PHYTOCHEMICAL PROFILING AND ANTICANCER ACTIVITIES OF ETHANOLIC AND AQUEOUS PROPOLIS EXTRACT OF *TRIGONA APICALIS.*

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Abstract
Propolis is an important structure that stores and protects honey in the stingless beehive. Despite its known benefits, there is lack of research that looks into the antioxidant and anticancer effects of propolis produced by a Malaysian stingless bee species, *Trigona apicalis*. Propolis was extracted using ethanol and aqueous and tested for antioxidant assay. The total phenolic and flavonoid contents were determined for both extracts. The in vitro cytotoxicity was assessed manually against the HeLa cell lines and the extracts were analyzed using GC-MS for potential compounds present. Ethanolic extract of propolis (EEP) was revealed to be a better scavenger of DPPH and ABTS with higher phenolic content; 85 µg gallic acid equivalents/g. Meanwhile, the aqueous extract of propolis (AEP) has higher flavonoid content; 147.44 µg quercetin equivalents/g. EEP and AEP have IC_{50} of 31.25 and 120 µg/mL respectively during a viability test against HeLa cells and the proliferation activity was constantly at a lower rate as compared to the control which occurred in 5 days. Both propolis extracts induced late apoptosis and EEP arrested at G0/G1 phase while AEP arrested at S phase. Several compounds have been identified, whereby 1,6-cyclodecadine,1-methyl-5-methylene-8-(1-methylethyl)-s-(E,E) was predominantly detected in EEP, while 9-isopropyl-1-methyl-2-methylene-5-oxatricydo[5.4.0.0(3,8)] undecane was major compound in AEP. While further studies are required to validate the above findings, propolis produced by local stingless bee species seems a suitable research candidate for discovery of potential anticancer agent.

Keywords: Antioxidant, Anticancer, Phytochemical Profiling, Propolis*, Trigona Apicalis*

Introduction

Propolis is a resinous substance, which is produced by bees through mixing its saliva with various sources of plant exudates and tree barks. Its biological characteristics are dependent on its phytochemical contents which are influenced by geographical zone and seasons, including surrounding plant sources (1). Propolis has been well known for its therapeutic properties since ancient time. It possesses of a broad range of biological activities such as antimicrobial, anti-inflammatory, antioxidant, anticancer, wound healing, antiviral and hepatoprotective effects (1-3). The chemical constituent of propolis consists of a group of polyphenols which are also generally exist in honey (2, 4). In addition, propolis comprises alcohol, esters, ketones, amino acids and inorganic compounds (2, 5, 6). The biological activities would depend upon the available chemical constituent in the propolis and efficiency of extraction solvent utilized (6, 7). Studies have confirmed the ability of a polar propolis extract as a good agent to terminate the free radical components, such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (8). A pure aqueous extract of propolis also illustrates a promising result in scavenging free radical and demonstrates an efficient condition to yield good amount of phenolic compounds (9, 10). Moreover, propolis in the form of ethanolic extract (EEP) revealed a dose-dependent relationship of cytotoxic and apoptotic effect against human breast cancer (MCF-7), human epithelial colorectal adenocarcinoma (Caco-2) and human cervical cancer (HeLa) cell lines (11). However, there is yet any studies that investigate the effects of local *Trigona apicalis* stingless bee propolis on HeLa cells. Hence, this study aimed to explore the potentials of local stingless bee propolis extracts on

antioxidant activities and anticancer properties against HeLa cell lines.

Materials and Methods

Preparation of propolis extracts

Raw propolis of *T. apicalis* was gathered from a stingless bee farm located in Kuala Kangsar, Perak, a state situated in northern region of Malaysia. Ten (10) g of raw propolis was extracted using 100 mL of either ethanol (ethanolic extract of propolis) or water (aqueous extract of propolis) at a ratio of 1:10 extract to ethanol or water, respectively. The solution was stirred continuously using a magnetic stirrer at room temperature for 72 hours. The extraction process was repeated three (3) times for each sample and the suspension was collected after centrifugation (Hettich EBA 21 Centrifuge, England) at 20,000 rpm for ten 10 minutes. The supernatant was then combined and concentrated in a rotary evaporator (EYELA N-1100, Japan) under reduced pressure at 40 ºC. One hundred (100) mg of extract was later weighed and diluted in 1 mL of isopropyl alcohol to establish the stock solution, which was later used to produce a series of different propolis extract concentrations (25, 31.25, 62.5, 120, 160 and 200 µg/mL), carried out using serial dilution.

Total phenolic and flavonoid determination

The total phenolic content (TPC) of the propolis extract was determined by the Folin-Ciocalteu method using gallic acid as standard (12). 1.5 g of Na_2CO_3 (1:20 mL g/v) and Folin-Ciocalteu (FC) were diluted in dH_2O (1:10 mL v/v). Samples were aliquoted in a ratio of 1 (samples): 5 (FC reagent): 4 (Na_2CO_3) and were incubated for 30 minutes. All samples were run in triplicate and inter-day manner and read at absorbance of 760 nm. In addition, the total flavonoid content (TFC) was measured spectrophotometrically at absorbance of 430 nm using quercetin as standard (13). Ten (10) µL of sample was mixed with 3 µL of $NaNO_3$ (5% w/v), and 40 µL of distilled water. After 5 minutes, 3 µL of 10 % (w/v) $AlCl_3$ and 20 µL of 1M NaOH. After incubation for 15 minutes, the samples were analysed in triplicate using the microplate reader (BMG Labtech, FLU0star Omega, Germany). Folin–Ciocalteu reagent, sodium carbonate, aluminium chloride, sodium hydroxide, sodium nitrate and gallic acid were supplied by Merck (Germany).

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was performed using an Agilent 19091S-433 equipped with HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). GC-MS spectroscopic detection, an electron ionization system with ionization energy of 70eV was used. Pure helium gas (99.999%) was used as a carrier gas at a constant flow rate of 2.0 mL/min. Mass transfer line and injector temperature were set at 280 °C and 290 °C, respectively. The oven temperature was programmed from 70 to 325 °C at 3 °C/min, then held isothermal for 20 min and finally raised to 280 °C at 20 °C/min. Diluted samples; 10000 ppm of 1 µL was injected in the split mode with a split ratio of 5:1 (v/v). The relative percentage of the chemical constituents in the extract was expressed as a percentage by peak area normalization.

Antioxidant capacities of propolis extract

Methods for scavenging assay of 1,1- diphenyl-2- picryl-hydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radicals were slightly modified (14). A fixed quantity of 150 µL of methanolic DPPH solution was added to each 7.5 µL sample solution. The mixture was incubated in room temperature and dark condition for 30 minutes. ABTS diammonium salt ethanolic solution and potassium persulfate were prepared individually and incubated in a dark room for 16 hours. Each extract of 1.25 µL was pipetted into a 125 µL ABTS solution and incubated for 6 minutes to initiate the reaction. The absorbance was read at 517 and 734 nm for DPPH and ABTS assay, respectively, and trolox was used as the standard. All the tests were performed in triplicate and inter-day manner and the results were averaged. DPPH, ABTS, diammonium salt, trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) with 97% purity was obtained from Sigma–Aldrich (Germany).

Maintaining and sub-culturing of HeLa cells

HeLa cells were maintained in the DMEM solution with 10% FBS, 1 % Sodium pyruvate and 1% Penicillin streptomycin. The monolayer of cells was sub-cultured after 80% of confluency was reached and maintained until the stable condition was achieved for treatment.

Viability and proliferation of HeLa cells

EEP and AEP were diluted according to the required concentrations (25, 31.25, 62.5, 120, 160 and 200 µg/mL), with DMSO and deionized water to reach a fixed final concentration of DMSO (0.1%). DMSO final concentration was fixed at 0.1% since at this concentration it was shown to be non-toxic to HeLa cell lines. The cells were later treated with the abovementioned series of propolis extract concentration to determine the IC_{50} of HeLa cells viability. Later, the cells were cultured and maintained in the same concentrations for 5 days to observe the prolonged effects of the extract against HeLa cells proliferation.

Flow cytometry analysis of HeLa cells apoptosis

A negative control was set up by incubating HeLa cells without the propolis extracts (untreated cells). Five (5) µL of FITC-Annexin-V and 1 µL of the 100 µg/mL propidium iodide working solution were added to each

100 µL of cell suspension with a cell density of 1 x 10^6 cells/ mL. The cells were incubated for 15 minutes at room temperature. After the incubation period, 400 µL of Annexin-V binding buffer was added and mixed gently. The sample were kept on ice and the stained cells were analysed immediately by flow cytometry, measuring the fluorescence emission between 530 and 575 nm.

Cell cycle analysis

The cells were stained based on the manufacturer protocol of BD cycle test TM Plus DNA Reagent kit. The pellet of treated HeLa cell lines that was collected from the culture plate was resuspended in 1 mL of buffer solution (PBS with 100 µg/ mL RNase A, 50 µg/ mL PI and 0.1% Triton X-100) and vortexed slowly, then centrifuged for 5 minutes at 10,000 rpm for 3 times. The total number of cells was counted manually under the microscope and adjusted to 1 x 10^6 cells/mL using the buffer solution. Five hundred (500) µL of cells were aliquoted in a new centrifuge tube and were centrifuged for 5 minutes at 400 rpm. The supernatant was discarded and 250 µL of solution A was added. The tube was gently mixed and incubated at room temperature for 10 minutes. Finally, 200 µL of solution C was added and incubated for 10 minutes in a cold and dark condition. The samples were analyzed within 3 hours and the results were analyzed with Modfit software (Verity Software House).

Statistical analysis

All data were shown as mean ± standard error of mean (SEM). The analysis of variance (ANOVA) and post-test Dunnett were used to compare the treatment with the control. Whilst, the dataset for proliferation of cells were compared within control and treatment by using one-way ANOVA and the data for apoptosis and cell cycle study were analyzed by comparing the treatment with the control via independent t-test analysis (SPSS). The results were considered significant at $p < 0.05$.

Results

Total phenolic and flavonoid contents

Total phenolic content (TPC) of the ethanolic extract of propolis (EEP), expressed as Gallic Acid Equivalent (GAE) was 85 mg/g of dry extract, and TPC for aqueous extract of propolis (AEP) was 76 mg/g GAE of dry extract. On the other hand, Total flavonoid content (TFC) was found higher in the AEP compared to the EEP, expressed as Quercetin Equivalent (QE), with its value of 147.44 mg/g and 133 mg/g QE of dry extract, respectively.

Chemical composition of propolis extracts

Table 1 shows the GC-MS profile of the propolis extracts and the phytochemical compounds detected were based on the MS database library on the equipment. Several compounds have been identified, where 1,6-cyclodecadine,1-methyl-5-methylene-8-(1-methylethyl) -s-(E,E) was predominantly detected in EEP, besides 4-(1,3,3-trimetyl-bicyclo[4.1.0]hept-2-yl) -but-3-en-2-one and lanosterol. In contrast, 9-isopropyl-1-methyl-2-methylene-5-oxatricydo[5.4.0.0(3,8)] undecane was the major compound found in AEP, besides aromadendrine oxide-(2) and androsta-1,4-dien-3-one,17-hydroxy-17-methyl-(17 alpha).

Table 1: GC-MS of compounds identified by MS library

Extracts	RT (min)	Area (%)	Compound ID	Quality
EEP	8.197	8.44	1,6-Cyclodecadine,1-methyl-5-methylene	96
	9.092	6.18	-8-(1-methylethyl)-,s-(E,E)	35
	18.569	22.24	4-(1,3,3-Trimetyl-bicyclo[4.1.0] hept-2-yl)-but-3-en-2-one Lanosterol	49
	21.665	27.1	(+)-(z)-Longipinane	53
AEP	10.141	20.78	Aromadendrine oxide- (2)	42
	10.231	18.21	Androsta-1,4-dien-3-one,17-	38
	10.439	50.16	hydroxy-17-methyl-,(17 alpha)- 9-isopropyl-1-methyl-2-methylene -5-oxatricydo[5.4.0.0(3,8)]undecane	66

EEP = Ethanolic extract of propolis; AEP = Aqueous extract of propolis; RT = retention time

Antioxidant potential of propolis extracts

Evaluation of the propolis extracts exhibited that both have the potentials as antioxidant agent as shown in Table 2. EEP demonstrated the lowest IC_{50} value to scavenge both DPPH and ABTS free radicals, which are 1.305 mg/mL and 0.407 mg/mL, respectively. These findings correspond to the higher total phenolic content compared to that of AEP, measured at 85 mg/g GAE of dry extract. In order to determine the correlation between antioxidant activity and the phytochemical composition of the propolis extracts, the equivalent values of TFC and TPC were put into comparison against the antioxidant capacities. The analysis shows that both the propolis extracts have significant correlation ($p < 0.01$) between antioxidant activity and TPC and TFC, with positive correlation coefficient values (r^2) ranging

from 0.879 to 0.991.

Table 2: Comparison of antioxidant activities and polyphenolic contents between ethanolic (EEP) and aqueous extract of propolis (AEP)

Unit	Parameters	Type of extract	
		EEP	AEP
IC_{50} (mg/mL)	DPPH	1.305	2.414
	ABTS	0.407	0.47
mg/g GAE of dry extract	Phenolic content	85	76
mg/g QE of dry extract	Flavonoid content	133	147.44

EEP = Ethanolic extract of propolis; AEP = Aqueous extract of propolis; DPPH = 1,1- Diphenyl-2- picryl-hydrazyl; ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); GAE = Gallic acid equivalent; QE = Quercetin equivalent

Cytotoxicity activity of propolis extracts against HeLa cells

HeLa cells treated with EEP and AEP extracts for 72 hours, exhibited a dose-dependent relationship in both treatments as shown in Figure 1. The efficiency of propolis against HeLa cells is convincing when it needs low IC_{50} concentration, as demonstrated in the Figure 1 (a) (31.25 µg/mL) and Figure 1 (b) (120 µg/mL) for EEP and AEP respectively, in inhibiting the growth of HeLa cells compared to the untreated cells (control; $p < 0.05$). However, the trend of viability of AEP-treated HeLa cells did not decrease rapidly as compared to EEP. In AEP-treated group, 40% of live cells existed even at higher concentration of 160 µg/mL. Compared to EEP-treated group, viability of HeLa cells dropped to about 7% at concentration of 125 µg/mL of EEP. A long-term effect of propolis was measured in the form of proliferation rate that has been simplified in the graph presented in Figure 2. At day 5, HeLa cells treated with EEP and AEP were observed to proliferate at a lower rate as compared to the control ($p < 0.01$).

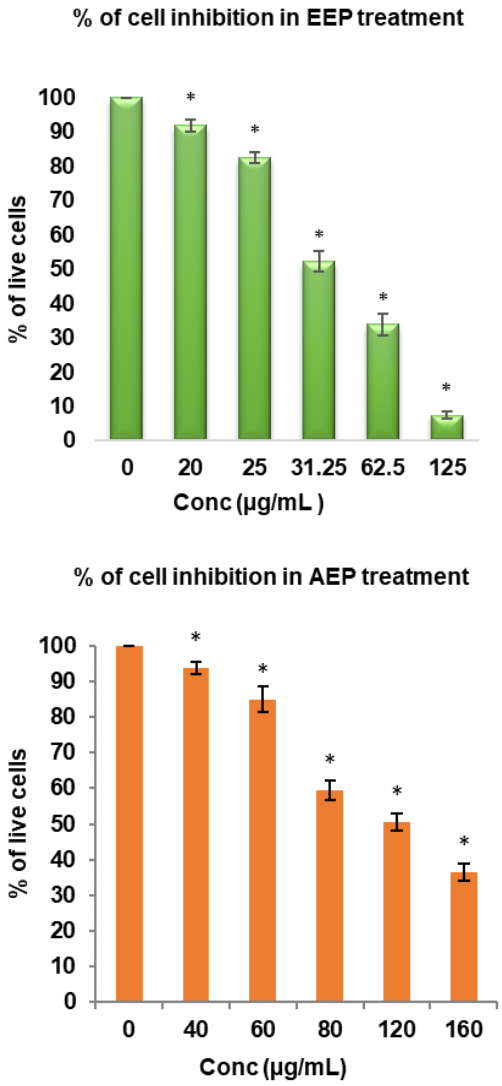

Figure 1: Viability of HeLa cells (%) when treated with (a) ethanolic extract of propolis (EEP) and (b) aqueous extract of propolis (AEP) for 72 hours of treatment. Results are mean ± SEM and significant at *$p < 0.05$ compared to control (untreated)

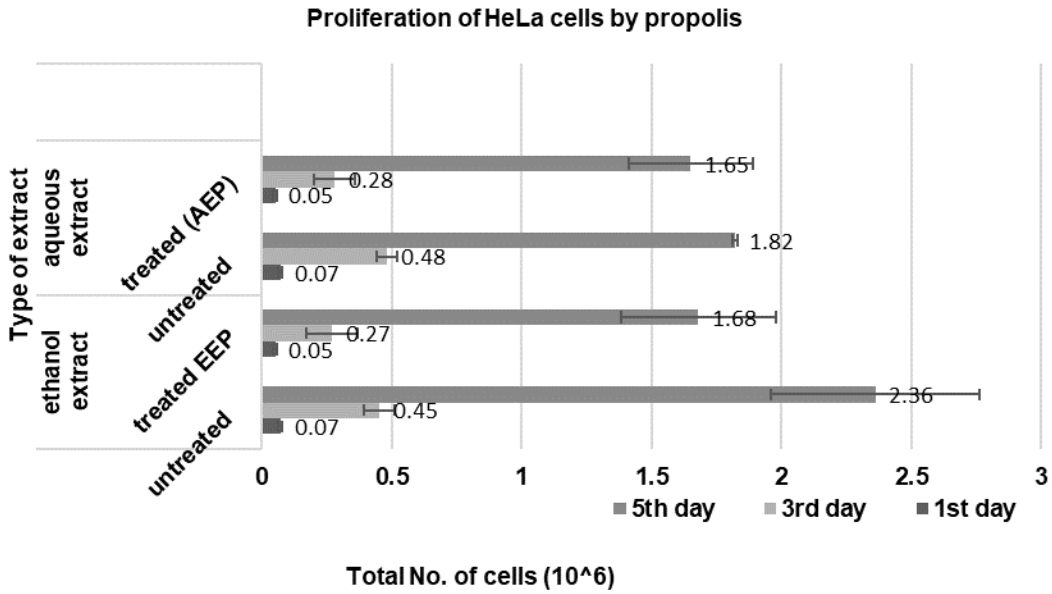

Figure 2: Effects on HeLa cells proliferation expressed in total no. of cell (x 10^6) within 5 days duration after treatment with ethanolic extract of propolis (EEP) and aqueous extract of propolis (AEP). Results are mean ± SEM and statistically significant different at *p < 0.01 compared to control (untreated)

Apoptosis and cell cycle arrest of HeLa cells by propolis extracts

EEP and AEP induced HeLa cell death at the late apoptosis phase as tabulated in Table 3. The IC_{50} concentration of EEP in treated cells caused an increase in the percentage of dead cells which is 30.57% (sum of early and late apoptosis). Similarly, the IC_{50} concentration of AEP extract also resulted in a significant increment of 17.4% HeLa cells death compared to the control ($p < 0.05$). The dose-dependent relationship was observed in both propolis extracts, as the percentage of cell death increased significantly up to 89.3 and 28.3 %, at a higher concentration of EEP (62.5 µg/mL) and AEP (200 µg/mL), respectively, compared to the untreated cells ($p < 0.05$).

Table 3: Tabulated data represents the cytotoxic effects of EEP and AEP on HeLa cells

HeLa cells conditions		Viable cells (%)	Apoptosis		Debris (%)
			Early (%)	Late (%)	
EEP	Untreated	88.13 ±7.24	0.77 ± 0.14	4.1 ± 1.05	0
	IC_{50} dose (31.25 µg/mL)	67.6 ± 3.15 *	6.6 ± 2.9	23.97± 0.7 *	1.83± 0.8
	Highest dose (62.5 µg/mL)	2.47 ± 1.09 *	16.6 ± 7.7	72.67± 6.81 *	1.17±0.31
AEP	Untreated	93.07±1.93	1.37 ± 0.58	5.5 ± 1.36	0.2 ± 0.1
	IC_{50} dose (120 µg/mL)	82.13± 2.03 *	2.9 ± 1.4	14.5 ± 0.6 *	0.5 ± 0.2
	Highest dose (200 µg/mL)	71.4 ± 5.9 *	7.36 ± 5.3	20.97 ± 0.69 *	0.17±0.07

Data represents Mean ± Standard Error of Mean (SEM). * Significant different ($p < 0.05$, One Way ANOVA, post-test Dunnett). EEP = Ethanolic extract of propolis; AEP = Aqueous extract of propolis.

In contrast to apoptosis, cell cycle arrest served the purpose to monitor cell cycle condition through every checkpoint. This analysis was carried out and the difference in cell cycle phase distribution of cells is presented in Table 4. In EEP-treated group, total number of cell was significantly higher at stage G0/G1 and lower at stage S and G2/M, compared to the untreated group ($p < 0.05$). Meanwhile in AEP-treated group, the total number of cells was significantly lower at stage G0/G1 and higher at stage S and G2/M, compared to the untreated group ($p < 0.05$).

Table 4: Tabulated data represents the cytotoxic effects of EEP and AEP on every stage of the cell cycle phase of HeLa cells

HeLa cells conditions	Percentage (%) of HeLa cells at different cell cycle phases		
	G0/G1	S	G2/M
Untreated	54.13 ± 1.8	35.15± 1.5	10.71 ± 0.28
Treated with EEP	70.60 ± 2.15* ↑	20.42 ± 2.04* ↓	8.97 ± 0.19* ↓
Untreated	68.76 ± 2.4	21.05± 2.8	10.19 ± 0.76
Treated with AEP	58.19 ± 1.84* ↓	29.68 ± 1.64* ↑	12.13 ± 0.39 ↑

Data represents Mean ± Standard Error of Mean (SEM). * Significant different ($p < 0.05$, Independent t-test analysis). Arrows indicated an increment or reduction of total number of cells. EEP = Ethanolic extract of propolis; AEP = Aqueous extract of propolis

Discussion

GC-MS analysis of the *Trigona apicalis* propolis extracts have detected some potential compounds that may be responsible for its biological activities. Prior studies on propolis of stingless bees have mentioned the presence of caffeic acid, coumaric acid, ferulic acid, quercetin, pinocembrin, chrysin, galangin, saponin, naringin and apigenin (15). Meanwhile, compounds reported in propolis of honeybee also listed the presence of caffeic acid, ferulic acid, galangin, chrysin alongside with benzoic acid, p-coumaric acid, kaempferol, pinobanksin, and naringenin (16), which show some degree of similarities between propolis of stingless bee and honeybee. Lanosterol is a tripertene, which was found in EEP, and has been reported to have positive pharmacological effects (4). Furthermore, aromadendrene oxide–(2) that was found in AEP has been reported to possess an antimicrobial effect (17). EEP exhibited a better scavenger than AEP in the antioxidant assays, where it scavenged 50% of the free radicals at the concentration of 1.305 mg/mL compared to 2.414 mg/mL of AEP. Nevertheless, a study reported that EEP has scavenging activity as high as 80% for DPPH assay and 89% for ABTS assay at concentration of 150 µl/mL (18). Variation in the antioxidant capacity may be due to the different geographical locations and species of stingless bee that contribute to the diversity of chemical content of the propolis extracts (19-21).

Since free radicals play a role in cancer development, the antioxidant properties of propolis extract may be linked to the presence of possible anticancer compound(s) (22). This study has proven the presence of potent anticancer ingredient(s) for both EEP and AEP by their ability to inhibit 50% growth of the HeLa cell at a relatively low concentration. The finding is supported by an in vitro study using HeLa cells, where prenylated benzophenone and isoprenylated benzophenone compounds isolated from the Brazilian propolis showed decent cytotoxic effects on HeLa cells at a concentration of 0.18 µM (22). In addition, the correlation analysis performed in this study has indicated that the antioxidant capacities on DPPH and ABTS are strongly correlated with the TFC and TPC, which have been exhibited in a few prior studies (23, 24).

The fact that both extracts induced late apoptosis in the HeLa cells, may indicate that the propolis extracts may have the ability as potent antiproliferative agent. Even across species, stingless bee reveals the anticancer characteristics like the study from Indonesian stingless bee (25), which showed 50% inhibition against cancer cell lines such as KATO-III and BT474 by the propolis produced by *T. incisa* and *T. fuscobalteta*. The importance of cell cycle analysis is concerning the DNA checkpoint mechanisms. When damage is detected, the cycle is abrupted and the cell attempts to complete DNA replication signaling cyclin-dependent kinases (CDKs) for the beginning of mitotic cell division.

Otherwise, the cell will repair the damaged DNA but may kill itself if the damage is unrepairable (26). Both EEP and AEP have blocked the HeLa proliferation by arrested the cycle at G0/G1 and S phase respectively. This finding is similar to the previous study of *T. incisa* propolis extract that arrest SW620 at G1 subphase (27). The potential of the stingless bee propolis in the local region of Malaysia is yet to be reported, but some general research on honey like Tualang honey reported to have an antinociceptive effect (28) and boosted CD4 count by causing viral load reduction in HIV positive subjects (29). Since the compounds in honey and propolis may overlap, the prior recorded data may be useful for future studies on stingless bee propolis.

Conclusion

Research on the bio products of stingless bee is considered new and there is still lack of medicinal properties recorded within the Asian region, including Malaysia. Interestingly current study managed to explore the potential of the two polar extracts which possess potential benefits for a huge scale production within the industry, especially in regards to the aqueous extract, since it is cheaper than any other organic solvents, yet exhibited relatively worthy antioxidant activity that is similar to ethanolic extract. Thus, it is crucial to bear in mind the significant role of polarity index of solvents used in determining the potency of anticancer activity of a propolis extract. The pharmacological findings of current study demonstrated that propolis produced by local stingless bee species has the potentials to be developed into a potent anticancer and chemo-preventive agent, particularly against the cervical cancer. Nevertheless, there are still much more to be explored in terms of the major phytochemical compounds responsible for the bioactivities, and the molecular mechanism involved that contributes to its effect.

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Competing interests

The authors declare that they have no competing interests.

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