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Research Article

Ethanolic Extract of Propolis from *Tetragonula laeviceps*: Selective Cytotoxicity for MCF-7 Breast Cancer cells

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ABSTRACT

Introduction: Many patients with breast cancer fail to respond to conventional chemotherapeutic agents; these agents are also associated with numerous adverse events and toxicities. These concerns have led to the ongoing search for natural ingredients with antitumor effects. As such, the aim of this study was to explore the anti-cancer properties of an ethanolic extract of propolis (EEP), a natural product derived from the stingless bee, from *Tetragonula laeviceps*. **Methods:** Bioactive components of EEP were identified by gas chromatography–mass spectrometry (GC–MS) and antioxidant capacity was tested by 2.2-diphenyl-1-picrylhydrazyl (DPPH) analysis. Selective cytotoxic actions of EEP on both MCF-7 and Vero cells were then evaluated using the MTT assay. Polyphenols were identified as the major components of EEP from *T. laeviceps*. **Results:** our results indicated that EEP was selectively toxic for human MCF-7 breast cancer cells and had only limited impact on African Green Monkey kidney Vero cells. **Conclusion:** EEP from *T. laeviceps* has bioactive components that may selectively inhibit the proliferation of cancer cells. As such, EEP may be useful source material to be used for the development of novel anti-cancer agents.
Key words: Breast Cancer, MCF-7 Breast Cancer Cells, Propolis, Proliferation.

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INTRODUCTION

Breast cancer is the most common form of malignancy in women worldwide.[1,3] The Global Cancer Observatory (2018) estimates that mortality from breast cancer in Asia may reach as high as 49%; this rate exceeds that of any other region in the world.[3] The development of early detection methods has not reduced the incidence of breast cancer in Indonesia, where breast cancer remains at the top of the list among all malignancies.[4,5] Therapeutic modalities that are currently in use include surgery, chemotherapy, radiotherapy, and targeted therapy.[6]

Inherent toxicity, adverse events, and resistance to standard drugs used to treat breast cancer continue to be highlighted as critical negative features of current chemotherapeutic modalities.[7] As such, many researchers have refocused their efforts toward improving our understanding of natural ingredients that may exert antitumor effects with minimal toxicity. An ideal candidate for anti-cancer therapy would be one that has the capacity to inhibit the growth or to eliminate cancer cells while promoting no harm to normal cells.[8]

Propolis is a natural resin that contains more than 300 bioactive compounds. Many researchers are intrigued with the possibility of using propolis as source material for alternative therapies for breast cancer due to its capacity to promote apoptosis selectively among cancer cells. As such, propolis may be an ideal candidate for development as a natural chemotherapeutic agent for the treatment of breast cancer.[9,10]

Propolis is produced by *T. laeviceps*, which is a species of stingless bees found primarily in Southeast Asia, including in Indonesia. Previous research has suggested that propolis resin has therapeutic efficacy as an antitumor, antioxidant, anti-inflammatory and anti-microbial agent.[11,12] Here, we present our findings that characterize the selective cytotoxic effects of propolis in experiments that target cultured MCF-7 breast cancer cells.

MATERIALS AND METHODS

Cell culture

Human epithelial breast adenocarcinoma MCF-7 and African green monkey (Vero) kidney cell lines were provided by the Hasanuddin University Medical Research Center Laboratory.[13] Both cell lines were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin and 1% amphotericin. The cells were grown in 5% CO_2 at a temperature of 37°C.

Propolis extraction

Propolis from *T. laeviceps* was obtained from a local beekeeper in Subang, West Java, Indonesia. Raw propolis was cleaned and immersed in 70% ethanol at a ratio of 1:20. The propolis–ethanol suspension was shaken at a speed of 200 rpm in rotary shaker while kept in dark for a period of one week. Whatman filter paper No. 1 was used to separate the extract and the residue of propolis-ethanol suspension to obtain Ethanolic Extract of Propolis (EEP).[14] Maceration was carried out for 24 hours to obtain a colorless extract; this was subjected to evaporation and then drying at a temperature of 45°C–50°C to obtain EEP powder. The extraction process was carried out at the Natural Pharmaceutical Pharmacy Laboratory, Research Center for Bioscience and Biotechnology, Bandung Institute of Technology, Bandung, Indonesia.

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GC–MS and 2.2-diphenyl-1-picrylhydrazyl (DPPH) analysis

The chemical composition of the EEP from *T. laeviceps* was analyzed with GC–MS[15] at the Forestry Research and Development Laboratory, Ministry of Forestry, Bogor, Indonesia. DPPH analysis was performed to assess antioxidant activity;[16] this was carried out at the Biopharma Research Center, Bogor Institute of Agriculture, Bogor, West Java, Indonesia.

Cytotoxicity testing

Viability of cells in each culture after treatment with EEP was evaluated using the MTT Cell Proliferation Assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells in 100 µl complete medium were plated in a well in a 96-well plate at a density of 50,000 cells/well. After 24 hours, cells 100 µl EEP was added at 0, 50, 100, 150, and 200 µg/ml. Cells were incubated for 48 hours in three replicate test wells. The medium was discarded and replaced with fresh medium and 10 ml of MTT reagent was added. Cells were incubated for 4 hours before adding 100 ml of solubilization solution to each well. Cells were then incubated overnight. Samples from each well were read on a microplate reader (Multiskan™ FC, Thermo Fischer Scientific, Waltham, MA, USA) at a wavelength of 570 nm. Viability was calculated with the formula: (OD_{570} cell treatment – OD_{570} blank)/ OD_{570} control – OD_{570} blank) × 100. Half minimal inhibitory concentration (IC_{50}) values were obtained as per algorithms in GraphPad Prism 6. The selectivity index was identified as the ratio of IC_{50} MCF-7 to IC_{50} Vero.

Statistical analysis

All data obtained are in the form of mean ± SD. Differences in mean values between groups were tested using one-way analysis of variance with the Tukey Multiple Comparison Test or the Kruskal–Wallis non-parametric test. Results from independent samples were evaluated with a t-Test or Mann–Whitney U Test; these were used to compare results from two test groups only. All data were analyzed using GraphPad Prism 6 software with a significance value of $p < 0.05$.

RESULTS

Identification of bioactive substances in EEP from T. laeviceps

Preliminary studies by GC-MS reveal that polyphenols are the dominant biochemical components of EEP from *T. laeviceps*. Polyphenols comprise 58% of the dry weight of this extract; these compounds include phenolic acid, pyrocatecol, methylphenol, methoxyphenol, ethylguaiacol, ethylphenol, dimethoxyphenol, cresol, and guaiacol (Figure 1); other components include fatty acid esters, aromatics, and benzofuran. DPPH analysis showed that 14.53 ppm concentration (IC_{50}) of EEP was able to inhibit half of free radicals 2.2-diphenyl-1-picrylhydrazyl compared with 2.54 ppm concentration of vitamin C (IC_{50}) as control.

EEP from T. laeviceps shows selective cytotoxic effects on MCF-7 breast cancer cells

MCF-7 cells treated with EEP from *T. laeviceps* (0–200 µg/ml) for 48 hrs demonstrated a dose–dependent reduction in viability, with an IC_{50} value of 79.45 µg/ml (Figure 2(a)). We tested the effects of EEP *T. laeviceps* on African green monkey kidney Vero cells. The results include a minor cytotoxic effect at 50 µg/ml EEP, although overall the IC_{50} value was calculated at >1000 µg/ml; we observed no consistent impact with regard to cell death following the increase in treatment dosages (Figure 2(b)). One-way ANOVA revealed significant differences with a p value = 0.0044 compared to the control group.

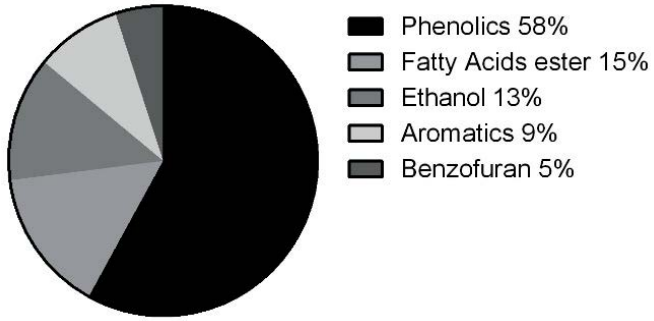

Figure 1: Bioactive substances identified in EEP from T. *laeviceps* by GC-MS in which phenolics compound comprised as major component.

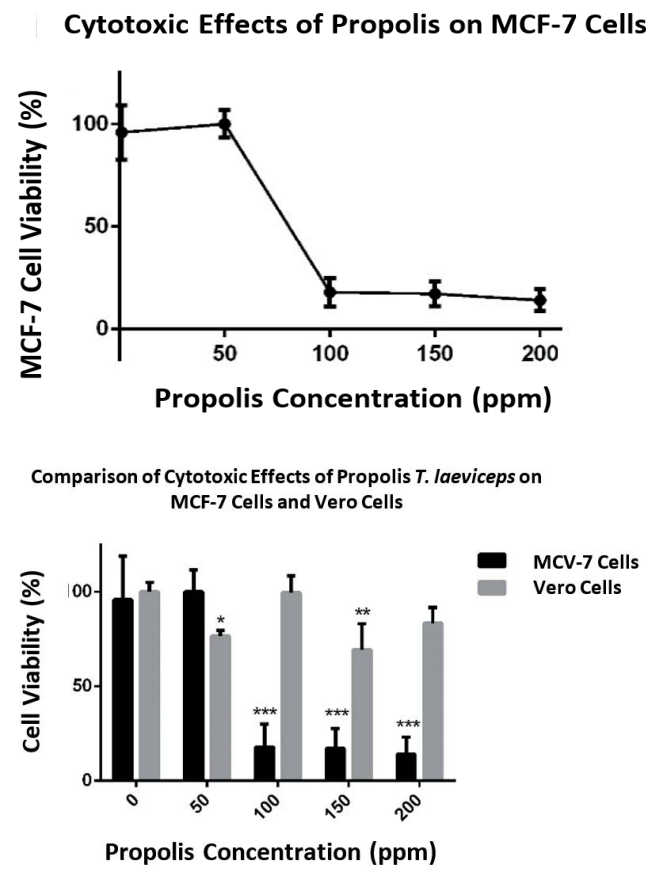

Figure 2: MTT cell proliferation assay. (a) Cytotoxic effect of EEP from T. *laeviceps* on MCF-7 breast cancer cells. Shown here is the dose-dependent response with an IC_{50} value of 79.45 µg/ml; $p < 0.0001$ compared to control group, one-way ANOVA. (b) EEP from T. *laeviceps* has selective cytotoxic impact on MCF-7 breast cancer cells compared to results when targeting Vero cells. The Tukey Multiple Comparison test shows a significant difference in the percentage of cell viability with a ***p value < 0.001 at concentrations of 100 µg/ml, 150 µg/ml, and 200 µg/ml compared to MCF-7 cell control. Interestingly, the EEP inhibited growth of Vero cells, with *p < 0.033 and **p < 0.002 at concentrations of 50 µg/ml and 150 µg/ml compared to 0. All experiments were performed in triplicate.

DISCUSSION

The antitumor, antioxidant and anti-inflammatory activities of polyphenols have been widely reported [17–20]. Niedzwiecki *et al.* (2016)[21] reported that polyphenols found primarily in plants are capable of inhibiting tumor proliferation, growth, angiogenesis, and

metastasis and can promote inflammation that leads to programmed cell death, or apoptosis.

The fatty acid ester derivative phloridzin was identified as a pro-apoptotic agent that targeted liver cancer cells, suppressing the expression of mediators involved in cell cycle regulation, DNA topoisomerase activity, and epigenetic mechanisms.[22] Bandyopadhyay *et al.* (2012)[23] reported that the polyaromatic compound, pyrenyl ether, was cytotoxic for HT-29 colon cancer cells and Hela cervical cancer cells and was more effective than the chemotherapeutic agent, cisplatin Furthermore, Soleimani *et al.* (2015)[24] reported that several compounds derived from benzofuran showed potent cytotoxic activity and were capable of promoting apoptosis in cells of the MCF-7 breast cancer line.

Cytotoxic effects observed may result from the combination of bioactive substances identified in EEP. Niedzwiecki *et al.* (2016)[21] reported that phenols were among the predominant bioactive materials that were effective chemo-preventive and chemotherapeutic agents. Furthermore, Vatansever *et al.*, (2010)[25] reported that propolis from Turkey could trigger cell death *via* the apoptotic pathway specifically by activating the caspase 6,8 and 9 cascade in MCF-7 cells. Kustiawan *et al.*, (2015)[26] found that propolis from *T. incisa* contains moderately potent cardol compounds which may function as anti-cancer agents by triggering cessation of the cell cycle that ultimately results in apoptosis. Similarly, phenol compounds are reported to be capable of modulating levels of intracellular kinases, regulators of cell proliferation, transcription factors, the tumor suppressor protein, p53, and reactive oxygen species via pathways that promote cell death.[27]

Polyphenols identified in EEP may also target proteins that play critical roles in modulating cell death pathways including Fas/FasL,[20,28] NFkβ,[29] Bak,[30] Bax,[31] and p53.[32] Other substances typically identified in propolis extracts may inhibit the cancer cell cycle by modulating cyclin B1[33] and by promoting cellular necrosis.[34-36]

Selective cytotoxic effects of propolis have been reported previously. Xuan *et al.*, (2014)[10] performed a comparative study on the efficacy of propolis used to target breast cancer cells (MCF-7 and MDA-MB-231) and human venous endothelial cells (HUVECS). Among their findings, propolis was minimally cytotoxic toward normal HUVECS cells. Demir *et al.* (2016)[37] reported the selectivity of Turkish propolis in a study that examined its impact on A549 lung cancer cells compared to normal fibroblasts. This selectivity may be due to one or more of the bioactive components contained in propolis extracts. Choudhari *et al.* reported the EEP might promote loss of viability of cancer cells to a greater extent than normal cells due to synergistic activity among phenol compounds and other components.[38]

CONCLUSION

The results of this study indicate that bioactive compounds in EEP from *T. laeviceps* promote selective cytotoxicity when used to treat MCF-7 breast cancer cells in experiments performed *in vitro*. These preliminary studies require further evaluation so that the biomolecules that promote critical differentiation between human cancer and normal cells can be identified and developed as novel anti-cancer therapeutic agents.

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ETHICS APPROVAL

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia (712/UN4.6.4.5.31/PP36/2019).

CONFLICTS OF INTEREST

The authors declare that there are no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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