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In Vitro Cytotoxicity Comparison of Stingless Bee Propolis and Cisplatin against ORL-48 and HGF

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ABSTRACT

Objectives: To assess the impact of Malaysian *Heterotrigona itama* (HI) propolis on the proliferation of oral squamous cell carcinoma cell lines (ORL-48) and Human Gingival Fibroblasts (HGF) through comparative treatment at different concentrations and time intervals.

Method: The ORL-48 and HGF cell lines were treated with varying HI propolis and cisplatin concentrations. Subsequently, the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay evaluated the in vitro cytotoxicity of HI propolis and cisplatin against ORL-48 and HGF cell lines.

Result: This study discovered that HI propolis could inhibit the proliferation of ORL-48 cells in a concentration-dependent manner. From the increased treatment concentrations, the HI propolis administration to HGF cells demonstrated a proportional rise in the cell proliferation percentage of HGF cells. Nevertheless, cisplatin treatment significantly decreased the cell viability of ORL-48 cells with a half maximal inhibitory concentration (IC_{50}) of 0.0036 mg/ml at 72 h ($p < 0.05$). Meanwhile, no significant reduction in cell viability was observed in HGF cells.

Conclusion: Propolis-based HI exhibited in vitro cytotoxic activity against human cancer cell lines while demonstrating lower cytotoxicity towards normal cells.

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1. INTRODUCTION

The sixth most common cancer worldwide is oral cancer, a prevalent form of head and neck cancer (Johnson, et al. 2020, Vigneswaran and Williams 2014). Furthermore, oral carcinogenesis is a multifaceted and intricate phenomenon arising from the various genetic mutation occurrences in the squamous epithelium. Oral cancer generally encompasses oral squamous cell carcinoma, verrucous carcinoma, benign oral cavity tumour (gingiva or tongue), and minor salivary gland carcinoma (Abati, et al. 2020). From the mentioned cancer types, oral squamous cell carcinoma accounts for most cases (ranging from 90 to 92%), with verrucous and other oral cancer types making up the remaining cases. Oral squamous cell carcinoma is typically treated with either surgery or radiotherapy (Le and Hanna 2018). Hence, the chemotherapy integration with the multimodal approach for squamous cell carcinoma management of the head and neck (SCCHN) has been effectively implemented. This process aims to enhance both cure rates and clinical results.

In most current chemotherapy studies, cisplatin has been the main topic for over 50 years following its involvement in radio-sensitisation. Nonetheless, these therapies produced adverse effects, which reduced the quality of life while leading to mortality (Le and Hanna 2018). In the past three decades, the mortality rates of these cancers exhibited an upward trend. Therefore, novel chemo-preventive and chemotherapeutic strategies were necessary for managing oral cancers. Propolis, often known as bee glue, is a resinous substance gathered by honeybees from the buds and exudates of various trees (Dezmirean, et al. 2021). This substance primarily comprises resin, beeswax, essential oils, pollen grains, micronutrients, and trace amounts of vitamins (Pasupuleti, et al. 2017). The primary function of propolis is to seal any cracks or openings within the hive, thus safeguarding it from potential bacterial and fungal infections (Bankova, de Castro and Marcucci 2000). Due to the antibacterial and anti-inflammatory properties of propolis, this naturally-occurring substance has been utilised for centuries in traditional medicine.

Propolis derived from stingless bees is known for its potent antibacterial, anti-inflammatory, and antiproliferative properties (Zulhendri, et al. 2022, Bonamigo, et al. 2017, Cornara, et al. 2017). Globally, the stingless bee is the most extensive assemblage of eusocial bees, with over 500 documented species. The species is widespread in tropical and subtropical regions, including tropical America, Africa, Australia, and Southeast Asia. In the latter region, this population is 50 times that of honey bees. Honey production begins with the collecting of nectar from plants by bees, which is then transformed and processed using specific substances from the bees. As opposed to hexagonal-shaped combs, stingless bees utilise resin pots to store their nectar. Currently, Malaysia has 32 documented species of stingless bees, with *Heterotrigona itama* (HI) and *Geniotrigona thoracica* being the most prevalent domesticated species (Kelly, et al. 2014).

Over 300 chemical compounds, including flavonoids, terpenes, phenolic acid, cinnamic acid, caffeic acid, and various esters, have been identified in propolis from numerous geographical regions. A diverse range of biological activities, including antifungal, antiviral, antioxidant, and immune-stimulating, have also been significantly demonstrated. Propolis has been extensively employed in various disease models, reporting the potential for safeguarding the immune response against leishmaniasis. Miranda *et al*. (2015) discovered that tissue repair in experimental leishmaniasis could be expedited (achieved through cell migration regulation, cytokine production, and collagen deposition) using a combination of nitric oxide and Brazilian propolis extract (Miranda, et al. 2015). Thus, the study suggested a promising therapeutic approach that warranted further *in vivo* investigations as a potential treatment option for cutaneous leishmaniasis. Over the years, propolis-based studies also uncovered its antitumor properties, in which propolis possessed various action mechanisms that conferred anti-cancer and chemo-preventive properties.

The *in vitro* cytotoxic effects against various human cell lines, such as colon cancer, prostate carcinoma, malignant melanoma, and astroglia cells, were successfully demonstrated in several studies (Elumalai, et al. 2022, Forma and Bryś 2021). In addition, the biological and pharmacological behaviours

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of propolis depended on its chemical constituents, geographical zone, plant source, and time of year. Teerasripreecha *et al*. (2012) proved that propolis extracts from *Apis mellifera* beehives in Thailand exhibited antiproliferative and cytotoxic effects against numerous cancer cell lines, including human breast carcinoma (BT474), human hepatocellular carcinoma (Hep-G2), gastric carcinoma (KATO-III), and colon adenocarcinoma (SW620) (Teerasripreecha, et al. 2012). Conversely, the *Trigona sirindhornae* propolis extract in the study produced cytotoxic effects on head and neck squamous cell carcinoma (HNSCC) cells. Regarding oral cancer, a group of researchers have established 3 cell lines obtained from untreated primary human oral squamous cell carcinomas of the oral cavity namely, ORL-48, -115 and -136 (Hamid et al., 2007). Since existing studies on the Malaysian HI propolis impact on ORL-48 cell lines were limited, this study assessed the potential anti-cancer properties of HI propolis and cisplatin against ORL-48 and HGF cell lines. This study emphasised the significance of propolis derived from stingless bees as a potentially valuable natural resource for advancing novel antitumor medications. Cytotoxicity testing enables researchers to observe the growth, morphological effects, reproduction and proliferation of cells on treatment with substances having an unknown cytotoxic state. This test also offers many ways of detecting cell damage like monitoring morphological changes, cell growth and measurement of metabolic properties. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) is one of the cytotoxic assays widely used in assessing cell viability and proliferation assay (Chee, et al. 2021). Additionally, this study introduced fresh opportunities for comparative analysis concerning the chemical constituents in propolis extracts, cell death patterns, and mechanisms of action.

2. METHODOLOGY

2.1 Ethics exemption

This study was exempted from ethics review by the Universiti Teknologi MARA (UiTM) Research Ethics Committee (REC) on the 7th of April 2021 with reference number: REC/04/2021(UG/EX/179).

2.2 Propolis sample collection and preparation

The propolis samples were extracted and prepared following the prescribed methodology with minor modifications (Al-Masoodi, et al. 2022, Omar, et al. 2019). First, HI propolis was obtained from Aasta Stingless Bee Honey Farm in Raub, Pahang, Malaysia. Subsequently, the HI propolis was dried and crushed with a blender. The propolis powder was then incorporated into a 70% ethanol solution and dissoluted in a shaking incubator at 200 rpm for seven days. Following the solution filtration using a Whatman® grade 1 qualitative filter paper, the ethanolic propolis extracts were filtered using a polytetrafluoroethylene (PTFE) 0.45 μm filter and evaporated in a rotary device under reduced pressure. The evaporated filtrate was dried in a laboratory freeze dryer and stored at -20°C. Fig 1 (see below) summarises the propolis extraction process in this study. The propolis was dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml concentration as a stock solution. Additionally, the DMSO concentration in the culture medium was regulated to 0.1% (v/v) to ensure its non-toxicity for the cells. Finally, the initial stock solution was diluted with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) to achieve the requisite concentrations in this study.

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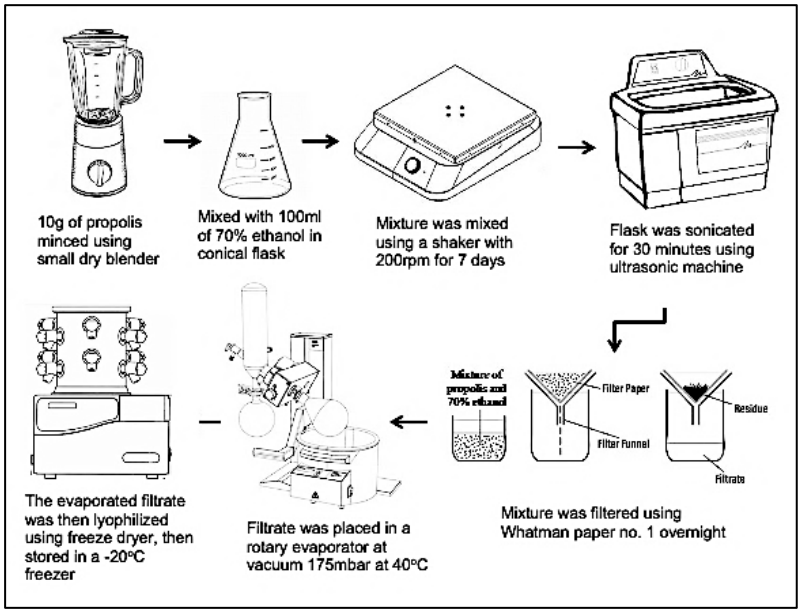

Fig. 1. Schematic flow of extraction method for propolis.

2.3 Cell culture

The ORL-48 was acquired from the Cancer Research Malaysia and Foundation, Subang Jaya Medical Centre (CARIF, Selangor, Malaysia). Subsequently, the Medical Ethics Committee, Faculty of Dentistry, Universiti Malaya [supported by the Malaysian Ministry of Health (MOH)], approved the cell line development through the informed consent of a 79-year-old female patient with a gum tumour. The clearance was given under "Oral Cancer and Precancer in Malaysia" with DP OP0306/0018/L (Hamid, et al. 2007), and the *in vitro* ORL-48 passage expansion was established from passage 30 (P30).

The Delbecco's modified Eagle medium/F-12 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, Gaithersburg, MD, USA) was used to sustain the cells. Meanwhile, the HGF cell line (ATCC PCS-201-018) was purchased from ATCC (American Type Culture Collection), USA and cultured following the ATCC protocol [Primary Gingival Fibroblast; Normal, Human, Adult (HGF), 2021]. The *in vitro* HGF passage expansion was also established from passage 5 (P5). Both cell lines were cultured at 37°C in a 5% CO_2 atmosphere. At 70 to 80% confluence, the cells were then passaged using 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA). Lastly, the sub-culturing process followed the Cell Culture Basics Handbook, 2014 until ORL-48 from P30 reached P35 and HGF from P5 to P8 was ready for Di Methyl Thiazoldiphenyl-Tetrazoliumbromide (MTT) assay to measure cell viability, cell proliferation and drug cytotoxicity (Khor et al., 2017).

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2.4 Cell counting

The cell counting was conducted following the protocol of Mat Nafi *et al*. (2019) (Mat Nafi, et al. 2019). In brief, the adherent cells were collected by aspirating the culture medium and rinsing with 0.01 mol/L phosphate buffer saline (PBS). Subsequently, the cells were incubated with 1 to 1.5 mL of 0.05% (w/v) trypsin in the same PBS at room temperature for 1 to 2 mins. The trypsin solution was then substituted with 1 ml of culture medium. These cells were also dissociated through gentle agitation, harvested, and the resulting cell suspension was further diluted as necessary to enable counting of a 10 µL aliquot using a haematocytometer. Finally, cell positioning at four large corner squares of the haemocytometer is counted, with the number of cells calculated as follows:

$$Cell\ count = \frac{Number\ of\ cells\ in\ 4\ squares\ x\ 10^4}{4} \quad (1)$$

2.5 Positive Control Preparation (Cisplatin)

A 1 mg of cisplatin (Sigma-Aldrich, Malaysia) was dissolved in 1 ml of 1% DMSO to obtain 1mg/ml of cisplatin stock. The cisplatin concentration was obtained from Khoo *et al*.'s (2019) study (Khoo, et al. 2019). Consequently, the cisplatin formula unit conversion is provided as follows:

$$Concentration\ (mg/ml) = Molarity\ (mol/L) \times Molecular\ weight * (g/mol) \quad (2)$$

**Molecular weight of cisplatin = 301.1 g/mol*

2.6 Cytotoxicity Assessment

This study investigated the cytotoxic effects of HI propolis on ORL-48 and HGF cell lines. As previously reported with slight modifications by Khor *et al*. (2017), the well-established MTT assay was employed to assess the cytotoxicity of HI propolis (Khor, et al. 2017). The ORL-48 and HGF cells were seeded into 96-well plates at a density of 1,000 cells per well and were incubated overnight at 37°C in 5% CO_2. Subsequently, the prepared HI propolis, serum-free DMEM (negative control), and cisplatin (positive control) were directly subjected to the cells. The propolis stock concentration began at 100 mg/ml using the method modified by Chee *et al*. (2021) (Chee, et al. 2021). Therefore, propolis was diluted to 11 concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.39063, 0.19531, and 0.09766 mg/ml. These concentrations were transferred in separate wells, with DMEM/F-12 supplemented with 10% foetal bovine serum and 1% antibiotic (penicillin-streptomycin).

The incubation period was evaluated and observed after 24, 48, and 72 h intervals. The media was then withdrawn after the exposure time, during which the cells were rinsed in PBS and treated with 0.5 mg/ml MTT in culture media for 4 h. Subsequently, the purple formazan crystals of the viable cells were dissolved in 100 µl of 100% DMSO and measured at 570 nm wavelength by a microplate reader. The IC_{50} value, representing the extract concentration (mg/ml) producing 50% growth inhibition, was determined by plotting the % of cell viability against the extract concentration. Moreover, the tests examined the cisplatin and complete culture media DMEM/F-12 as positive and negative controls, respectively. This process is followed by plotting the obtained results, which the following formula is utilised to determine the percentage of inhibition:

$$\%\ of\ cell\ viability = \frac{OD\ treated\ sample}{OD\ untreated\ sample} \times 100\% \quad (3)$$

**OD = Optical density*

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2.7 Statistical analysis

The statistical analysis was performed using Statistical Package of Social Science (SPSS) software version 24 (SPSS® Inc, USA). In addition, the MTT assay data were analysed, with the results expressed as median from three independent replications. Finally, the data were analysed by one-way analysis of variance and followed by Tukey's test (significance level was set to $p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Cytotoxic Effect Comparison of HI Propolis and Cisplatin on ORL-48 Cells for 24, 48 and 72 h

An MTT assay was employed to assess the cytotoxic impact of HI propolis on ORL-48 cells at different time intervals. A graph was then plotted based on the obtained result. Three separate experiments used the means and standard errors of the means (SEM) to express all data. Compared to the control, the dose-dependent HI propolis reduced the viability of the ORL-48 cells (see Table 1 and Fig. 2). Furthermore, the results revealed increased treatment time intervals, resulting in commensurate percentage inhibition of ORL-48 cells. Nevertheless, the lowest maximal half inhibitory concentration (IC_{50}) value was observed at 48 and 72 h of incubation, which were 4.58 and 6.24 mg/ml, respectively ($p < 0.05$).

Table 1. Cytotoxicity effect summary of HI propolis on ORL-48 cells after treatment for 24, 48, and 72 h, which is evaluated by MTT assay (below).

Item no.	**Concentration *(mg/ml)***	**Cell viability *(%)***		
		24h	48h	72h
1	100.00	67.21 ± 10.9*	43.26 ± 5.2*	37.58 ± 11.0*
2	50.000	72.76 ± 12.1	38.11 ± 0.8*	50.96 ± 7.5*
3	25.000	61.04 ± 2.5*	46.55 ± 2.7*	40.71 ± 7.2*
4	12.500	80.80 ± 8.8	47.70 ± 3.2*	43.08 ± 10.1
5	6.250	68.95 ± 1.3	40.86 ± 1.2*	38.62 ± 6.9*
6	3.125	77.49 ± 10.8	48.96 ± 3.1*	29.28 ± 2.9*
7	1.563	75.59 ± 10.5	45.54 ± 5.7*	31.94 ± 0.6*
8	0.781	90.87 ± 4.2	70.61 ± 23.5*	35.18 ± 2.7*
9	0.391	98.73 ± 13.2	72.77 ± 7.0	54.88 ± 6.9*
10	0.195	103.49 ± 5.5	81.33 ± 0.7	65.28 ± 4.5*
11	0.098	94.85 ± 9.5	87.14 ± 1.1	85.80 ± 6.5
12	0.000	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0

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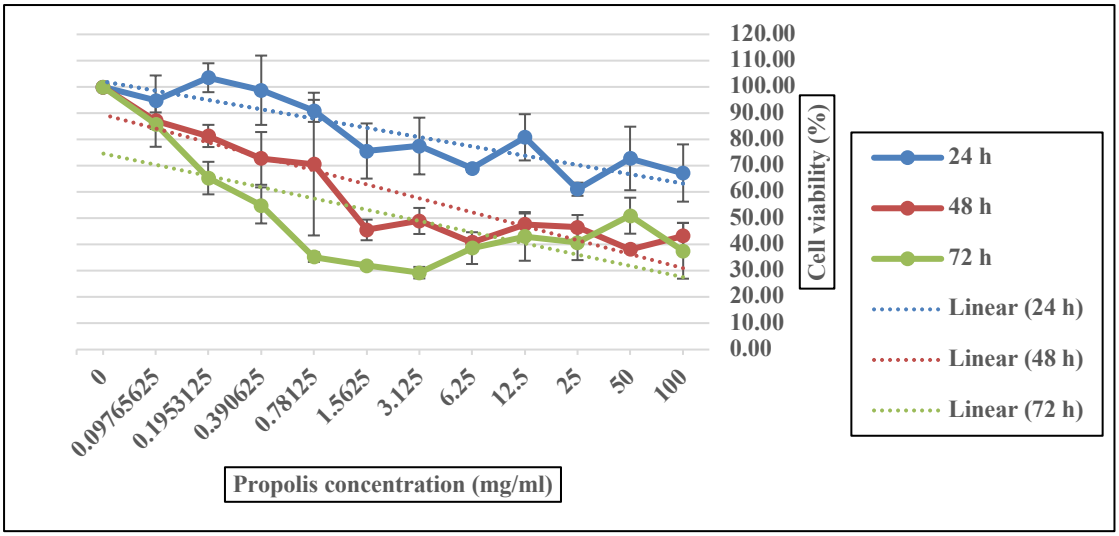

Fig. 2. Graph indicating cytotoxic evaluation oh HI propolis on ORL-48 cells measured via MTT assay (above).

In Table 2, the number of live ORL-48 cells after 24 h of cisplatin exposure and the 24, 48, and 72 h observation periods were tabulated. A similar trend was observed as the cisplatin cytotoxic effect was dose-dependent based on a corresponding decrease in live cells relative to the control group. The observed pattern displayed a slight fluctuation, in which the value increased, followed by a rise of 0.0048 mg/ml at 24 and 48 h and then decreased (see Fig. 3). At the 72-h mark, cell viability increased at a dosage of 0.0042 mg/ml, followed by a decrease in live cells.

Table 2. The % summary of cell viability for ORL-48 cells in a 24 h cisplatin post-exposure solution (below).

Item no.	Concentration *(mg/ml)*	Cell viability *(%)*		
		24h	48h	72h
1	0.0060	89.85 ± 3.0	63.53 ± 1.1*	52.80 ± 1.9*
2	0.0054	86.93 ± 1.9*	63.83 ± 1.2*	54.62 ± 1.2*
3	0.0048	86.90 ± 2.2*	65.09 ± 2.2*	52.08 ± 2.9*
4	0.0042	85.85 ± 0.2*	65.19 ± 1.1*	52.00 ± 3.2*
5	0.0036	85.92 ± 1.8*	64.24 ± 0.6*	48.66 ± 2.7*
6	0.0030	88.46 ± 0.4*	66.51 ± 3.4*	56.98 ± 4.3*

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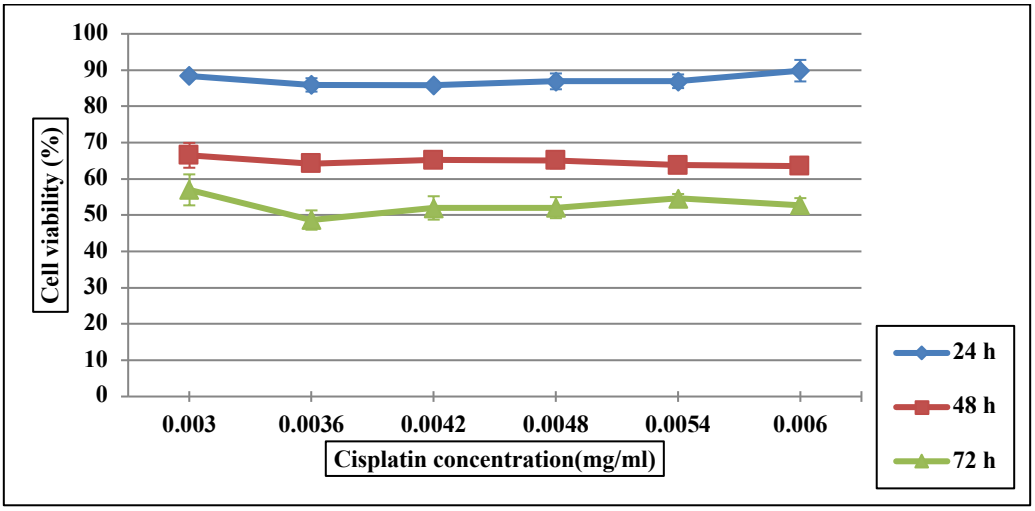

Fig. 3. Graph indicating proportional correlation % cell viability of ORL-48 cells on 24 h cisplatin exposure and different observation period (above).

A comparative study in Northeast China evaluated the antiproliferative efficacy of a distinct Chinese propolis type from the Changbai Mountains (CBMP) at a significantly reduced range of treatment concentrations from 0.00625 to 0.075 mg/ml. Jiang *et al*. (2020) presented that CBMP exhibited antiproliferative properties, and the IC_{50} value of CBMP against SGC-7901 cells was determined at 0.06664 mg/ml (Jiang, et al. 2020). The study also indicated a higher cytotoxicity degree than this study. On the contrary, the examined propolis origin and cancer cell lines were dissimilar. In a separate study, Mat Nafi *et al*. (2019) differentiated the propolis from HI, GT, *Lepidotrigona terminate* (LT), and *Tetrigona apicalis* (TA) species. Based on IC_{50} values of 0.005, 0.004, and 0.008 against MDA-MB-231, SK-UT-1, and HeLa cells, respectively, HI propolis successfully inhibited cell proliferation and generated the highest cytotoxic effect. Alternatively, the propolis from other stingless bees produced weak cytotoxicity (Mat Nafi, et al. 2019). Although the cancer cell lines in this study differed from other studies, higher toxicity to cancer cells was still indicated. Hence, the propolis hive location and the extraction technique could affect how cytotoxic the effect was against different cancer cell types.

3.2 Cytotoxic effect comparison of HI propolis and Cisplatin on HGF cells for 24, 48, and 72 h.

The cytotoxicity of HI propolis towards HGF cells was evaluated using the MTT test after HGF cells were exposed to varying HI propolis concentrations for 24, 48, and 72h. This process was necessary to assess the cytotoxicity level of HI propolis. Cells not treated with HI propolis [negative control (DMEM)] concluded 100% cell growth. Table 3 and Fig. 4 illustrate that the MTT experiment findings reveal a dose-dependent pattern of substantial cell proliferation in HGF cells following exposure to HI propolis treatments. Moreover, cell viability variations were generated at greater doses (48 h mark). Interestingly, the amount could not suppress HGF cell growth by 50% for all time intervals.

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Table 3. Cytotoxicity effect summary of HI propolis on HGF cells after treatment for 24, 48, and 72 h, which is evaluated by an MTT assay (below).

Item no.	Concentration *(mg/ml)*	Cell viability *(%)*		
		24h	48h	72h
1	100.00	185.10 ± 18.3	155.91 ± 48.9	143.60 ± 10.0
2	50.000	196.76 ± 23.3	196.60 ± 29.4	102.35 ± 6.5
3	25.000	127.70 ± 17.3	186.54 ± 23.9	98.97 ± 21.8
4	12.500	125.54 ± 23.3	121.43 ± 19.0	90.26 ± 12.6
5	6.250	115.62 ± 3.8	125.26 ± 25.4	87.07 ± 5.0
6	3.125	108.08 ± 6.3	109.73 ± 7.9	96.10 ± 8.3
7	1.563	100.77 ± 7.3	111.43 ± 6.1	97.66 ± 13.3
8	0.781	121.71 ± 10.0	105.15 ± 0.5	91.22 ± 13.2
9	0.391	88.11 ± 5.6	102.35 ± 1.6	88.64 ± 7.0
10	0.195	87.68 ± 3.6	99.49 ± 3.1	93.47 ± 2.9
11	0.098	88.06 ± 0.3	92.12 ± 2.0	92.78 ± 3.5
12	0.000	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0

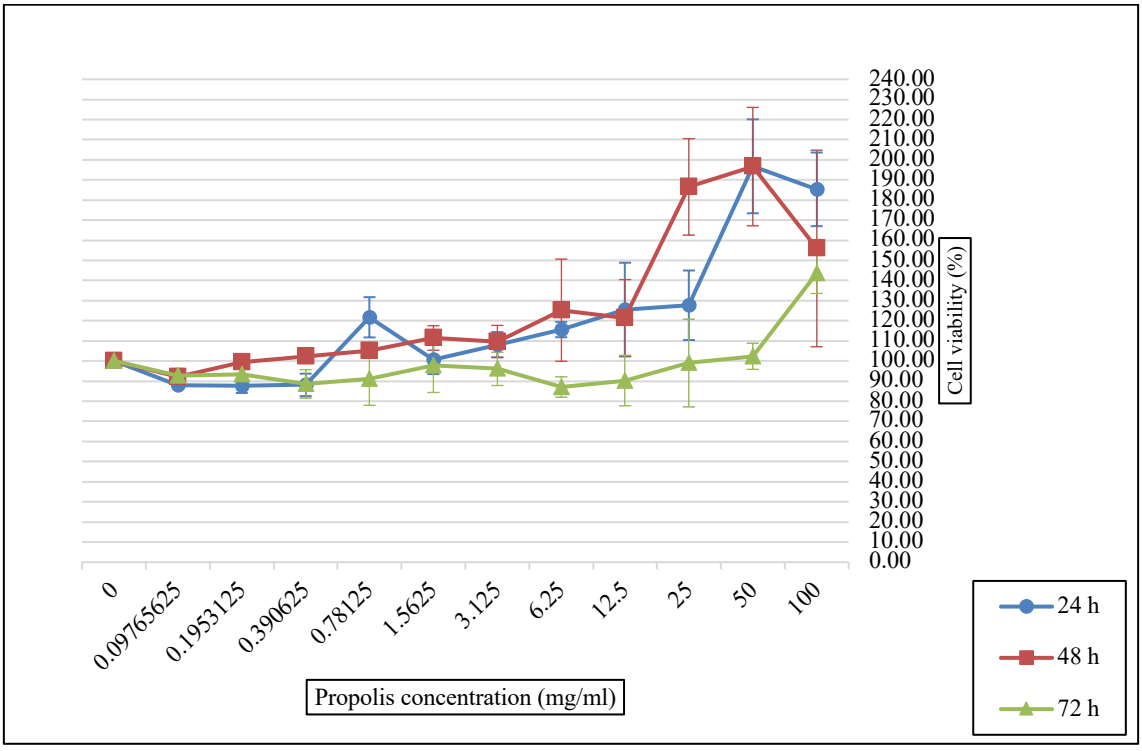

Fig. 4. Graph indicating cytotoxic evaluation of HI propolis on HGF cells measured by MTT assay (above).

For elucidating the anti-cancer efficacy of cisplatin in HGF cells, the relative vitality at different concentrations was assessed using the MTT assay (see Table 4). Fig. 5 depicts the results of an *in vitro*

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analysis of the cisplatin therapy effects on HGF cells. The cell viability of HGF gradually reduced as the cisplatin concentration and treatment duration increased. This study could not determine the IC_{50} values of cisplatin in HGF cells as higher cisplatin dosage was necessary to achieve a 50% inhibition of cell growth.

Table 4. The % summary of cell viability of HGF cells in a 24 h cisplatin post-exposure solution (below).

Item no.	Concentration *(mg/ml)*	Cell viability *(%)*		
		24h	48h	72h
1	0.0060	86.18 ± 1.4	83.66 ± 1.6*	68.33 ± 1.3*
2	0.0054	87.31 ± 0.7	85.59 ± 2.1*	70.41 ± 1.6*
3	0.0048	90.94 ± 7.9	79.99 ± 4.7*	78.22 ± 11.8
4	0.0042	87.05 ± 0.0	80.05 ± 2.5*	69.41 ± 0.8*
5	0.0036	87.45 ± 1.8	81.28 ± 3.3*	72.98 ± 1.2*
6	0.0030	88.58 ± 3.6	87.11 ± 4.6*	86.25 ± 16.7

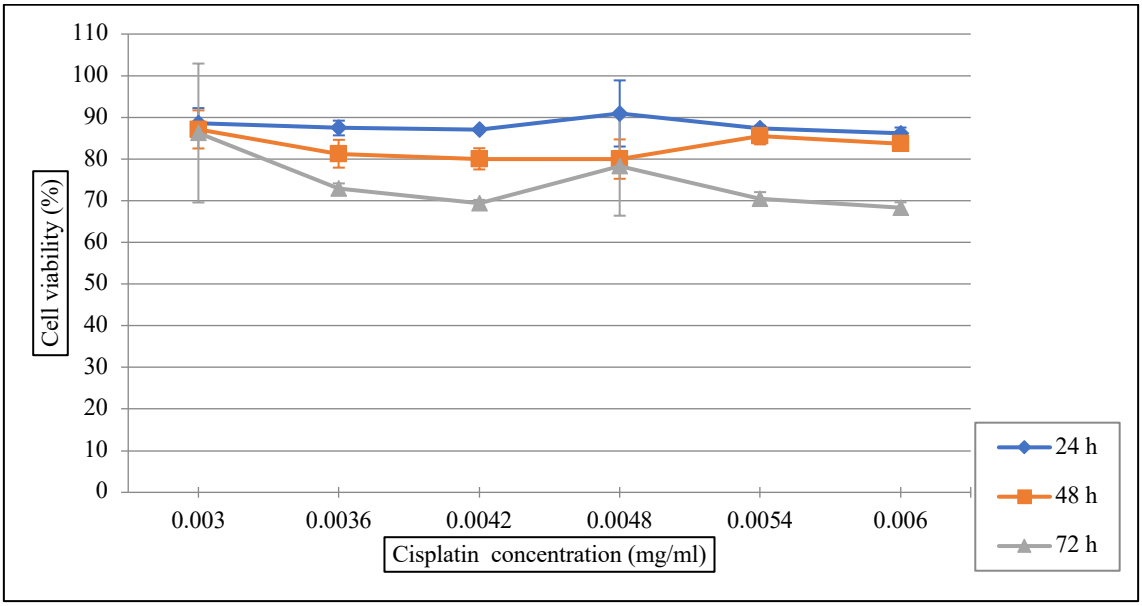

Fig. 5. Graph indicating proportional correlation % cell viability of HGF cells on 24h cisplatin exposure and different observation duration (above).

The HGFs are the most prevalent cell types in the gingival and periodontal connective tissues, which promote periodontal tissue healing and contribute to inflammatory periodontal disorders (Bartold, Walsh and Narayanan 2003). In this study, HI propolis was administered to HGF cells, which revealed high cell viability of more than 80% at all concentrations ranging from 0.098 to 100 mg/ml. Thus, the HI propolis was not cytotoxic at these concentrations, suggesting that the HI propolis compound acquired high selectivity for only cancerous cells. This result was comparable to Zi Yun *et al.*'s (2021) study, where Malaysian "kelulut" honey treatment increased the vitality of HGF cells by more than 70% at all concentrations (3.125–200 mg/ml) (Chee, et al. 2021).

The viability of the cells increased in a dose-dependent manner in line with the HI propolis concentration. At 50 mg/ml, the cell viability of HGF was at its maximum (196.76 ± 23.3 and 196.60 ± 29.4% after 24 and 48 h, respectively). Meanwhile, this value was 143.60 ± 10.0% at 72 h ($p < 0.05$) at 100 mg/ml. The propolis potentially produced wound-healing capabilities as HI propolis promoted the HGF

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cell growth. Since HI propolis promoted HGF cell development, this process suggested that propolis possessed wound-healing abilities. Therefore, this observation was consistent with a study by Jacob *et al*. (2015), where Malaysian propolis from stingless honey bee *Trigona spp*. generated a more favourable effect on fibroblast migration and proliferation assays than the control (Jacob, et al. 2015).

4. LIMITATIONS AND RECOMMENDATIONS

Although a linear trendline was portrayed in this study, the actual MTT assay findings of HI propolis on the ORL-48 and HGF cell lines displayed erratic swings (particularly at higher dosages above 6.25 mg/ml). The larger concentrations led to higher standard deviation values as each replication variability increased. Alternatively, cisplatin produced a more stable linear horizontal graph with modest standard deviations for ORL-48 and HGF cell lines. This outcome was possible due to the HI propolis causing unnatural fluctuations in absorbance values, potentially interfering with the MTT experiment. A perceptible increase in colour intensity was also observed, altering the colourimetric absorbance of the MTT experiment as the concentration increased. This concern was crucial as any modifications in absorbance values generated by chemical interactions could result in an incorrect interpretation of the chemosensitivity results for the MTT assay. Thus, data misinterpretation could result in false-positive or false-negative results, thus causing the cytotoxic potential of HI propolis to be underestimated or overestimated (Ulukaya, Colakogullari and Wood 2004).

In improving outcome accuracy, the substances employed in the colourimetric test should be examined for potential unanticipated interactions with the dye of the assay. Another possible effect involved the solvent, which could rearrange or separate the powdered extracted propolis. Additionally, stock propolis could lose some of its hydrophobic properties and become less soluble when diluted with 1% DMSO. As Wang *et al*. (2018) stated, this issue could prevent the solvent from penetrating the bigger solid propolis matrix, leaving the particles floating in the solution (Wang, et al. 2018). The reconstituted 1% DMSO solutions also included precipitations, particularly at higher concentrations. Furthermore, the solvent should be biocompatible and not harmful to the cells, as this factor could affect the readings. Consequently, future studies should examine the use of better solvents.

An increasing corpus of research presented that over-subculturing altered the characteristics of cell lines over time. Compared to lower-passage cells, cell lines with large passage numbers exhibited changes in cell shape, responsiveness to stimuli, growth rates, protein expression, transfection, and signalling. Among the other conclusions drawn from this study, both cell types were more inhibited after 48 h. According to the data, therapy that lasted for 48 or 72 h acquired a higher IC_{50} value than treatment that ended after 24 h (no discernible effects). As insufficient therapy concentrations were observed, less effective inhibition of cell proliferation was produced.

MTT assay is just of many ways of indicating cellular toxicity and in this study other parameters were not explored due to the limitations posed by the COVID 19 pandemic.

5. CONCLUSION

This study successfully demonstrated the longer treatment durations on ORL-48 cells. Thus, propolis was a more effective oral cancer treatment than cisplatin and DMEM as positive and negative controls, respectively. Although propolis and cisplatin were less damaging to normal cells, the comparison concentrations were inconclusive. Nonetheless, propolis revealed a considerable HGF proliferation, while cisplatin did not. Several studies also reported that propolis did not have the same potentially fatal side effects as cisplatin, including ototoxicity, gastrotoxicity, myelosuppression, and nephrotoxicity. Consequently, HI propolis was deemed an appropriate material for investigating the expression of genes

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associated with anticancer processes. Moreover, this study possessed the potential to serve as a viable candidate for the inhibition of oral cancer cell proliferation and dissemination.

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AUTHORS' CONTRIBUTIONS

Ikmal Hisham Ismail. Contributed to the conception and design of the study, data analysis, interpretation, financial acquisition, manuscript writing and final approval of manuscript. **Syahmirul Saharuzaman, Faatin 'Afeefah Ahmad Naqiyuddin and Amiyah Bismelah** are contributed to the collection and assembly of data, data analysis and interpretation and writing of the manuscript. **Khor Goot Heah and Wan Iryani Wan Ismail** are participated in the study design, data analysis and interpretation. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declared no conflicts of interest in conducting this study.

REFERENCES

Abati, S., Bramati, C., Bondi, S., Lissoni, A., & Trimarchi, M. (2020). Oral cancer and precancer: A narrative review on the relevance of early diagnosis. *International Journal of Environmental Research and Public Health,* 8 (17), 9160. https://doi.org/10.3390/ijerph17249160.

Al-Masoodi, O. H. H., Said Gulam Khan, H. B., Baharuddin, I. H. & Ismail, I. H. (2022). In-vitro comparison of antibacterial activities on stingless bee propolis using selected extraction methods. *Compendium of Oral Science,* 9 (2), 23-43. https://doi.org/10.24191/cos.v9i2.19230.

Bankova, V. S., de Castro, S. L., & Marcucci, M. C. (2000). Propolis: Recent advances in chemistry and plant origin. *Apidologie 31* (1), 3-15. https://doi.org/10.1051/apido:2000102.

Bartold, P. M., Walsh, L. J., & Narayanan, A. S. (2003). Molecular and cell biology of the gingiva. *Periodontology, 24* (1), 28-55. https://doi.org/10.1034/j.1600-0757.2000.2240103.x.

Bonamigo, T., Campos, J. F., Alfredo, T. M., Balestieri, J. B. P., Cardoso, C. A. L., Paredes-Gamero, E. J., Souza, K. de P., & Santos, E. L. D. (2017). Antioxidant, cytotoxic, and toxic activities of propolis from two native bees in Brazil: Scaptotrigona depilis and Melipona quadrifasciata anthidioides. *Oxidative Medicine and Cellular Longevity, 2017*, 1038153. https://doi.org/10.1155%2F2017%2F1038153.

Chee, Z. Y., Mohd Nor, N. H., Berahim Z., & Ponnuraj K. T. (2021). Cytotoxic evaluation of Malaysian kelulut honey on human gingival fibroblast cell line using MTT assay. *Journal of Dentistry Indonesia, 28* (2), 88-93. https://doi.org/10.14693/jdi.v28i2.1244.

Cornara, L., Biagi, M., Xiao, J., & Burlando, B. (2017). Therapeutic properties of bioactive compounds from different honeybee products. *Frontiers in Pharmacology, 8*. https://doi.org/10.3389/fphar.2017.00412.

Dezmirean, D. S., Paşca, C., Moise, A. R. & Bobiş, O. (2021). Plant sources responsible for the chemical composition and main bioactive properties of polar-type propolis. *Plants, 10* (1), 22. https://doi.org/10.3390/plants10010022.

Elumalai, P., Muninathan, N., Megalatha, S. T., Suresh, A., Kumar, K. S., Jhansi, N., Kalaivani, K., & Krishnamoorthy, G. (2022). An insight into anticancer effect of propolis and its constituents: A review of molecular mechanisms. *Evidence Based Complement Alternative Medicine, 2022*, 5901191. https://doi.org/10.1155%2F2022%2F5901191.

Forma, E., & Bryś, M. (2021). Anticancer activity of propolis and its compounds. *Nutrients, 13* (8), 2594. https://doi.org/10.3390%2Fnu13082594.

Hamid, S., Lim, K. P., Zain, R. B., Ismail, S. M., Lau, S. H., Mustafa, W. M. W., Abraham, M. T., Nam, N. A., Teo, S. H., & Cheong, S. C. (2007). Establishment and characterization of Asian oral cancer cell lines as in vitro models to study a disease prevalent in Asia. *International Journal of Molecular Medicine, 19* (3), 453-460. https://doi.org/10.3892/ijmm.19.3.453.

Jacob, A., Parolia, A., Pau, A., & Amalraj, F. D. (2015). The effects of Malaysian propolis and Brazilian red propolis on connective tissue fibroblasts in the wound healing process. *BMC Complementary and Alternative Medicine, 15*, 294. https://doi.org/10.1186/s12906-015-0814-1.

Jiang, X. S., Xie, H. Q., Li, C. G., You, M. M., Zheng, Y. F., Li, G. Q., Chen, X., Zhang, C. P., & Hu, F. L. (2020). Chinese propolis inhibits the proliferation of human gastric cancer cells by inducing apoptosis and cell cycle arrest. *Evidence-Based Complementary and Alternative Medicine,* 2020. https://doi.org/10.1155/2020/2743058.

Johnson, D. E., Burtness, B., Leemans, C. R., Lui, V. W. Y., Bauman, J. E., & Grandis, J. R. (2020). Head and neck squamous cell carcinoma. *Nature Reviews Disease Primers, 6* (92). https://doi.org/10.1038/s41572-020-00224-3.

Kelly, N., Farisya, M. S. N., Thevan, K., & Marcela, P. (2014). Species diversity and external nest characteristics of stingless bees in Meliponiculture. *Pertanika Journal of Agricultural Science, 37* (3), 293-298.

Khoo, X. H., Paterson, I. C., Goh, B. H., & Lee, W. L. (2019). Cisplatin-Resistance in Oral Squamous Cell Carcinoma: Regulation by Tumor Cell-Derived Extracellular Vesicles. *Cancers, 11* (8), 1166. https://doi.org/10.3390/cancers11081166.

Heah, K. G., Mohd Shobri, N. R., Khoruddin, N. A., Suhaimi, S. N., Mohd Yusof, Y. P., & Thean H. T. (2017). A review on di methyl thiazoldiphenyl-tetrazoliumbromide (MTT) assay in cell viability. *Research Journal of Applied Sciences*, *12* (7), 372-378. https://dx.doi.org/10.36478/rjasci.2017.372.378.

Le, X., & Hanna, E. Y. 2018. Optimal regimen of cisplatin in squamous cell carcinoma of head and neck yet to be determined. *Annals of Translational Medicine, 6* (11). https://doi.org/10.21037/atm.2018.05.10.

Mat Nafi, N. E., Md Zin, N. B., Pauzi, N., Abdul Khadar, S, S., Anisava, A. R., Mohd Badiazaman, A. A., & Mohd, K. S. (2019). Cytotoxicity, antioxidant and phytochemical screening of propolis extracts from four different Malaysian stingless bee species. *Malaysian Journal of Fundamental and Applied Science,* 307-312. https://doi.org/10.11113/mjfas.v15n2-1.1542.

Miranda, M. m., Panis, C., Cataneo, A. H. D., da Silva, S. S., Kawakami, N. Y., de França Lopes, L. G., Morey, A. T., Yamauchi, L. M., de Jesus Andrade, C. G. T., Cecchini, R., da Silva, J. J. N., Sforcin, J. M., Conchon-costa, I., & Pavanelli, W. R. (2015). Nitric oxide and Brazilian propolis combined accelerates tissue repair by modulating cell migration, cytokine production and collagen deposition in experimental leishmaniasis. *PloS one, 10* (5), e0125101. https://doi.org/10.1371/journal.pone.0125101.

Omar, S., Enchang, F. K., Nazri, M. U. I. A., Ismail, M. M., & Ismail. W. I. W. (2019). Physicochemical profiles of honey harvested from four major species of stingless bee (kelulut) in North East Peninsular of Malaysia. *Malaysian Applied Biology, 48* (1).

Pasupuleti, V. R., Sammugam, L., Ramesh, N., & Gan, S. H. (2017). Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxidative Medicine and Cellular Longevity,* 2017. https://doi.org/10.1155/2017/1259510.

Teerasripreecha, D., Phuwapraisirisan, P., Puthong, S., Kimura, K., Okuyama, M., Mori, H., Kimura, A., & Chanchao, C. (2012). In vitro antiproliferative/cytotoxic activity on cancer cell lines of a cardanol and a cardol enriched from Thai Apis mellifera propolis. *BMC Complementary and Alternative Medicine, 12* (27). https://doi.org/10.1186/1472-6882-12-27.

Ulukaya, E., Colakogullari, M., & Wood, E. J. (2004). Interference by anti-cancer chemotherapeutic agents in the MTT-tumor chemosensitivity assay. *Chemotherapy, 50* (1), 43-50. https://doi.org/10.1159/000077285.

Vigneswaran, N., & Williams, M. D. (2014). Epidemiological trends in head and neck cancer and aids in diagnosis. *Oral and Maxillofacial Surgery Clinics of North America, 26* (2), 123-141. https://doi.org/10.1016%2Fj.coms.2014.01.001.

Wang, H., Zhong, Q., Yang, T., Qi, Y., Fu, M., Yang, X., Qiao, L., Ling, Q., Liu, S., & Zhao, Y. (2018). Comparative characterization of SHED and DPSCs during extended cultivation in vitro. *Molecular Medicine Reports,* 6551-6559. https://doi.org/10.3892/mmr.2018.8725.

Zulhendri, F., Perera, C. O., Chandrasekaran, K., Ghosh, A., Tandean, S., Abdulah, R., Herman, H., & Lesmana, R. (2022). Propolis of stingless bees for the development of novel functional food and nutraceutical ingredients: A systematic scoping review of the experimental evidence. *Journal of Functional Foods, 88*, 104902. https://doi.org/10.1016/j.jff.2021.104902.

11. APPENDIX

A. Statistical Analysis Results SPSS - One-Way ANOVA

Table S1. The HI propolis concentration effects on ORL-48 cell viability, examined using one-way ANOVA

ANOVA					
OD24	**Sum of Squares**	***df***	**Mean Square**	***F***	**Sig.**
Between Groups	0.041	11	0.004	5.480	0.000
Within Groups	0.016	24	0.001		
Total	0.057	35			

Table S2. The ORL-48 cell viability was determined by post-hoc Tukey test.

Multiple Comparisons

Dependent Variable:

Tukey HSD						
(I) cont		**Mean Difference (*I-J*)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	**Upper Bound**
12.00	1.00	.07707*	0.02117	0.046	0.0007	0.1534
	2.00	0.04054	0.02117	0.740	-0.0358	0.1169
	3.00	.10334*	0.02117	0.003	0.0270	0.1797
	4.00	0.02632	0.02117	0.979	-0.0500	0.1027
	5.00	0.07299	0.02117	0.070	-0.0034	0.1493
	6.00	0.05291	0.02117	0.385	-0.0234	0.1293
	7.00	0.05738	0.02117	0.279	-0.0190	0.1337
	8.00	0.02145	0.02117	0.996	-0.0549	0.0978
	9.00	0.00298	0.02117	1.000	-0.0734	0.0793
	10.00	-0.00821	0.02117	1.000	-0.0846	0.0681
	11.00	0.01210	0.02117	1.000	-0.0642	0.0884

One-way ANOVA was performed to analyse the concentration effects of HI propolis towards the cell viability of ORL-48 cells. The one-way ANOVA revealed a statistically significant interaction between the effects of the concentration of HI propolis and the cell viability of ORL-48 cells. ($F = (11,24) = 5.480$, $p <$

0.05). Post-hoc Tukey test was performed to identify which concentration significantly reduced the cell viability of ORL-48 cells.

B. Two-way ANOVA

Table S3. Two-way ANOVA in assessing the effects of HI propolis concentration and duration on ORL-48 cell viability.

Tests of Between-Subject Effects

Dependent Variable: OD

Source	Type III Sum of Squares	*df*	Mean Square	*F*	Sig.
Corrected Model	.455[a]	35	.013	17.416	.000
Intercept	3.639	1	3.639	4870.679	.000
Concentration	.292	11	.027	35.467	.000
Incubation	.065	2	.032	43.421	.000
Concentration * Incubation	.099	22	.005	6.026	.000
Error	.054	72	.001		
Total	4.149	108			
Corrected Total	.509	107			

[a]R Squared = .894 (Adjusted R Squared = .843)

A two-way ANOVA was performed to analyse the concentration effects of HI propolis and different time intervals on the cell viability of ORL-48 cells. The two-way ANOVA revealed a statistically significant interaction between the concentration effects of HI propolis and the different time intervals. ($F = (22,72) = 6.026$, $p < 0.05$).

C. Morphological Appearance

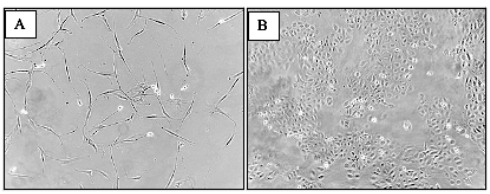

Fig. S2. Morphological appearance under an inverted light microscope after 24 h.

(A) HGF cells (p34), spindle-shaped; cells are bipolar and refractile
(B) ORL-48 cells (p6), polygonal-shaped epithelial-like cells. Magnification × 100.

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D. Equipment List:

- Refrigerator and freezer (-80˚C) (Sanyo)
- Laboratory blender (Philips, Eindhoven, Netherlands)
- Centrifuge (Kubota)
- Orbital laboratory shaker (SK-600, Lab Companion, Jeiotech)
- Cell culture hood (BSC Lab 2)
- Sterilizer (Sanyo)
- Lyophilizer (Labconco Freezone 2.5, USA)
- Rotary evaporator (Buchi R-210, Switzerland)
- Ultrasonic bath (UC-10, Lab Companion. Jeiotech)
- Inverted microscope (Nikon Eclipse)

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