

DEVELOPMENT OF GUAVA LIPOSOME SERUM AND EVALUATION OF FREE RADICAL-SCAVENGING CAPACITY

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ABSTRACT: The aims of the study were to formulate serum containing liposome with guava leaf (*Psidium guajava* Linn.) extract as for cosmetic. Free radical scavenging and antityrosinase property in term of IC₅₀ of the extract from guava leaf was used as an outcome of the anti-wrinkle and whitening ability. The first part of this study was to evaluate the percentage of free radical inhibition and antityrosinase activity of the extract from guava leaf by DPPH method and dopachrome method respectively. The second part of this study was formulation of the serum containing liposome with guava leaf extract and evaluated free radical-scavenging capacity of the formulation compare to the crude extract by analyzing the phenolic compound in term of gallic acid. According to our study, the free radical scavenging property of the guava leaf extract was found effectively (IC₅₀ = 53.1889 µg/ml) with antityrosinase activity calculated in the same term (IC₅₀ = 9.864 mg/ml). The quantity of gallic acid were found in the extract and serum 3.0743 µg/mg of extract and 1.041 µg/ml respectively and percent of recovery were 67.73.

Keywords: Guava leaf extract, Liposome, Gallic acid, Antioxidant, Antityrosinase

INTRODUCTION: Guava (*Psidium guajava* L. Myrtaceae) which is well known to be rich in tannins is widely cultivated in the tropics and subtropics for its edible fruits and also has been used as folk medicine for treatment of diarrhea and stomachache in Thailand. According to the scientific support of their pharmacological activity, the anti-diarrhea antipyretic, antimicrobial and bio-antimutagenic properties of guava leaf extract have been demonstrated. In addition, guava juice exhibited hypoglycemic effects in mice. There is an important role of oxidative stress in the development of cancer and diabetes. As noted above, the infusion of guava leaves has potential as a functional drink. Recently, the study of the antioxidant activity and free radical-scavenging effects of guava leaf extracts has been demonstrated by Chen and Yen¹⁾ and Tachakittirungrod *et al*²⁾.

We studied antioxidant and anti-tyrosinase capacities of guava leaf extracts and guava liposome serum in order to evaluate some of the cosmeceutical claims in these particular properties such as stability and efficacy of the entrapment liposome.

MATERIALS AND METHODS:

Chemicals

Cholesterol, gallic acid and rutin were purchased from Fluka Chemicals (Buchs, Switzerland). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), L-dopa, kojic acid, mushroom tyrosinase, soybean phosphatidylcholine and trolox were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, chloroform, ethanol, methanol, and phosphoric acid were purchased from Merck (Darmstadt, Germany). Carbopol, triethanolamine, aloe vera, D-panthenol (vitamin B₅), vitamin E acetate, silk protein and yoghurt extract were obtained from Specialty Natural Product Company (Chonburi, Thailand). All other reagents were analytical grade available.

Plant material

Guava leaves were kindly provided by Dr. Supaporn Pitiporn, Chaophraya Abhaibhubejhr Hospital, Prachinburi province, Thailand. All of guava leaves used in this study were collected in March, 2007.

Crude extracts

Guava leaves extracts were obtained by

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maceration. Briefly, guava leaves were washed by tap water and dried at room temperature for 7 days. The dried guava leaves were ground into powder. Then dried guava leaves powder was extracted with 95% ethanol (100 g dried powder/500 ml of solvent) in the ultrasonic bath at 37 °C for 1 h. The filtrates of each time were collected and pooled. The solvent was then removed under vacuum at 45°C by using a rotary evaporator and 50°C by putting on water bath. The obtained concentrated guava leaves extracts were stored in a desiccator at room temperature.

Diphenyl-picrylhydrazyl (DPPH) radical scavenging assay

This assay detects free radicals scavenging capacity of the test samples through the scavenging of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical into the reduced DPPH formazan form, which can be determined by using a spectrophotometer. The assay was performed using a previously described method³⁾ with some minor modifications. Five microliters of each sample; guava leaves extracts as test sample, methanol and ethanol as negative control and rutin as positive control, were allowed to react with 195 µl of 100 µM DPPH ethanolic solution in a 96-well microplate. The plate was then incubated at 25 °C for 30 min after which the absorbance was measured at 535 nm using a Zenyth 200 rt microplate spectrophotometer (Anthos Labtec Instruments GmbH, Salzburg, Austria). Scavenging capacity of the samples was expressed as the concentration of the samples, which scavenged free radical by 50% (IC₅₀).

Inhibition of tyrosinase assay

Tyrosinase-inhibition activity was determined by using L-dopa as a substrate. Twenty microliters of 1000 units/ml of mushroom tyrosinase solution, 20 µl of phosphate buffer (pH 6.8), and 20 µl of sample with or without enzyme were mixed. The assay mixture was pre-incubated at 25 °C for 10 min and then 20 µl of 0.85 mM L-dopa was added. The amount of dopachrome was measured at 475 nm in a Zenyth 200 rt microplate spectrophotometer (Anthos Labtec Instruments GmbH, Salzburg, Austria). The data were expressed as a percentage of inhibition of

tyrosinase activity. Kojic acid was used as a standard tyrosinase inhibitor control.

HPLC analysis of phenolic compound

The content of phenolic compound in term of gallic acid in guava leaves extract and liposome serum were determined by HPLC, performed with an Agilent 1100 series HPLC-DAD (Waldbronn, Germany) consisting of an isocratic pump (flow rate: 1.0 ml/min) and the detection wavelength of the diode array detector was set at 270 nm. The analyses were carried out on an Inertsil ODS-3 RP-18 column (250 mm × 4.6 mm i.d., 5 µm, E. Merck Co., Darmstadt, Germany). Extracts were filtered through a 0.45 µm filter before use. The elution solvent was H₃PO₄ adjusted to pH 3.0 by phosphoric acid and acetonitrile (90/10 v/v). Phenolic compounds were identified by comparison of their retention time (Rt) values and UV spectra with that of standard gallic acid at 9.5 min and determined by peak areas from the chromatograms. All analyses were run in triplicate and mean values were calculated.

Liposomes preparation

Guava liposomes were prepared by thin film method⁴⁾. Briefly, a stock solution of each lipid; soybean phosphatidylcholine and cholesterol was prepared by dissolving the lipids in chloroform. An exact amount of each lipid solution was mixed in a round-bottom flask. The lipid mixture was evaporated to dryness and kept overnight in a desiccator to remove chloroform residue. Then the lipid film formed was resuspended in Tris-buffered saline containing guava leaf extract (pH 7.4) for 1 h at room temperature (25 °C) to completely hydrate the film. After hydration, the guava liposomes dispersion formed was sonicated for 30 min. Then the excess amount of guava leaf extract in guava liposomes dispersion was washed out (3 times) by Tris-buffered saline (pH 7.4) with ultracentrifugation subsequently. The total lipid concentration of guava liposomes was then adjusted to 2 mM.

Serum containing liposomes preparation

Serum base was prepared by utilizing carbopol as gel forming agent. Briefly, carbopol was dispersed in distilled water. After hydration,

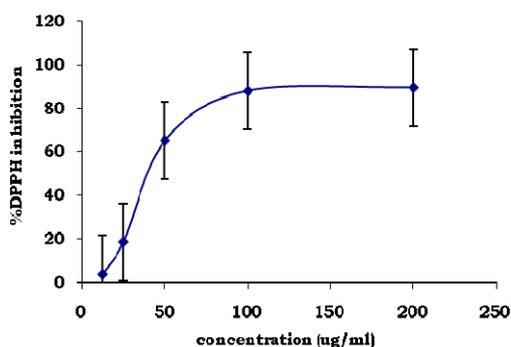


Figure 1 Correlation of %DPPH inhibition and concentration of guava leaves extract

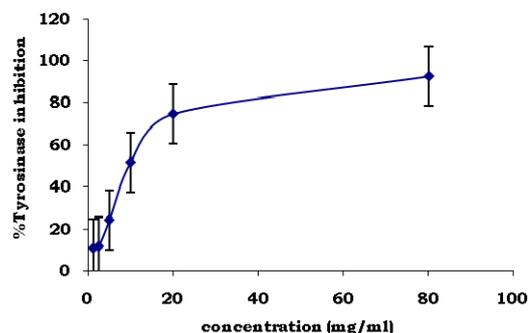


Figure 2 Correlation of %tyrosinase inhibition and concentration of guava leaves extract

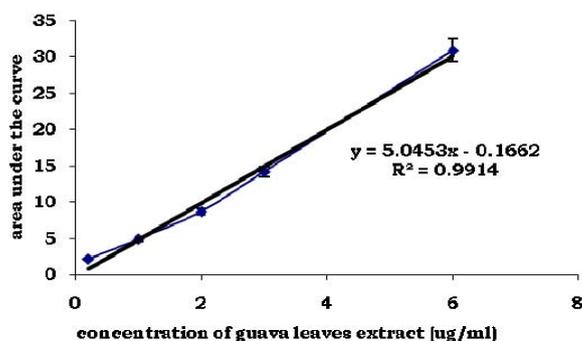


Figure 3 Correlation of area under the curve and concentration of guava leaves extract

triethanolamine was added to the gel to adjust the viscosity and pH to 7.4. Then aloe vera, D-panthenol, vitamin E acetate, silk protein and yoghurt extract were added to the gel. Serum containing guava liposomes was prepared by the same method with the addition of guava liposomes. The final concentration of guava liposomes in serum was adjusted to 5% w/w.

Entrapment efficiency of guava liposomes

Free guava leaves extracts were separated from guava leaves extracts associated to liposomes by centrifugation using Ultracentrifuge (Beckman, USA) operated at 60,000 rpm, 4 °C for 6 h. The entrapment efficiency was determined indirectly by measuring the concentration of gallic acid, a marker of phenolic compounds in guava leaves extracts, in the supernatant after centrifugation using high-performance liquid chromatography (HPLC).

TEM investigation

Characteristics and particle sizes of prepared guava liposomes suspension and serum entrapping guava liposomes have been investigated by using a transmission electron microscope (TEM 1200s Jeol, Japan) operated at 80 kv with negative staining technique in 2.5% uranyl acetate.

RESULT:

Free radical scavenging ability of guava extracts (DPPH assay)

HPLC analysis determined that guava extracts contained high levels of phenolic compounds in term of gallic acid as the chemical marker. This compound has been reported to be a good scavenger of free radicals. In the present study guava extracts were evaluated for their abilities to neutralize the stable free radicals such as DPPH radicals (Figure 1). The IC₅₀ value (the concentration that scavenges 50% of the DPPH radical) for guava extract was 53.2 µg/ml. In

addition, rutin showed very high activity towards DPPH radicals with IC_{50} 12.5 μ g/ml.

Inhibition of tyrosinase capacity

Guava leaves ethanol extract clearly showed tyrosinase inhibitory activity in a concentration-dependent manner (Figure 2). The IC_{50} value (the concentration that inhibits 50% of the mushroom tyrosinase) for guava extract was 9.9 mg/ml. However, the inhibitory activity of this extract was four times weaker than a reference inhibitor, kojic acid (IC_{50} = 0.25 mg/ml).

Quality of phenolic compound in Guava leaves extract and liposome serum

The results showed that guava leaves ethanol extract and liposome serum contained gallic acid at retention time 9.367 and 9.239 min with concentration 3.1 and 1.0 μ g/ml, respectively compared with the calibration curve of standard gallic acid (Figure 3).

Physicochemical properties of guava liposomes and serum containing guava liposomes

Transmission electron micrograph (TEM) of guava liposomes illustrates the spherical shape of liposomes entrapping guava leaf extract with the particle size ranging from 100 to 500 nm (Figure 4A). It also clearly shows that guava liposomes

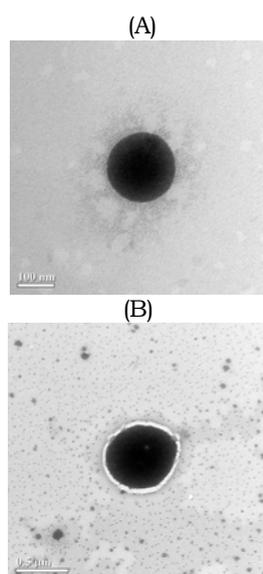


Figure 4 Transmission electron micrograph of guava liposomes (A) and serum containing guava liposomes (B).

were stable in serum preparation as shown in figure 4B.

DISCUSSION:

Free radical scavenging ability of guava extracts (DPPH assay)

This result was similar to that of a previous study where the ethanol guava extract were investigated and compared on their antioxidant activity by ABTS radical scavenging assay. Tachakittirungrod *et al.*²⁾ demonstrated the percent yield of ethanol extract and Trolox equivalence antioxidant capacity (TEAC) in the different tissues of guava. The ethanol guava extract from the leaves showed the highest antioxidant capacity with the TEAC value of 4.91 ± 0.050 mM/mg, followed by the stem and fruit pulp with the TEAC values of 1.96 ± 0.02 and 0.90 ± 0.01 mM/mg, respectively.

Inhibition of tyrosinase capacity

Takashi⁵⁾ reported the effect of anti-pigmentation of oral guava extract on the color changes induced by UV-B irradiation. As a result, guinea pig-treated guava extract decrease the number of dopa-positive melanocytes in dopa-reacted split epidermal sheets as compared to the number seen in controls. Guava extract inhibited the theophylline-induced melanogenesis in the cell and inhibited tyrosinase activity which is key enzyme of pigmentation.

Quality of phenolic compound in Guava leaves extract and liposome serum

Tachakittirungrod *et al.*²⁾ reported the total phenolic content of guava leaf fractions in term of gallic acid equivalent concentration (GAE) whereas Liang *et al.*⁶⁾ analyzed several polyphenolic compounds from the leaves of guava by HPLC-UV analysis and HPLC mass spectrometry. The results showed that guava leaves extract contained a mixture of phenolic compounds; gallic acid, quercetin, procatechuic acid, chlorogenic acid, caffeic acid, kaempferol and ferulic acid. It could be estimated that the phenolic compounds present in the guava leaves played an important role in antioxidant activity, directly through the mechanism of reduction of oxidized intermediates in the chain reaction.

Physicochemical properties of guava liposomes and serum containing guava liposomes

Microscopic examination showed that the prepared empty liposomes and liposomes entrapping guava leaf extract were spheroids with unilamellar structure. The liposomal guava possessed the highest drug entrapment efficiency (approximately 95%) with particle size range of 100 - 500 nm.

CONCLUSION: Our data have demonstrated the correlation between the phenolic content of the guava leaves fraction and free radical-scavenging capacity of guava leaves extract and liposome serum. Guava leaves extract showed the higher level of antioxidant activity than liposome serum equivalent to gallic acid concentration. The high antioxidant activity of such extract was attributed to radical-scavenging and reducing mechanisms. The results demonstrated that the phenolic content existing in guava leaf fractions play an important role in the antioxidant activity directly through the reducing activity mechanism, with some mutual action among the active compounds through free radical-scavenging activity.

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