

***In Vitro* Cytotoxicity of Thai Stingless Bee Propolis from Chanthaburi Orchard**

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Abstract

Stingless bee propolis has a complex chemical composition and has been reported to possess various pharmacological properties. Chanthaburi province is the major source of stingless bee propolis production in Thailand. In the present study, the cytotoxic activities of the crude extracts of 3 different stingless bee (*Tetragonula pagdeni*, *Lepidotrigona ventralis*, and *Lepidotrigona terminata*) propolis from the same orchard were determined by sulphorhodamine assay against 4 human cancer cell lines (KB, HepG2, Caco-2, and SK-MEL-28) and a normal human cell line (Hs68). *T. pagdeni* propolis extract expressed the highest cytotoxicity on cancer cells, but a low toxicity on normal cells. On the contrary, *L. ventralis* and *L. terminata* propolis extracts demonstrated low cytotoxicity against cancer cell lines. Compounds from *T. pagdeni* propolis were investigated and elucidated as gamma and alpha mangostin. The cytotoxicity of these pure compounds was comparable to doxorubicin, a positive control. These compounds may be responsible for the cytotoxicity activity on cancer cells of *T. pagdeni* propolis from a Chanthaburi plantation.

Keywords: Cytotoxicity, stingless bees, propolis, Chanthaburi, sulphorhodamine

Introduction

Cancer is one of the main fatal diseases affecting mankind. Several natural products play principal roles in cancer chemotherapy, such as paclitaxel, from genus *Taxus*, etoposide, derived from the lignans of *Podophyllum* spp., and vinblastine and vincristine, from *Catharanthus roseus*. These compounds are cytotoxic and act predominantly by obstructing cell proliferation via various mechanisms [1]. In Thailand, numerous natural products from plants and mushrooms, as well as animals are traditionally employed against cancer [2-6].

Propolis (bee glue) is a bee product that exhibits anticancer activity and is also extensively used for antiviral, antibacterial, antifungal, and anti-inflammatory treatments [7-9]. Propolis was also listed in the Chinese Pharmacopoeias in 2005 [10]. In Thailand and India, stingless bee propolis is commonly used for the treatment of illnesses such as toothache, acne and inflammation. The antiproliferative, antimicrobial, and antioxidant activities of some species of stingless bee propolis have been scrutinized [5,10,11]. Due to the range of activities, propolis has been applied in pharmaceutical products and the food industry as nutraceuticals and food supplements.

The biological activities and chemical composition of propolis are variable based on bee species and the flora at the site of bee collection. For instance, propolis from Brazil mostly consists of diterpenic acids and prenylated *p*-coumaric acids, while those from North America and European regions typically contain major compounds of cinnamic acids and their esters, flavones, and flavanones [10-13]. In addition, dissimilar races of bees collected from the same area have fluctuating potency. For example, the propolis of *Apis mellifera carnica* and *Apis mellifera anatolica* exhibited an inferior antibacterial activity to that of

Apis mellifera caucasica. For stingless bee species, the propolis of *Trigona incisa*, *Timia apicalis*, *Trigona fusco-balteata*, and *Trigona fuscibasis*, collected from similar gardens in Indonesia, possessed dissimilar degrees of cytotoxicity on cancer cell lines [14].

Cytotoxicity assessment depends on mammalian cell lines being grown under conditions such that they actively increase and undergo mitotic division. The degree of cell multiplication and development is measured by the formation of a color intensity of which is directly related to the amount of existing cells. The most basic investigation is to compare the proportion of growth of a cancer cell line in the absence and presence of the test material after a specified period [15]. Two well-known methods are 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), or 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) assay, and the sulphorhodamine B (SRB) assay. The MTT and XTT assays contain reagents that produce colors based on the activity of mitochondria. If the function of these activities is obstructed by variations in cellular stages of glucose, NADH, and other factors, inconstant outcomes occur, and a false negative is observed as though the cells were not proliferating. Because of these restrictions, the sulphorhodamine B (SRB) assay was established. This method depends on the uptake of SRB, charged by basic amino acids in the cells. The amount of dye taken up after fixing will contribute to a more intense absorbance when the cells are lysed. In addition, the SRB assay is reproducible, sensitive, and more rapid, and also has a stable end-point, better linearity, and a better signal-to-noise ratio than the MTT or XTT assays [1].

Although Thai stingless bee propolis is increasingly used, the study of propolis has been limited. Thus, the aim of this work was to compare the cytotoxic effect of propolis from stingless bee species from the same orchard in Thailand. *Tetragonula pagdeni* Schwarz, *Lepidotrigona ventralis* Smith, and *Lepidotrigona terminata* Smith, which are commercially cultivated in artificial hives in Chanthaburi orchard and used in several preparations, were chosen. In addition, the active compounds from the species producing the strongest cytotoxicity activity were elucidated.

Materials and methods

Sample and preparation

Stingless bee propolis from *Tetragonula pagdeni* Schwarz, *Lepidotrigona ventralis* Smith, and *Lepidotrigona terminata* Smith were collected from the same orchard in December from Makhm district, Chanthaburi province, Thailand, in 2014, and kept in the dark at 0 °C until use. The stingless bees were identified by Dr. Chama Inson, Department of Entomology, Faculty of Agriculture, Kasetsart University. The voucher specimens (*Tetragonula pagdeni* No.1214003, *Lepidotrigona ventralis* No.1214001, and *Lepidotrigona terminata* No.1214002) were deposited at the Faculty of Pharmaceutical Sciences, Burapha University, Thailand.

Propolis from each species (10 g) was separately cleaned, cut into small pieces, and sonicated with methanol (200 ml) at 40 °C for 30 min. The suspension was centrifuged at 5,000 rpm 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. The crude residue was kept and stored in the dark at 0 °C.

Cell Lines

Human cancer cells, colon adenocarcinoma Caco-2 (ATCC HTB-37), melanoma SK-MEL-28 (ATCC HTB-72), hepatocellular carcinoma HepG2 (ATCC HB-8065), papilloma carcinoma KB (ATCC CCL-17), as well as normal human fibroblast Hs68 (ATCC CRL-1635), were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). All cell types were grown in an incubator at 37 °C with 5 % CO₂ in Dulbecco's modified Eagle's medium with a low glucose concentration (DMEM-LG; Gibco, Grand Island, NY, United States) containing 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) and 10 % fetal bovine serum (HyClone, Cramlington, UK). Medium was changed every 3 days until it reached 80 % confluence. Subculturing was done by 0.25 % trypsin-EDTA (Gibco).

Cytotoxicity by sulforhodamine B (SRB) assay

Cytotoxicity of various extracts on cell lines was determined by the SRB assay. In brief, cells were washed 3 times with PBS and detached with 0.25 % trypsin-EDTA. Viable cell density was counted by trypan blue exclusion and diluted with DMEM-LG to make concentrations of 5×10^4 cells/ml. The cells were plated 24 h prior to testing in 96-well plates at a density of 5,000 cells/well in 100 μ l of medium. Each extract was dissolved in dimethyl sulfoxide (DMSO) and diluted in medium to produce 5 concentrations of 20, 100, 200, 1,000, and 2,000 μ g/ml for crude extract, and 2, 10, 20, 100, and 200 μ g/ml for pure compounds. Doxorubicin (Sigma-Aldrich, St. Louis, United States) was used as a positive control in this study. After 24 h, the cells were treated with the extracts and pure compounds by adding 100 μ l/well of each concentration in triplicate. Then, they were further incubated for 24 h before being subjected to the SRB assay.

The SRB assay was performed to assess growth inhibition by a colorimetric technique, as described previously [16]. Cells were fixed by adding 100 μ l of ice-cold 10 % trichloroacetic acid (TCA, Sigma-Aldrich) at 4 °C for 1 h. Plates were washed 3 times with tap water and stained with 100 μ l of 1 % SRB (Sigma-Aldrich) for 30 min. Then, the microplates were washed with 1 % acetic acid and rinsed 4 times to removed unbound dye. The plates were dried at room temperature, and 100 μ l of 10 mM Tris base pH 10.5 was added to dissolve the dye. Then, the plates were shaken gently for 20 min on a gyratory shaker and the absorbance (OD) of each well was read on a VersaMax plate reader (Molecular devices, United States) at 510 nm.

Separation of active compound by preparative thin layer chromatography

The propolis extract from *Tetragonula pagdeni* that displayed the strongest activity was chosen to isolate the bioactive compounds, following the method described by Vongsak *et al.* [17]. In brief, the crude residue was subjected to column chromatography (3 \times 20 cm, Silica gel) with 80:20, hexane and ethyl acetate as a mobile phase. The sub-fractions were combined together to obtain 2 main fractions for examination by thin-layer chromatography. The first fraction was subjected to preparative thin-layer chromatography (pTLC) with 50:50, hexane and ethyl acetate to obtain compound 1. The second fraction was subjected to pTLC with dichloromethane (triple run) as a mobile phase to obtain compound 2. The isolated pure compounds were identified by proton and carbon nuclear magnetic resonance by using the Bruker Topspin software on a Bruker AVANCE 400 spectrometer (Bruker, Rheinstetten, Germany). The NMR data were compared with previous studies [9,17] and reported as gamma and alpha mangostin, respectively.

Statistical analysis

The results were reported as mean \pm standard deviation (SD) ($n = 3$). The IC₅₀ of the different propolis bee species extract were statistically investigated using one-way analysis of variance (ANOVA) with least significant difference (LSD) by using SPSS for Windows® 16.0. A statistical probability (p value) of less than 0.05 indicated a statistically significant difference between groups.

Results and discussion

In this study, 3 kinds of stingless bee propolis (from *Tetragonula pagdeni*, *Lepidotrigona ventralis*, and *Lepidotrigona terminata*) were screened in human cancer cells (papilloma carcinoma KB, hepatocellular carcinoma HepG2, colon adenocarcinoma Caco-2, and melanoma SK-MEL-28), as well as in normal human fibroblast (Hs68), for cytotoxicity, at a fixed concentration of 100 μ g/ml. Considerable growth-inhibitory activity of cells was observed with *T. pagdeni* propolis extracts, which were able to kill more than 50 percent of all human cancer cell lines (**Figure 1**). In order to determine 50 % inhibitory concentrations (IC₅₀) of active crude extracts, concentration-dependent assays were carried out in a concentration range from 20 to 2,000 μ g/ml, the inhibitory effect being concentration dependent. The most promising activity against human cancer cells was still that of *T. pagdeni* propolis extracts, with IC₅₀ values of 33.38 ± 3.30 to 80.81 ± 2.68 μ g/ml. The IC₅₀ values of normal human fibroblast cells were 228.75 ± 10.64 μ g/ml (**Table 1**). *L. ventralis* and *L. terminata* propolis extracts showed low cytotoxicity

in both cancer and normal cell lines. In Indonesia, stingless bee (*Trigona incisa*, *Timia apicalis*, *Trigona fusco-balteata*, and *Trigona fuscibasis*) propolis extract was reported to have different degrees of cytotoxicity against HepG2, SW620, Chago-I, KATO-III, and BT474 at a concentration of 20 µg/ml by MTT assay [14]. The cytotoxicity of stingless bee (*Trigona laeviceps*) propolis extract from Samut Songkram province in Thailand was also tested on cancer cell lines (HepG2, SW620, Chago-I, KATO-III, and BT474). However, the IC₅₀ values that were toxic to normal cell lines and cancer cell lines were not significantly different, and the active compound was not reported [5].

The extract of *T. pagdeni* propolis that exhibited the highest activity in this study was subjected to separation and identification of the active compounds. Using preparative thin layer chromatography, 2 major active constituents, gamma mangostin and alpha mangostin, were elucidated (**Figure 2**). These active compounds expressed comparable cytotoxicity to the positive control, doxorubicin, against cancer cell lines, with IC₅₀ values of 2.84 ± 0.22 to 15.12 ± 0.69 and 1.63 ± 0.05 to 7.07 ± 0.17 for gamma mangostin and alpha mangostin, respectively (**Table 1**). The amounts of gamma and alpha mangostin in the extract were determined according to the previous method by HPLC, and found to be 1.27 and 3.07 % w/w, respectively [18]. Polyphenols, especially mangostin derivatives, are known to have anticancer activity [6,19], and may contribute to the activity of stingless bee propolis from Chanthaburi province. Previously, the cytotoxic mechanism of these xanthone derivatives were investigated. Gamma mangostin augmented the cell cycle arrest in the G1 phase, and consequently stimulated apoptosis. Alpha mangostin induced apoptotic effect via disruption of mitochondrial membrane pathways and caspase activation on the human melanoma SK-MEL-28 cell line [20].

One of the factors that could affect the chemical composition of propolis is flora at the site of bee collection. The chemical constituents of honeybee propolis from Chiang Mai were reported to be phenylallylflavanone and its derivatives. These compounds were found to be secondary metabolites from *Styrax* spp., which is widely distributed over that area [21]. The presence of mangostin derivatives in stingless bee propolis may be due to there being plenty of mangosteen trees in the garden, as propolis was produced by resin or the exudate of trees integrated with beeswax and bee enzymes [22,23]. Collection period and amount of active compounds could also affect the chemical composition, and should be investigated in a further study.

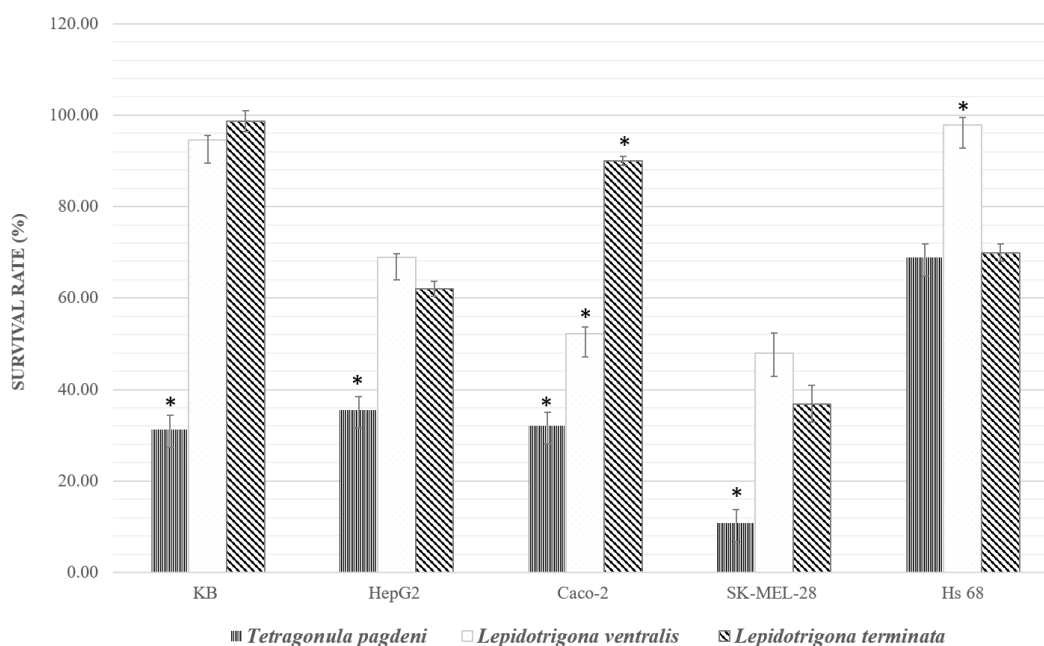


Figure 1 Effect of propolis crude extracts of stingless bees (100 $\mu\text{g/ml}$) on growth inhibition of human cancer cell lines (KB, HepG2, Caco-2, SK-MEL-28) and normal human fibroblast cells, Hs68. Data were shown as the mean \pm SD, $n = 3$. Statistical analysis was investigated using one-way analysis of variance (ANOVA) with least significant difference (LSD) by SPSS for Windows[®] 16.0. A statistical probability (p value) of less than 0.05 indicated a statistically significant difference.

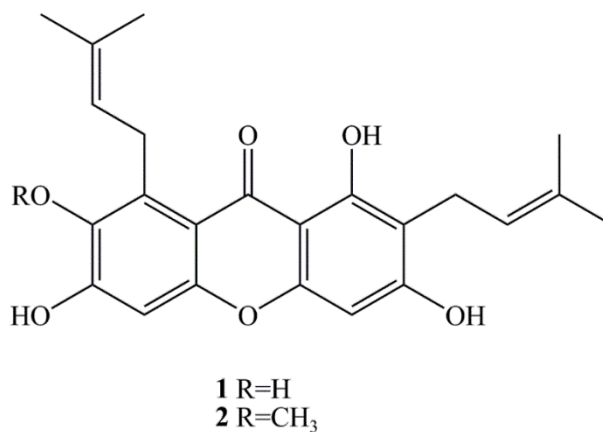


Figure 2 Chemical structure of active compounds, gamma mangostin (1) and alpha mangostin (2), from *T. pagdeni* propolis extract.

Table 1 Cytotoxicity (IC₅₀ value in µg/ml) of 4 cancer cell lines and normal human fibroblast cells against propolis crude extracts of stingless bees and active compounds.

Propolis extracts of bees / Compounds	Cancer cell lines				Normal cells
	KB	HepG2	Caco-2	SK-MEL-28	Hs68
<i>T. pagdeni</i>	62.41 ± 2.70	80.81 ± 2.68	73.44 ± 2.37	33.38 ± 3.30	228.75 ± 10.64
<i>L. ventralis</i>	565.19 ± 18.63	428.00 ± 70.29	96.58 ± 14.93	153.38 ± 18.98	> 1000
<i>L. terminata</i>	264.78 ± 11.91	198.42 ± 14.25	259.10 ± 2.27	74.30 ± 5.99	327.99 ± 10.97
Gamma mangostin	6.25 ± 0.35	15.12 ± 0.69	6.32 ± 0.38	2.84 ± 0.22	7.67 ± 0.33
Alpha mangostin	1.63 ± 0.05	3.34 ± 0.34	7.07 ± 0.17	5.00 ± 0.14	19.95 ± 0.50
Doxorubicin	2.04 ± 0.12	54.66 ± 1.89	11.13 ± 2.10	2.06 ± 0.08	65.93 ± 4.43

Data were shown as the mean ± SD, *n* = 3

Conclusions

This study has demonstrated that the propolis extract of *T. pagdeni* exhibited the strongest cytotoxicity against human cancer cell lines among the extracts studied. The finding may be applied as a geographical indication of propolis from mangosteen orchards and contributes to better understanding of natural products from *T. pagdeni* propolis, which is one of the most used bee products in indigenous medicine from Chanthaburi province.

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