.79	nd	768.55±29.5	870.94±8.91
	FRAP assay (mM $FeSO_4$/mg sample)	0.37±0.00	nd	4.57±0.02	11.22±0.52

GTGV = gelatin dessert containing guava leaf extract, GTB = gelatin dessert without guava leaf extract, GV = guava leaf extract, nd = not detected. Ascorbic acid was used as a positive control in the antioxidant assays.

Although the antioxidant contents and the antioxidant activity of guava leaf extract from this study agreed well with previous studies,[4-7] both determinations on gelatin dessert containing guava leaf extract (GTGV) resulted in lower antioxidant contents and antioxidant activity than anticipated. As stated by Strauss et al.[20] that polyphenols could react with side chain amino groups of peptides under oxidizing conditions leading to cross-links formation in proteins. The phenolic compounds which were cross-linked with gelatin could not be extracted completely, thus, resulting in lower values. Future studies were suggested.

Conclusion

This is the first study on the determination of antioxidant contents and antioxidant activity of a complex matrix, i.e. gelatin dessert containing guava leaf extract. The crucial sample preparation step was the extraction by ultrasonication, avoiding acidic condition.

Acknowledgments

The authors would like to thank Prof. Emeritus Dr. Nuntavan Bunyapraphatsara for the provision of equipment. This study was financially supported by Mahidol University.

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