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Cytotoxic Prenylflavanones from Philippine Stingless Bee (*Tetragonula biroi* Friese) Nests

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The dichloromethane crude extract and compounds, glyasperin A (**1**), propolin E (**2**) and propolin A (**3**), obtained from Philippine stingless bee (*Tetragonula biroi* Friese) nests, were evaluated for their cytotoxic potentials. The anti-proliferative activities of crude extract and compounds **1**-**3** against human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a normal cell line, human dermal fibroblast neonatal (HDFn), were evaluated using the PrestoBlue® cell viability assay. The crude extract was most anti-proliferative against HCT-116 cells (IC_{50} = 1.410 µg/mL). Glyasperin A (**1**) exhibited the strongest effect on MCF-7 cells (2.378 µg/mL). Propolin E (**2**) was most cytotoxic against HCT-116 cells (2.279 µg/mL), while propolin A (**3**) was most inhibitory against MCF-7 cells (2.815 µg/mL). Comparing the colorectal cancer cell lines, HCT-116 was generally more susceptible under crude extract, **2**, and **3** (IC_{50} = 1.410, 2.279, 3.013 µg/mL, respectively) than HT-29 (5.620, 5.320, 5.359). Comparing the activities of propolin E and propolin A against HCT-116 cells, **2** (IC_{50} = 2.279 µg/mL) was more cytotoxic than **3** (3.013). The crude extract was more cytotoxic than Zeocin against HCT-116 cells. To our best of knowledge, this is the first report on the anti-proliferative properties of crude extract and compounds from *T. biroi*.

Keywords: Cytotoxicity, Glyasperin A, Philippine stingless bee, Propolin A, Propolin E, *Tetragonula biroi* Friese.

INTRODUCTION

Propolis or "bee glue" is a resinous material collected by honeybees from tree buds, sap flows or other botanical exudates and is used for sealing and coating bee hives. For many centuries, aside from its use as food, beverage and food supplement, it has long been used extensively in traditional medicine because of its wide range of therapeutic values for the treatment of common cold, flu, upper respiratory tract infections, as dermatological preparations for wounds, burns, acne, herpes simplex, genitalis and neurodermatitis, as mouthwash and toothpaste to prevent caries and gingivitis and for cosmetic applications [1,2]. Propolis was shown to exhibit a broad spectrum of biological activities which include antimicrobial [3,4], antifungal [5,6], free radical scavenging [7,8], anti-HIV [9,10], anti-inflammatory [11,12] and anti-herpes [13,14]. A recent study reported that propolis, at a concentration of 0.01 % (w/v), exhibited antiproliferative and cytotoxic actions against human fibroblasts proliferation in cell culture, followed by mild cell necrosis [15]. High concentrations of ethanolic extracts of propolis were found to have an *in vitro* cytotoxic and genotoxic effect on human peripheral lymphocytes [16]. Recently, focus has been given to the understanding of actual chemical constituents of propolis and their corresponding biological activities.

The chemical composition of propolis is highly variable depending on the geographical area, prevailing environmental conditions and the plant species abundant around the bee hives [1,17-20]. The ethanolic extracts of propolis from different regions in Java were tested for cytotoxicity against tumor cell lines (T47D, MCF-7, Hela, Myeloma and Vero) using the MTT assay [21]. The propolis extract from Batang, Central Java showed the most potent activity against T47D and MCF-7 with IC_{50} values of 34.67 and 37.8 µg/mL, respectively, while the propolis extract from Sukabumi, West Java showed the most potent activity against Hela cell with an IC_{50} of 147.34 µg/mL. However, the propolis extracts were inactive against myeloma and Vero cells. Different types of propolis from Europe, New Zealand, Brazil and China were found to contain secondary metabolites such as flavonoids, phenolic acids, terpenoids, steroids and amino acids [3]. Propolis has been reported to contain β-amylase [22], polyphenolic compounds, flavones,

phenolic acid, esters [23-25] and fatty acids [26]. Artepillin C, isolated from Brazilian propolis was reported to exhibit cytotoxicity against HuH13 (human hepatocellular carcinoma) cells [27]. In another study, artepillin C exhibited potent cytocidal effects and induced marked levels of apoptosis in human leukemia cell lines of different phenotypes [28]. Clerodane diterpenes, 15-oxo-3,13Z-kolavadien-17-oic acid and 15-oxo-3Z,I3E-kolavadien-17-oic acid from Brazilian propolis damaged and retarded the growth of HuH13, KB, HeLa and HLC-2 cells with ID_{50} of 20-30 μg/mL [29]. Glyasperin A was earlier reported as a constituent of Philippine propolis [30]. Propolins with cytotoxic activities have been isolated from propolis. Propolins A-F, isolated from Taiwanese propolis, effectively induced human melanoma cell apoptosis and acted as strong antioxidant agents [31]. Propolin G, isolated from the Taiwanese propolis extract was reported as a potent-caspase-dependent inducer of apoptosis in brain cancer cells where the compound demonstrated a protective effect against oxidative stress in rat cortical neurons [32]. Another study reported that propolin H inhibited the proliferation of human lung carcinoma cell lines [33].

Using liquid chromatography-mass spectrometry (LC-MS), Philippine propolis afforded artepillin C and pinobanksin-3-O-hexanoate and these were identified as possible phenolic compounds present in the propolis of *T. biroi* and exudates from avocado (*Persea americana* Mill), jackfruit (*Artocarpus heterophyllus* Lam), mango (*Mangifera indica* L.), pili (*Canarium ovatum* Engl) and rambutan (*Nephelium lappaceum* L.) [34]. Other researches have been conducted to evaluate the bioactivities of Philippine propolis. In a study aimed at discovering novel antimicrobial drugs that suppress bacterial virulence *via* quorum sensing (QS) inhibition, the ethanol extract of Philippine propolis was tested for its quorum sensing inhibitory potential in *Chromobacterium violaceum* and *Pseudomonas aeruginosa* [35]. Recently, a patch containing Philippine honey and propolis was developed for possible rapid and effective anti-microbial and wound-healing applications [36]. The potential of propolis against tooth decay caused by *Streptoccocus mutans* was recently studied in Philippines [37].

Reviews on the chemical constituents and biological properties of propolis have been provided by several authors [6,32,38-41]. Previously, the isolation of glyasperin A, propolin E, propolin A, propolin H, squalene, a mixture of lupeol, α-amyrin and β-amyrin and another mixture of urs-12-en-3-one, olean-12-en-3-one and lup-12-en-3-one from dichloromethane extracts of propolis collected from the stingless bee (*Tetragonula biroi* Friese) hives in San Roque, Sorsogon, Philippines was reported [30]. This study was expanded and we report herein the cytotoxicity potentials of the dichloromethane crude extract as well as those of the compounds, glyasperin A (**1**), propolin E (**2**) and propolin A (**3**), obtained from *T. biroi* bee hives, against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116) and a normal cell line, human dermal fibroblast, neonatal (HDFn). To the best of our knowledge, this is the first report on the anti-proliferative properties of dichloromethane crude extract of propolis obtained from Philippine stingless bee (*Tetragonula biroi* Friese) nests and compounds **1-3**, isolated from this extract, against the human cancer-cell lines used.

EXPERIMENTAL

The Philippine propolis used in this study was collected from bee (*Tetragonula biroi* Friese) hives in San Roque, Sorsogon, Philippines in May 2012.

Isolation and structure elucidation: The extraction of crude extract and the isolation and structure elucidation of compounds **1-3** from *T. biroi* were reported previously [30].

Preparation of compounds for cytotoxicity tests: The crude extract and compounds **1-3** from *T. biroi* were dissolved in dimethyl sulfoxide to make 4 mg/mL stock solution. Working solutions were prepared in complete growth medium to a final non-toxic DMSO concentration of 0.1 %.

Preparation of cell lines for cytotoxicity tests: The effects on the cell proliferation of compounds **1-3** from dichloromethane extracts of *T. biroi* were tested on the following human cell lines: breast cancer (MCF-7) and colon cancer (HCT-116 and HT-29) (ATCC, Manassas, Virginia, U.S.A.) and human dermal fibroblast-neonatal (HDFn; Invitrogen Life Technologies, U.S.A.), which are routinely maintained at the Cell and Tissue Culture Laboratory, Molecular Science Unit, Center for Natural Science and Environmental Research, De La Salle University, Manila, Philippines. Following standard procedures [42,43], cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, USA) containing 10 % fetal bovine serum (FBS, Gibco®, USA) and 1x antibiotic-antimycotic (Gibco®, USA) and kept in an incubator (37 °C, 5 % CO_2, 98 % humidity). At about 80 % confluence, the monolayers were prepared for cell counting and inoculation. The cells were washed with phosphate-buffered saline (PBS, pH 7.4, Gibco®, USA), trypsinized with 0.05 % Trypsin-EDTA (Gibco®, USA) and resuspended with fresh complete media. Cells were counted following standard trypan blue exclusion method [42] using 0.4 % Trypan Blue Solution (Gibco®, USA). Cells were seeded in 100 μL aliquots into a 96-well microtiter plate (Falcon™, USA) using a final inoculation density of 1×10^4 viable cells/well. The plates were further incubated overnight (37 °C, 5 % CO_2, 98 % humidity) until cell attachment was achieved. These monolayer cultures were used for the cytotoxicity studies as described below.

Cell viability assay: The cytotoxicity of the crude extract and compounds from *T. biroi* nests was determined in an *in vitro* cell viability test using PrestoBlue® (Molecular Probes®, Invitrogen, USA). This bioassay is based on the ability of viable cells with active enzymes, mitochondrial reductases of the electron transport chain, to convert the resazurin dye (blue and non-fluorescent) to resorufin (red and highly fluorescent.) The conversion is proportional to the number of metabolically active cells and is determined quantitatively using either absorbance or fluorescence measurements. To the monolayers in the microtiter plate, 100 μL of filter-sterilized **1-6** were added to corresponding wells at two-fold serial dilutions to make final screening concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 μg/mL, respectively [43]. Wells with no com-pound added served as negative controls, wells with Zeocin™ (Gibco®, USA) served as positive controls and wells containing only cell culture media were used to correct for background color. The cells were further incubated (37 °C, 5 % CO_2, 98 % humidity) for 4 days. Ten microliters of PrestoBlue® was added to each well. The cells were incubated further for 2 h (37 °C,

5 % CO_2, 98 % humidity). Absorbance was measured using a BioTek ELx800 absorbance microplate reader (BioTek® Instruments, Inc., U.S.A.) at 570 nm and normalized to 600 nm values (reference wavelength). Absorbance readings were used to calculate for the cell viability for each compound concentration following the equation below.

$$\text{Cell viability (\%)} = \frac{(\text{Absorbance of treated sample} - \text{Absorbance of blank})}{(\text{Absorbance of negative control} - \text{Absorbance of blank})} \times 100$$

Non-linear regression and statistical analyses were done using GraphPad Prism 7.02 (GraphPad Software, Inc.) to extrapolate the half maximal inhibitory concentration, IC_{50}, the concentration of the compound which resulted in a 50 % reduction in cell viability. The cytotoxicity (anti-proliferative potential) of **1**-**6** was expressed as IC_{50} values. All tests were performed in triplicates and data were expressed as means ± SEM. The extra sum-of-squares F test was used to evaluate the differences in the best-fit parameter (half maximal inhibitory concentration) among data sets (treatments) and to determine the differences among dose-response curve fits following the software's manual. One-way ANOVA was used to determine differences in IC_{50} under different treatments, followed by Tukey's multiple comparison post hoc test to evaluate differences between pairs of data. Results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Dichloromethane crude extract, as well as those of the compounds, glyasperin A (**1**), propolin E (**2**) and propolin A (**3**), obtained from the hives of the Philippine stingless bee, *Tetragonula biroi*, were evaluated for their anti-proliferative activities against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a human normal cell line, human dermal fibroblast-neonatal (HDFn), using the *in vitro* PrestoBlue® cell viability assay. Zeocin, a known anti-cancer drug was used as positive control. The % cell viability as a function of the logarithmic values of compound concentration is shown in Figs. 1 and 2. Overall, the curves follow the typical sigmoidal curve which is characteristic of an inhibitory dose-response relationship between treatments and cell viability. Fig. 1 shows the anti-proliferative effects per cell line while Fig. 2 shows the effects per treatment. The extrapolated IC_{50} values are summarized in Table-1 and Fig. 3.

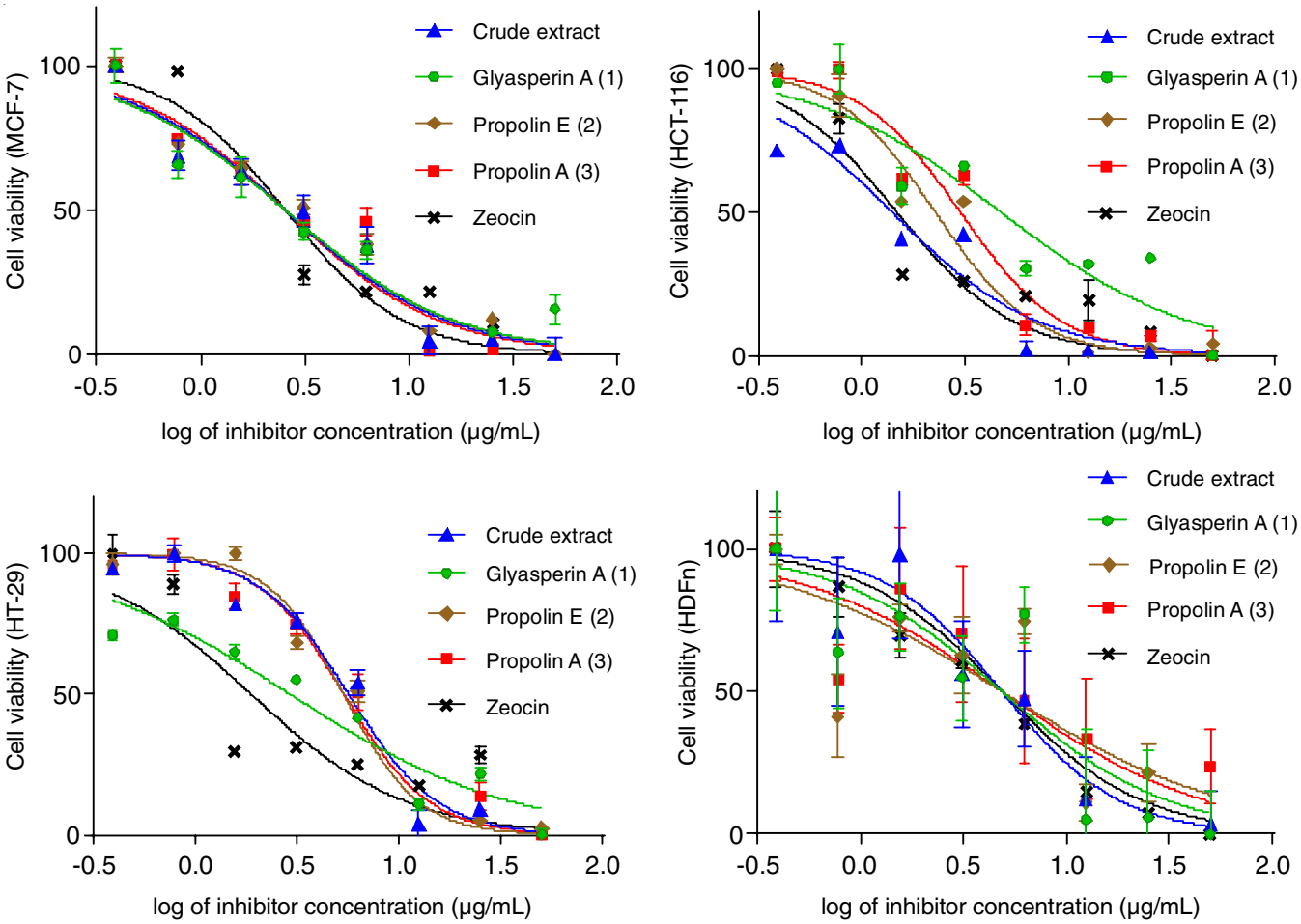

Fig. 1. Dose-response curves showing the cytotoxic activities of crude extract, compounds **1-3** and Zeocin on the cell viability of MCF-7, HCT-116, HT-29 and HDFn. Each plot shows the effects per cell line. Data represents means ± SEM. GraphPad Prism 7.02 was used to perform extra sum-of-squares F test to (A) evaluate if the best-fit parameter (half maximal inhibitory concentration) differs among data sets (treatments) and (B) determine the differences among the dose-response curve fits. The results are: MCF-7 (A) F(DFn, DFd) = F(5, 132) = 0.9687, p = 0.4395 and (B) F(10, 132) = 1.238, p = 0.2726; HCT-116 (A) F(5, 131) = 9.85, p < 0.0001 and (B) F(10, 131) = 6.588, p < 0.0001; HT-29 (A) F(5, 131) = 11.94, p < 0.0001 and (B) F(10, 131) = 12.2, p < 0.0001; HDFn (A) F(5, 132) = 1.177, p = 0.3237 and (B) F(10, 132) = 1.273, p = 0.252

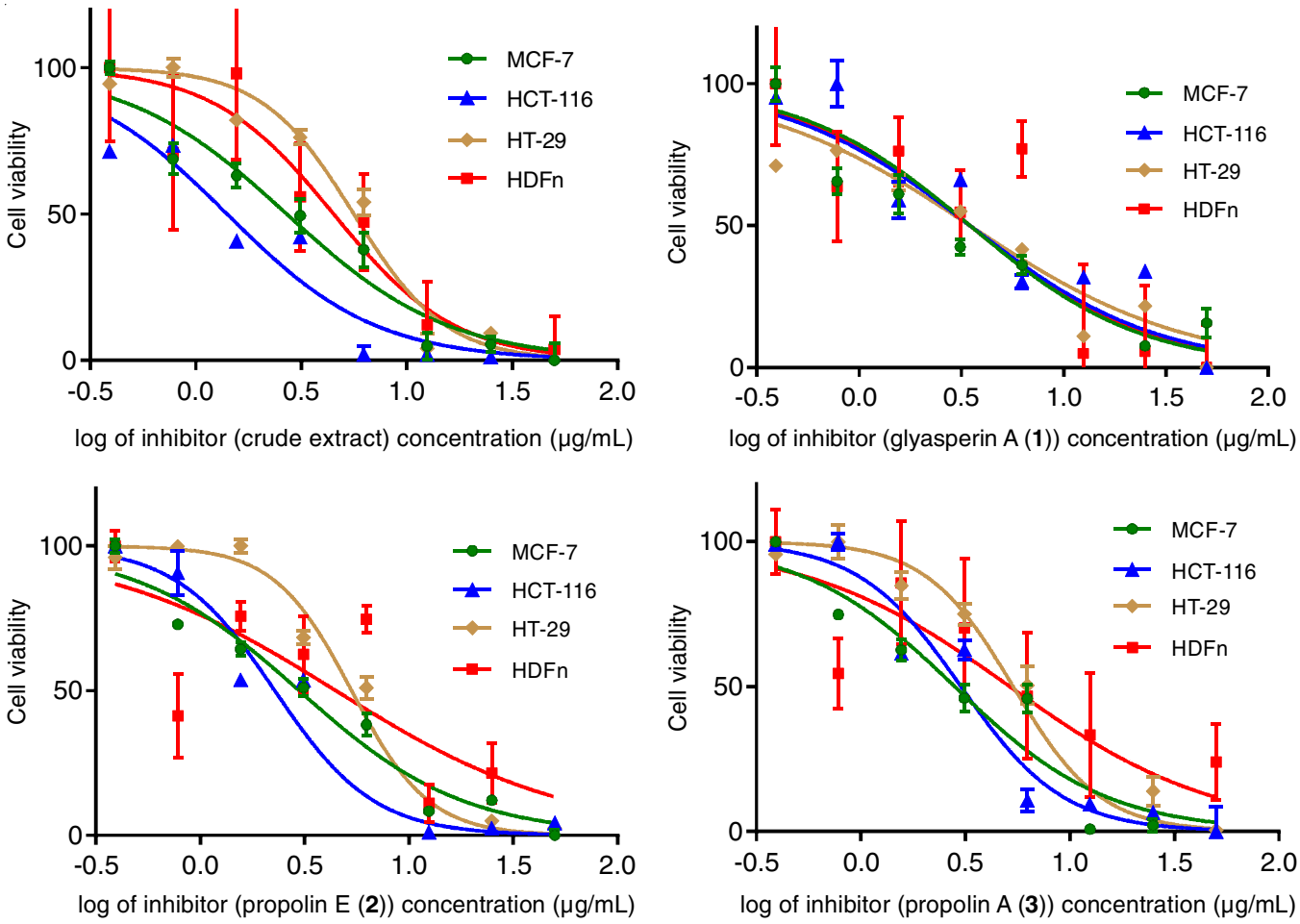

Fig. 2. Dose-response curves showing the cytotoxic activities of crude extract, compounds **1-3** and Zeocin on the cell viability of MCF-7, HCT-116, HT-29 and HDFn. Each plot shows the effects per treatment. Data represents means ± SEM. GraphPad Prism 7.02 was used to perform extra sum-of-squares F test to (A) evaluate if the best-fit parameter (half maximal inhibitory concentration) differs among data sets (treatments) and (B) determine the differences among the dose-response curve fits. The results are: crude extract (A) F(DFn, DFd) = F(3, 87) = 11.1, $p < 0.0001$ and (B) F(6, 87) = 5.72, $p < 0.0001$; **1** (A) F(3, 87) = 1.975, p = 0.1236 and (B) F(6, 87) = 1.086, p = 0.3771; **2** (A) F(3, 88) = 8.615, $p < 0.0001$ and (B) F(6, 88) = 8.087, $p < 0.0001$; **3** (A) F(3, 88) = 3.545, p = 0.0178 and (B) F(6, 88) = 2.972, p = 0.0109

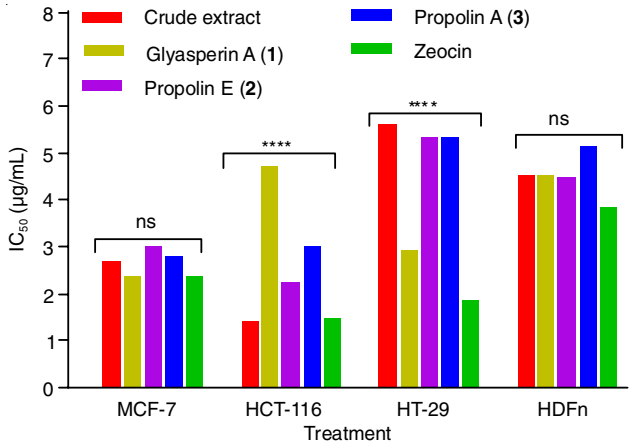

Fig. 3. Comparative cytotoxic activities (IC_{50}) of crude extract, compounds **1-3** (collected from *Tetragonula biroi* Friese bee hives) and Zeocin against MCF-7, HCT-116, HT-29 and HDFn cells

TABLE-1
CYTOTOXIC ACTIVITIES (IC_{50}) OF CRUDE EXTRACT, COMPOUNDS **1-3** (FROM *Tetragonula biroi* Friese BEE HIVES) AND ZEOCIN AGAINST MCF-7, HCT-116, HT-29 AND HDFn CELL LINES

Sample	MCF-7	HCT-116	HT-29	HDFn
Crude extract	2.704	1.410	5.620	4.506
1	2.378	4.736	2.916	4.537
2	2.983	2.279	5.320	4.474
3	2.815	3.013	5.359	5.166
Zeocin	2.382	1.487	1.873	3.863

*IC_{50} values (µg/mL) were extrapolated from dose-response curves generated from nonlinear regression analysis performed using GraphPad Prism 7.02. For each cell line, one-way ANOVA was conducted to determine differences between data sets (treatments). Results of the Tukey's multiple comparison post hoc test are discussed in this section. Treatments: crude extract, glyasperin A (**1**), propolin E (**2**) and propolin A (**3**). Cell lines: human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and normal cell line, human dermal fibroblast neonatal (HDFn).

The crude extract and compounds **1**, **2** and **3**, exhibited strong anti-proliferative effects against the breast cancer (MCF-7) cell line, with IC_{50} values of 2.704, 2.378, 2.983 and 2.815 µg/mL, respectively. One-way ANOVA, followed by Tukey's multiple comparison post hoc test revealed that there are no pairwise differences between crude extract and compounds **3**, and **2** and **3** ($p > 0.05$).

The crude extract exhibited the strongest anti-proliferative effect against HCT-116 (IC_{50} = 1.410 µg/mL), followed by **2**, **3** and **1** (IC_{50} values of 2.279, 3.013 and 4.736 µg/mL, respectively). Pair-wise comparisons revealed significant differences between all pairs of treatments from *T. biroi* ($p < 0.0001$).

Glyasperin A (**1**) was most cytotoxic against HT-29 (IC_{50} = 2.916 µg/mL). Compounds **2** and **3** and crude extract exhibited nearly the same anti-proliferative effects (5.320, 5.359 and 5.620 µg/mL, respectively) and the post hoc test verified that there are no significant differences between crude extract and **2**, crude extract and **3**, and **2** and **3** ($p > 0.05$).

Moderate cytotoxicity was observed in HDFn cells with IC_{50} values of 4.506, 4.537, 4.474 and 5.166 µg/mL for crude extract, compounds **1**, **2** and **3**, respectively. However, multiple comparison post hoc test results revealed that there are no statistical differences between pairs of treatments from *T. biroi* ($p > 0.05$).

Comparing the overall cytotoxic effects of propolin E (**2**) and propolin A (**3**) against the cell lines tested, the data showed that compounds **2** and **3** are not statistically different when used against MCF-7, HT-29 and HDFn cells ($p > 0.05$). Hence, it is only against HCT-116 that compound **2** (IC_{50} = 2.279 µg/mL) is more cytotoxic than compound **3** (IC_{50} = 3.013 µg/mL) ($p < 0.0001$) although both values indicate strong anti-proliferative effects.

The study also revealed that the cytotoxic activities of crude extract and **1-3** were a function of the specific type of cancer cells targeted. Comparing the two colorectal cancer cell lines, HCT-116 and HT-29, the IC_{50} values of crude extract and **1-3** for HCT-116 were generally lower as verified statistically where the results for the two cell lines were found significantly different ($p < 0.0001$) for all pairwise analyses made. This implies that HCT-116 cells are more responsive to the treatments used. Differences in treatment response between colon cancer cell lines were also observed in previous studies [44-46]. It was reported that variations in the expression profiles of genes associated with drug sensitivity in HCT-116 and HT-29 cells could be an important factor dictating how the cells respond to inhibitory compounds [47]. In a previous work, differences in the sensitivity of HCT-116 and HT-29 cells against known metabolic stressor molecules, ribavirin and metformin, were found to be due to the genetic and metabolic activities of the cell lines [48]. Another study showed that variations in cellular response in four human colon cancer cells (HCT-116, HT-29, HCT-15, KM-12) are related to the inhibition of signal transduction by 17-allylamino-17-demethoxygeldanamycin, an inhibitor of the hsp90 molecular chaperone [49].

Overall, crude extract, compounds **1**, **2** and **3** exhibited the strongest anti-proliferative effects against MCF-7 cells, followed by HT-116 and HT-29. The samples also exhibited cytotoxic activities against the normal cell line, HDFn. The known anti-cancer drug, Zeocin, showed strong but varying anti-proliferative activities as expected. In general, crude extract, compounds **1**, **2** and **3** showed varying, but promising cytotoxic properties. The US National Cancer Institute has defined the active cytotoxic limits of natural products as 20 µg/mL or less for crude extracts and 4 µg/mL or less for pure compounds [50]. Pure compounds that exhibit active cytotoxicity may have some potential for further drug development [43]. The dichloromethane extract of Philippine propolis and the isolated compounds (**1-3**) exhibited high cytotoxic activities against the cancer cell lines tested with IC_{50} values ranging from 1.410 to 5.620 µg/mL. Therefore, the results proved that crude extract, compounds **1**, **2** and **3,** isolated from the dichloromethane extracts of *T. biroi* bee hives can be further evaluated for the treatment of cancer, especially human breast cancer and colorectal cancer (HCT-116 type) (Fig. 3).

Previous studies revealed that propolis crude extract, as well as the compounds, glyasperin A, propolin E and propolin A, exhibited cytotoxic activities.

Propolis from different parts of the world have been reported to exhibit cytotoxic properties against different cancer cell lines. Results of our studies corroborate earlier findings on the cytotoxicity of propolis. The ethanolic extract of Indonesian propolis was reported to be cytotoxic against the breast carcinoma cell, MCF-7, with an IC_{50} value of 37.8 µg/mL, while the extract of Philippine propolis used in this study gave 2.704 µg/mL [21]. The difference in cytotoxicities of Indonesian and Philippine propolis may be attributed to different chemical compositions of the extracts due mainly to the different geographical sources where the samples were obtained. The ethanolic extract of Chinese propolis (EECP) showed antitumor effects against MCF-7 and MDA-MB-231 cells by inducing apoptosis, regulating the levels of ANXA7, p53 and NF-κB p65, upregulating intra-cellular ROS and decreasing mitochondrial membrane potential [51]. Supplementation of propolis with radiotherapy treatment was reported to offer protection against DNA damage caused by ionizing radiation in breast cancer patient leukocytes [52]. New Zealand propolis yielded phenolic compounds, pinocembrin, pinobanksin-3-O-acetate, tectochrysin, dimethylallyl caffeate, 3-methyl-3-butenyl caffeate, benzyl ferulate and benzyl isoferulate, which showed good broad-spectrum activity in anti-proliferative assays against three other gastro-intestinal cancer cell lines, HCT-116 colon carcinoma, KYSE-30 oesophageal squamous cancer and NCI-N87 gastric carcinoma [53].

Glyasperin A, isolated from the acetone extract of the leaves of *Macaranga gigantea*, was shown to exhibit cytotoxic activity against murine leukemia P-388 cells with an IC_{50} value of 6.0 µM [54]. In another study, the same compound gave IC_{50} values of < 8 µg/mL against oral squamous cell carcinoma (HSC-2) [55]. Propolin A exhibited cytotoxic properties against human melanoma, C6 glioma, and HL-60 with IC_{50} values of 6.0, 3.5 and 7.5 µg/mL, respectively [56]. In another study, propolins A and B induced cytotoxicity effects against human melanoma A2058 cells and were also found as strong anti-oxidants [57].

Although compounds **1-3** have been shown to exhibit cytotoxic properties in previous studies, the cancer cell lines tested and/or the type and polarities of the extracting solvents were different from those used in this study. Thus, comparison of the cyto-toxic activities of compounds **1-3** with earlier studies could not be made completely.

Conclusion

The dichloromethane crude extract from Philippine stingless bee (*Tetragonula biroi* Friese) nests, as well as the isolated

compounds, glyasperin A (**1**), propolin E (**2**) and propolin A (**3**), exhibited strong to moderate cytotoxic activities, based on their anti-proliferative effects against three human cancer cell lines, breast (MCF-7) and colon (HT-29 and HCT-116), and a normal cell line, human dermal fibroblast - neonatal (HDFn). The crude extract was found most cytotoxic against HCT-116 cells (IC_{50} = 1.410 µg/mL). Glyasperin A (**1**) exhibited the strongest effect on MCF-7 cells (IC_{50} = 2.378 µg/mL). Propolin E (**2**) was most potent against HCT-116 cells (IC_{50} = 2.279 µg/mL) while propolin A (**3**) was most anti-proliferative against MCF-7 cells (IC_{50} = 2.815 µg/mL). The samples also exhibited cytotoxic activities against the normal cell line, HDFn (IC_{50}< 6 µg/mL). Zeocin, as expected, exhibited varying anti-proliferative effects with the strongest activity seen in HCT-116 cells (IC_{50} = 1.487 µg/mL). The two colorectal cancer cell lines responded well under the treatments. However, HCT-116 cells were generally found more susceptible compared to HT-29 cells with the IC_{50} values of crude extract, **2** and **3** for HCT-116 lower (IC_{50} = 1.410, 2.279, 3.013 µg/mL, respectively) than HT-29 (IC_{50} = 5.620, 5.320, 5.359 µg/mL, respectively). Comparing propolin E (**2**) and propolin A (**3**), the results showed strong to moderate anti-proliferative effects although significant difference was only seen in HCT-116 cells where**2** (IC_{50} = 2.279 µg/mL) was more cytotoxic than **3** (IC_{50}= 3.013 µg/mL). Overall, crude extract, **1**, **2** and **3** exhibited potentially strong cytotoxic activities against the human cancer cell lines, MCF-7, HT-116 and HT-29.

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