RESEARCH ARTICLE

Cytotoxic Activity of Propolis Extracts from the Stingless Bee *Trigona Sirindhornae* Against Primary and Metastatic Head and Neck Cancer Cell Lines

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Abstract

Background: Propolis, a resinous substance produced by the honeybee, has a wide spectrum of potent biological activities. However, anti-cancer activity of propolis obtained from *Trigona sirindhornae*, a new species of stingless bee, has not yet been reported. This study concerned cytotoxicity of propolis extracts from *T. sirindhornae* against two head and neck squamous cell carcinoma (HNSCC) cell lines. **Materials and Methods:** A dichloromethane extract of propolis (DMEP) was prepared generating 3 fractions: DMEP-A, DMEP-B, and DMEP-C. Genetically-matched HNSCC cell lines derived from primary (HN30) and metastatic sites (HN31) in the same patient were used to study cytotoxic effects of the DMEPs by MTT assays. The active compounds in the DMEPs were analyzed by reverse-phase high performance liquid chromatography. **Results:** DMEP-A exhibited cytotoxic activity on HN30 cells with significantly decreased viability at 200 µg/ml compared with the control ($p<0.05$). However, no significant cytotoxic effect was evident in HN31 cells. DMEP-B and DMEP-C significantly decreased the viability of both cell lines from 100–200 µg/ml and 50–200 µg/ml, respectively ($p<0.05$). Interestingly, HN31 cells were more toxically sensitive compared with the HN30 cells when treated with DMEP-B and DMEP-C. IC_{50} values for DMEP-B with HN30 and HN31 cells were more than 200 µg/ml and 199.8±1.05 µg/ml, respectively. The IC_{50} of DMEP-C to HN30 and HN31 cells was found to be 114.3±1.29 and 76.33±1.24 µg/ml, respectively. Notably, apigenin, pinocembrin, p-coumaric acid, and caffeic acid were not detected in our propolis extracts. **Conclusion:** *T. sirindhornae* produced propolis displays cytotoxic effects against HNSCC cells s. Moreover, DMEP-B and DMEP-C differentially inhibited the proliferation of a metastatic HNSCC cell line.

Keywords: Cytotoxic activity- HNSCC cell lines-*Trigona sirindhornae* propolis

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Introduction

Propolis is a resinous material that bees collect from various parts of plants and use for sealing hive pores and protecting against microbes in the bee hive (Oses et al., 2016). More than 300 constituents have been identified in different propolis types (Wagh, 2013). The main chemical components of propolis are flavonoids, various phenolic and aromatic compounds, amino acids, minerals, and sugars. The active compounds extracted from propolis including phenolic, flavonoids, flavones, and fatty acids have diverse therapeutic effects such as anti-microbial, anti-inflammation, wound healing, and anti-cancer activities (Sforcin, 2016). Thus, the biological effects of propolis depend on the extracted active constituents and geographic regions from where the propolis was collected (Oses et al., 2016).

Propolis and its extracted compounds are cytotoxic to various tumor cells (Watanabe et al., 2011). The ethanolic extracts of propolis from *Trigona laeviceps* demonstrated anti-proliferative activities on human colon carcinoma cell lines including CACO-2, HCT116, HT-29, and SW480 cells, and caused marked dose-dependent growth inhibition (Ishihara et al., 2009; Umthong et al., 2009). Moreover, the crude hexane and dichloromethane extracts of *Apis mellifera* propolis from Thailand displayed anti-proliferative and cytotoxic activities on cancer cell lines that were derived from breast cance (BT474), undifferentiated lung carcinoma (ChaCo), liver hepatoblastoma (Hep-G2), gastric carcinoma (KATO-III), and colon adenocarcinoma (SW620) (Teerasripreecha et al., 2012). In addition, Turkish propolis was cytotoxic to bladder cancer cells by decreasing cell division (Erhan Eroglu et al., 2008). Recently, a pilot study on the effect of propolis on the oral health of head and neck cancer patients implied that a water based propolis extract efficiently

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prevents and heals radiotherapy induced mucositis in patients (Javadzadeh Bolouri et al., 2015). Moreover, caffeic acid phenethyl ester (CAPE), an active component in propolis, was used to treat oral cancer cells in vitro (Kuo et al., 2015). The results indicated that CAPE effectively suppressed the proliferation and survival of these cells.

The anti-tumorigenic effects of propolis are related to geographic location and bee species, however, their cytotoxicity on head and neck squamous cell carcinoma (HNSCC) cells is unclear. Therefore, this study investigated the cytotoxic activity of propolis that was extracted from *Trigona sirindhornae* (Michener and Boongird, 2004), a newly identified species of stingless bee that is typically found in Thailand. We hypothesized that *T. sirindhornae* propolis would be cytotoxic to HNSCC cell lines. We compared the cytotoxic effects of propolis fractions on primary and metastatic head and neck cancer cell lines.

Materials and Methods

Chemicals

Apigenin, pinocembrin, p-coumaric acid, caffeic acid, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Propolis collection

Propolis samples were collected from *T. sirindhornae* located in Chantaburi province, in eastern Thailand, during the summer. The samples were kept desiccated and in the dark until processed.

Propolis extraction

Propolis extraction was performed with modifications from a previous report. (Banskota et al., 1998) Propolis (100 g) was cut into small pieces and extracted using 80% (v/v) ethanol (400 ml) at 25°C with stirring for 18 h. The solution separated into an ethanolic and an aqueous phase (lower part). The aqueous phase was collected and reextracted with 80% ethanol. The pooled extracts were evaporated using a rotary vacuum evaporator (Rotovapor R-215, BUCHI Labortechnik, AG, Switzerland) at 40°C. A viscous residue was obtained and dissolved in methanol/water (60:40, v/v). An equal volume of hexane was added and stirred until the methanolic phase (lower part) separated. The methanolic extract was fractioned with 80% (v/v) ethyl acetate into 3 fractions: A, B, and C. The aqueous phase of each fraction was collected for further extraction with dichloromethane to yield the dichloromethane extract of propolis (DMEP). The DMEP was reextracted with dichloromethane. The pooled DMEP from fraction A, B, and C was designated DMEP-A, DMEP-B, and DMEP-C, respectively. The DMEPs were evaporated and kept at -20 °C until used for the experiments.

Cell culture

We aimed to determine effects of propolis extracts on cancer cells. Therefore genetic-matched HNSCC cell lines derived from primary and metastatic sites of the same patient were used in our study (Cardinali et al., 1995). HN30 and HN31 cells were obtained from primary pharynx lesions and lymph node metastases, respectively. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and an anti-fungal agent. The cells were cultured in a 37°C humidified 5% CO_2 atmosphere. The cells were passaged with 0.25% trypsin-EDTA when reaching 90-100% confluence. Only cultures with at least 95% cell viability were used in the experiments.

MTT assay

Cells were seeded in 96-well plates at a density of 2,000 cells/well/100µl and incubated in a 37 °C humidified 5% CO_2 atmosphere. The cells were treated with a range of concentrations (50, 100, and 200 µg/ml) of the DMEP fractions diluted in growth medium. Cells in growth medium served as control. After a 72 h incubation, the amount of viable cells in each treatment group were determined using thiazolyl blue tetrazolium bromide (MTT, Sigma). The medium was removed, 150 µl of fresh media was added, followed by adding 50 µl/well of 2 mg/ml MTT solution. The plates were incubated for 4 h at 37°C in a 5% CO_2 incubator. The precipitated formazan crystals were solubilized in DMSO (200 µl/well). The absorbance of the resulting solution was measured at 570 nm by a microplate reader (Tecan, Switzerland) and converted to percent of viable cells compared with control. Cell viability (%) was determined as follows: cell viability (%) = (Abs570 treated cells/ Abs570 control cells) × 100%. DMEP-A, DMEP-B, and DMEP-C were each evaluated using 3 independent experiments.

The inhibitory concentration (IC_{50}) values were calculated by non-linear regression with a one phase exponential association equation using Prism GraphPad version 5.0 (GraphPad Software, San Diego, CA).

High performance liquid chromatography (HPLC) analysis

Reverse-phase HPLC (RP-HPLC) analysis of the DMEP fractions was performed using an HPLC Spectra system equipped with a P4000 pump and a quaternary gradient pump system, a UV6000LP diode-array detector, and an AS3000 autosampler (Thermo Separation Products, Fremont, CA). The column was a Hypersil C18 (250 mm x 4.6 mm, 5-µm particle size). DMEPs (1–5 µg/ml) were dissolved in acetonitrile and filtered through a 0.22 µm filter. Twenty microliters of each sample solution were injected into the HPLC. The flavonoids and cinnamic acid derivatives in the DMEP extracts were identified using flavonoid standards for apigenin, pinocembrin, and cinnamic acid derivatives (p-coumaric acid and caffeic acid). Each standard was dissolved in acetonitrile to 0.3 µg/ml. Twenty microliters of the standard solutions and samples were injected into the HPLC. Elution was performed at a flow rate of 1ml/min using a linear gradient of 0.1% trifluoroacetic acid in an acetonitrile-water mixture. Chromatograms were recorded at 220

and 254 nm.

Statistical analyses

The results are presented as the means and standard error of the mean (SEM) of three identical experiments. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni posttest using Prism GraphPad 5.0. A p-value <0.05 was considered as statisticaly significant.

Results

Cytotoxicity of T. sirindhornae propolis fractions on HNSCC cell lines

The three propolis extract fractions were tested for their cytotoxicity on HN30 and HN31 cell lines (Figure 1). The results showed that DMEP-A reduced HN30 cell viability in a dose-dependent manner (Figure 1A). Moreover, HN30 cell viability was significantly decreased when exposed to 200 µg/ml DMEP-A compared with control ($p<0.05$). Although DMEP-A treatment of HN31 cells resulted in lower cell numbers compared with control, these differences were not significant ($p>0.05$). DMEP-A (200 µg/ml) exhibited a significant cytotoxic effect on HN30 cells compared with that of HN31 cells ($p<0.05$). In contrast, the remaining 2 DMEP fractions were significantly cytotoxic to both HNSCC cell lines. DMEP-B significantly decreased the viability of HN30 cells at 100 and 200 µg/ml compared with control

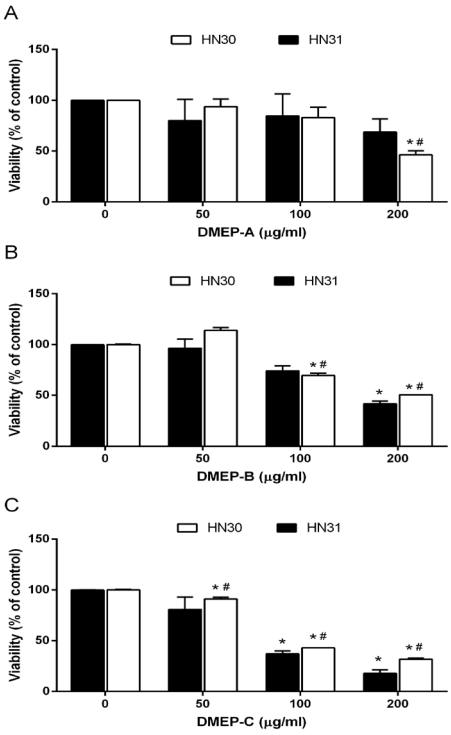

Figure 1. *Cytotoxic* Activity of DMEPs on HN30 and HN31 Cell Measured by MTT assay. Cells were treated with DMEP-A (A), DMEP-B (B) and DMEP-C (C) at a range of concentrations; 50, 100, and 200 µg/ml for 72 h. Bars represent means±SEM (n=3). * indicates $p<0.05$ compared to the control, # indicates $p<0.05$ compared to its counterpart cell line at the same concentration.

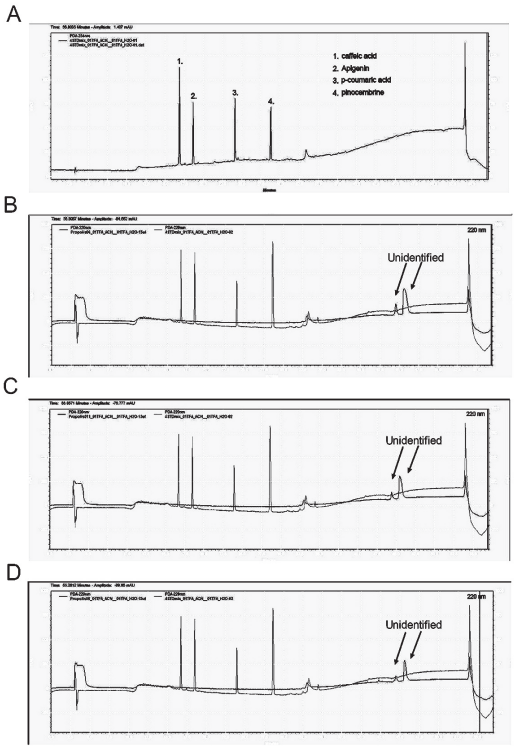

Figure 2. RP-HPLC Analysis of Propolis from *T. Sirindhornae* (column: 250 mm X 4.6 mm, 5 µM,λ 250 nm). Standard compounds: caffeic acid (1), apigenin (2), p-coumaric acid (3), and pinocembrin (4) (A). Component analysis of DMEP-A (B), DMEP-B (C) and DMEP-C (D) are indicated by the black lines.

($p<0.05$). In addition, DMEP-B significantly decreased the viability of HN31 cells at 200 µg/ml compared with control ($p<0.05$). DMEP-B (100 µg/ml) was more toxic to HN30 cells compared with HN31 cells ($p<0.05$). However, DMEP-B (200 µg/ml) was more toxic to HN31cells than it was to HN30 cells ($p<0.05$) (Fig. 1B). DMEP-C from 50 to 200 µg/ml significantly decreased the viability of HN30 cells compared with control ($p<0.05$). DMEP-C significantly reduced HN31 cell viability at 100 and 200 µg/ml compared with control ($p<0.05$). Moreover, DMEP-C was more toxic to HN31 cells than it was to HN30 cells at 100 and 200 µg/ml ($p<0.05$) (Figure 1C).

Although it is relatively difficult to obtain IC_{50} in the low range of concentrations, our results suggested that DMEP-B-treated HN30 cells had an IC_{50} value greater than 200 µg/ml that was higher than that of the HN31 cells at 199.8±1.05 µg/ml. Interestingly, DMEP-C showed IC_{50} values against HN30 and HN31 cells at 114.3±1.29 and 76.33±1.24 µg/ml, respectively.

HPLC analysis of the propolis extracts

To determine the active components in our propolis extracts, HPLC analysis was performed. HPLC chromatograms of the extracts were compared to the apigenin, pinocembrin, p-coumaric acid, and caffeic acid standard chromatograms (Figure 2A). However, the HPLC spectrum of the fractions did not exhibit the presence of any of the standard compounds (Figure 2B-D). There were 2 unidentified peaks in DMEPs that had much longer retention times when compared with the standards.

Discussion

In present study, propolis from *T. sirindhornae* was evaluated to determine its in vitro cytotoxic activity on primary and metastatic HNSCC cell lines. There are many bee species that produce propolis, especially stingless bees, such as *Melipona fasciculate*, (Liberio et al., 2011) *Tetragonula carbonaria* (Massaro et al., 2011) and *A. mellifera* (Teerasripreecha et al., 2012). Importantly, the bioactivities of propolis are reported to depend on the geographical region where it is produced (Oses et al., 2016), season during which it is collected, (Tomazzoli et al., 2015) and the extraction sovents used (Kubiliene et al., 2015). Thus, the effects of propolis from *T. sirindhornae*, a newly identified stingless bee that is typically found in Thailand, are of interest because they have not yet been reported.

In our study, propolis was initially extracted with ethanol and sequentially fractioned with ethyl acetate into 3 fractions that were individually extracted with dichloromethane, generating the dichloromethane extracts of propolis (DMEPs). All of the DMEP fractions were cytotoxic to the HNSCC cell lines we used, as shown by our MTT assays.

Propolis extracts from different solvents demonstrate diverse biological activities. Chloroform-extracted propolis displayed anti-microbial activity against oral pathogens (Liberio et al., 2011). A methanolic extract of propolis demonstrated both anti-microbial effects and anti-proliferative and cytotoxic effects against a colon carcinoma cell line (SW620) (Umthong et al., 2009). Furthermore, crude propolis extracted using hexane and then dichloromethane exhibited anti-proliferative and cytotoxic effects on various carcinoma cell lines (Teerasripreecha et al., 2012). Thus, the active compounds in crude propolis extracts that result in their specific bioactivities depend on the extraction solvents used.

In our study, *T. sirindhornae*-associated DMEPs displayed significant cytotoxicity against HNSCC cell lines at different doses. Among these, DMEP-C showed the highest cytotoxicity against both primary (HN30) and metastatic (HN31) cell lines in a dose-dependent manner. Interestingly, the IC_{50} values indicated that HN31 cells were more toxically sensitive to DMEP-B and DMEP-C compared to HN30 cells. Our data support those of a report on the cytotoxicity of dichloromethane crude propolis extract from *A. mellifera* (Teerasripreecha et al., 2012). Their propolis extract exhibited in vitro anti-proliferative/cytotoxic effect on breast carcinoma (BT474), undifferentiated lung carcinoma (ChaCo), gastric carcinoma (KATO-III), colon adenocarcinoma (SW620), and liver hepatoblastoma (Hep-G2) cancer cells with different IC_{50} values.

The cytotoxic mechanisms that resulted in the anti-cancer effects demonstrated by crude propolis extracts have been investigated. A Chinese propolis extract exerted anti-cancer activity by increasing the cellular mRNA levels of the tumor suppressor genes p21CIP1 and p53 in a human colon cancer cell line (Ishihara et al., 2009). Additionally, the anti-tumor activity of crude Egyptian propolis was associated with the inhibition of cell cycle progression and induction of apoptosis in tumors that had been induced with an Ehrlich ascites carcinoma cell line in mice (El-khawaga et al., 2003).

The synergistic effect of the main components of propolis extracts including flavonoids (quercetin, pinocembrin, and caffeic acid) and cinnamic acid resulted in potent inhibition of proliferatation and induction of apoptosis in various cancer cells (Catchpole et al., 2015; Reddy et al., 2015; Seo et al., 2016). Therefore, the analysis of the bioactive compounds in the DMEPs was performed using HPLC. The presence of the flavonoids apigenin, pinocembrin, p-coumaric acid, and caffeic acid were not observed in the DMEPs from *T. sirindhornae*. A previous study has reported that DMEP contains several flavonoids as its major components, including galangin and apigenin (Chaurasiya et al., 2014). Galangin inhibits growth and induces apoptotic mechanisms in HNSCC (Zhu et al., 2014), hepatocellular carcinoma (Wang et al., 2014) and colon cancer cells (Ha et al., 2013). Apigenin synergizes with tumor necrosis factor-related apoptosis-inducing ligand leading to apoptosis in anaplastic thyroid carcinoma cells (Kim et al., 2015). Moreover, apigenin itself inhibits the proliferation and invasion of osteosarcoma cells (Liu et al., 2015). However, based on our HPLC analyses, DMEP cytotoxic activity cannot be attributed to flavonoids or cinnamic acid derivatives as previously presumed. This suggests that the cytotoxicity of our DMEPs may be attributed to compounds in the unidentified peaks in the DMEP HPLC chromatograms. These findings agree with the anti-cancer effects of propolis extracts from China (Sun et al., 2012) and Thailand (Teerasripreecha et al., 2012) that were not enriched in flavonoid or cinnamic acid.

Recently, propolis produced by different species of bees located in various countries have been reported to have anti-cancer effects (Banskota et al., 1998; Erhan Eroglu et al., 2008; Ishihara et al., 2009; Umthong et al., 2009; Teerasripreecha et al., 2012; Catchpole et al., 2015). Our studies demonstrated that propolis extracts from *T. sirindhornae* also have a cytotoxic effect on HNSCC cell lines. These data suggest that distinct bee species may have the potential to produce propolis with significant in vitro cytotoxic activity.

The current studies provide evidence that the dichloromethane extract of different fractions of T. sirindhornae differentially inhibit the proliferation of a metastatic HNSCC cell line. It is of interest whether the DMEP of *T. sirindhornae* would be useful in the chemotherapy of advance staged human HNSCC. To address this issue, identification of the active component in the extracts and investigation of the precise molecular mechanism by which the extracts might reduce HNSCC progression should be performed.

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