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RESEARCH ARTICLE

Cytotoxicity, antioxidant and phytochemical screening of propolis extracts from four different Malaysian stingless bee species

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Abstract

Propolis is a plant-derived substance collected by stingless bee's product from various sources, including plant resins with combination of bee's saliva and wax. Propolis has been used to treat several diseases since ancient times and it is an important source of bioactive natural compound and drug derivatives. The aim of this study was to evaluate biological and chemical profiles of ethanolic extracts from propolis produced by *Heterotrigona itama* (HI), *Geniotrigona thoracica* (GT), *Lepidotrigona terminate* (LT), and *Tretrigona apicalis* (TA). Cytotoxicity activity was evaluated by using 3-(4,5-Dimethylthiazol-2-YI)-2,5-Diphenyltetrazolium Bromide (MTT) assay against three cancer cell lines. *H. itama* extracts showed the highest cytotoxicity effect with IC_{50} of 5 µg/mL, 4 µg/mL and 8 µg/mL for MDA-MB-231, SK-UT-1 and HeLa, respectively. Other species only possessed moderate to weak cytotoxicity effect against tested cells. Phytochemical screening was carried out by thin layer chromatography (TLC) analysis and visualized by derivatives agents in order to detect the presence of terpenoids, steroids, saponins, essential oils and phenol. It was found that *H. itama* (HI) possessed the highest antioxidant activity with the lowest IC_{50} of 30 µg/mL with percetange of inhibition at 85.69 % evaluated by (2,2-diphenyl-1-picryl-hydrazyl-hydrate) DPPH scaveging assay. In conclusion, bee species was considered as important factor in selecting the quality of propolis. It was found that *H. itama* produced the most active extract compared to other species. The data obtained from this study would be the basis for further investigation on therapeutic application especially for cytotoxic activity, antioxidant and phytochemical screenings for four Malaysian stingless bees of propolis.

Keywords: Propolis, stingless bee propolis, cytotoxicity, TLC, antioxidant

INTRODUCTION

Propolis is a complex resinous mixture collected from plant exudates by stingless bees to protect their nest or hive from invader including abiotic factors (Sanpa *et al*., 2015). They use propolis in thin layer on the internal nest as a defence system by repairing combs to strengthen the thin borders of the comb, including embalming dead bees and intruders in order to protect their hive (Bankova *et al*., 2000). Propolis resin collected by bees is influenced by three sources including plant exudates, substances that secreted from bee's metabolism, and materials that presented during propolis elaboration (Marcucci, 1995). It is characterized as a lipophilic material, hard and brittle when cold, but soft, pliable and very sticky when in warm condition. Hence, it is named as a bee glue.

The raw propolis is a complex mixture produced by bee-released and plant-derived compound, typically composed of 50 % resins, 30 % waxes, 10 % essential and aromatic oils, 5 % pollen and 5 % of various organic compounds (Park *et al.,* 2002; Pietta *et al.,* 2002; Wagh, 2013; Huang *et al.,* 2014). Propolis has more than 150 constituents and rich in biochemical constituents, including mixture of phenolic, flavonoid aglycones, polyphenols, and ketones (Marcucci, 1995). There are numerous biological properties of propolis that have been reported, for instance anti-bacterial, anti-viral, anti-fungal, anti-ulcer, anti-tumor and anti-inflammatory properties, in which they provide beneficial effects on human health (Szliszka *et al*., 2009; Wagh, 2013; Chang *et al*., 2017).

Propolis is used for antibacterial activity by previous study due to its ability to inhibit the growth of various bacteria. The presence of esters, aromatic acids and flavonoids compounds in propolis is good for bactericidal action (Marcucci, 1995). In addition, the presence of esters, flavonoids and phenolics compounds such as caffeic acids and aromatic acid in the propolis will give effect on antiviral activity including the ability to inhibit the reproduction of the influenza viruses A & B, vaccinia virus and also Newcastle disease virus (Marcucci, 1995). More than that, it can give effect to antifungal activity and inhibit 50 % of HeLa cell growth due to caffeic acid, quercetin and phenyl ester which contained in the propolis resin (Marcucci, 1995).

Bioactive compounds and the color of propolis can vary depending on the diversity of geographical location, plant sources or types of resins and bee species (Huang *et al*., 2014). The specificity of local flora is responsible for difference species of bees to collect the pollen and thus, the chemical composition of propolis may be different. Malaysia is located at megabiodiversity region and possesses rich flora and fauna

diversities, including stingless bee species (Sakagami *et al*., 1990; Norowi *et al*., 2010). Since 2012 until now, stingless bees have higher production value of propolis of about 66 million per year and became highest demanded due to its nutrition also the medicinal properties (Norowi et al., 2010; Omar et al., 2016). Five species of stingless bees are recorded to be the most abundance species and have commercial value in Malaysia, including *Heterotrigona itama,* followed by *Geniotrigona thoracica, Tetragonula laeviceps, Lepidotrigona terminata* and *Tetragona apicalis* (Salim *et al*., 2012; Kelly *et al*., 2014). Stingless bee species namely as *H. itama* is the most abundance species inhabited in both urban and forest areas (Ab Hamid *et al*., 2016).

Stingless bees are lacked in functional sting under family Apidae and belong to the tribe Meliponini that commonly used as a pollinator. They have diversity of morphologies, such as nest architecture, habitat, color and body size population which are actively depended on the weather and types of the species (Chinh, & Sommeijer, 2005; Kelly *et al*., 2014; Ab Hamid *et al*., 2016). This meliponini species do not migrate in long distance, show rapid population growth and change their food types to others (Nagamitsu & Inoue, 2002). The different species of these bees have their own habitat properties and climatic conditions (Ab Hamid *et al*., 2016). All the four species used in this study have aggressive worker defence (Roubik, 2016). These bees are also known as one of the highly eusocial bees after honey bees and at least 600 stingless bees species belong under 60 genera (Rasmussen & Cameron, 2009). Stingless bees forage their resources, including pollen, resin and nectar, oil, water, muds and sand particles, in which they utilise the pollen and nectar for food resources while resin is used for constructing their nest (Roubik, 2006; Saufi & Thevan, 2015). They have specific structure that is located at tibia of hind legs to keep their collected resources (Pangestika *et al*., 2017).

About 17-32 species of stingless bees are established in Malaysia, yet only two species of them known as *H. itama* and G. *thoracica* are mostly used in the meliponiculture and research studies (Kelly *et al*., 2014). Previous study recorded that *H. itama* and *T. terminata* preferred tree trunk such as rubber trees and few forest hardwood trees with the range between 71cm to 164 cm while *G. thoracica* preferred tree trunk ranging between 82 cm to 129 cm (Kelly et al., 2014). The nest requirement is depended on the types of the stingless bee species. The shapes of nest entrance by *H. itama* are funnel and round-ringed with a brown or light brown in color, while *L. terminata* has a funnel shape with light brown color for nest entrance. However, the nest entrance of the stingless bee namely *G. thoracica* has a brown or black mount-shape entrance with the widest entrance compared to other species that present in Malaysia (Kelly *et al.,* 2014; Saufi & Thevan, 2015). The nest entrance for *L. terminata* forms a sticky resin that applied on outside of it (Roubik, 2016).

G. thoracica species is larger species with the body length of 6.67-10.80 mm (Jalil, 2014; Saufi & Thevan, 2015) while *H. itama* is smaller species with body length between 3-7.5 mm compared to stingless bees (Pangestika *et al*., 2017). On the other hand, *T. apicalis* is also relatively small species with ~6 mm of body size (Schwarz *et al*., 1937), meanwhile *L. terminata* has medium to large body size of 4.0–5.5 mm (Pangestika *et al*., 2017). The species *H. itama* is easily identified in comparison to others by their size and color of their body. Although *H. itama*, *G. throcica* and *T, apicalis* have black colored body, *H. itama* has monotone of color with black colored and uniform sepia tinged wings, while *T. apicalis* and *G. thoracica* have split colored wings with black color at base and white or clear color on the apex region. All species in genus Heterotrigona have a mandible with single tooth towards inner edge (Schwarz *et al*., 1937). Species namely as *L. terminata* has yellow brownish body on mesoscutellum with tessellation shape on the cuticle, yellow scales on the dorsal and black wings (Jalil, 2014; Pangestika *et al*., 2017). The morphology of the stingless bee species is different according to different species types as described in Table 1.

Table 1 Morphology of the stingless bee species (Schwarz, 1937; Jalil, 2014).

Species / Characteristics	*Heterotigona itama*	*Geniotrigona thoracica*	*Lepidotrigona terminata*	*Tetrigona apicalis*
Mandible	Single tooth towards inner edge of edentate apex	1st large, 2nd small denticle	Big (coarse) teeth	Two small (fine) teeth
Wing membrane	Monotone, black	Dark at base and clear or white at apex	Dark at base and clear or white at apex	Monotone, clearly black
Wing humuli (Some species may have asymmetrical of humuli)	7 on both wings	9 on both wings	6 -8	7 on both wings
Scutellum	Short	Short	Short	Short
Propodeum	Middle region, feathery silver while tomentum on each side	Rear of propodeum with hairless, shiny bare spot	Rear of propodeum with hairless, shiny bare spot	-
Hind tibia	Absence of plumose hairs	Has plumose hairs on posterior rim	Has plumose hairs on posterior rim	Has plumose hairs on posterior rim
Hind basitarsis	With silky patch on inner face of hind basitarsus	No sericeous patch on hind basitarsus	No sericeous patch on hind basitarsus	No sericeous patch on hind basitarsus
Molar space	Equal to flagellar width	Very long	Equal to flagellar width	Equal to flagellar width

According to the previous study on the nest structure of the stingless bees, the cell arrangement of the nest is in horizontal comb and in a cluster form. The internal nest is made up of brood cells and layers, pollen pots and honey pots (Roubik, 2006). The nest of these bees consists of lying queen, gynes (virgin queen), drones (males) and worker bees. Only the worker bees are involved in the foraging activity while the others are worked on their job at the nest (Saufi & Thevan, 2015). The nest entrances give in functions to defence their nest from invader, foraging and physio-chemical regulation (Biemeijer *et al*., 2005; Roubik, 2006). The specific structure of the nest entrance is the arrangement or the thickness of the resin surrounded the internal nest of this species due to the nest age, species types and micro environment factors including abiotic factors and invaders (Roubik, 2006).

Most of these scientific reports of propolis are done on honey bees. Reports on stingless bee are still lacking. Previous study shown that *H. itama* possessed higher antioxidant and antibacterial properties compared to *G. thoracica* (Ibrahim *et al*., 2016). These two species also showed different chemical profiles. To the best of our knowledge, there is no report found on therapeutic properties of propolis produced by *Lepidotrigona terminate* and *Tretrigona apicalis.* The aim of this study was to investigate the chemical profiles of propolis from different species, namely *Heterotrigona itama* (HI), *Geniotrigona thoracica* (GT), *Lepidotrigona terminata* (LT) and *Tretrigona apicalis* (TA). This study would also evaluate the antioxidant activity and cytotoxic effect against several tumour cell lines.

EXPERIMENTAL

MATERIALS AND METHODS

Preparation of ethanolic extracts propolis (EEP)

The extraction method was used and based on the method described by (Szliszka *et al.,* 2009; Xuan *et al.,* 2014) with a slight modification. Each sample was cleaned and frozen in −20°C, then ground to powder. Propolis powder was frozen again at -18 °C after grinding. The extracts were prepared by mixing 21 g of crude propolis with 70 mL of 95% (v/v) ethanol for at least 3 days at 37 °C, with occasional shaking. The ethanolic extract was filtered through a Whatman filter paper No 1 and dried using rotary evaporator, under reduced pressure at 60 °C. The crude propolis extracts were kept cool (-20 °C) prior analysis. Propolis extracts (EEP) produced by *Heterogona itama* were coded as HI, *Geniotrigona thoracica* as GT, *Lepidotrigona terminate* as LT, and *Tretrigona apicalis* as TA.

Cell cultures and maintenance

Cytotoxicity assay was carried out against MDA-MB-231 (ATCC® HTB-26™), (SK-UT-1ATCC® HTB-114™) and HeLa (ATCC® CCL-2™) cell lines. All cells were cultured in DMEM or RPMI 1640 medium, respectively and supplemented with 10% (v/v) FBS and 100U/mL of penicillin, 100 μg/mL of streptomycin at 37˚C under humidified 95–5% (v/v) air and supplemented with 5% CO_2. Media for cells were replaced every three days and subcultured after cells reaching more that 80% confluent. The cells were then transferred into culture flask and incubated in humidified at 37˚C in 5% CO_2 incubator for overnight. Then, in order to maintain and harvest the cells, cells were washed with phosphate buffer saline (PBS) twice and then, old growth media were discarded from flask and trypsin was added into the flask to quickly and gently detach adherent cells. Dislodged cells were incubated for 10 minutes at 37˚C. After that, cells were resuspended into complete medium and transferred into new flask and incubated in 5% CO_2 incubator at 37˚C.

Cell counts

Hemocytometer was used to count the cells for cytotoxicity test of propolis extracts against three cancer cell lines. Approximately 10-20 µL of cells were placed to the side of coverslip of Neubauer counting chamber. Cell positionings at four large corner squares of the hemacytometer were counted. The number of cells was calculated according the formula below:

$$\text{Cell no} = \frac{cell\ no\ in\ 5\ squares\ x\ dilution\ factor\ x\ 10^4}{5}$$

Cell viability assay

Cytotoxicity effect of EEP was carried out using 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium (MTT) assay that followed the report by Szliszka *et al*. (2009) with some modifications. For each of the cell lines, 1x10^5 cell/well was seeded in a 96-well plate and incubated at 37°C in 5% CO_2 overnight prior to treatment with extracts at various concentrations (100-0µg/mL). After 72h of incubation, 20 µL of MTT reagent (5mg/mL) was added and incubated for an additional 4h at 37°C. After that, the old media were discarded and 100 µL of 100% DMSO was added to solubilize the formazan crystals. Absorbances were measured at 570 nm and reference at 630 nm by microplate reader (Tecan, Switzerland). The concentration of extract that resulted in 50% growth inhibition (IC_{50}) was determined from a graph of percentage of cell viability against the concentration of the extract. Doxorubicin and cisplatin were used as the positive control. The percentage of inhibition was determined using the formula:

$$Percentage\ of\ inhibition = \frac{(OD\ untreated\ sample - OD\ treated\ sample)}{(OD\ untreated\ sample)}\ x\ 100\%$$

Phytochemical screening by thin layer chromatography (TLC)

Propolis extracts were screened for the presence of various classes of compounds according to method by Ibrahim *et al*., (2016). The extracts were dissolved in 1 mL of methanol and spotted on thin-layer chromatography (TLC) plates coated with silica gel G (Merck $60F_{254}$) of 0.05 mm thickness. Plates were developed in mobile phase of toluene: ethyl acetate: acetic acid: methanol (8: 2: 0.1: 0.2 v/v). After development and drying, the plates were sprayed with derivatization reagents such as vanillin-sulphuric acid and iodine for detection of the respective classes of compounds.

DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

The ability of extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined by DPPH free radical scavenging assay. The scavenging effect of extracts for DPPH was performed and based on the method described by Rosli, *et al.* (2016) with slight modification. Trolox, water soluble α-tocopherol (vitamin E) analogue and Quercetin were served as the standard references. One mM of DPPH solution was prepared by diluting 5 mg of DPPH in 100 mL of methanol. Then, 25 µL of standard and sample solution (500, 250, 150, 125, 62.5, 31.25, 15.625 and 7.8125 µg/mL) were added into a 96-well plate. Then, 200 µL of 1 mM DPPH solution was mixed into each well and incubated at room temperature in the dark for 30 minutes. After 30 minutes of incubation period, the absorbance was read at 517 nm by plate reader spectrophotometer. The blank samples used were 50 µL of DMSO and 200 µL of 1 mM DPPH. The ability of propolis extracts and positive controls to scavenge the DPPH free radical was calculated using the formula, as shown in equation below:

Inhibition % = [(A Blank-A Sample)/ A Blank] x 100 %

The lower absorbance was indicated for a higher scavenging activity followed by decreasing the intensity of purple to yellow colour. The radical scavenging activities of crude extracts were compared via IC_{50}values, in which concentration inhibition that could scavenge the 50 % of DPPH free radical.

RESULTS

Cytotoxicity effect of propolis extracts produced by different stingless bee species

Propolis is a complex resinous substance manufactured by stingless bee that collected from natural sources and has various pharmacological properties (Wagh, 2013; Xuan *et al.,* 2014). In order to evaluate the cytotoxicity effect of propolis extracts, the MTT assay was used in this study. The MTT assay is a colorimetric assay based on assessing the cell metabolic activity and linear relationship between metabolically active cells and the color produced, thus allowing an accurate quantification of changes in the rate of cell death or proliferation. SK-UT-1, MDA-MB-231 and HeLa cell lines were used to assess the cytotoxic potential of propolis extracts for initial screening of cell viability. The biochemical mechanism of the MTT assay involves NAD(P)H-dependent cellular oxidoreductase enzyme that will convert the yellow tetrazolium MTT [3-(4, 5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan) (Bahuguna, *et al.*, 2017). The formed formazan can be dissolved with dimethyl sulfoxide (DMSO) to give a purple color with characteristic absorption bands at 570 nm and reference at 630 nm. Intensity of purple color is directly proportional to the cell number and thus, indicating the cell viability. This method is widely used in both cell viability and cytotoxicity tests because it is easy-to-use, safe and has high reproducibility. The MTT assay is the best known method for determining mitochondrial dehydrogenase activities in the living cells.

The cytotoxicity effects of extracts at different concentrations by MTT assay were presented in Figure 1. The IC_{50} value was obtained from the plot between the concentrations of extracts versus percent of

cell viability after 72h incubation. The value was used to describe the cytotoxicity effect of extracts towards cell lines. The extracts or compounds were considered to be cytotoxic by American National Cancer Institute when (IC_{50}) was less than 30µg/mL (Suffness & Pezzuto cited in Halim *et al.*, 2017). From the graph, it was shown that *Heterotrigona itama* coded as HI showed the highest cytotoxicity effect compared to other species with concentration of extracts required for 50% inhibition meanwhile the cell viability (IC_{50}) was the lowest value for all cell lines with the value of 5 µg⁄mL, 4 µg⁄mL and 8µg⁄mL for MDA-MB -231, SK-UT-1 and HeLa (Figure 1 (A), 1 (B) and 1 (C)). While LT showed no cytotoxicity effect against MDA-MB-231 (IC_{50} 59 µg/mL) and SK-UT-1(IC_{50} 45 µg/mL) (Figure 1 (A) and 1 (B)). Propolis that produced by TA also showed no cytotoxicity effect against HeLa cell line with IC_{50} of 68 µg/mL (Figure 1 (C)). The result of cytotoxic effects against three cancer cells was demonstrated by doxoribucin and cisplatin and shown to be very cytotoxic. The commercial drugs such as doxoribucin and cisplatin were mostly used in treating cancer treatment by chemotherapy to treat any diseases and hence, they were used as a positive control for cell viability test in the present study. Doxoribucin showed IC_{50}1µg/mL for all cell lines, whereas cisplatin with IC_{50} of 1 µg/mL, 2 µg/mL and 4 µg/mL for MDA, SK-UT-1 and HeLa, respectively (Figure 1 (A), 1 (B) and 1 (C)). MTT assay revealed that both HI and GT extracts showed highest cytotoxicity effect against all cancer cell lines, while LT and TA showed no cytotoxic against tested cell lines.

Phytochemical Screening by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is most common and versatile method for identifying and separating compounds due to its low cost, simplicity, short development time, high sensitivity and good reproducibility. The derivative spray reagents such as vanillin-sulphuric acid and iodine vapor were used for detecting compounds presented in the propolis extracts. Table 2 shows the presence of terpenoids, phenols and steroids in propolis extracts when reacting with vanillin-sulphuric acid whereas the presence of both unsaturated and aromatic compounds were detected in all extracts when reacting with iodine vapor in all extracts. However, saponin was absent in GT, LT and TA while it was present in HI.

Meanwhile, essential oils were only present in HI and GT but not in LT and TA. Previous report mentioned about the presence of coumarins in propolis methanol extract of Malaysian HI (Ibrahim *et al.*, 2016). However, in this present study, the presence of coumarins was only observed in propolis produced by LT and they were absent in other extracts. Previous study stated that coumarins could be used to treat cancer and resulted in the side effects by radiotherapy (Marshall *et al.*, 1991; Agarwal, 2000).

Overall, the chemical composition of ethanolic propolis extract produced by *Heterotrigona itama* (HI) was more complex compared to other Malaysian stingless bee species.

Table 1: Phytochemical test (vanillin-sulphuric acid spray reagent and iodine) of the ethanol extracts of propolis produced by stingless bee ,*Heterogona itama* (HI), *Geniotrigona thoracica* (GT), *Lepidotrigona terminate* (LT), and *Tretigona apicalis* (TA).

Constituents	Color Detected	HI	GT	LT	TA	Chemical/Spray Reagent
Saponins	Dark Bluish	+	-	-	-	
Essential Oils	Red & Brown	+	+	-	-	Vanillin-sulphuric acid
Terpenoids	Purple	+	+	+	+	
Phenols	Pink & Red	+	+	+	+	
Steroids	Red	+	+	+	+	
Coumarins	Light Blue	-	-	+	-	UV 366 nm
Unsaturated and Aromatic Compounds	Yellow-Brown	+	+	+	+	Iodine

Remarks: +, detected, -, not-detected

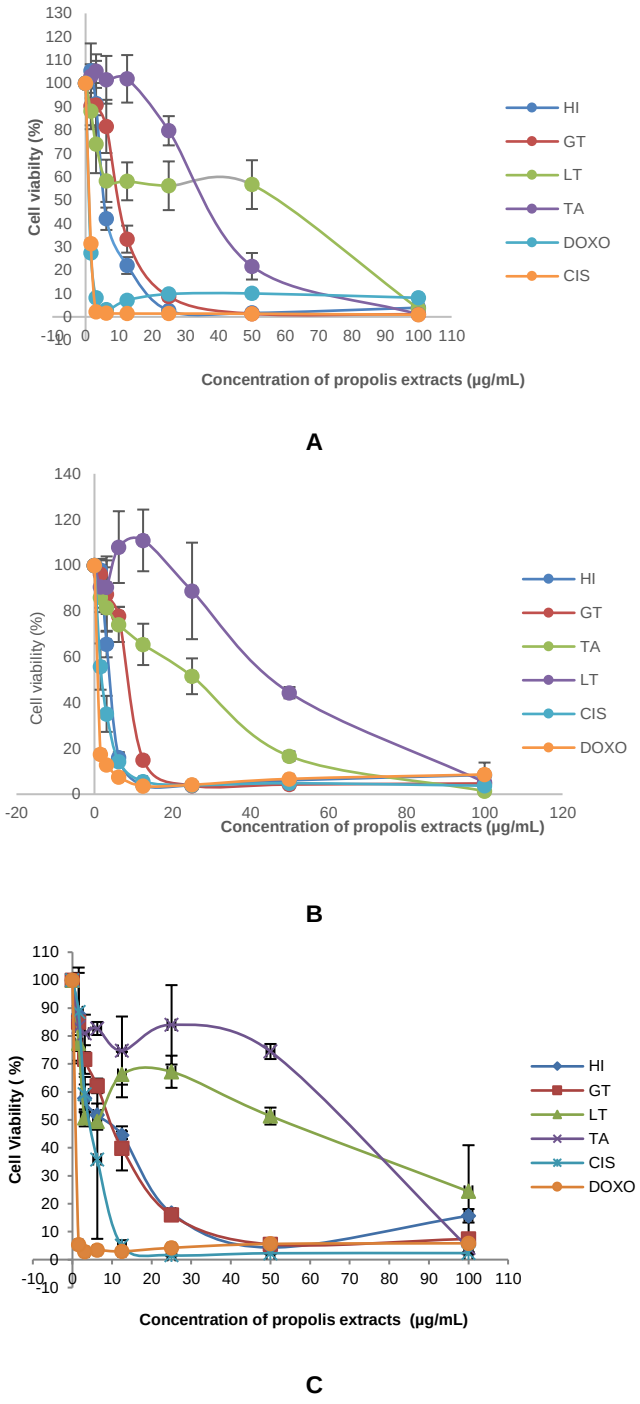

Figure 1 Cytotoxicity effect of propolis extracts evaluated using MTT assay against (A) MDA-MB-231 cells (B) SK-UT-1 cells (C) HeLa cells for 72h incubation produced by *Heterogona itama* (HI), *Geniotrigona thoracica* (GT), *Lepidotrigona terminate* (LT), and *Tretigona apicalis* (TA).

DPPH free radical scavenging activity assay

The antioxidant potential of propolis extracts was determined by using DPPH free radical scavenging assay. This assay has several advantages which are easy to use high sensitivity and enable to analyse large numbers of samples in a short time. The parameter used for this study was 50% inhibition of the concentration (IC_{50}) propolis extracts required to capture the DPPH free radical by scavenging the oxidation reaction chain. The IC_{50} value was obtained from the graph by the plot 50 % of percentage inhibition versus various concentrations of extracts. Smaller IC_{50} value of the extract was indicated for the increase in scavenging activity of the DPPH radical, which resulting in increase of antioxidant activity (Ibrahim *et al.*, 2016). In order to measure

antioxidant activity, a stable free-radical DPPH was used to evaluate the antioxidant activity of HI, GT, LT and TA propolis extracts at concentrations in the range between (7.8125-500 µg/mL) with quercetin and trolox as standard references.

In colorimetric analysis of DPPH for determination of antioxidant activity, HI propolis extract showed the highest antioxidant activity at IC_{50} of 30 µg/mL and highest percentage of inhibition at 85.69%, followed by GT with 40 µg/mL and 82.22% at concentration of 150 µg/mL. However, LT showed weak antioxidant activity at IC_{50} of 128 µg/mL with 80.47% of inhibition percentage at concentration of 500 µg/mL. In contrast, TA showed inactive antioxidant activity (Figure 2). Scavenging activity of HI was most proportional to that of quercetin and trolox with IC_{50} of 10 µg/mL and 9 µg/mL, respectively (Figure 2). However, HI and GT showed antioxidant potential compared to other species. The differences in antioxidant activity could be due to the phenolic, flavonoid or other compound contents of propolis extracts, which were related to the potential antioxidants properties.

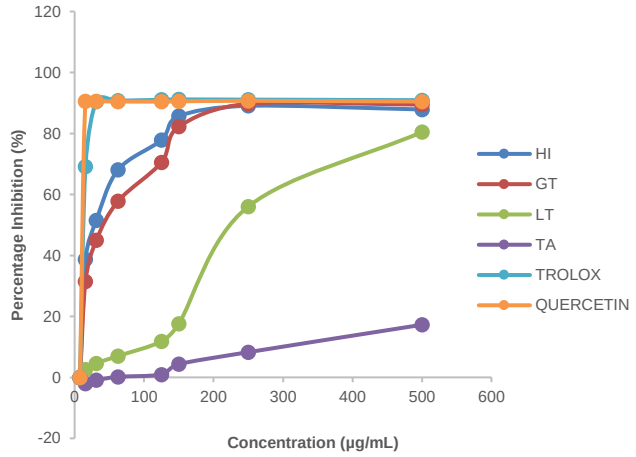

Figure 2. Percentage of DPPH inhibition against concentration of propolis produced by *Heterogona itama* (HI), *Geniotrigona thoracica* (GT), *Lepidotrigona terminate* (LT), and *Tretigona apicalis* (TA), trolox and quercetin (µg/mL).

DISCUSSION

This study was first reported on the cell viability assay by four stingless bee propolis ethanol extracts (EEP) against tumor cell lines. The ability of propolis extracts namely *H. itama* (HI), *G. thoracica* (GT), *L. terminata* (LT) and *T. apicalis* (TA) against triple negative breast cancer cells (MDA-MB-231), uterine leiomyosarcoma cells (SK-UT-1) and cervical cancer cells (HeLa) was evaluated. Doxorubicin and cisplatin were used as positive control. Doxorubicin is a commercial drug used to treat certain types of bladder, breast, lung, stomach and ovarian cancer (Primeau *et al.*, 2005; Brayfield, 2014) whereas cisplatin is a platinum-based and commonly used in the chemotherapy drug to treat various types of cancers, including small cell lung cancer, ovarian cancer, lymphomas and germ cell tumors (Oun *et al*., 2018).

The cytotoxicity assay test was found that propolis extract namely *Heterotrigona itama* coded as HI showed a highest cytotoxicity effect with the lowest concentration of extracts required for 50% inhibition the cell viability (IC_{50}) compared to other species. In contrast, we found that LT extract showed no cytotoxicity effect against MDA-MB-231 and SK-UT-1 cell lines while propolis produced by TA also showed no cytotoxicity effect against HeLa cell lines. The different abilities of propolis extracts on cytotoxicity effect against tumor cells might be due to the chemical compound presented in the propolis was different as different types of stingless bee species were existed. The color of propolis and chemical composition from different species were depended on the availability of plant sources and the age of the nest (Brown, 1989; Bankova *et al*., 2000). The types of local flora and foraging activity were depended on the types of the species of meliponini bees. Therefore, compound presented in the propolis resin was different with the differences in species. They also have time-specific competition with each other for food resources.

The flight intensity of meliponini species to collect food supply was influenced by the time of the day and the abiotic factors (Sajap *et al*., 2015). As previous study proposed flight intensity of G. *thoracica* has high tolerance towards weather conditions such as relative humidity, light intensity and air temperature. However, flight intensity by *H. itama* species was highly influenced by light intensity and temperature (Sajap *et al*., 2015). These two species showed the same flight activity pattern; which actively forage at early morning and reduce in food collected at noon, yet the time of the foraging activity was different. The differences of the time flight activity for both colonies were because of high food resources at the morning (Pierrot & Schlindwein, 2003) and the low temperature and/or low humidity factors, but higher temperature at noon which reduced the food hunting of the stingless bees for preventing dehydration to occur.

Previous study also claimed that the size of the species was correlated with the types of the plant intake by these species. The smaller size of the stingless bee species preferred the smaller plant due to the ability of this species to enter into floral nectaries to collect the nectar and pollen, while larger species preferred larger plant since they were able to collect large quantities of food resource (Sawatthum *et al*., 2017). Thus, the chemical composition of propolis was strongly influenced by the source materials, species of bees and types of the extraction.

The chemical composition of ethanolic propolis extract produced by *Heterotrigona itama* (HI) was more complex compared to other stingless bee species because of the major presence of the chemical compound itself. In contrast, *T. apicalis* (TA) showed lesser compound presented in the extract. The different chemical compounds contained in all propolis extracts might be due to some species that were preferred to forage at certain plant or flower.

The developed thin layer chromatography (TLC) method is a straightforward and efficient way for screening propolis samples at preliminary stage and for quality purposes. It is significant to qualitatively determine biologically active compounds in propolis using the specific detection reagents. The phytochemical screening of propolis extracts by TLC analysis showed the presence of terpenoids, steroids, saponins, essential oils, phenol, unsaturated and aromatic compounds that visualized by derivatives agents of vanillin-sulphuric acid and iodine vapor. Study by a previous researcher claimed that terpenoid compound was effective against cancer and inflammation due to its ability to block the inhibit proliferation, induce intrinsic apoptosis (targeting Bcl2) invasion, and inhibit metastasis (Yadav *et al*., 2010). Phenolic chemical composition was also reported to exhibit anti-oxidant activity and anti-proliferative activity, as well as can induce apoptosis (Dolečková *et al*., 2012; Estévez *et al*., 2014).

The complex chemical composition contained in HI extract compared to other species, as shown in TLC analysis resulted in highest antioxidant activity by scavenging DPPH method. However, GT also showed good source of antioxidant due to its antioxidant properties. This propolis extracts has the ability to inhibit 50% of oxidation chain reaction. The reasons behind the different radical scavenging activities exhibited by different types of propolis might due to their specific pollen foraging activities and different diets, which could contribute to the different compounds found in the stingless bee itself (Nagamitsu & inove, 2002). From this study, we concluded that the chemical composition presented in the propolis extracts was influenced by the types of stingless bee species, resulting in different abilities to inhibit the tested tumour cells and antioxidant activity.

CONCLUSION

The findings were showed that *Heterotrigona itama* produced the most active extract in term of cytotoxicity and has potential to be antioxidant agent compared to propolis produced by other Malaysian stingless species. Tests for phytochemical screening by thin layer chromatography (TLC) of four different species of propolis extracts revealed the presence of terpenoids, phenols and steroids but essential

oils were only presented in *Heterotrigona itama* (HI) and *Geniotrigona throracica* (GT), whereas saponin was only presented in *Heterotrigona itama* (HI) and coumarins were presented in propolis produced by *Lepidotrigona terminate* (LT). The differences in chemical composition were probably due to the resin plant origin, climate, and resin collection time by the bees. From this study, it was indicated that bee species played a role in determining the chemical and biological profiles of particular propolis and should put into account in decision of further development for propolis.

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