Sains Malaysiana 52(4)(2023): 1145-1160
http://doi.org/10.17576/jsm-2023-5204-09

Antiproliferative and Anti-Tyrosinase Activities of Propolis from *Tetragonula laeviceps* and *Tetragonula pegdeni* in Thailand

(Aktiviti Antiproliferasi dan Anti-Tirosinase Propolis daripada *Tetragonula laeviceps* dan *Tetragonula pegdeni* di Thailand)

Thitipan Meemongkolkiat[1,*], Songchan Puthong[2], Phanthiwa Khongkarat[3], Preecha Rod-im[4], Orawan Duangphakdee[4], Preecha Phuwapraisirisan[5] & Chanpen Chanchao[1]

[1]*Department of Biology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand*
[2]*Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand*
[3]*Program in Biotechnology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand*
[4]*Native Honeybee and Pollinator Research Center, Ratchaburi Campus, King Mongkut's University of Technology Thonburi, Ratchaburi 70150, Thailand*
[5]*Center of Excellence in Natural Products, Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand*

Received: 7 August 2022/Accepted: 27 March 2023

ABSTRACT

Propolis is one of the economic bee products with biological activities, but these activities can vary according to the local plants and bee species. This study aimed to evaluate the cytotoxic and antityrosinase activity of the methanol-, hexane-, and dichloromethane-partitioned propolis extracts (MPE, HPE, and DPE, respectively) of two dominant stingless bee species in Thailand (*Tetragonula laeviceps* and *Tetragonula pegdeni*) sourced from four locations in Ratchaburi province and one location in Chiangmai province. Their antiproliferative/cytotoxic activity, as the relative cell viability, was screened against the liver (Hep-G2) and gastric carcinoma (KATO-III) cancer cell lines in comparison to the untransformed lung fibroblast (WI-38) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Interestingly, DPE7 was the extract that showed great potential anticancer properties because it was significantly higher cytotoxic to cancer cell lines (Hep-G2 and KATO-III, with IC_{50} values of 36.40 and 35.15 µg/mL, respectively) than to normal cell lines (WI-38, with IC_{50} values of 46.52 µg/mL). Although DPE7 did not exhibit the highest antityrosinase activity, its moderate antityrosinase activity (IC_{50} of 1.388 mg/mL) considered it possible for further purification though not as effective as Kojic acid (IC_{50} of 0.0432 mg/mL). Besides, the different bioactivities in propolis from different sites were expected due to the different flora in each location. However, further studies are needed to better understand the properties and safety aspects of selected partitioned extracts.

Keywords: Antiproliferative activity; antityrosinase activity; *Tetragonula laeviceps*; *Tetragonula pegdeni*; Thai propolis

ABSTRAK

Propolis adalah salah satu produk lebah dengan aktiviti biologi, tetapi aktiviti ini boleh berbeza-beza mengikut tumbuhan tempatan dan spesies lebah. Kajian ini bertujuan untuk menilai aktiviti sitotoksik dan antitirosinase bagi ekstrak propolis melalui pemisahan metanol, heksana dan diklorometana (masing-masing MPE, HPE dan DPE) daripada dua spesies lebah tanpa sengat yang dominan di Thailand (*Tetragonula laeviceps* dan *Tetragonula pegdeni*) yang diperoleh dari empat lokasi di wilayah Ratchaburi dan satu lokasi di wilayah Chiangmai. Aktiviti antiproliferasi/sitotoksik mereka sebagai kebolehidupan sel relatif, telah disaring terhadap sel kanser hati (Hep-G2) dan karsinoma

gastrik (KATO-III) berbanding dengan titisan sel fibroblas paru-paru tidak tertransform (WI-38) menggunakan asai 3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium bromida. Menariknya, DPE7 ialah ekstrak yang menunjukkan sifat antikanser yang berpotensi besar kerana tinggi sitotoksik terhadap titisan sel kanser (Hep-G2 dan KATO-III, dengan nilai IC_{50} masing-masing 36.40 dan 35.15 g/mL) berbanding titisan sel normal (WI-38, dengan nilai IC_{50} 46.52 g/mL). Walaupun DPE7 tidak menunjukkan aktiviti antitirosinase tertinggi, aktiviti antitirosinase sederhananya (IC_{50} sebanyak 1.388 mg/mL) mungkin boleh dipertimbangkan untuk penulenan selanjutnya walaupun tidak berkesan seperti asid Kojic (IC_{50} sebanyak 0.0432 mg/mL). Selain itu, bioaktiviti yang berbeza dalam propolis dari tapak kajian yang berbeza telah dijangka akibat daripada flora yang berlainan di setiap lokasi. Walau bagaimanapun, kajian lanjutan diperlukan untuk memahami sifat dan aspek keselamatan bagi ekstrak pembahagian terpilih dengan lebih lagi.

Kata kunci: Aktiviti antiproliferasi; aktiviti antitirosinase; propolis Thai; *Tetragonula laeviceps; Tetragonula pegdeni*

Introduction

Cancers are one of the major leading causes of fatality to humans, with 9.6 million affected patients globally in 2018 (WHO 2018). This study used the Hep-G2 and KATO-III cancer cell lines that originated from liver (hepatocellular carcinoma) and stomach cancers, respectively (Park et al. 1997). These cancers are two of the most common and leading global causes of death, with over 250,000 new cases and an estimated 600,000 deaths per year (WHO 2018). Additionally, according to the World Cancer Report, skin cancer represents 30% of all newly diagnosed malignancies worldwide (Aziz et al. 2005). Melanin is the dark pigment in hair and skin and is crucial for protecting human skin against radiation. Malignant melanoma is a type of skin cancer that begins in cells known as melanocytes. Melanocytes proliferate rapidly, which increases tumor growth and melanin pigmentation (Ahmed, Qadir & Ghafoor 2020; Lerner 1955). Several recent studies demonstrated that certain natural bee products can limit tumor cell growth and induce apoptosis of cancer cells (Premratanachai & Chanchao 2014), suggesting the possible use of these natural compounds (or their active components) as an alternative medical treatment for human tumors. When chemotherapy and radiotherapy are employed systemically or over a large area of tissue to kill cancerous cells, they usually destroy various untransformed (normal) tissues, including the gastrointestinal system, heart, lungs, kidney, and brain (Sporn 1996; Topçul & Çetin 2015). This results in unwanted side effects that can limit the treatment (time and/or dose) and its efficacy. Consequently, it is critical to develop new anticancer medicines that can overcome the rise in the incidence of cancer patients throughout the world.

Numerous natural products, including stingless bee products (honey, bee pollen, and propolis), had a long history of being used in traditional medicine dating back to 300 BC including the anticancer activity of propolis and extracts (Banskota et al. 2002; Rozman et al. 2022). Stingless bees are social insects in the family Apidae. There are more than 600 described species spread across all the tropical and subtropical areas of the globe (Lavinas et al. 2019; Roubik 2023). Propolis is one of the main and most widely appreciated stingless bee products. It is mainly derived from the plant resins collected by bees and is used to construct and repair hives. Moreover, propolis from various geographical locations and bee species, as well as their extracts, had been reported as an interesting source of various biological activities, such as antibacterial, free radical scavenging, and anticancer properties (Khalil 2006). The main bioactive compounds in propolis are reported to be phenolic acids, terpenes, cinnamic acid, caffeic acid, and flavonoids. These diverse types of chemical components in propolis possessed a crucial role in the anti-cancer activities vary in their size and polarity and so were obtained in different crude extracts or subsequent fractions (Teerasripreecha et al. 2012).

Among the various bee species in Thailand, the propolis of *T. laeviceps* from Samut Songkram province exhibited an anticancer potential against five human cancer cell lines, namely: ductal carcinoma (BT-474), colon adenocarcinoma (SW620), liver cancer (Hep-G2), lung cancer (ChaGo), and gastric cancer (Kato-III). Propolis from *Tetragonula sirindhornae*, *Lepidotrigona ventralis*, and *Lepidotrigona terminata* from eastern Thailand (Chantaburi province) promoted cytotoxicity and inhibited cell migration in head, neck (HN30), colorectal cancer (Caco-2), melanoma (SK-MEL-28), and papilloma carcinoma (KB) cancer cells (Campos et al. 2021). Additionally, propolis of *Apis mellifera* from Nan province, Thailand was found to have a cytotoxic

action against the BT474, Chaco I, Hep-G2, and SW-620 cell lines (Umthong et al. 2011). Given that the studies developed in Thailand described different anticancer properties of the cancer cell lines for each type of propolis in different locations and provided data on its safety, which may guide perspectives on complete information in the locations where it is not yet available.

However, standardization of the pharmacological and therapeutic properties of propolis is complicated by its significant chemical variability between bee species and botanical sources. This may especially be the case for countries like Thailand that are situated in a hot and humid climatic zone, as well as in a floral biodiversity hotspot, which support a diverse variety of plant species. In Thailand, there are approximately 15,000 species of plants, which account for 8% of the estimated total numbers of plants found globally (OEPP 1992; Phumthum et al. 2018). Indeed, the biological effects of propolis have been shown to depend on the extracted active constituents and geographic regions from where the propolis was collected (Reis et al. 2019).

Ratchaburi and Chiangmai provinces in Thailand were selected in this study because they are significant sources of bee production in Thailand. The forest type in Ratchaburi is an interconnection between four biogeographical areas, including Indo-Chinese, Sino-Malayan, Indo-Burmese, and Eastern Indian, which are diverse and provide habitats for a vast array of animals and insects (Chanlabut & Nahok 2022). Several bee and stingless bee species had been collected for scientific purposes in this province (Sooklim et al. 2022). The vegetation in Ratchaburi is classified as deciduous dipterocarp, mixed deciduous, and seasonal rain forest. Depending on the forest type, the dominant tree species include *Pterocarpus macrocarpus*, *Shorea obtusa*, and *Afzelia xylocarpa* (Chaiyo, Garivait & Wanthongchai 2012). While Northern Thailand is the primary location for beekeeping operations, beekeepers move their hives to farms in the Chiang Mai-Lamphun valley each season in search of a variety of nectar sources (Narjes & Lippert 2021; Seanbualuang 2012). Chiangmai province is well-known as the main exporter of fruits such as *Mangifera indica* and *Dimocarpus longan* (Pott et al. 2004; Wongsiri, Thapa & Kongpitak 1998). Among bee species, *Tetragonula laeviceps* and *Tetragonula pegdeni* were selected in this study because they are the most common stingless bee species found across Thailand (Rattanawannee & Duangphakdee 2020).

This research aimed to determine the *in vitro* cytotoxicity and antityrosinase activities of propolis from *T. laeviceps* and *T. pegdeni* collected from Ratchaburi and Chiangmai provinces. The partitioned crude extracts were prepared using sequential partitioning with three different polarity solvents. Each partitioned extract was screened for its cytotoxic effect against two human cancer cell lines (Hep-G2 and KATO-III) in comparison to the untransformed (normal) cell line (WI-38) using the 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay. Changes in the morphology of the treated cells were observed by light microscopy. In addition, the tyrosinase inhibitory activities of the partitioned extracts were also measured using mushroom tyrosinase. Increasing tyrosinase levels appear to contribute to the accumulation of melanocytes and potentially cause skin cancer (Khongkarat et al. 2020). Finally, 11 phenolic compounds in the propolis extracts were identified and quantified. This study is the first to demonstrate these two activities in the propolis of *T. laeviceps* and *T. pegdeni* collected from two specific regions in Thailand and suggests some extracts could potentially be used as complementary ingredients in pharmaceutical agents against cancer cells.

Materials and Methods

Sample collection

Propolis was gathered from the two stingless bee species, *T. laeviceps* and *T. pegdeni*, from distinct sites in Suan Pheung, Chom Bueng (two sites), and Ban Kha district in Ratchaburi province, Thailand, in April 2021 (Table S1) and in San Patong, Chiangmai province in May 2021. To ensure that the propolis had been taken from the specific flora around each site, none of the chosen hives had been relocated for more than a year. The plant species discovered in the vicinity of the sampling locations were previously noted (Meemongkolkiat et al. in press). Approximately 150-300 g of propolis from each selected hive was taken, kept in the dark by wrapping with aluminum foil, and stored at -20 °C until used.

Propolis extraction and partition

The extraction of propolis was conducted as previously reported (Umthong, Puthong & Chanchao 2009) with modifications. Propolis (80 g) was cut into small pieces and suspended in 350 mL of 80% (v/v) methanol (MeOH) at 25 °C with shaking at 100 rpm for 18 h and then centrifuged at 3,834.7 × g (7,000 rpm) for 15 min at 4 °C before the supernatant was collected. This process was repeated three more times in the same

manner. To obtain the crude MeOH extract (CME), all the supernatants were combined and evaporated under reduced pressure at 40-45 °C using a rotary evaporator (Heidolph, Germany), and the obtained residue was maintained at 4 °C in the dark until used. Next, the CME was dissolved in 80% (v/v) MeOH, slowly adding more MeOH until it was no longer sticky, and then sequentially partitioned as above except with an equal volume of hexane (low polarity) rather than MeOH; and finally, by dichloromethane (DCM; medium polarity) to yield the hexane-partitioned extract (HPE) and DCM-partitioned extract (DPE), respectively. Finally, for the residual DCM-extracted solution, the MeOH phase was evaporated as above to yield the MeOH-partitioned extract (MPE). A total of 30 partitioned extracts were obtained. All the partitioned extracts were kept at 4 °C in the dark until used to determine their cytotoxic and anti-tyrosinase activities as described below.

CELL CULTURE

The hepatoblastoma or liver cancer (Hep-G2, ATCC_ HB8065) and gastric carcinoma or stomach cancer (KATO-III, ATCC_ HTB103) cell lines were used for screening for the *in vitro* antiproliferative activity. These cancer cell lines were cultured in complete media (RPMI 1640 medium (Invitrogen) containing 10% (v/v) fetal bovine serum (FCS; Gibco)). Also, the untransformed (normal) lung fibroblast line WI-38 (ATCC No. CCL-75) was used for comparison to check for selective specificity towards cancer cells rather than all dividing cells. This normal cell line was cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% (v/v) FCS. All cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University and were incubated at 37 °C in a humidified air atmosphere containing 5% (v/v) CO_2.

CYTOTOXICITY: MTT ASSAY

The potential cytotoxicity (reduced relative cell viability) of each partitioned extract was ascertained using the MTT surrogate cell viability assay, as previously reported (Teerasripreecha et al. 2012). The Hep-G2 and KATO-III cells were seeded at 5×10^3 cells in each well of 96-well plates containing 200 µL of medium and incubated at 37 °C in a humidified air atmosphere enriched with 5% (v/v) CO_2 for 24 h in order to let the cells attach to the bottom of each well. The WI-38 cells were seeded at 1×10^4 cells/well and cultured in the same manner. Then, the cells were treated with the respective partitioned extract (2 µL/well) dissolved in dimethylsulfoxide (DMSO) to a final concentration of 100 µg/mL for the cytotoxicity screening activity. Also, the cells were treated with various concentrations of doxorubicin as a positive control, or the DMSO solvent alone as a control. The cells were then cultured as described above for another 48 h prior to the addition of 10 µL of a 5 mg/mL solution of MTT into each well and cultured for a further 4 h to allow formazan formation. After this, the supernatant (media) was carefully removed and 150 µL of DMSO was added to each well to solubilize the formazan crystals before measuring the absorbance at 540 nm (A_{540}). The relative viable cell number (RVCN) was then calculated as reported (Teerasripreecha et al. 2012). The cytotoxic activity of the partitioned extract was expressed as the cell viability (%) relative to the control.

Any partitioned extract that provided a less than 50% relative cell viability of the cancer cell lines was selected to further investigate the half maximal inhibitory concentration (IC_{50}). To evaluate the cytotoxic IC_{50} value, the qualified samples were dissolved in DMSO to give a serial concentration ranging from 12.5 - 200 µg/mL. Then, the different sample dilutions were assayed as detailed above and the relative percentage of viable cells was calculated. The IC_{50} value was determined from plotting the proportion (%) of the relative number of viable cells against the concentration of the partitioned extracts. Four replications of each experiment were performed.

In addition, the cell morphology of the three cell lines after treatment with DMSO alone as a control or with the respected partitioned extracts dissolved in DMSO were observed and photographed after 48 h of incubation using a Nikon Eclipse TS100 microscope coupled with a DS-L3 imaging system at 200 × magnification.

IN VITRO ANTITYROSINASE ACTIVITY

The antityrosinase activity of the 30 partitioned propolis extracts was determined as previously reported (Khongkarat et al. 2020) with slight modification. The inhibition of mushroom tyrosinase activity by the extracts was assessed spectrophotometrically using L-DOPA as the substrate, while kojic acid was used as the standard tyrosinase inhibitor (Masuda et al. 2005). Initially, the extracts were dissolved and diluted to different concentrations (0.1 - 2.5 mg/mL) in DMSO. Subsequently, 10 µL of the selected mixture was mixed with 30 µL of 80 mM phosphate buffer pH 6.8 (PB) and 120 µL of 2.5 mM L-DOPA in 80 mM PB and then pre-incubated at room temperature for 10 min. Next, 40 µL of

0 (control) or 165 units/mL mushroom tyrosinase in 80 mM PB was added to each well, incubated for another 10 min, and finally the absorbance of the reaction mixture was measured at 492 nm using a microplate reader. The inhibition of tyrosinase activity as a percentage of the uninhibited control activity was calculated as previously reported (Khongkarat et al. 2020). The concentration at which half the original tyrosinase activity was inhibited (IC_{50}) was calculated by plotting the inhibition percentages against the concentrations of the sample. All tests were carried out in triplicate. Kojic acid at various concentrations (6.25 - 200 µg/mL) was used as a positive control, and exhibited an IC_{50} value of 0.0432 mg/mL ($y = 0.7264x + 16.547$; $r^2 = 0.882$).

DATA ANALYSIS

All data were illustrated as the mean ± standard deviation (SD), derived from three replications. The significance of any difference between means was ascertained using one-way analysis of variance (ANOVA) and Tukey's test of multiple comparisons using the SPSS program version 22.0. A probability value at or less than 0.05 was statistically significant.

IDENTIFICATION AND QUANTIFICATION OF COMPOUNDS BY HPLC

The quantification and identification of 11 phenolic compounds [gallic acid (GA), 4-hydroxybutyl acrylate (4-HBA), catechin, syrigic acid, epicatechin, coumaric acid, sinapic acid, vitexin, rutin, kaempferol, and chrysin] in the two selected partitioned extracts was performed by HPLC analysis with an Elipse XDB C18 column (250 mm × 4.6 mm id, 5 µm particle size). The HPLC analysis of the DPE1 and DPE7 samples was conducted by the Food Research and Testing Laboratory (FRTL), Chulalongkorn University. Initially, solutions of 1 mg/mL partitioned extracts were dissolved in 70% (w/v) MeOH and filtered through a 0.45 µm membrane filter. The respective sample solution (25 µL) was injected into the HPLC system (Agilent 1100 HPLC apparatus) equipped with an automatic injector and diode array detector. Gradient elution was determined at a flow rate of 1.0 mL/min and column temperature of 25 °C using a gradient of 1% (v/v) formic acid in MeOH at different ratios with a total analysis time of 75 min. The detection wavelength was set at 280 nm. For identification of the compounds, comparison of the retention time and ultraviolet spectrum were performed between the samples and standards.

RESULTS

Although *T. laevicpes* and *T. pegdeni* are genetically and morphologically similar, they can be distinguished by their external morphology and the emplacement patterns of their brood nest structures (Rattanawannee & Duangphakdee 2020). *T. pegdeni* forms clustered brood cells by connecting tiny cerumen threads, while *T. laeviceps* brood cells form a horizontal comb (Figure S1). According to the plant survey conducted in each location, the most common plants in all four areas in Ratchaburi province were *Pterocarpus macrocarpus* and *Eucalyptus globulus*. In contrast, different principal (prevalent) species of plants were found in each region near the hives especially between the sampling locations in Ratchaburi and Chiangmai provinces (Meemongkolkiat et al. in press).

The characteristics (yield, weight, and appearance) of the obtained 30 partitioned crude extracts were summarized previously in Meemongkolkiat et al. (in press). Each partitioned extract was preliminarily screened for potential *in vitro* cytotoxic activity against the KATO-III and Hep-G2 cancer cell lines as well as the WI-38 normal cell line at a single concentration of 100 µg/mL. Crude extracts that resulted in less than 50% RVCN were considered to have an appropriate cytotoxic activity, whereas those with a RVCN of more than 100% were considered stimulatory. From the results, all the DPE and HPE extracts induced less than a 50% RVCN of the cancer cell lines at this concentration, suggesting a strong cytotoxic activity (Table S2), except for DPE2 against Hep-G2 cells (60.49% RVCN). The RVCN of the cell lines after treatment with DPE at 100 µg/mL ranged from 26.64 - 60.49% for Hep-G2, 16.35 - 44.50% for KATO-III, and 28.43 - 38.22 % for WI-38. At the same time, a high cytotoxic activity was also detected in the HPE extracts at this concentration with the RVCN ranging from 24.01 - 34.60% for Hep-G2, 14.89 - 19.45% for KATO-III, and 30.26 - 38.59% for WI-38. In contrast, all the CME extracts were essentially inactive at this concentration with a much weaker cytotoxic activity. That is the RVCN ranged from 64.34 - 122.79% for Hep-G2, 79.16 - 128.27% for KATO-III, and 97.07 - 126.14% for WI-38 (Table S2). Thus, the DPE and HPE extracts were selected for further estimation of the cytotoxic IC_{50} value against the cancer and control cell lines.

The DPE and HPE extracts were assayed for *in vitro* cytotoxicity over a concentration range from 12.5 - 200 µg/mL on the two cancer and one normal cell lines using the MTT assay, with the IC_{50} (µg/mL) values

summarized in Table 1. Most of the screened partitioned extracts not only showed a cytotoxic effect against the two selected cancer cell lines but also against the normal cell line (Table 1). The IC_{50} for the normal cell line (WI-38) of the DPE2, DPE5, DPE6, DPE9, HPE9, and HPE10 extracts were significantly lower than that in at least one of the two cancer cell lines, suggesting a higher toxicity to the normal cell line than the cancer cell lines. Thus, those extracts would potentially not be suitable for anti-cancer application. However, seven partitioned extracts exhibited significantly higher IC_{50} values (lower activity) against the WI-38 cell line than against both cancer cell lines, indicating they are potential suitable candidates for further application (Table 2).

Among the extracts in Table 2, only the IC_{50} values for DPE1, HPE3, and DPE7 against the WI-38 cell line were significantly higher ($p \leq 0.05$) than those against the Hep-G2 and KATO-III cancer cell lines, and ranged from 1.62-fold lower (KATO-III) to only 1.27-fold lower (Hep-G2) than that for the WI-38 normal cell line (Table 2 & Figure 1). Although DPE1 was more cytotoxic than HPE3 against the Hep-G2 and KATO-III cell lines, these differences were not significant ($p \geq 0.05$; Table 2). However, the IC_{50} value for HPE3 against WI-38 was significantly higher than that for DPE1 ($p \leq 0.05$). Although DPE1 displayed a significantly greater cytotoxic activity against the Hep-G2 and KATO-III cell lines than DPE7 ($p \leq 0.05$), DPE1 also exhibited a significant cytotoxic effect against the WI-38 normal cell line compared to DPE7 ($p \leq 0.05$). In addition, DPE7 also exhibited a significantly lower ($p \leq 0.05$) cytotoxicity than HPE3 against the normal cell line (Table 2 & Figure 1), but HPE3 presented a higher cytotoxicity than DPE7 against the cancer cell lines ($p \leq 0.05$). However, these three extracts were less inhibitory than doxorubicin, which had a more than 10-fold greater cytotoxicity against the cancer and normal cell lines (Table 2). Overall, those three extracts (DPE1, DPE7, and HPE3) may be useful, or contain compound(s) that upon enrichment would be useful for application in anticancer treatment.

TABLE 1. Average *in vitro* IC_{50} values (µg/mL) against two cancer (Hep-G2 and KATO-III) and one normal (WI-38) cell line, based on the RVCN, after 48 h *in vitro* treatment with 20 partitioned extracts of propolis from two stingless bee species. The final concentration of each partitioned extract ranged from 12.5 - 200 µg/mL

Location	Bee species	Extract	IC_{50} value (µg/mL)		
			Hep-G2	KATO-III	WI-38
Chombueng1	*T. laeviceps*	DPE1	15.52 ± 2.04^{a}	14.71 ± 3.93^{a}	23.90 ± 1.76^{c}
		HPE1	23.93 ± 1.19^{b}	14.70 ± 1.35^{a}	$22.41 \pm 2.42^{b,c}$
	T. pegdeni	DPE2	$40.30 \pm 0.84^{c,d,e}$	88.23 ± 8.39^{e}	29.78 ± 1.81^{d}
		HPE2	24.13 ± 0.42^{b}	15.73 ± 2.02^{a}	$18.60 \pm 0.81^{a,b}$
Chombueng2	*T. laeviceps*	DPE3	$21.60 \pm 1.05^{a,b}$	15.52 ± 2.06^{a}	24.21 ± 0.18^{c}
		HPE3	$20.83 \pm 1.09^{a,b}$	22.72 ± 1.57^{a}	$32.73 \pm 0.73^{d,e}$
	T. pegdeni	DPE4	$37.28 \pm 2.67^{c,d,e}$	$40.22 \pm 0.48^{b,c}$	$23.32 \pm 0.38^{b,c}$
		HPE4	14.22 ± 1.32^{a}	13.57 ± 0.77^{a}	15.71 ± 3.31^{a}
Suan pueng	*T. laeviceps*	DPE5	99.63 ± 2.19^{f}	94.33 ± 8.59^{e}	75.77 ± 3.63^{j}
		HPE5	34.91 ± 2.67^{c}	35.33 ± 4.21^{b}	$36.89 \pm 1.74^{e,f}$
	T. pegdeni	DPE6	96.75 ± 2.52^{f}	103.69 ± 1.88^{f}	$43.75 \pm 1.92^{g,h,i}$
		HPE6	$42.12 \pm 1.65^{c,d,e}$	49.63 ± 1.98^{d}	$32.77 \pm 1.46^{d,e}$
Bankha	*T. laeviceps*	DPE7	$36.40 \pm 1.30^{c,d}$	35.15 ± 1.69^{b}	46.52 ± 0.31^{i}
		HPE7	$41.34 \pm 1.01^{c,d,e}$	23.02 ± 2.28^{a}	$42.02 \pm 3.39^{g,h,i}$
	T. pegdeni	DPE8	$20.26 \pm 1.34^{a,b}$	33.25 ± 1.57^{b}	$21.49 \pm 0.35^{b,c}$
		HPE8	$42.30 \pm 2.33^{c,d,e}$	20.84 ± 1.44^{a}	$39.22 \pm 0.97^{f,g}$
Sanpatong (Chiangmai)	*T. laeviceps*	DPE9	45.49 ± 0.62^{e}	$48.65 \pm 1.55^{c,d}$	$41.55 \pm 1.74^{f,g,h,i}$
		HPE9	$43.33 \pm 0.71^{d,e}$	$46.11 \pm 1.67^{c,d}$	$41.13 \pm 1.23^{f,g,h}$
	T. pegdeni	DPE10	$44.14 \pm 10.28^{d,e}$	$48.41 \pm 2.19^{c,d}$	$44.67 \pm 2.11^{h,i}$
		HPE10	$43.13 \pm 0.99^{c,d,e}$	$48.31 \pm 0.20^{c,d}$	$42.84 \pm 1.22^{g,h,i}$

Data are shown as the mean ± SD, derived from three repeats. Means within a column followed by a different letter are significantly different. MPE: methanol partitioned extract; DPE: dichloromethane partitioned extract; HPE: hexane partitioned extract.

TABLE 2. Average *in vitro* IC_{50} values (µg/mL) against the Hep-G2, KATO-III, and WI-38 cell lines, based on the RVCN, after 48 h *in vitro* treatment with the seven partitioned extracts that showed a higher IC_{50} value against the WI-38 cell line than the Hep-G2 and KATO-III lines

Location	Bee species	Extract	IC_{50} value (µg/mL)		
			Hep-G2	KATO-III	WI-38
Chombueng1	*T. laeviceps*	DPE1	15.52 ± 2.04[b]	14.71 ± 3.93[b]	23.90 ± 1.76[a]
Chombueng2	*T. laeviceps*	DPE3	21.60 ± 1.05[a]	15.52 ± 2.06[b]	24.21 ± 0.18[a]
		HPE3	20.83 ± 1.09[b]	22.72 ± 1.57[b]	32.73 ± 0.73[a]
	T. pegdeni	HPE4	14.22 ± 1.32[a]	13.57 ± 0.77[a]	15.71 ± 3.31[a]
Suan pueng	*T. laeviceps*	HPE5	34.91 ± 2.67[a]	35.33 ± 4.21[a]	36.89 ± 1.74[a]
Bankha	*T. laeviceps*	DPE7	36.40 ± 1.30[b]	35.15 ± 1.69[b]	46.52 ± 0.31[a]
		HPE7	41.34 ± 1.01[a]	23.02 ± 2.28[b]	42.02 ± 3.39[a]
-	-	*Doxo	0.551 ± 0.02	1.431 ± 0.02	0.785 ± 0.06

Data are shown as the mean ± SD, derived from three repeats. Means between a column followed by a different letter are significantly different. MPE: methanol partitioned extract; DPE: dichloromethane partitioned extract; HPE: hexane partitioned extract. *Doxo = doxorubicin, which was used as a positive control

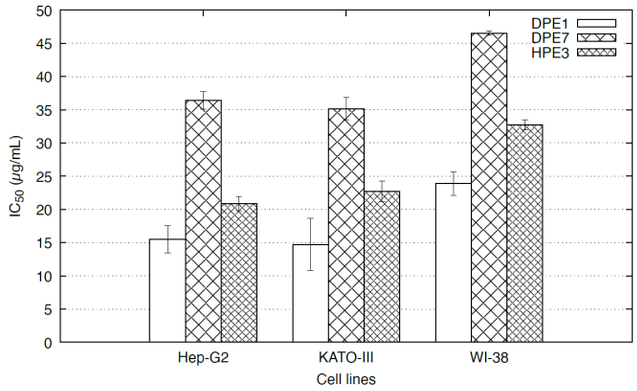

FIGURE 1. Average *in vitro* cytotoxic IC_{50} values (µg/mL), based on the RVCN, of the three partitioned extracts (DPE1, HPE3, and DPE7) on the two cancer and one normal cell lines in tissue culture, as determined by the MTT assay

MORPHOLOGY OF THE HEP-G2, KATO-III, AND WI-38 CELLS AFTER *IN VITRO* EXPOSURE TO THE DPE1, HPE3, AND DPE7 PARTITIONED EXTRACTS

The three cell lines were cultured for 48 h in complete medium supplemented with DMSO alone (control), doxorubicin (positive control), or containing the respective extract (DPE1, HPE3, or DPE7) at its obtained cytotoxic IC_{50} value and then examined for their cell morphology (Figure 2). For the Hep-G2 cell line, the untreated cells were normal spindle shaped, branching, and spreading on the surface of the culture plate (Figure 2(a)). At 200X magnification, clear colony formation was evident in the control cells, whereas the smaller colonies and a reduction in the cell density were detected

in the DPE1-, HPE3-, DPE7-, and doxorubicin-treated cells (Figure 2(d), 2(g), 2(j), and 2(m)). Therefore, these three partitioned extracts may inhibited the proliferation of Hep-G2 cells by inhibiting migration/invasion. Possible apoptotic signs, such as irregular cell shape and shrinkage, were found in all the partitioned extract-treated cells but not in the control cells (Figure 2(d), 2(g), and 2(j)).

For the KATO-III cell line, the untreated cells were observed in three forms: adherent cells, non-adherent cells, and spheroid cell clusters (Figure 2(b)). After 48 h exposure to DPE1, there were flat cells, cell debris, and a reduction in the cell size and adherent cell density compared to control (Figure 2(e)). In addition, cell blebbing and shrinkage were prevalent in the HPE3-, DPE7-, and doxorubicin-treated cells (Figure 2(h),

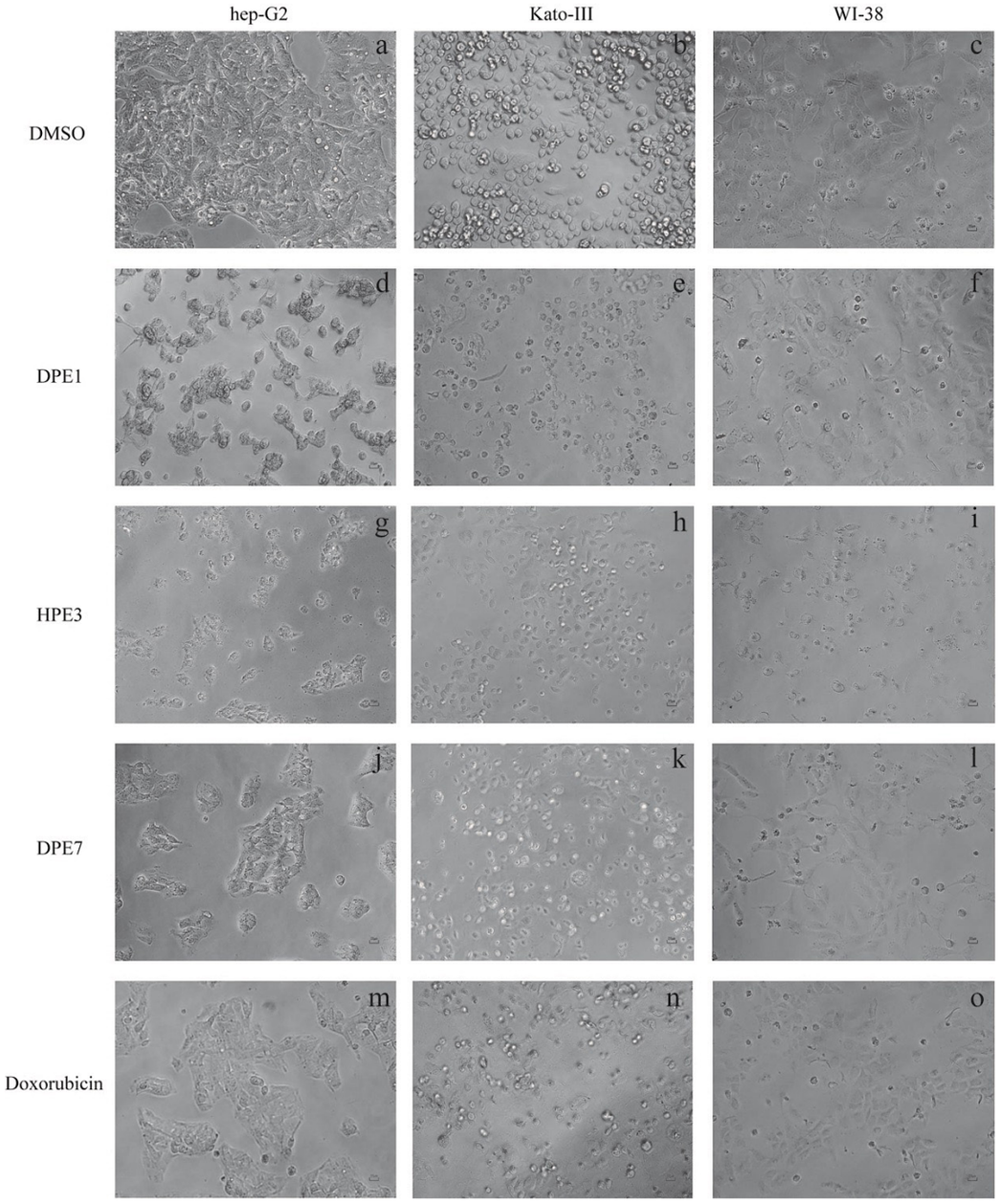

FIGURE 2. Representative images of the morphology of the Hep-G2 (a, d, g, j, and m), KATO-III (b, e, h, k, and n), and WI-38 (c, f, i, l, and o) cells treated with DMSO or doxorubicin (control), or with DPE1, HPE3, or DPE7 dissolved in DMSO at their obtained cytotoxic IC_{50} value (Figure 1) after 48 h at 200× magnification. Images shown are representative of those seen from at least three such fields of view per sample and

2(k), and 2(n)). Lose of cell adhesion and a reduction in the adhered cell number were visible in the DPE7- and doxorubicin-treated cells. Also, the DPE7- and doxorubicin-treated KATO-III cells had more vacuoles (Figure 2(k) and 2(n)).

For the normal cell line (WI-38), the untreated cells were mostly flat, spindle shaped, and attached to the substratum (Figure 2(c)). On the other hand, cells treated with any of the three extracts (DPE1, HPE3, and DPE7) showed a significant reduction in cell numbers and cell adhesion, a loosening of attached cells, and a large amount of cell debris, especially in the HPE3-treated cells (Figure 2(i)). The decreased RVCN (potential cell mortality) and increased cell debris was more common in the HPE3-treated cells than in the DPE1- and DPE7-treated cells. Although morphological examination of the DPE1- and DPE7-treated cells revealed a cytotoxic effect against WI-38 cells, an intact monolayer of the normally spread cells was still observed with similar shaped cells to the control, except that a lower adherent cell density was detected in the DPE1- and DPE7-treated cells (Figure 2(f) and 2(l)), suggesting a minimal cytotoxicity.

ASSESSMENT OF TYROSINASE INHIBITION ACTIVITIES

The tyrosinase inhibition activity of each extract was assessed using the *in vitro* mushroom tyrosinase inhibition assay in comparison to kojic acid as the standard reference, with the results, as IC_{50} values, summarized in Table 3. Among the extracts, MPE3 and DPE4 exhibited the most potent tyrosinase inhibition activities (IC_{50} values of 0.865 and 0.873 mg/mL, respectively), although these were over 20-fold less effective than kojic acid (IC_{50} = 0.0432 mg/mL).

Although DPE1 and DPE7 did not exhibit the highest tyrosinase inhibition, with IC_{50} values that could be classified as a moderate activity (IC_{50} of 1.165 and 1.388 mg/mL, respectively), their combined cytotoxic and tyrosinase inhibition activities showed a good activity against the Hep-G2 and KATO-III cancer cell lines compared to the untransformed WI-38 cell line. Thus, DPE1 and DPE7 are interesting candidates for further investigation to identify the pure compound(s) responsible for the respective biological activities, since the pure compound(s) or mixtures thereof may be markedly more potent.

TABLE 3. The antityrosinase activity (as IC_{50} values) of the 30 partitioned extracts of propolis from two stingless bee species

Location (District)	Species	MPE		DPE		HPE	
		IC_{50} (mg/mL)	Code name	IC_{50} (mg/mL)	Code name	IC_{50} (mg/mL)	Code name
Chombueng1	*T. laeviceps*	> 2.5	MPE1	$1.165^{b,c} \pm 0.020$	DPE1	ND	HPE1
	T. pegdeni	$1.840^{e} \pm 0.013$	MPE2	> 2.5	DPE2	ND	HPE2
Chombueng2	*T. laeviceps*	0.865 ± 0.0597^{a}	MPE3	$1.143^{b} \pm 0.063$	DPE2	> 2.5	HPE3
	T. pegdeni	> 2.5	MPE4	0.873 ± 0.068^{a}	DPE4	ND	HPE4
Suan pueng	*T. laeviceps*	> 2.5	MPE5	$2.373^{g} \pm 0.021$	DPE5	ND	HPE5
	T. pegdeni	> 2.5	MPE6	$1.484^{d} \pm 0.011$	DPE6	ND	HPE6
Bankha	*T. laeviceps*	$1.349^{b,c,d} \pm 0.021$	MPE7	$1.388^{c,d} \pm 0.076$	DPE7	> 2.5	HPE5
	T. pegdeni	> 2.5	MPE8	$2.354^{g} \pm 0.122$	DPE8	ND	HPE8
Sanpatong (Chiangmai)	*T. laeviceps*	> 2.5	MPE9	$2.143^{f,g} \pm 0.026$	DPE9	ND	HPE9
	T. pegdeni	$2.035^{e,f} \pm 0.119$	MPE10	$2.275^{g} \pm 0.076$	DPE10	ND	HPE10

Data are shown as the mean ± SD, derived from three repeats. Means within and between a column followed by a different letter are significantly different. MPE: methanol partitioned extract; DPE: dichloromethane partitioned extract; HPE: hexane partitioned extract; ND: Not detected

PHENOLIC COMPOUNDS IDENTIFIED BY HPLC ANALYSIS

Figure S2 and Table 4 summarize the compounds detected in DPE1 and DPE7, which included various quantities of 4-HBA, epicatechin, syringic acid, sinapic acid, cormaric acid, rutin, kaempferol, and chrysin in both extracts. In addition, GA and vitexin were only found in DPE7 at concentrations of 6.451 ± 0.038 and 1.084 ± 0.068 µg/mL, respectively, while DPE1 had the highest concentration of kaempferol (11.130 ± 0.048 µg/mL) and DPE7 had the highest concentration of 4-HBA (21.376 ± 0.251 µg/mL). Interestingly, there was a single unidentified peak in DPE7 that had a much higher proportional concentration when compared with the standards.

TABLE 4. Compounds identified in selected partitioned extracts (DPE1 and DPE7) by HPLC analysis (Peak numbers are indicated in Figure S2; LD means below the limit of detection)

Peak	Retention time (min)	Compound	DPE1 (µg/mL)	DPE7 (µg/mL)
1	10.489	GA	LD	6.451 ± 0.038
2	20.895	Catechin	LD	LD
3	21.360	4-HBA	3.674 ± 0.019	21.376 ± 0.251
4	24.844	Epicatechin	0.358 ± 0.011	0.391 ± 0.012
5	25.911	Syringic acid	2.545 ± 0.008	2.362 ± 0.033
6	27.070	Sinapic acid	0.465 ± 0.066	0.400 ± 0.011
7	30.833	Cormaric acid	0.268 ± 0.013	0.249 ± 0.006
8	33.101	Vitexin	LD	1.084 ± 0.068
9	36.665	Rutin	0.824 ± 0.012	1.931 ± 0.046
10	47.794	Kaempferol	11.130 ± 0.048	4.168 ± 0.053
11	55.206	Chrysin	0.957 ± 0.029	1.089 ± 0.013

DISCUSSION

Bees collect propolis from different source plants in various ecosystems, selecting appropriate representatives of the local flora. Thus, 'propolis' had no specific chemical meaning but rather is comprised of a mixture of secondary plant metabolites that vary by plant species and season, and they did not all have the same composition over any region or between regions. Although the cytotoxic activity of propolis from Thailand has been reported in some regions (Umthong, Puthong & Chanchao 2009), this was the first study to investigate the *in vitro* cytotoxic effects of propolis from *T. laeviceps* and *T. pegdeni*, and these were obtained from four localities in Ratchaburi and Chiangmai provinces.

Numerous studies demonstrated that different samples of propolis extracts significantly inhibit cell growth and decrease tumor cell differentiation or proliferation (Choudhari et al. 2013; Kustiawan et al. 2014). Additionally, the cytotoxicity might vary significantly amongst propolis samples. Vatansever et al. (2010) demonstrated that the ethanol extract of propolis (EEP) at a concentration of 125 µg/mL was cytotoxic against the MCF-7 cell line but the degree of cytotoxicity (as RVCN) varied between seven distinct EEP samples taken from the same region. These findings suggested that the chemical composition and pharmacological activity of propolis differ according to its geographical and botanical origin. Different plant compounds were present in propolis at low concentrations and in varying amounts depending on the botanical species visited by the bees and the collection period in the area from which the resin was harvested (Watanabe et al. 2011).

The results in this study demonstrated that only three of the screened partitioned extracts (DPE1, DPE3, and DPE7) displayed a lower cytotoxicity, in terms of the IC_{50} values based on the RVCN (Figure 1), against

the normal cell line (WI-38) than against the two cancer cell lines (Hep-G2 and KATO-III). The cytotoxic IC_{50} (mg/mL) value for the two cancer cell lines of each three partitioned extracts were close, but they were also cytotoxic against the untransformed WI-38 cell line. A similar effect had been reported for the crude extract of *A. mellifera*, where it inhibited the development of several cancer cell lines but also affected the growth of normal cells (Najafi et al. 2007). Additionally, the reported cytotoxic IC_{50} values of the propolis extract from Samut Songkram province in Thailand were not significantly different between the normal and cancer cell lines (Umthong et al. 2011). Consequently, further enrichment is needed to exclude the possibility of compounding effects caused by catatonic agents at high bioactive concentrations being mixed with the desired bioactivity components.

With respect to the solvents used for the extraction, the more virulent cytotoxic components in the propolis from Ratchaburi and Chiangmai provinces, Thailand, are likely to be non-polar or low polar chemicals due to the low cytotoxicity of the MPEs against the two cancer cell lines, while both the DPEs and HPEs provided a significant cytotoxic activity against the two selected cancer cell lines (Table S2). This result was in keeping with Castro et al. (2009), who showed the main chemical components in Brazilian propolis was prenylated benzophenone (hyperibone A), a low polarity compound that was found in the crude hexane extracts (CHE). Moreover, the CHEs and DCEs of propolis from Nan province, Thailand also showed strong antiproliferative/cytotoxic activities across five cancer cell lines with the main bioactive components being cardanol and cardol (Teerasripreecha et al. 2012). Thus, the type of solvent employed will affect the composition and biological activities of the obtained extract.

Factors that cause the divergent cytotoxic activities between different propolis samples include the flora nearby the hive and the bee species. The presence of different floral species in the vicinity of the different propolis samples in this study supported that the bioactivity depended upon the plant species surrounding the hive as well as the bee species. The effects of the plant resin source on the biological activity and chemical composition of propolis had been described by Trusheva et al. (2011), who reported that alk(en) ylresorcinols and prenylflavanoids were the compounds that could be identified from the specific plant sources of East Java, Indonesian propolis (*Macarangaa tanarius* L. and *Mangifera* L.) and possess antioxidant activity.

Additionally, it was discovered in this study that propolis from different locations, and from the same location but different bee species, had variable cytotoxic and tyrosinase activities. When comparing these two stingless bee species in the same area, most of the samples significantly differed from each other in both their cytotoxic and anti-tyrosinase activities. However, there was no apparent correlation between these two activities and bee species. This might indicate that these two bee species had a different floral preference in the same location. Several studies had been conducted on the foraging preferences of bees for various plant types (Brian 1957; Pangestika, Atmowidi & Kahono 2017). For instance, *Bombus lucorum* in Scotland foraged in exposed habitats, while *Bombus pratorum* likely to visited more frequently in sheltered habitats (Brian 1957). The three species of stingless bees showed different preferences for visiting the flowers. *Tetragonula laeviceps* preferred Poaceae plants, *Lepidotrigona terminata* preferred Euphorbiaceae plants, and *Heterotrigona itama* selected Solanaceae plants as their nutrient sources (Pangestika, Atmowidi & Kahono 2017). Their choice of visiting is an adaptive behavior for bees to reduce the energy redundantly spent for choosing other plants (Grüter & Ratnieks 2011). Thus, the bioactivities in this study might have been diverse since each colony was in close proximity to different varieties of plants.

Although the pigment melanin in human skin is a major defense mechanism against ultraviolet light, melanin hyperpigmentation can be a major consequence of skin cancer (Rao et al. 2013). Expression of tyrosinase is mainly limited to melanoma or melanocytes and its expression is noted to increase during tumorigenesis (Vargas et al. 2011). Thus, inhibition of this enzyme might help to establish a highly specific treatment for skin cancer. Although the antityrosinase activity of MPE3 and DPE4 (IC_{50} of 0.865 and 0.873 mg/mL, respectively) was around 20-fold lower than the reference standard kojic acid (IC_{50} = 0.043 mg/mL), they still displayed a higher activity than the other extracts, suggesting their potential as tyrosinase inhibitors. Khongkarat et al. (2020) also reported that the antityrosinase activity of the DCM-partitioned extracts of bee pollen exhibited much higher IC_{50} values than kojic acid, yet the enriched compounds from this partitioned extract had a higher activity than kojic acid. Tyrosinase inhibitors are mainly phenolic compounds (Gheibi & Taherkhani 2014). Thus, further studies are required to validate the bioactivity of these propolis samples as isolated pure compound(s) to determine their potency as tyrosinase inhibitors.

Previous studies had shown that higher concentrations of phenolic compounds could possibly reflect the presence of (an) active compound(s) in the samples (López et al. 2014). Among the 11 phenolic profiles found in DPE7, GA, 4-HBA, and kaempferol might be responsible for the cytotoxic activity found against the two cancer cell lines, based upon their similar concentration in both extracts and their higher concentration in each extract compared to the other phenolics. In accord, GA had been reported to prevent the development and progression of various types of cancers (Premratanachai & Chanchao 2014). For instance, GA inhibited the proliferation of and induced apoptosis in prostrate (Kaur et al. 2009) and ovarian (He et al. 2016) cancer cells. In addition, 4-HBA inhibited the proliferation of human K-562 leukemia cells after 24 - 72 h of treatment at 100 µM (Seidel et al. 2014). Moreover, most studies on the anticancer potency of kaempferol had reported a cytotoxic/antiproliferation activity against different cancer cells, including those derived from breast, ovarian, gastric, lung, and pancreatic cancers (Imran et al. 2019). Likewise, the high contents of kaempferol in DPE1 and DPE7 (Figure S2) may account for the observed cytotoxic activity of these extracts. However, based on the HPLC analyses in this study, the cytotoxic activity in DPE7 cannot be solely attributed to the identified phenolics as previously presumed, but might be attributed to the compounds in the unidentified peaks in the DPE7 chromatogram (Figure S2).

Conclusion

The antityrosinase and cytotoxicity activities against two cancer (KATO-III and Hep-G2) and one normal (WI-38; untransformed) cell lines of the partitioned extracts of *T. laeviceps* and *T. pegdeni* propolis from four different locations in Thailand were evaluated in this study. The cytotoxicity of DPE1, HPE3, and DPE7 was significantly more pronounced against the two cancer cell lines than the untransformed one. Meanwhile, MPE3 and DPE4 provided the highest antityrosinase activity. Interestingly, DPE1 and DPE7 can be considered potent inhibitors of cancer cells and so candidates for cancer treatment due to their high toxicity to the cancer cell lines and moderate antityrosinase activity. Taken together, these observations support further exploration for a new anticancer drug from stingless bee propolis based on geographic regions, variety of vegetation, and bee species. However, further investigations are needed to identify the pure compound(s) responsible for the cytotoxic activity of these partitioned extracts, whilst their use in compound-based nanoparticles could bring a more alternative perspective on cancer chemoprevention strategies.

Acknowledgements

This research was supported by Ratchadapisek Somphot Fund for Postdoctoral Fellowship (Chulalongkorn University) and Thailand Science Research and Innovation Fund Chulalongkorn University (CUFRB65_food(6)_114_23_44).

References

Ahmed, B., Qadir, M.I. & Ghafoor, S. 2020. Malignant melanoma: Skin cancer-diagnosis, prevention, and treatment. *Critical Reviews in Eukaryotic Gene Expression* 30: 291-297.

Aziz, M.H., Reagan-Shaw, S., Wu, J., Longley, B.J. & Ahmad, N. 2005. Chemoprevention of skin cancer by grape constituent resveratrol: Relevance to human disease? *The FASEB Journal* 19: 1193-1195.

Banskota, A.H., Nagaoka, T., Sumioka, L.Y., Tezuka, Y., Awale, S., Midorikawa, K., Matsushige, K. & Kadota, S. 2002. Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines. *Journal of Ethnopharmacology* 80: 67-73.

Brian, A.D. 1957. Differences in the flowers visited by four species of bumble-bees and their causes. *Journal of Animal Ecology* 26: 71-98.

Campos, J.F., dos Santos, H.F., Bonamigo, T., de Campos Domingues, N.L., de Picoli Souza, K. & dos Santos, E.L. 2021. Stingless bee propolis: New insights for anticancer drugs. *Oxidative Medicine and Cellular Longevity* 2021: 2169017.

Castro, M.L., do Nascimento, A.M., Ikegaki, M., Costa-Neto, C.M., Alencar, S.M. & Rosalen, P.L. 2009. Identification of a bioactive compound isolated from Brazilian propolis type 6. *Bioorganic & Medicinal Chemistry* 17: 5332-5335.

Chaiyo, U., Garivait, S. & Wanthongchai, K. 2012. Structure and carbon storage in aboveground biomass of mixed deciduous forest in western region, Thailand. *GMSARN International Journal* 6: 143-150.

Chanlabut, U. & Nahok, B. 2022. Forest structure and carbon stock of Suan Phueng nature education park in Ratchaburi province, Western Thailand. *Biodiversitas* 23: 4314-4321.

Choudhari, M.K., Haghniaz, R., Rajwade, J.M. & Paknikar, K.M. 2013. Anticancer activity of Indian stingless bee propolis: An *in vitro* study. *Evidence-based Complementary and Alternative Medicine* 2013: 928280.

Gheibi, N. & Taherkhani, N. 2014. Inhibitory effects of quercetin and kaempferol as two propolis derived flavonoids on tyrosinase. *Biotechnology & Health Science* 1: e22242.

Grüter, C. & Ratnieks, F.L.W. 2011. Flower constancy in insect pollinators: Adaptive foraging behaviour or cognitive limitation? *Communicative & Integrative Biology* 4: 633-636.

He, Z., Chen, A.Y., Rojanasakul, Y., Rankin, G.O. & Chen, Y.C. 2016. Gallic acid, a phenolic compound, exerts anti-angiogenic effects via the PTEN/AKT/HIF-1α/VEGF signaling pathway in ovarian cancer cells. *Oncology Reports* 35: 291-297.

Imran, M., Salehi, B., Sharifi-Rad, J., Aslam Gondal, T., Saeed, F., Imran, A., Shahbaz, M., Tsouh Fokou, P.V., Umair Arshad, M., Khan, H., Guerreiro, S.G., Martins, N. & Estevinho, L.M. 2019. Kaempferol: A key emphasis to its anticancer potential. *Molecules* 24: 2277.

Kaur, M., Velmurugan, B., Rajamanickam, S., Agarwal, R. & Agarwal, C. 2009. Gallic acid, an active constituent of grape seed extract, exhibits anti-proliferative, pro-apoptotic and anti-tumorigenic effects against prostate carcinoma xenograft growth in nude mice. *Pharmaceutical Research* 26: 2133-2140.

Khalil, M. 2006. Biological activity of bee propolis in health and disease. *Asian Pacific Journal of Cancer Prevention* 7: 22-31.

Khongkarat, P., Ramadhan, R., Phuwapraisirisan, P. & Chanchao, C. 2020. Safflospermidines from the bee pollen of *Helianthus annuus* L. exhibit a higher *in vitro* antityrosinase activity than kojic acid. *Heliyon* 6: e03638-e03638.

Kustiawan, P.M., Puthong, S., Arung, E.T. & Chanchao, C. 2014. *In vitro* cytotoxicity of Indonesian stingless bee products against human cancer cell lines. *Asian Pacific Journal of Tropical Biomedicine* 4: 549-556.

Lavinas, F.C., Macedo, E.H.B.C., Sá, G.B.L., Amaral, A.C.F., Silva, J.R.A., Azevedo, M.M.B., Vieira, B.A., Domingos, T.F.S., Vermelho, A.B., Carneiro, C.S. & Rodrigues, I.A. 2019. Brazilian stingless bee propolis and geopropolis: Promising sources of biologically active compounds. *Revista Brasileira de Farmacognosia* 29: 389-399.

Lerner, A.B. 1955. Melanin pigmentation. *The American Journal of Medicine* 19: 902-924.

López, B.G.C., Schmidt, E.M., Eberlin, M.N. & Sawaya, A.C.H.F. 2014. Phytochemical markers of different types of red propolis. *Food Chemistry* 146: 174-180.

Masuda, T., Yamashita, D., Takeda, Y. & Yonemori, S. 2005. Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*. *Bioscience, Biotechnology, and Biochemistry* 69: 197-201.

Meemongkolkiat, T., Khongkarat, P., Rod-im, P., Duangphakdee, O. & Chanchao, C. in press. The contribution of phenolics to the antioxidant potentials of propolis from *Tetragonula laeviceps* and *T. pegdeni* and its correlation to the dominant plant sources in different regions in Thailand. *Accepted for publication in Journal of Apicultural Research on 9 November 2022*.

Najafi, M.F., Vahedy, F., Seyyedin, M., Jomehzadeh, H.R. & Bozary, K. 2007. Effect of the water extracts of propolis on stimulation and inhibition of different cells. *Cytotechnology* 54: 49-56.

Narjes, M.E. & Lippert, C. 2021. Regional differences in farmers' preferences for a native bee conservation policy: The case of farming communities in Northern and Eastern Thailand. *PLoS ONE* 16: e0251206.

OEPP. 1992. *Thailand Country Study on Diversity*. Ministry of Science: Technology and Environment Bangkok, Thailand.

Pangestika, N.W., Atmowidi, T. & Kahono, S. 2017. Pollen load and flower constancy of three species of stingless bees (Hymenoptera, Apidae, Meliponinae). *Tropical Life Sciences Research* 28: 179-187.

Park, J.G., Yang, H.K., Kim, W.H., Chung, J.K., Kang, M.S., Lee, J.H., Oh, J.H., Park, H.S., Yeo, K.S., Kang, S.H., Song, S.Y., Kang, Y.K., Bang, Y.J., Kim, Y.I. & Kim, J.P. 1997. Establishment and characterization of human gastric carcinoma cell lines. *International Journal of Cancer* 70: 443-449.

Phumthum, M., Srithi, K., Inta, A., Junsongduang, A., Tangjitman, K., Pongamornkul, W., Trisonthi, C. & Balslev, H. 2018. Ethnomedicinal plant diversity in Thailand. *Journal of Ethnopharmacology* 214: 90-98.

Pott, I., Konrad, S., Scherer, R., Wiriyacharee, P. & Mühlbauer, W. 2004. Quality of five Thai mango cultivars (*Mangifera indica* L.) using a solar drying system. *CMU Journal* 3: 1-10.

Premratanachai, P. & Chanchao, C. 2014. Review of the anticancer activities of bee products. *Asian Pacific Journal of Tropical Biomedicine* 4: 337-344.

Rao, A.R., Sindhuja, H.N., Dharmesh, S.M., Sankar, K.U., Sarada, R. & Ravishankar, G.A. 2013. Effective inhibition of skin cancer, tyrosinase, and antioxidative properties by astaxanthin and astaxanthin esters from the green alga *Haematococcus pluvialis*. *Journal of Agricultural and Food Chemistry* 61: 3842-3851.

Rattanawannee, A. & Duangphakdee, O. 2020. Southeast Asian meliponiculture for sustainable livelihood. In *Modern Beekeeping Bases for Sustainable Production*, edited by Ranz, R.E.R. London: Intech Open. pp. 1-17.

Reis, J.H.D.O., Barreto, G.D.A., Cerqueira, J.C., Anjos, J.P.D., Andrade, L.N., Padilha, F.F., Druzian, J.L. & Machado, B.A.S. 2019. Evaluation of the antioxidant profile and cytotoxic activity of red propolis extracts from different regions of northeastern Brazil obtained by conventional and ultrasound-assisted extraction. *PLoS ONE* 14: e0219063.

Roubik, D.W. 2023. Stingless bee (Apidae: Apinae: Meliponini) ecology. *Annual Review of Entomology* 68: 231-256.

Rozman, A.S., Hashim, N., Maringgal, B. & Abdan, K. 2022. A comprehensive review of stingless bee products: Phytochemical composition and beneficial properties of honey, propolis, and pollen. *Applied Sciences* 12: 6370. 10.3390/app12136370

Seanbualuang, P. 2012. Basic knowledge of beekeeping. *Naresuan University Journal* 20: 93-100.

Seidel, C., Schnekenburger, M., Dicato, M. & Diederich, M. 2014. Antiproliferative and proapoptotic activities of 4-hydroxybenzoic acid-based inhibitors of histone deacetylases. *Cancer Letters* 343: 134-146.

Sooklim, C., Samakkarn, W., Thongmee, A., Duangphakdee, O. & Soontorngun, N. 2022. Enhanced aroma and flavour profile of fermented *Tetragonula pagdeni* Schwarz honey by a novel yeast *T. delbrueckii* GT-ROSE1 with superior fermentability. *Food Bioscience* 50: 102001.

Sporn, M.B. 1996. The war on cancer. *The Lancet* 347: 1377-1381.

Teerasripreecha, D., Phuwapraisirisan, P., Puthong, S., Kimura, K., Okuyama, M., Mori, H., Kimura, A. & Chanchao, C. 2012. *In vitro* antiproliferative/cytotoxic activity on cancer cell lines of a cardanol and a cardol enriched from Thai *Apis mellifera* propolis. *BMC Complementary and Alternative Medicine* 12: 27.

Topçul, M.R. & Çetin, İ. 2015. The biology of cancer metastasis. In *Cancer: Disease of the age*, edited by Ahmed M El-Sharkawy. Foster City: OMICS Group eBooks. pp. 1-20. 10.4172/978-1-63278-039-3-040

Trusheva, B., Popova, M., Koendhori, E.B., Tsvetkova, I., Naydenski, C. & Bankova, V. 2011. Indonesian propolis: Chemical composition, biological activity and botanical origin. *Natural Product Research* 25: 606-613.

Umthong, S., Puthong, S. & Chanchao, C. 2009. *Trigona laeviceps* propolis from Thailand: Antimicrobial, antiproliferative and cytotoxic activities. *The American Journal of Chinese Medicine* 37: 855-865.

Umthong, S., Phuwapraisirisan, P., Puthong, S. & Chanchao, C. 2011. *In vitro* antiproliferative activity of partially purified *Trigona laeviceps* propolis from Thailand on human cancer cell lines. *BMC Complementary and Alternative Medicine* 11: 1-8.

Vargas, A.J., Sittadjody, S., Thangasamy, T., Mendoza, E.E., Limesand, K.H. & Burd, R. 2011. Exploiting tyrosinase expression and activity in melanocytic tumors: Quercetin and the central role of p53. *Integrative Cancer Therapies* 10: 328-340.

Vatansever, S.H., Sorkun, K., Gurhan, S.I.D., Ozdal-Kurt, F., Turkoz, E., Gencay, O. & Salih, B. 2010. Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines. *Acta Histochemica* 112: 546-556.

Watanabe, M.A.E., Amarante, M.K., Conti, B.J. & Sforcin, J.M. 2011. Cytotoxic constituents of propolis inducing anticancer effects: A review. *Journal of Pharmacy and Pharmacology* 63: 1378-1386.

WHO. 2018. *Cancer: Key Facts*. Accessed 2022 Mar 16. https://www.who.int/news-room/fact-sheets/detail/cancer

Wongsiri, S., Thapa, R. & Kongpitak, P. 1998. Longan: A major honey plant in Thailand. *Bee World* 79: 23-28.

*Corresponding author; email: lek_tues_ti@hotmail.com

TABLE S1. Propolis collection sites in Ratchaburi province, Thailand with the coordinates and the distances between hives of *T. laeviceps* and *T. pegdeni* in each area

District in Ratchaburi	Coordinates				Distances between species at each site (m)
	T. laeviceps		*T. pegdeni*		
	Longtitude	Latitude	Longtitude	Latitude	
Suan Phueng	13°31’21.5”N	99°14’44.8”E	13°31’17.4”N	99°14’44.7”E	125.41
Chom Bueng	13°34’38.9”N	99°31’11.0”E	13°34’34.6”N	99°31’12.5”E	134.65
Chom Bueng	13°35’22.6”N	99°30’28.1”E	13°35’24.0”N	99°30’26.2”E	76.07
Ban Kha	13°22’03.2”N	99°25’12.5”E	13°22’01.8”N	99°25’13.5”E	103.89

TABLE S2. Relative viable cell number (% of control) of two cancer (liver hepatoblastoma (Hep-G2) and gastric carcinoma (KATO-III)) and normal human diploid fibroblast (WI-38) cell lines after 48 h *in vitro* treatment with 30 partitioned extracts of propolis from two stingless bee species. Screening was tested at a final concentration of 100 µg/mL

Location	Bee species	Extract	Cell lines		
			Hep-G2	KATO-III	WI-38
Chombueng1	*T. laeviceps*	MPE1	111.69 ± 5.55	113.45 ± 0.95	106.72 ± 1.75
		DPE1	29.26 ± 1.08	16.35 ± 0.45	36.98 ± 0.20
		HPE1	26.76 ± 0.33	14.89 ± 0.91	30.26 ± 0.28
	T. pegdeni	MPE2	122.79 ± 5.88	128.27 ± 5.79	115.22 ± 5.13
		DPE2	60.49 ± 3.77	44.50 ± 7.71	28.78 ± 1.70
		HPE2	25.87 ± 0.80	16.28 ± 0.95	34.78 ± 3.38
Chombueng2	*T. laeviceps*	MPE3	64.34 ± 4.91	101.17 ± 6.02	109.03 ± 11.97
		DPE3	30.39 ± 1.71	18.45 ± 2.88	34.43 ± 0.37
		HPE3	34.60 ± 0.96	16.55 ± 1.12	33.52 ± 1.20
	T. pegdeni	MPE4	109.92 ± 5.34	98.13 ± 8.82	113.08 ± 4.71
		DPE4	29.50 ± 1.12	22.34 ± 4.69	29.86 ± 2.41
		HPE4	29.27 ± 0.49	16.81 ± 0.42	32.82 ± 2.29
Suan pueng	*T. laeviceps*	MPE5	117.34 ± 4.60	79.16 ± 5.30	97.07 ± 10.45
		DPE5	32.19 ± 2.69	37.81 ± 5.91	36.64 ± 3.21
		HPE5	28.41 ± 0.55	17.87 ± 0.56	31.04 ± 1.14
	T. pegdeni	MPE6	72.35 ± 8.99	105.81 ± 6.80	126.14 ± 10.63
		DPE6	29.69 ±1.35	37.88 ± 3.88	35.07 ± 1.49
		HPE6	27.94 ± 1.39	17.84 ± 1.90	33.05 ± 0.57
Bankha	*T. laeviceps*	MPE7	79.30 ± 0.33	119.08 ± 5.77	101.56 ± 2.70
		DPE7	48.36 ± 3.15	18.60 ± 0.70	37.56 ± 0.99
		HPE7	26.46 ± 1.13	17.56 ± 0.27	34.45 ± 0.76
	T. pegdeni	MPE8	102.09 ± 8.83	104.18 ± 3.09	100.00 ± 8.01
		DPE8	34.42 ± 1.15	17.18 ± 0.60	37.57 ± 1.39
		HPE8	31.69 ± 1.93	19.45 ± 0.25	38.59 ± 1.21
Sanpatong (Chiangmai)	*T. laeviceps*	MPE9	86.96 ± 3.49	85.38 ± 7.51	91.96 ± 2.11
		DPE9	26.64 ± 0.58	16.76 ± 0.84	38.22 ± 1.67
		HPE9	33.08 ± 0.47	16.67 ± 0.91	31.17 ± 1.71
	T. pegdeni	MPE10	102.07 ± 8.52	103.76 ± 7.20	107.08 ± 8.41
		DPE10	30.44 ± 3.57	15.16 ± 1.42	28.43 ± 1.23
		HPE10	24.01 ± 0.66	15.14 ± 0.52	34.01 ± 1.62

Data are shown as the mean ± SD, derived from three repeats. MPE: methanol partitioned extract; DPE: dichloromethane partitioned extract; HPE: hexane partitioned extract

FIGURE S1. Brood cell arrangement of stingless bees in this study. (A) Cell arrangement as a horizontal comb in T. laeviceps and (B) brood cell clusters found in T. pegdeni

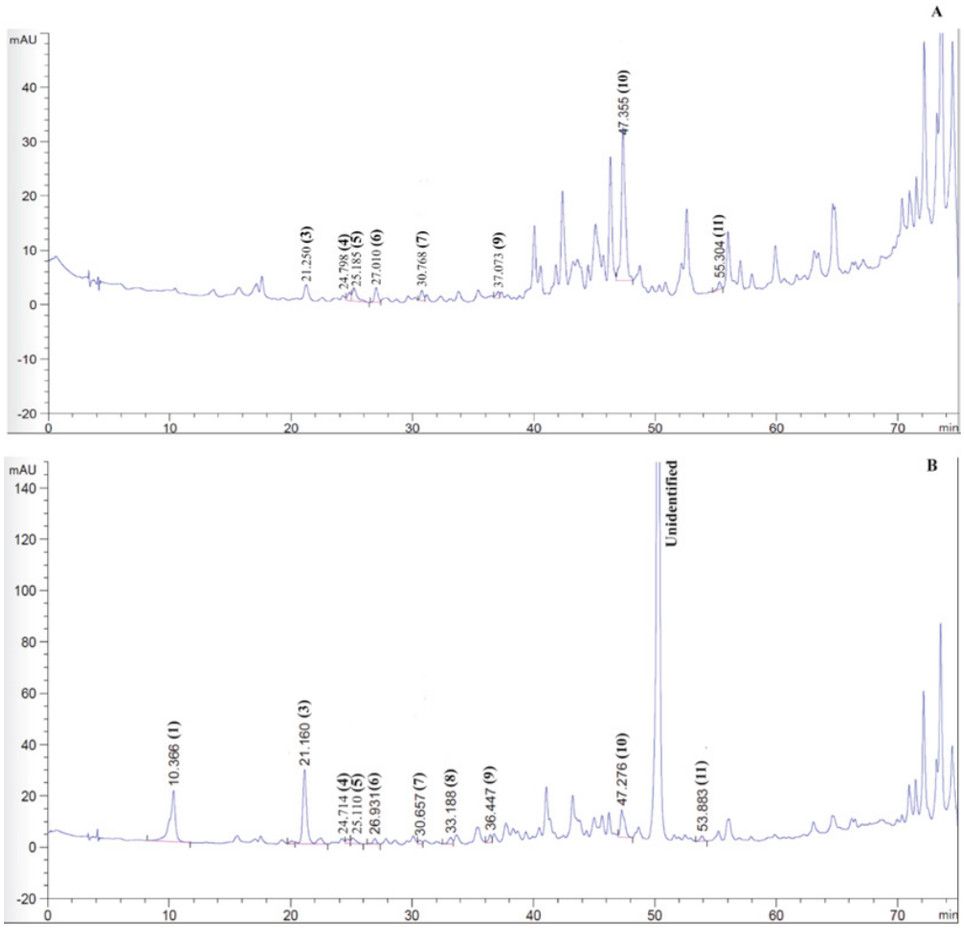

FIGURE S2. Representative HPLC chromatograms showing the 11 detected phenolic acids in (A) DPE1 and (B) DPE7. The number of the peaks in each chromatogram refer to the phenolic compounds with the indicated retention times (min) as: (1) GA, (2) catechin, (3) 4-HBA, (4) epicatechin, (5) syringic acid, (6) sinapic acid, (7) coumaric acid, (8) vitexin, (9) rutin, (10) kaempferol, and (11) chrysin