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In vitro cytotoxicity of Indonesian stingless bee products against human cancer cell lines

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PEER REVIEW

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Comments

This is a valuable study work in which the authors threw light on the relationship between stingless bee products and human's most serious diseases, which is cancer using human cancer cell lines in a new way, and showed that the propolis and honey from some species of stingless bees should have anticancer activities.

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ABSTRACT

Objective: To screen crude extracts of propolis, bee pollen and honey from four stingless bee species [*Trigona incisa* (*T. incisa*)], *Timia apicalis*, *Trigona fusco-balteata* and *Trigona fuscibasis*) native to East Kalimantan, Indonesia for cytotoxic activity against five human cancer cell lines (HepG2, SW620, ChaGo-I, KATO-III and BT474).

Methods: All samples were extracted with methanol, and then subpartitioned with *n*-hexane and ethyl acetate. Each crude extract was screened at 20 μg/mL for *in vitro* cytotoxicity against the cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In addition, four previously shown bioactive components from propolis (apigenin, caffeic acid phenyl ester, kaempferol and naringenin) and two chemotherapeutic drugs (doxorubicin and 5-fluorouracil) were used to evaluate the sensitivity of the cell lines.

Results: Overall, crude extracts from propolis and honey had higher cytotoxic activities than bee pollen, but the activity was dependent upon the extraction solvent, bee species and cell line. Propolis extracts from *T. incisa* and *Timia apicalis* showed the highest and lowest cytotoxic activity, respectively. Only the HepG2 cell line was broadly sensitive to the honey extracts. For pure compounds, doxorubicin was the most cytotoxic, the four propolis compounds the least, but the ChaGo-I cell line was sensitive to kaempferol at 10 μg/mL and KATO-III was sensitive to kaempferol and apigenin at 10 μg/mL. All pure compounds were effective against the BT474 cell line.

Conclusions: Propolis from *T. incisa* and *Trigona fusco-balteata* contain an *in vitro* cytotoxic activity against human cancer cell lines. Further study is required, including the isolation and characterization of the active antiproliferative agent(s).

KEYWORDS

Antiproliferative activity, Bee product, Cancer cell lines, Cytotoxicity, Ethyl acetate extract, *n*-Hexane, Honey, Methanol, Propolis

1. Introduction

Propolis, bee pollen and royal jelly are bee products that have been ascribed several medical properties in both traditional medicine and more recently in conventional medicine[1]. Bee pollen is a granular composite of pollen collected by bees from various flowers and then compacted with a sticky substance produced by the bee. Propolis, often called bee glue, is produced by worker bees from collected plant resins or exudates from phloem-feeding insects, and

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is used to assemble, protect, or repair the bee hives[2]. Royal jelly is secreted by nurse bees and fed to all bee larvae during their first 3 days of development, but the continuous feeding to larvae at sufficient levels thereafter promotes the developmental switch to queen and not worker bees[3].

In recent decades, propolis has attracted increasing attention and use in foods, beverages, supplements and cosmetics for both medicinal treatment and beneficial health reasons (preventative medicine). It is used to prevent or reduce some diseases or symptoms, such as inflammation, heart disease and cancer[4-6]. Propolis has been shown to have various biological activities, such as antibacterial, anti-inflammatory, antioxidant and anticancer properties, in support of its ancient use as a folk medicine in many regions of the world[7,8]. The chemical components of propolis depend on the resin from the vegetation within the foraging region of the bees and the plants the bees select for collection from, since honeybees preferentially target certain plants within range of their beehives as sources of propolis. Thus, propolis has been found to have a seasonal, geophysical regional and bee species specificity to its composition and bioactivity[9]. For example, *Apis mellifera* (*A. mellifera*) propolis collected in temperate regions contains many kinds of flavonoids and phenolic acid esters, particularly pinocembrin, pinobanksin, galangin, chrysin and caffeic acid phenyl ester (CAPE), as the main bioactive compounds[7,10]. The propolis from these regions was shown to have been collected from the bud exudates of members of the *Populus* genus[7,10]. However, the chemical composition of *A. mellifera* propolis has been found to be quite complicated with more than 300 identified compounds, such as polyphenols, phenolic aldehydes, sesquiterpene quinones, coumarins, amino acids, steroids and inorganic compounds, and to vary depending on the collecting location, time and plant source[4]. In contrast to *A. mellifera* propolis, the propolis from *Tetragonula carbonaria*, a stingless bee native to Australia, contained several isomers of pimaric acid and gallic acid as its main components[11,12]. Also, it was reported that eucalypt resin, especially that from *Corymbia torelliana*, shaped the chemical constituents in this stingless bee propolis[12].

Besides *A. mellifera* propolis, Sawaya *et al*. reported the antioxidant activity of propolis from three stingless species[13], which were *Scaptotrigona* spp., *Scaptotrigona depilis* and *Scaptotrigona bipunctata*. Samples were collected monthly over a one-year period. *Scaptotrigona* spp. propolis was collected from the northeastern region of Brazil, while the rest was collected from the southeastern region of the country. Using the 1,1-diphenyl-2-picrylhydrazyl free radical scavenging method (DPPH), the composition of the samples and the antioxidant activity was assayed and found to vary according to the bee species, geographic region and month of collection.

Recently, nine species of stingless bees were recorded in the Mulawarman University Botanical Garden, Samarinda, Indonesia [*Trigona apicalis* (*T. apicalis*), *Trigona drescheri*, *Trigona fuscibasis* (*T. fuscibasis*), *Trigona fuscobalteata* (*T. fuscobalteata*), *Trigona incisa* (*T. incisa*), *Trigona itama*, *Trigona laeviceps*, *Trigona melina* and *Trigona terminate*][14]. Thirty nine plant species from 13 families were reported to act as their pollen source, whilst 22 plant species from 17 families belonging to forest plants and crops were reported to act as their nectar source[14]. The products of those stingless bees were honey (15.4%), beebread (20.9%) and propolis (63.7%).

The existence of stingless bees in the Mulawarman University Botanical Garden is likely to be important in terms of the economics and ecology of the region, since these bees are essential in pollination and can also make useful bee products that can be harvested and applied in food products. However, no research on the bioactivities of products from stingless bees in this region has been reported, yet it is likely to be of interest given that the biological activities of propolis can vary greatly across different phytogeographical areas, time periods[4], and within the same region. Moreover, the bioactivity of bee products from different races or species of bees can also be different. For example, the propolis of *Apis mellifera caucasica*, *Apis mellifera anatolica* and *Apis mellifera carnica* collected from the same apiary in East Anatolia contained different chemical compositions and had different antimicrobial activities[15]. Hence, in this research, the *in vitro* cytotoxic activity of the propolis, bee pollen and honey from four stingless bees collected from within the same area (Mulawarman University Botanical Garden, Samarinda, Indonesia) was evaluated. Crude methanol, *n*-hexane and ethyl acetate extracts of those bee products were prepared and tested for their *in vitro* cytotoxic activity against five human cancer cell lines. In addition, the sensitivity of these five cell lines to four pure compounds (apigenin, CAPE, kaempferol and naringenin) previously reported to be some of the main bioactive components in *A. mellifera* propolis were evaluated in comparison with doxorubicin and 5-fluorouracil (5-FU), two standard chemotherapeutic drugs.

2. Materials and methods

2.1. Sample collection

Propolis, bee pollen and honey were each harvested from four stingless bee species [*T. incisa*, *T. apicalis*, *T. fuscobalteata* and *Trigona fuscibisca* (*T. fuscibisca*)] collected in Mulawarman University Botanical Garden, Samarinda, East Kalimantan, Indonesia in February, 2013. All samples were kept at -20 °C until used.

2.2. Sample preparation

Propolis was cut into small pieces and ground. Bee pollen was collected by sifting with a sieve. Honey was filtered to remove the residual comb and solid matter, such as bee remains, and then by a sifter with the honey liquid being used directly. The samples (Table 1 for amounts) were then separately extracted in three times the volume of 96% (v/v) methanol at room temperature (RT) with continuous shaking (7400 Tübingen; Edmun Buchler, Germany) at 100 r/min for 24 h. This process was repeated until the color of extract was

almost clear (maximum of 7 d) and the extracts were pooled.

Table 1

Yields of the crude extracts of bee products.

Source		Methanol extraction[a]		IME extraction[b]			
		Initial (g)	IME yield (g) (%)	Initial (g)	CHE yield (g) (%)	CEE yield (g) (%)	CME yield (g) (%)
Propolis	*T. incisa*	50	45.8 (91.6)	5.0	0.2 (4.8)	2.5 (50.8)	1.9 (37.2)
	T. apicalis	46	39.2 (85.2)	2.0	0.3 (16.0)	1.1 (53.0)	0.6 (30.5)
	T. fuscibisca	50	46.3 (92.5)	5.0	0.5 (10.0)	3.6 (72.0)	0.9 (18.0)
	T. fuscobalteata	50	43.7 (87.2)	10.0	0.7 (6.9)	6.9 (68.5)	1.8 (17.5)
Bee pollen	*T. incisa*	20	8.1 (40.7)	1.0	0.1 (10.0)	0.2 (20.0)	0.2 (22.0)
	T. apicalis	27	7.3 (27.2)	3.7	0.1 (3.2)	0.2 (5.1)	2.3 (62.4)
	T. fuscibisca	25	8.2 (33.0)	2.7	0.2 (7.0)	0.2 (7.8)	1.8 (65.2)
	T. fuscobalteata	25	7.6 (30.6)	3.5	0.1 (2.9)	0.1 (2.9)	1.5 (42.9)
Honey	*T. incisa*	150	54.8 (36.5)	28.0	0.3 (1.1)	8.4 (30.0)	2.9 (10.4)
	T. apicalis	150	48.6 (32.4)	28.0	0.2 (0.7)	3.3 (11.8)	3.7 (13.2)
	T. fuscibisca	150	58.2 (38.8)	28.0	0.3 (1.1)	3.3 (11.8)	2.4 (8.6)
	T. fuscobalteata	150	59.2 (39.4)	28.0	0.3 (1.1)	3.9 (13.9)	3.8 (13.6)

[a]: The initial amount of sample extracted by methanol, and the yield of the obtained initial methanol extract (IME). [b]: The amount of IME extracted with hexane/methanol and subsequent extraction of the methanol portion with ethyl acetate, with the yields obtained of the crude hexane extract (CHE), crude ethyl acetate extract (CEE) and crude methanol extract (CME).

The respective pooled methanol (MeOH) extracts were then filtered through Whatman filter paper No. 2 (Sigma-Aldrich, Germany) and the filtrate was evaporated at 40 °C and dried in a vacuum oven to near dryness to yield the initial methanol extract (IME) (Table 1). A portion (see Table 1 for amounts) of each filtrate was then dissolved in the minimal volume of 60% (v/v) aqueous MeOH required for complete solvation, and then extracted (partitioned) at RT with an equal volume of *n*-hexane and left to phase separate. The two phases were harvested separately and the upper *n*-hexane phase was solvent evaporated as above to leave the crude hexane extract (CHE). The lower MeOH phase was further partitioned at RT with an equal volume of ethyl acetate (EtOAc) and then left to phase separate and harvested. The upper (EtOAc) phase was evaporated to the yield crude EtOAc extract (CEE), whilst on solvent evaporation the lower phase yielded the crude MeOH extract (CME) (Table 1). Each crude extract was then wrapped with aluminum foil and kept at RT until used. The fractionation yields obtained are summarized in Table 1.

2.3. Cell culture

The human cancer derived cell lines used in this study were derived from ductal carcinoma (BT474, ATCC No. HTB 20), lung undifferentiated cancer (ChaGo I, National Cancer Institute), liver hepatoblastoma ($HepG_2$, ATCC No. HB8065), gastric carcinoma (KATO-III, ATCC No. HTB 103) and colon adenocarcinoma (SW620, ATCC No. CCL 227). All cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, and were used at passage number 115 for BT474, 286 for ChaGo I, 57 for $HepG_2$, 134 for KATO-III and 160 for SW620. Cell lines were cultured in complete medium (CM; RPMI 1640 medium containing 5% (v/v) fetal calf serum) at 37 °C with 5% (v/v) CO_2, seeding at 1×10^5 cells/25-cm^2 flask in 5 mL CM, and repassaging when at 70%-80% confluency.

2.4. Cell counts

Adherent cells were harvested by removing the CM, washing with 0.01 mol/L phosphate buffer saline (PBS (pH 7.4) with 0.01% EDTA but without Ca^{2+} and Mg^{2+}) and then incubating with 1-1.5 mL of 0.05% (w/v) trypsin in the same PBS at RT for 1-2 min. The trypsin solution was then replaced with CM (1.5-2 mL) and the cells dissociated by gentle agitation, harvested and the cell suspension further diluted as required such that a 10 μL aliquot could be counted using a hematocytometer.

2.5. Determination of the extract cytotoxicity by the surrogate MTT assay

The potential cytotoxicity (anti-proliferative and/or reduced cell viability) of each crude extract was assayed using the surrogate cell viability 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay, as previously reported[16]. In brief, cultured cells (5 000 cells) in 200 μL of CM were transferred into each well of a flat 96 well plate and then 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 cultured cells were then treated with the test extract (triplicate wells per condition) at a final concentration of 1, 0.1, 0.01, 0.001 and 0 (solvent control) μg/mL by the addition of 2 μL of CM serial dilutions of the respective crude extract dissolved in dimethyl sulfoxide (stock concentration of 20 μg/mL). The cells were then cultured as above for another 48 h prior to the addition of 10 μL of a 5 mg/mL of MTT solution into each well. The incubation was continued for another 4 h before the media was removed. A mixture of dimethyl sulfoxide (150 μL) and 0.1 mol/L glycine (25 μL) was then added to each well and mixed to lyse the cells and dissolve the formazan crystals, prior to measuring the absorbance at 540 nm. Three replications of each experiment were performed. The number of viable cells was then assumed from the ratio of the absorbance at 540 nm of the respective test sample to that of the control, taking the control to be 100% viability. Thus, the assay approximates the sum of any differential proliferation and cell viability, and so is referred to as the cytotoxicity without discrimination of the two activities.

2.6. Data analysis

Data are presented as the mean±SD, which derived from the indicated number of independent repeats. The significance of any difference between means was tested using a one-way analysis of variance (ANOVA) and Duncan's multiple range tests (DMRT) when parametric, or a One-way Kruskal-Wallis analysis of variance and Mann-Whitney U test with Holm correction when non-parametric. Significance was accepted at the $P<0.05$ level.

3. Results

3.1. Cytotoxic activity of the crude extracts against five human cancer cell lines

Each crude extract was screened for potential *in vitro* cytotoxic activity against the five human cancer derived cell lines at a single concentration of 20 μg/mL. Those crude extracts that had a relative MTT level (assumed viable cell number) of less than 50% of that of the control were deemed to have a sufficient cytotoxic activity, whilst those with a greater than 130% relative viable cell number were deemed to be stimulatory. Note, however, this single high dose assumes no hormesis, where compounds at low concentrations are cytotoxic but not at higher concentrations, such as the potentially high concentration of 20 μg/mL used here.

The obtained cytotoxic activities (and stimulatory activities) varied not only with the solvent used in the extraction, bee product source and bee species, as expected, but were also was markedly variant between the five different cell lines (Tables 2-4).

With respect to the bee product source, overall, the bee pollen was the least effective source with only two [crude *n*-hexane extract of bee pollen (CHEB) and crude EtOAc extract of bee pollen (CEEB)] of the three extracts from two (*T. fuscobalteata* and *T. fuscobisca*) of the four bee species showing a less than 50% relative viable cell number after a 48 h exposure to the extracts, each on a different two of the five cell lines (Table 3). However, these four cases were fairly strongly cytotoxic with only a 8% relative viable cell number for SW620 with the CHEB extract from *T. fuscobalteata*, and the other three cases having a 20%-29% viable cell number. In contrast, the honey extracts showed the highest stimulatory activity, where six and four of the extracts resulted in a more than 130% viable cell number in the ChaGo-I and SW620 cell lines, respectively. Overall, the most cytotoxically active source was the propolis with all cell lines, bee species and solvent extracts showing a less than 50% viable cell number in at least some cases, although only the SW620 cell line had a reduced viable cell level to below 10% in three cases (Table 2). Note, however, an increased relative viable cell number to over 130% was seen for three cell lines/bee species, with the $HepG_2$ line being the most sensitive to this positive stimulation. For the honey extracts, the $HepG_2$ line was the most cytotoxically sensitive (but note it also had two cases of enhanced viable cell number), followed by the ChaGo-I cell line but with no cases of the viable cell number falling below 10%. The SW620 and KATO-III cell lines were insensitive to any honey extract tested (Table 4).

With respect to the bee species, a significant variation in the cytotoxic activity was observed for the bee product, extraction solvent and tested cell line between the different bee species. Overall, the products from *T. fuscobalteata* and then *T. fuscibasis* were more cytotoxic than those from *T.*

Table 2

Relative viable cell number (% of control) of five cancer cell lines after 48 h *in vitro* treatment with crude extracts of propolis from four stingless bee species.

Bee species	Extract	Cell lines				
		$HepG_2$	SW620	ChaGo-I	KATO-III	BT474
	CMEP	59.000±0.042^{a}	8.000±0.008^{A}	33.000±0.083^{aa}	18.000±0.001^{AA}	33.000±0.020*
T. incisa	CHEP	74.000±0.062^{b}	69.000±0.248	35.000±0.041^{bb}	50.000±0.017	22.000±0.031**
	CEEP	108.000±0.160	6.000±0.003^{B}	15.000±0.011^{cc}	17.000±0.001^{BB}	83.000±0.004
	CMEP	125.000±0.076	78.000±0.010	85.000±0.054	53.000±0.015	73.000±0.024
T. apicalis	CHEP	129.000±0.090	46.000±0.021	38.000±0.220	43.000±0.011	84.000±0.004
	CEEP	90.000±0.064	125.000±0.164	170.000±0.099	95.000±0.004	96.000±0.027
	CMEP	49.000±0.049^{c}	15.000±0.016	16.000±0.006^{dd}	20.000±0.008^{CC}	38.000±0.069***
T. fuscobalteata	CHEP	119.000±0.043	133.000±0.058	93.000±0.005	76.000±0.029	65.000±0.053
	CEEP	39.000±0.016^{d}	7.000±0.018	18.000±0.024^{ee}	18.000±0.003^{DD}	29.000±0.044****
	CMEP	146.000±0.044	18.000±0.101^{C}	26.000±0.018	45.000±0.040	55.000±0.010
T. fuscibasis	CHEP	43.000±0.058	124.000±0.282	96.000±0.142	57.000±0.011	19.000±0.048
	CEEP	114.000±0.050	50.000±0.086^{D}	86.000±0.286	40.000±0.016	89.000±0.072

Data are shown as the mean±SD, derived from three repeats. Means within a column followed by a different letter are significantly different. CMEP: crude MeOH extract of propolis. CHEP: crude *n*-hexane extract of propolis. CEEP: crude EtOAc extract of propolis.

Table 3

Relative viable cell number (% of control) of five cancer cell lines after 48 h *in vitro* treatment with crude extracts of pollen from four stingless bee species.

Bee species	Extract	Cell lines				
		HepG2	SW620	ChaGo-I	KATO-III	BT474
T. incisa	CMEB	104.000±0.113	135.000±0.088	182.000±0.131	107.000±0.030	110.000±0.022
	CHEB	127.000±0.139	114.000±0.121	139.000±0.101	95.000±0.006	95.000±0.053
	CEEB	105.000±0.034	75.000±0.192	62.000±0.218	88.000±0.006	63.000±0.017
T. apicalis	CMEB	129.000±0.056	154.000±0.082	180.000±0.077	99.000±0.039	113.000±0.067
	CHEB	171.000±0.246	118.000±0.155	159.000±0.116	103.000±0.056	111.000±0.023
	CEEB	104.000±0.019	125.000±0.038	78.000±0.147	117.000±0.039	55.000±0.021
T. fuscobalteata	CMEB	119.000±0.213	152.000±0.143	159.000±0.046	108.000±0.014	114.000±0.036
	CHEB	80.000±0.025	8.000±0.022	20.000±0.026^{aa}	115.000±0.018	78.000±0.064
	CEEB	95.000±0.036	94.000±0.115	57.000±0.014^{bb}	93.000±0.012	76.000±0.011
T. fuscibasis	CMEB	79.000±0.085	135.000±0.101	152.000±0.075	106.000±0.009	113.000±0.010
	CHEB	116.000±0.053	76.000±0.034	84.000±0.075	106.000±0.050	76.000±0.067
	CEEB	22.000±0.080	104.000±0.148	67.000±0.078	106.000±0.036	29.000±0.039

Data are shown as the mean±SD, derived from three repeats. Means within a column followed by a different letter are significantly different. CMEB: crude MeOH extract of bee pollen. CHEB: crude *n*-hexane extract of bee pollen. CEEB: crude EtOAc extract of bee pollen.

Table 4

Relative viable cell number (% of control) of five cancer cell lines after 48 h *in vitro* treatment with crude extracts of honey from four stingless bee species.

Bee species	Extract	Cell lines				
		HepG2	SW620	ChaGo-I	KATO-III	BT474
T. incisa	CMEH	14.000±0.123[a]	71.000±0.047	30.000±0.190	65.000±0.005	38.000±0.034
	CHEH	18.000±0.053[b]	62.000±0.531	70.000±0.205	119.000±0.060	100.000±0.025
	CEEH	26.000±0.064[c]	109.000±0.477	89.000±0.045	101.000±0.019	70.000±0.034
T. apicalis	CMEH	23.000±0.057[d]	114.000±0.199	45.000±0.019	63.000±0.029	28.000±0.009
	CHEH	23.000±0.033[e]	78.000±0.275	93.000±0.086	117.000±0.003	82.000±0.021
	CEEH	119.000±0.022	98.000±0.417	83.000±0.032	118.000±0.023	91.000±0.015
T. fuscobalteata	CMEH	16.000±0.041[f]	80.000±0.114	40.000±0.111	69.000±0.038	63.000±0.020
	CHEH	18.000±0.041[g]	74.000±0.208	89.000±0.071	117.000±0.044	88.000±0.065
	CEEH	141.000±0.023	115.000±0.298	71.000±0.107	98.000±0.010	79.000±0.021
T. fuscibasis	CMEH	14.000±0.025[h]	104.000±0.116	55.000±0.035	82.000±0.021	102.000±0.028
	CHEH	20.000±0.054[i]	124.000±0.134	110.000±0.031	126.000±0.067	83.000±0.040
	CEEH	132.000±0.030	92.000±0.064	72.000±0.047	88.000±0.025	85.000±0.022

Data are shown as the mean±SD, derived from three repeats. Means within a column followed by a different letter are significantly different. CMEP: crude MeOH extract of honey. CHEP: crude *n*-hexane extract of honey. CEEP: crude EtOAc extract of honey.

incisa, with *T. apicalis* being the least cytotoxic source of bee products.

The solvent used to extract the bee source was also very variable in the cytotoxicity results with no clear trend between the level of cytotoxicity and the bee species or the cell line, but in general the overall weak trend was that the methanol extract yielded a slightly more cytotoxic extract, except perhaps for the hexane extract of products from *T. apicalis*. It should be noted, however, that the different CHEs represent the hexane subfractionation of an initial aqueous methanol extraction, and not a direct hexane extraction of the bee products including propolis. Thus, non-polar and weakly polar bioactive compounds present in the bee products that would be extracted in hexane (but not in the aqueous methanol) may be absent or at lower concentrations in these CHEs.

With respect to the cell lines used to measure the cytotoxic activity, overall the KATO-III cell line appeared the least sensitive of the five cell lines, with no cytotoxicity to below a 50% relative viable cell number in any bee pollen or honey extract, although it was probably the most sensitive cell line overall to the different propolis extracts. In addition, the SW620 cell line showed no significant level of cytotoxicity to any honey extract. The $HepG_2$ cell line appeared the most sensitive to cytotoxic activity in the honey extracts (the least cytotoxic bee product), yet was the least sensitive to cytotoxic activity in the propolis extracts (the most cytotoxic bee product), whilst the reverse trend was seen for the KATO-III cell line.

3.2. Cytotoxic activity of known pure compounds on each of the five human cancer cell lines

The dramatic variation in the apparent cytotoxicity of each crude extract of the bee products between the five different cell lines (Section 3.1) could question the relative sensitivity of these cell lines to different components of bee products and, therefore, the use of any one alone is in assays for screening for bioactive compounds. However, these were only crude extracts at one dose and so the results are potentially compounded by complex interactions between different components (synergistic or additive agonistic or antagonistic activities) within each crude extract. Accordingly, each of the same five cell lines were screened for the *in vitro* cytotoxicity of six known cytotoxic compounds as pure reagents. Of the six chosen compounds, apigenin, CAPE, kaempferol and naringenin have previously been reported to be the major bioactive compounds found in *A. mellifera* propolis[17-19], whilst 5-FU and doxorubicin are currently used chemotherapeutic drugs[20,21]. Each pure compound was tested over the range of 0.001-10 μg/mL (final concentration) for their *in vitro* cytotoxic activity against the five cancer cell lines in the same manner as the crude extracts of bee products using the MTT surrogate total viable cell number assay (Section 2.5).

As with the crude extracts of the bee products, the different cell lines showed different sensitivities to each of the six tested pure compounds (Figure 1). Although these appeared to be less marked between the different cell lines than that observed with the crude extracts of bee products, a direct comparison is not possible for various reasons, including that the concentrations used were quite different.

Doxorubicin was the most cytotoxic of all six tested compounds, with strong (<20% viable cell number) cytotoxic activity against the $HepG_2$, SW620 and ChaGo-I cell lines, although the dose-responses clearly differed between these three cell lines, and moderate cytotoxic (<50% viable cell number) activity against the KATO-III and BT-474 cell lines (again with different dose responses). The sensitivity to 5-FU across the cell lines was less marked, with strong sensitivity only seen in the ChaGo-I cell line, moderate sensitivity in the SW620 and $HepG_2$ lines, but only at high doses and with different dose responses, and a weak sensitivity in the KATO-III and BT474 lines. However, no significant increase in the viable cell number was noted with doxorubicin or 5-FU.

With respect to the four pure compounds known to be bioactive (including cytotoxic activity) ingredients in propolis, none showed any strong cytotoxic activity to any of the cell lines, with a moderate cytotoxic activity (30%-50% viable cell number) being observed only at the highest tested dose (10 μg/mL), but with variation between each cell line and each compound. In addition, at low concentrations (0.01 and 0.10 μg/mL) all four compounds appeared to be stimulatory (120%-140% viable cell number) and so may promote cell proliferation in the $HepG_2$ cell line and, to a lesser extent, in the KATO-III cell line, but not in the other

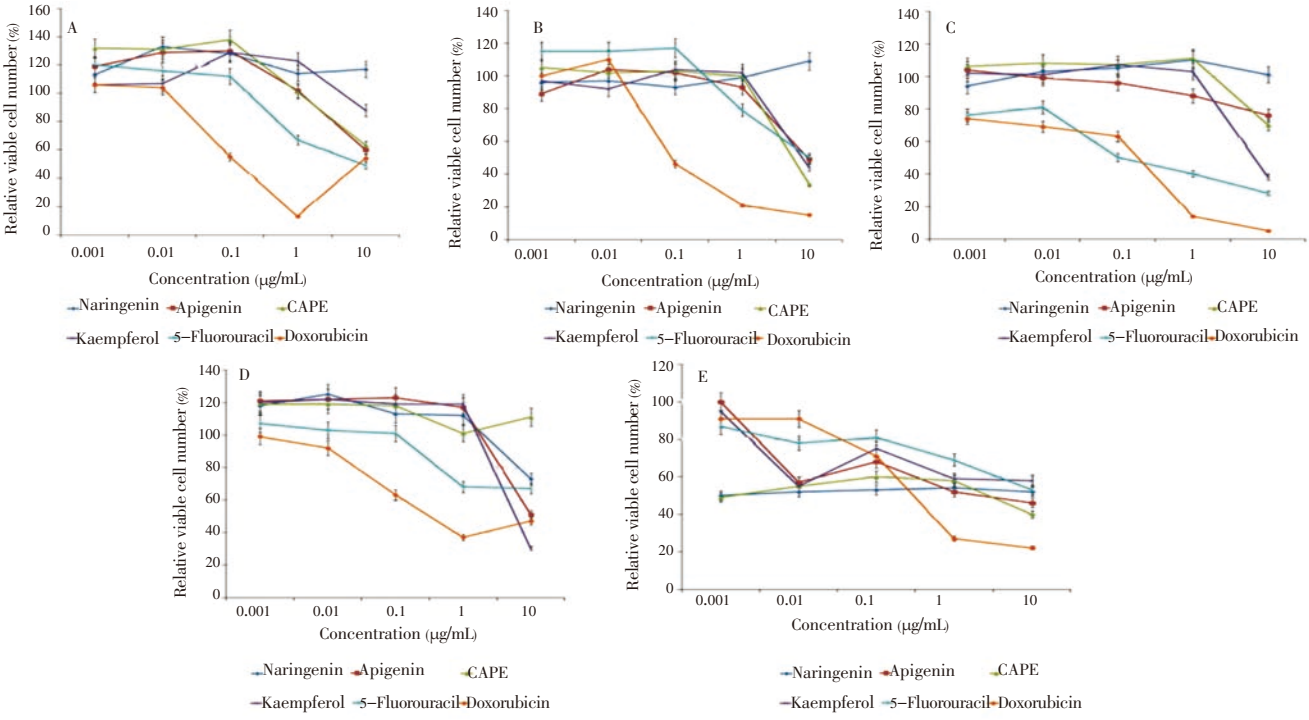

Figure 1. The sensitivities of different cell lines to each of the six tested pure compounds.

Relative viable cell number of the (A) HepG2, (B) SW620, (C) ChaGo-I, (D) KATO-III and (E) BT474 cell lines after a 48 h treatment with the indicated pure compounds and concentrations. The data are shown as the mean±SD percentage of viable cell numbers relative to that of the control, as determined by the surrogate MTT assay, and are derived from three replications.

three cell lines. Excluding the highest tested dose (10 μg/mL), no significant cytotoxicity was observed with any of these four compounds against any of the cell lines except for BT-474, which, in contrast, showed a moderate to weak level of cytotoxic activity (50%-70% viable cell number) against four compounds at all tested doses (0.01-10.00 μg/mL).

4. Discussion

There are many published studies suggesting that bee (mostly *Apis* sp., the honey bees) products exert a fairly diverse array of bioactivities, including anti-proliferation of cancer cell lines. However, so far very few studies have addressed the products of stingless bees, and none on the product of stingless bees native to Indonesia. In Thailand, antimicrobial and antiproliferative activities were reported from the crude extract and partial purified fractions of *Tetragonula laeviceps* propolis but the chemical structure of the active compounds has not been reported[22,23]. However, the major compounds in the geopropolis (propolis mixing with wax and soil in its constitution) of the stingless bee, *Melipona scutellaris*, in Brazil were identified as benzophenones, whilst, in contrast to honey bee propolis, flavonoids were absent. The geopropolis also had both antimicrobial and antiproliferative activities[24].

In this study, five different human cancer derived cell lines were found to be differentially sensitive in terms of the cytotoxic activity to the crude extracts of different bee products and from different species. Overall, the $HepG_2$ cell line was sensitive to the CMEH and CEEH extracts from all four tested stingless bee species. The crude extracts of propolis from *T. incisa* and *T. fuscobalteata* were cytotoxic against most of the five cell lines, whilst the crude bee pollen extracts, and especially the CHEP extracts, were the least cytotoxic.

The bioactivities of bee products depend on the bee species, extraction method, harvesting period, geography, season, and so on[4,25,26]. For example, the preliminary phytochemical screening of *A. mellifera* propolis extracts from several areas in Java for antimicrobial activity against *Mycobacterium tuberculosis* and *in vitro* antiplasmodial activity against *Plasmodium falciparum* strains D6 and W2, revealed that the bioactivity depended upon the locality of harvesting the propolis[25]. Furthermore, the free radical scavenging and anti-*Staphylococcus aureus* activities of *A. mellifera* propolis from Indonesia varied with the propolis source, due to variations in the levels of four prenylflavanones according to the location of the plant resin sources, in this case *Macaranga tanarius* L. and *Mangifera indica* L.[26].

This manuscript is the first study to report the antiproliferative effects of the crude extracts of Indonesian propolis from four species of stingless bees within the same region. Although they were only assayed as crude extracts, and only on transformed cell lines *in vitro*, with no comparison on untransformed cells, the observed cytotoxic activity is sufficient to merit their evaluation for application as crude extracts in addition to their enrichment and identification of the bioactive component(s) and their

potential application. For example, it has been previously reported that mixtures containing 25% (v/v) of a 90% minimum inhibition concentration of the ethanol extract of *A. mellifera* propolis with essential oils from aromatic plants, such as *Caryophyllus aromaticus*, *Zingiber officinale*, *Cinnamomum zeylanicum* and *Mentha piperita*, acted synergistically to inhibit the growth of *Staphylococcus aureus*[27]. This synergism was found in terms of the bacteristatic effects with all four essential oils, and for bactericidal effects with the *Zingiber officinale* and *Cinnamomum zeylanicum* oils. Although a diverse amount of research has supported that pure bioactive compounds in propolis offer the best bioactivity[16], the opposite trend that crude or unpurified extracts were more active, presumably due to synergism of compounds, has also been reported[28].

Regardless, the crude extracts of the different bee products from these four Indonesian stingless bee species showed quiet marked variations in their *in vitro* cytotoxic activities across the five different human cancer derived cell lines. Accordingly, these cell lines were tested in a similar manner for their sensitivity to four known major bioactive (including cytotoxic activity) compounds in *A. mellifera* propolis as pure compounds (apigenin, CAPE, kaempferol and naringenin), in comparison to two currently used chemotherapeutic drugs (doxorubicin and 5-FU) as reference controls. The results revealed that kaempferol was cytotoxic to the ChaGo-I and KATO-III cell lines, apigenin was cytotoxic to KATO-III, and both naringenin and CAPE were cytotoxic to BT474. Although their cytotoxic activity was rather low compared to that of doxorubicin and 5-FU, there is still the possibility of improving them by chemical modification. In the future, the potential synergistic effects between pure compounds from bee products should be evaluated including their *in vivo* activities and potential for nanoencapsulation of the more hydrophobic compounds. For example, a polymeric nanoparticle-encapsulated formulation of *A. mellifera* propolis (propolis nanofood), utilizing micellar aggregates of cross-linked and random copolymers of N-isopropylacrylamide with N-vinyl-2-pyrrolidone and poly (ethylene glycol) monoacrylate, has been used to overcome the poor aqueous solubility and minimal systemic bioavailability of the propolis nanofood[29]. The encapsulated propolis demonstrated a comparable *in vitro* therapeutic efficacy to that of the free propolis against a panel of human pancreatic cancer cell lines.

One final aspect of concern is the dose of the sample, either in the crude or pure form. The results of this research showed that a specific *in vitro* concentration is required for the cytotoxic activity, otherwise, it may promote the proliferation of cancer cells via hormesis[30]. Consequently, further studies are necessary to confirm the bioactivity *in vivo*, and on normal cells, of these bee products as crude extracts and isolated pure compounds.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Comments

Background

Propolis has played an important role in foods, beverages, supplements and cosmetics for both medicinal treatment and beneficial health reasons (preventative medicine). It is used to prevent or reduce some diseases or symptoms. Propolis has been shown to have various biological activities, such as antibacterial, anti-inflammatory, antioxidant and anticancer properties, in support of its ancient use as a folk medicine in many regions of the world.

Research frontiers

Stingless bee products have been used for sweetening and medicines from old times especially in the tropical regions. This research is dealing with the relationship between cytotoxic activity against human cancer cell lines, and stingless bee products (propolis, bee pollen and honey), in a new way using five stingless bee species native to Indonesia.

Related reports

Although there have been very few reports on stingless bees products, it is known empirically that these products would have similar dietetic qualities as the ones from honeybees. However, some reports say that stingless bee's honey have a little stronger anti-biotic functions than honeybee's honey, which is due to the structure of the nest. Stingless bee's honey is stocked in the honey-pots which are made of propolis, while honeybee's honey-pots are made of beewax alone. This research also shows a brief account of the function; stingless bee's honey should have a little high cytotoxic activities.

Innovations and breakthroughs

One of the most noticeable honeybee products is propolis which has been well known to have various effective bioactivities. In the present study, authors have indicated enlighteningly that stingless bee's propolis also should have the anti-cancer activities as well.

Applications

This valuable research shows that some of the stingless bee products such as propolis and honey have notable anticancer activity for men, and should encourage fairly further actual applications.

Peer review

This is a valuable study work in which the authors threw light on the relationship between stingless bee products and human's most serious diseases, which is cancer using human cancer cell lines in a new way, and showed that the propolis and honey from some species of stingless bees should have anticancer activities.

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