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Original Article

In vitro alpha glucosidase inhibition and free-radical scavenging activity of propolis from Thai stingless bees in mangosteen orchard

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ABSTRACT

The chemical component and biological activity of propolis depend on flora area of bee collection and bee species. In the study, the propolis from three stingless bee species, *Lepidotrigona ventralis* Smith, *Lepidotrigona terminata* Smith, and *Tetragonula pagdeni* Schwarz, was collected in the same region of mangosteen garden from Thailand. Total phenolic content, alpha glucosidase inhibitory effect, and free-radical scavenging activity using FRAP, ABTS, DPPH assays were determined. The most potent activity of propolis extract was investigated for bioactive compounds and their quantity. The ethanol extract of *T. pagdeni* propolis had the highest total phenolic content 12.83 ± 0.72 g of gallic acid equivalents in 100 g of the extract, and the strongest alpha glucosidase inhibitory effect with the IC_{50} of 70.79 ± 6.44 μg/ml. The free-radical scavenging activity evaluated by FRAP, ABTS, DPPH assays showed the FRAP value of 279.70 ± 20.55 μmol $FeSO_4$ equivalent/g extract and the IC_{50} of 59.52 ± 10.76 and 122.71 ± 11.76 μg/ml, respectively. Gamma- and alpha-mangostin from *T. pagdeni* propolis extract were isolated and determined for the biological activity. Gamma-mangostin exhibited the strongest activity for both alpha glucosidase inhibitory effect and free-radical scavenging activity. Using HPLC quantitative analysis method, the content of gamma- and alpha-mangostin in the extract was found to be 0.94 ± 0.01 and $2.77 \pm 0.08\%$ (w/w), respectively. These findings suggested that *T. pagdeni* propolis may be used as a more suitable raw material for nutraceutical and pharmaceutical products and these mangostin derivatives as markers.

Introduction

Overproduction of free radical species can be damaging to host cells and lead to various ailments such as inflammatory, aging and diabetes (Zhou et al., 2015). In the case of diabetes, alpha glucosidase is a vital enzyme essential for cleavage of maltose to glucose for the absorption into the blood stream in the small intestine. Therefore, alpha glucosidase inhibitors could regulate abnormally high stages of plasma glucose after carbohydrate ingestion (Ryu et al., 2011). Nowadays, α-glucosidase inhibitors, such as acarbose, voglibose and miglitol, have been accepted for clinical use in the treatment of type 2 diabetes. Nonetheless, some synthetic alpha glucosidase inhibitors have undesirable side effects, such as abdominal cramping, flatulence and diarrhea (Zhang et al., 2015). As a result, many scientists have turned their attention to natural alpha glucosidase inhibitors including mangosteen and bee products, which are utilized to develop nutraceuticals or lead compounds for antidiabetic management (Matsui et al., 2004; Ryu et al., 2011; Juárez-Rojop et al., 2014).

Natural products from bees have been extensively employed since ancient time because of its broad pharmacological activity (Dantas et al., 2014; Kustiawan et al., 2014). Propolis is one of the bee products that exhibits numerous biological activities such as antioxidant, anti-inflammatory, antitumor, antiviral, antibacterial, antifungal, antidiabetic activities and is also listed in the London Pharmacopoeias and Chinese Pharmacopoeias (Sforcin and Bankova, 2011; Zhang et al., 2015). However, the chemical and biological activities of propolis vary depending on bee species and the flora at site of bee collection. For instance, propolis from Europe and North America regions' main compounds comprises mostly flavanones, flavones, cinnamic acids and their esters, while that from Brazil comprises mainly prenylated *p*-coumaric acids, diterpenic acids (Bankova, 2005; Sforcin, 2007). Also, different races of honeybees collected at the same area demonstrated varying potency.

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Apis mellifera caucasica showed a superior antibacterial activity to *Apis mellifera carnica* and *Apis mellifera anatolica*; stingless bees – *Trigona incisa*, *Timia apicalis*, *Trigona fusco-balteata* and *Trigona fuscibasis* – from Indonesia revealed different degrees of cytotoxicity (Silici and Kutluca, 2005; Kustiawan et al., 2014).

Stingless bees, whose propolis is utilized for medicinal and nutraceutical purposes, are a large group of eusocial insect that play a part in plant pollination in tropical regions (Choudhari et al., 2012; da Cunha et al., 2013). In Thailand and India, stingless bee propolis is popularly applied for the treatment of maladies such as acne, diabetes and inflammation (Umthong et al., 2011; Choudhari et al., 2012). Antimicrobial, antiproliferative and antioxidant activities of several species of stingless bee propolis were also investigated (da Cunha et al., 2013; Dutra et al., 2014). Nevertheless, the study of propolis from Thai stingless bees, (*Lepidotrigona ventralis* Smith, *Lepidotrigona terminata* Smith, and *Tetragonula pagdeni* Schwarz (Apidae)), which are commercially cultivated in artificial hives in fruit gardens and marketed in several preparations in Thailand, is limited. Thus, the objective of the present work was to compare alpha glucosidase inhibitory effect and free-radical scavenging activity of propolis from three stingless bee species in the same area of Thai mangosteen orchard. The active compounds from the species that demonstrated the strongest activity were identified and quantified.

Materials and methods

Chemical products

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), α-glucosidase from *Saccharomyces cerevisiae*, acarbose, 4-nitrophenyl α-D-glucopyranoside (pNPG), iron(III)chloride hexahydrate, ferrous sulfate, iron(II) sulfate heptahydrate, were obtained from Sigma–Aldrich® (St. Louis, MO, USA), aluminum chloride, acetic acid, ascorbic acid, Folin–Ciocalteu reagent, gallic acid were purchased from Merck® (Darmstadt, Germany). Sodium bicarbonate and potassium persulfate were purchased from Ajax Finechem® (NSW, Australia). All reagents were of analytical grade. Deionized water was purified by Ultra Clear™ system (Siemen Water Technologies Corp®). Ethanol, ethyl acetate, dichloromethane, hexane, formic acid and HPLC grade methanol were purchased from Labscan® (Thailand). Standard gamma-mangostin (**1**) and alpha-mangostin (**2**) purity more than 98% were purchased from Chengdu Biopurify Phytochemicals Ltd, Sichuan, China.

Propolis sample and preparation

Propolis of *Lepidotrigona ventralis* Smith, *Lepidotrigona terminata* Smith, and *Tetragonula pagdeni* Schwarz were collected from an apiary in the mangosteen garden in December from Makham district, Chanthaburi province, eastern of Thailand in 2014, and kept in the dark at 4 °C until use. The stingless bees were identified by Dr. Chama Inson, Department of Entomology, Faculty of Agriculture, Kasetsart University. The voucher specimens (*Lepidotrigona ventralis* No. 1214001, *Lepidotrigona terminata* No. 1214003, and *Tetragonula pagdeni* No. 1214003) were deposited at Faculty of Pharmaceutical Sciences, Burapha University, Thailand.

Propolis from different bee species (10 g) was separately cleaned and cut into small pieces and was then sonicated with 80% ethyl alcohol (200 ml) at 40 °C for 30 min. The suspension was centrifuged at 3000 × *g* for 5 min at 20 °C. The supernatant was kept while the pellet was re-extracted using the same procedure. The supernatants were pooled together and evaporated in a rotary evaporator. Each extract was dewaxed by sonication with 100 ml of hexane at 40 °C for 20 min and centrifuged at 2000 × *g* for 5 min at 20 °C. The supernatant was discarded while the crude residue was kept and stored in the dark at 0 °C.

Separation of active compounds

The propolis extract that exhibited the strongest activity was selected to separate the bioactive compounds. The crude residue was subjected to column chromatography (3 cm × 20 cm, silica gel (0.063–0.200 mm, Merck 7734)) with 20% ethyl acetate in hexane as a mobile phase. The sub-fractions were pooled together to obtain two main fractions as monitored by thin-layer chromatography. The first fraction was subjected to preparative thin-layer chromatography (pTLC) with 50% ethyl acetate/hexane to obtain compound **1**. The second fraction was also applied to pTLC with dichloromethane (triple run) as a mobile phase to obtain compound **2**. Each pure compound was dissolved in 99.98% $CDCl_3$ or in 99.8% methanol-d_4 (*ca.* 5 mg in 0.7 ml) and transferred into 5 mm NMR sample tube (Promochem, Wesel, Germany). Spectra were recorded by the Bruker Topspin software on a Bruker AVANCE 400 spectrometer (Bruker, Rheinstetten, Germany).

Biological assay

Determination of contents of total phenolic compounds

Total phenolic content was determined using Folin–Ciocalteu reagent following method described by Vongsak et al. (2013b). Each sample (1000 μg/ml), 200 μl was mixed with 500 μl of the Folin–Ciocalteu reagent (diluted 1:10 with deionized water) and 800 μl of sodium bicarbonate solution (7.5%, w/v). The mixture was allowed to stand at room temperature for 30 min with intermittent shaking. The absorbance was measured at 765 nm using a UV–Visible spectrophotometer (Hitachi®, Japan). The same procedure was repeated for the gallic acid standard solution (500, 250, 125, 62.5, 31.25 and 0 μg/ml) and the quantification was made based on a standard curve generated with of gallic acid. The content of total phenolic compounds was calculated as mean ± SD ($n = 3$) and expressed as grams of gallic acid equivalents (GAE) in 100 g of the extract.

Alpha glucosidase inhibition assay

The α-glucosidase inhibitory activity was carried out spectrophotometrically using pNPG as substrate (Zhou et al., 2015). Samples, standard solutions (gamma- and alpha-mangostin) or positive control (acarbose) with different concentrations (50 μl, Tables 1 and 3), α-glucosidase (50 μl, 2 unit/ml) and phosphate buffer (50 μl, pH 7.0) were mixed and pre-incubated at 37 °C for 10 min, and then pNPG (50 μl, 20 mM) was added to start the reaction. After incubation at 37 °C for 30 min, the absorbance was measured at 405 nm using UV–Visible microplate reader (Metertech®, Taiwan). The percent inhibition of α-glucosidase activity was calculated as follows:

$$\text{Inhibition}\,(\%) = \frac{(A_1 - A_2)}{A_1} \times 100,$$

where A_1 is the absorbance of control, and A_2 is the absorbance of samples. The extent of inhibition was expressed as mean ± SD ($n = 3$) of the enzymatic activity (IC_{50}).

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out by the method of Al-Mansoub et al. (2014). Briefly, 150 μl FRAP working solution (300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in a ratio of 10:1:1) was added to 50 μl test samples. The reaction mixtures were incubated at 37 °C for 8 min; the absorbance was measured at