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RESEARCH ARTICLE

Cytotoxicity Effect of Aqueous Propolis Extract of *Geniotrigona thoracica* Sumatrans on Colo-201 Colon Cancer Cell Line and Senescence Colo-201 Colon Cancer Cell Line Induced by Low-dose Doxorubicin

Zahra Nabila Latama,[1] Achadiyani Achadiyani,[2] Mutiara Mila Kamilah,[3] Ramadhani Eka Putra,[4] Ida Kinasih,[5] Lia Faridah,[2,6] Savira Ekawardhani[2,6]

[1]Master of Biomedical Science Study Program, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia, [2]Department of Biomedical Science, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia, [3]Parasitology Laboratory of Human, Safety, and Environment (HSE), Universitas Padjadjaran, Bandung, Indonesia, [4]School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung, Indonesia, [5]Department of Biology, Faculty of Sciences and Technology, UIN Sunan Gunung Djati, Bandung, Indonesia, [6]International Collaboration Research Lab Ehime University-Universitas Padjadjaran, Bandung, Indonesia

Abstract

Propolis, a resinous compound honeybees produce, demonstrates an extensive spectrum of powerful biological properties. However, the anti-cancer activity of propolis derived from *Geniotrigona thoracica* Sumatrans has yet to be reported. Thus, we sought to investigate the cytotoxicity of aqueous propolis extracts from *Geniotrigona thoracica* Sumatrans against Colo-201 colon cancer cell line and senescence Colo-201 colon cancer cell line induced by low-dose doxorubicin. This study was conducted at the Parasitology Laboratory of Human, Safety, and Environment, Universitas Padjadjaran Bandung from January to May 2024. This study assessed cell viability using the WST-1 test. Non-induced Colo-201 cells were treated with an aqueous extract of propolis (AEP) 100 ppm, or 5-fluorouracil (5-FU) 5 mg/ml as the positive control or water as a vehicle on untreated control. Colo-201 senescence was induced by doxorubicin 0.1 µM for three days. Doxorubicin-induced Colo-201 senescence was then treated with AEP 100 ppm, with 5-FU 5 mg/ml as the positive control, or with the combination of AEP 100 ppm and 5-FU 5 mg/ml, or water as a vehicle on untreated control. The data were analyzed using SPSS version 25.0, a one-way ANOVA, and Tukey's post hoc test. The results showed that AEP has cancer-killing effects on Colo-201 cells and Colo-201 senescent cells induced by low-dose doxorubicin. AEP-treated Colo-201 cells and Colo-201 senescent cells induced by low-dose doxorubicin viability were significantly reduced to 37.15% and 13.72%, respectively, although slightly higher than those of the 5-FU-treated one at this concentration. There was also a decrease in the cancer-killing effect of 5-FU from 88.55% in non-induced Colo-201 cells to 41.5% in the doxorubicin-induced Colo-201 senescence model. In conclusion, aqueous extract of propolis from *Geniotrigona thoracica* Sumatrans showed cancer-killing-effects both on the Colo-201 colon cancer cell line and senescence Colo-201 colon cancer cell line induced by low-dose doxorubicin.

Keywords: Aqueous extract of propolis, Colo-201 cells, colon cancer cell line, fluorouracil, *Geniotrigona thoracica* Sumatrans, senescence, WST-1 assay

Introduction

Cancer is a broad term for many diseases affecting any body region. Other terminology used include malignant tumors and neoplasms. One distinguishing aspect of cancer is the rapid formation of aberrant cells that grow past their normal borders and eventually infiltrate neighboring sections of the body and spread to other organs; this process is known as metastasis. The leading cause of cancer-related death is widespread metastases. Cancer is the most common cause of death worldwide, accounting for approximately ten million deaths by 2022. In 2022, the most prevalent new cancer cases were lung cancer (2.48 million new cases), breast cancer (2.3 million new cases), colorectum (1.92 million new cases), prostate cancer (1.46 million new cases), and stomach cancer (968 thousand new cases). In Indonesia, based on Global

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Correspondence: Savira Ekawardhani. Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadajaran. Jln. Prof. Eyckman No. 38, Bandung 40161, West Java, Indonesia. E-mail: savira@unpad.ac.id

Conservatory Cancer 2022, the number of new cases of cancer was 408,661, with a death toll of 242,988.[1] Based on the Indonesia Basic Health Survey (*Riskesdas*) in 2018, the prevalence of cancer in Indonesia is 1.8 per 1000 population.[2] It is the second most prevalent new cancer case among men (29 thousand new cases) and the fourth among men and women (35 thousand new cases).[3]

In 2022, Hanahan[4] edited and expanded the list of cancer hallmarks as the understanding of cancer mechanisms deepened. In the updated list of cancer hallmarks, senescent cells have been added to the list of new cancer hallmarks. Cell senescence is characterized by irreversible cell cycle arrest by causing telomere shortening.[5] Many chemotherapeutic drugs, such as doxorubicin (Dox), affect the cellular states of cancer cells, including senescence induction.[6] Dox is frequently used for the treatment of breast, esophageal, liver, and other cancers because it triggers cell growth arrest with senescent markers.[7] Dox causes senescence through a p53-dependent mechanism and telomere disruption in various malignancies.[8] Nevertheless, cellular senescence suppresses proliferation activity; these cells will continue to be metabolically active and contribute significantly to developing drug resistance and tumor recurrence.[9] 5-fluorouracil (5-FU) is the primary chemotherapeutic drug used in the treatment of colorectal cancer. Cardiotoxicity is a relatively infrequent but concerning side effect of 5-FU therapy. This condition is especially troublesome in the context of colorectal cancer since 5-FU is one of the essential components of first-line treatments.[10]

Propolis is a natural substance produced by honey bees from numerous botanical resources. It has become recognized for its pharmacological benefits, including antibacterial, antiviral, anti-inflammatory, and anti-cancer properties.[11] For example, propolis from *Tetragonula sapiens* shows the prospect of decreasing diabetes and hypertension.[12] In recent years, substantial in vitro and in vivo research has revealed that propolis has anti-cancer potential. Propolis inhibits cancer cell proliferation, induces apoptosis by regulating different signaling pathways and halting the tumor cell cycle, induces autophagy and epigenetic modulations, and inhibits tumor invasion and metastasis. Propolis targets cancer-related signaling pathways, including p53, β-catenin, ERK1/2, mitogen-activated protein kinase (MAPK), and NF-κB.[13]

Propolis is a resinous mixed form that includes beeswax and partially digested exudates from plant leaves and buds. Its chemical composition changes substantially according to the bee species, geographic area, plant type, and meteorological conditions. Propolis is well known for its antioxidative, antibacterial, anti-inflammatory, and anti-cancer effects.[14] Studies have shown that the presence of caffeic acid phenethyl ester, artepillin C, and chrysin is responsible for propolis' anti-cancer properties. The majority of earlier research revealed that propolis and its active chemicals prevent cancer progression by targeting numerous signaling pathways, including phosphoinositide 3-kinases (PI3K)/AKT and MAPK signaling molecules, and causing cell cycle arrest. Propolis' anti-cancer actions are broad since it targets various cancer metabolic targets. Critical pathways for cancer treatment include preventing metastatic spread, inhibiting NF-κB nuclear localization, regulating gene expression, inactivating matrix metalloproteinases, activating tumor suppressors, and altering TNF-related apoptosis-inducing ligand resistance.[15]

In Indonesia, there are several types of honey-producing bees. A recent study revealed that Indonesian stingless bee propolis collected from South Sulawesi, Bintan, and Lampung may include a wide range of phytochemical substances and antioxidant compounds.[16] Another honey-producing bee is the bees from genus *Trigona*. *Trigona* sp. is a species of bee distributed on the islands of Sumatra, Kalimantan, Java, and Maluku. Propolis from *Trigona incisa* and *Trigona fusco-balteata*, native to East Kalimantan, Indonesia, has in vitro cytotoxic activity against human cancer cell lines.[17] *Trigona incisa* propolis had the highest in vitro cytotoxicity against the SW620 colon cancer cell line, with 6% cell survival at 20 μg/ml concentration. The following study on propolis of *Trigona incisa* revealed that it has moderate in vitro anti-cancer effect on human cancer cell lines, and cardol or 5-pentadactyl resorcinol was shown to be the main active component, causing apoptosis in SW620 cells within 6 hours and cell cycle arrest at the G1 subphase.[18] However, no research has been conducted on propolis produced from *Geniotrigona thoracica*

Sumatrans. Therefore, its anti-cancer activity is unknown. Thus, we sought to investigate the cytotoxicity of the aqueous extract of propolis of *Geniotrigona thoracica* Sumatrans on Colo-201 colon cancer cell line and senescence Colo-201 colon cancer cell line induced by low-dose doxorubicin.

Methods

Raw propolis used for this study was purchased from beekeepers in Sumatera, Indonesia. This propolis was used in this study because it had previously been checked in a certified laboratory using gas chromatography-mass spectrometry tools. Raw propolis purchased from beekeepers and processed by PT Liberta was extracted using the maceration method with water as the solvent. The resulting solution was thickened using a rotary evaporator to form a paste, which was then dried in a sterile environment to obtain an aqueous extract of propolis (AEP). The dried extract was dissolved in sterile distilled water, sonicated for 4×30 minutes, and filtered through a 0.22-micron membrane to ensure sterility. The final concentration of the AEP solution used in this research was 100 ppm.[19]

This study was conducted at the Parasitology Laboratory of Human, Safety, and Environment (HSE), Universitas Padjadjaran from January to May 2024. The Parasitology Laboratory of HSE at Universitas Padjadjaran provided the Colo-201 colon cancer cell line [ATCC CCL-224], which Ehime University Japan had given. Cells were cultured in RPMI 1640 [Corning 10-040-CV], 10% Panexin basic [PAN Biotech P04-96900], 100 U ml-1 penicillin, and 100 μgml^{-1} streptomycin [PAN Biotech P06-017100], and then incubated at 37°C in a humidified atmosphere with 5% CO^2 until 80–90% confluence. The medium for growth was discarded, and cells were treated with trypsin-EDTA (Servicebio G4001). Tripsynization was put off by adding an equal volume of growth medium. Cells were suspended and placed in a tube, then centrifuged at 500 ×g for 5 minutes. The supernatant was discarded, and the pellets were resuspended in a 4–5 ml growth medium. The cell suspension was transferred into a T-flask containing a growth medium with a cell density of 10,000/cm^2. Medium was renewed every two days. Cells were incubated at 37°C with 5% CO^2.[20]

For senescence induction, 0.1 μM Dox was used to induce senescence on the Colo-201 colon cancer cell line because previous studies have shown that 0.1 μM Dox-induced senescence in the cancer cell line.[21] Cells were seeded at a density of 10 or/cm^2 for 24 hours and then treated with 0.1 μM Dox for three consecutive days to induce senescence of Colo-201 cells. The Colo-201 senescent cells were treated with AEP, 5-FU, or both AEP and 5-FU.

This experimental research employed a post-test-only control randomized group design with n=3 and a group size of 5 for both Colo-201 cells and Colo-201 senescent cells. The treatment in the Colo-201 group included untreated cells (Colo-201 cells), treatment control (Colo-201 cells with 5-FU 5 mg/ml), colo-201 cells with Dox 0.1 μM, treated cells (Colo-201 cells with AEP 100 ppm), and colo-201 cells with a combination of Dox 0.1 μM, AEP 100 ppm, and 5-FU 5 mg/ml. The treatment on Colo-201 senescent cell group included untreated cells (Colo-201 cells), doxorubicin-induced colo-201 senescent cells, doxorubicin-induced Colo-201 senescent cells with AEP 100 ppm, doxorubicin-induced Colo-201 senescent cells with 5-FU 5 mg/ml, and doxorubicin-induced Colo-201 senescent cells with combination of AEP 100 ppm and 5-FU 5 mg/ml.

Cell viability was assessed with ten μl WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) in RPMI medium and incubated at 37°C for 4 hours. First, 100 μl cells were plated (20×103 cells per well) and incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO^2. After 24 hours, the medium was removed and replaced with 90 μl of fresh medium, ten μl of AEP (100 ppm) in RPMI, and 10 μl of 5-FU (5 mg/ml) in different plates and in triplicate, followed by 48 hours of incubation—the cells with no treatment served as the control. Ten μl of WST-1 was applied to each well. The plate was incubated for 4 hours in an incubator with 5% CO^2 at 37°C. The absorbance was measured at 450 nm using a microplate reader [MultiskanSky Go]. Statistical analyses were done with SPSS version 25.0 using one-way ANOVA (Tukey's multiple comparison test). Figures were made with GraphPad Prism 5 (San Diego, CA, USA), and data were presented as a mean±SD. A value of $p<0.05$ was considered statistically significant.[22]

Results

As shown in Figure 1, AEP showed cancer-killing effects on Colo-201 cells compared to the control. AEP 100 ppm could lower the viability of Colo-201 cells, which is 37.15%, slightly higher than those treated with 5-FU, which is 11.45% at this concentration. The Tukey's post hoc test showed significant differences from the control ($p<0.05$). Figure 1 also showed that 0.1 μM Dox did not affect the viability of the cell. Therefore, 0.1 μM Dox could be used as a senescence induction dose.

As shown in Figure 2, AEP showed cancer-killing effects on Dox-induced Colo-201 cells compared to Dox-induced Colo-201 cells without treatment. Interestingly, the senescence-induction protocol by 0.1 μM Dox for three days could result in decreased viability of Colo-201 cells to 20.75%. Tukey's post hoc test showed significant differences compared to the control ($p<0.05$) but did not show statistically significant differences among treatments.

There was also a decrease in the cancer-killing effect of 5-FU from 88.55% in non-induced Colo-201 cells to 41.5% in the Dox-induced Colo-201 senescence model, meaning that the doxorubicin-induced Colo-201 senescence model were more resistant to 5-FU (Table). AEP 100 ppm could lower the viability of Dox-induced Colo-201 cells to 13.72%, slightly higher than those treated with 5-FU with 8.63% at this concentration.

Discussion

This research found that administration of AEP in Colo-201 cells and Colo-201 senescent cells induced by low-dose doxorubicin resulted in reduced viability of Colo-201 cells (37.15%) and Colo-201 senescent cells induced by low-dose doxorubicin (13.72%). This research also showed a decrease in the cancer-killing-effect of 5-FU from 88.55% in non-induced Colo-201 cells to 41.5% in the doxorubicin-induced Colo-201 senescence model; this phenomenon was caused by the Colo-201 senescent cells model having a phenotype that was more resistant to 5-FU. Interestingly, the doxorubicin-induced Colo-201 cells also showed decreased cell viability (20.75%). This research employed an aqueous extract of propolis and WST-1 to assess cytotoxicity in the Colo-201 colon cancer cell line and senescence Colo-201 colon cancer cell line induced by low-dose doxorubicin which is a novel approach in experimental research. This study's

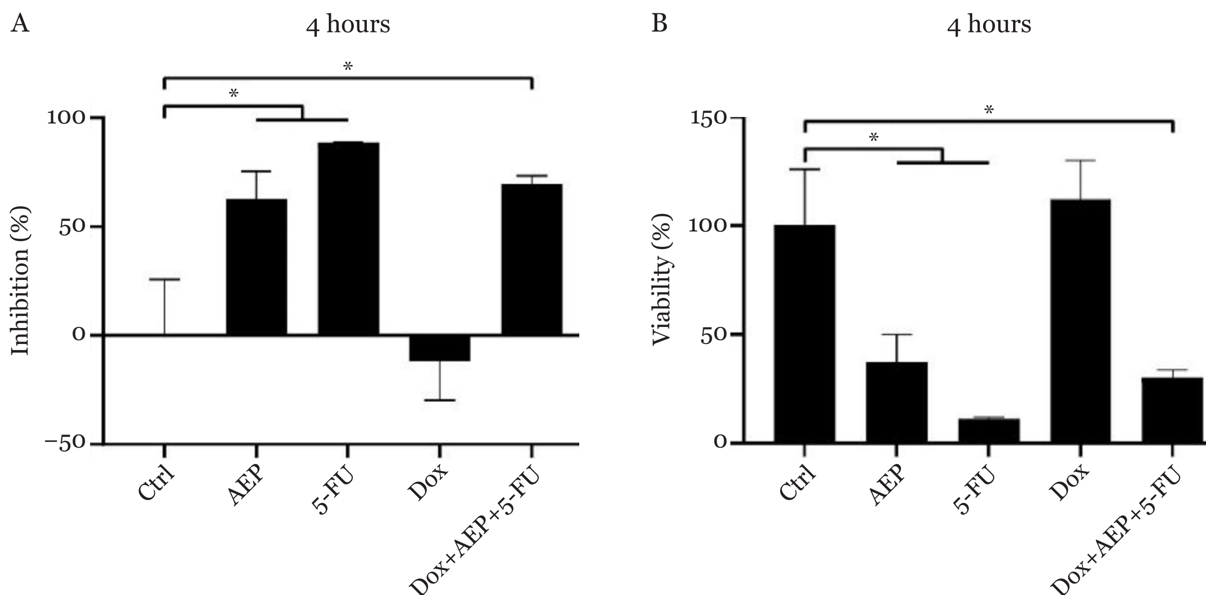

Figure 1 Colo-201 Cells Treated with Aqueous Extract of Propolis of *Geniotrigona thoracica* Sumatrans

Note: (A) percentage of cell growth inhibition; (B) percentage of viable cells; Ctrl: control; AEP: aqueous extract of propolis 100 ppm; 5-FU: 5-fluorouracil 5 mg/ml; Dox: doxorubicin 0.1 μM; *shows significant differences compared to the control based on the Tukey's post hoc test with $p<0.01$; the data is shown as means±SD with n=3

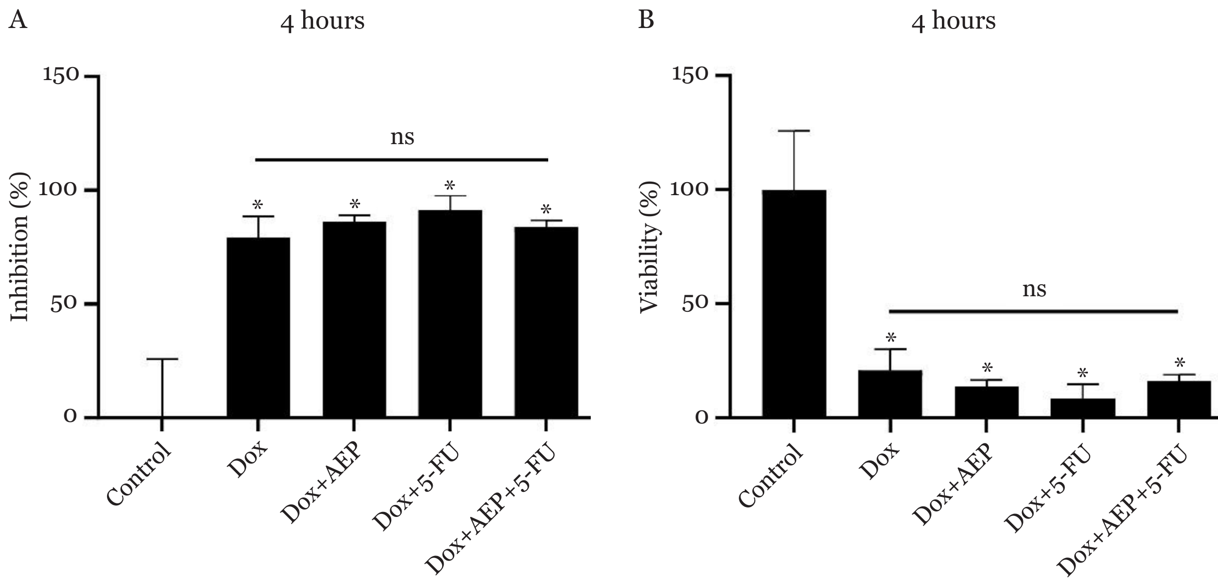

Figure 2 Doxorubicin-induced-senescence Colo-201 Cells Treated with Aqueous Extract of Propolis of *Geniotrigona thoracica* Sumatrans

Note: (A) percentage of cell growth inhibition; (B) percentage of viable cells; Control: Colo-201 cells; Dox: Colo-201 cells induced by 0.1 µM doxorubicin for 3 days; AEP: aqueous extract of propolis 100 ppm; 5-FU: 5-fluorouracil 5 mg/ml; *shows significant differences compared to the control based on the Tukey's post hoc test with $p<0.05$; ns: shows no statistically significant differences among treatments; the data is shown as means±SD with n=3

limitations are the necessity to characterize the half maximal inhibitory concentration, the effect of aqueous extract of propolis on non-cancerous cells, and the characterization of the compounds of aqueous extract propolis from *Geniotrigona thoracica* Sumatrans stingless bee.

Research on senescent cancer cell lines is still scarce. Senescent cancer cell lines can be obtained using senescence induction protocol using low-dose doxorubicin.[21] Administration of a combination of *Garcinia mycorrhiza* fruit extract and doxorubicin without a senescence-induction protocol has been shown not to affect cell viability.[23] Research into the effects of propolis on cancer cell lines and senescent cancer cell lines has never been carried out. Recent research revealed that curcumin, caffeine, and thymoquinone potentially induced apoptosis of both proliferative and senescent colon cancer HCT116 and breast cancer MCF7 cell lines.[24]

Table Differences in the Percentage of Viability Cells and Cancer-killing-effects of Colo-201 Cells and Doxorubicin-induced Colo-201 Senescence with Various Treatments

	Colo-201 Cells		Dox-induced Colo-201 Cells	
	Cell Viability	Cancer-killing-effects Compared to Untreated Colo-201 Cells	Cell Viability	Cancer-killing-effects Compared to Untreated Dox-induced Colo-201 Cells
Untreated Dox-induced Colo-201 cells			20.75%	
With AEP	37.15%	62.85%	13.72%	66.12%
With 5-FU	11.45%	88.55%	8.63%	41.5%
Untreated Colo-201 cells	100%		100%	

Note: AEP: aqueous extract of propolis 100 ppm; 5-FU: 5-fluorouracil 5 mg/ml; Dox-induced Colo-201 cells: Colo-201 cells induced by 0.1 µM doxorubicin for 3 days; cell viability: percentage of viable cells

Based on another study, the cytotoxicity of propolis extracts from *Trigona sirindhornae* against two head and neck squamous cell carcinoma (HNSCC) cell lines, a dichloromethane extract of propolis (DMEP) was prepared to generate three fractions: DMEP-A, DMEP-B, and DMEP-C. This study shows that *Trigona sirindhornae*-produced propolis displays cytotoxic effects against HNSCC cells. Moreover, DMEP-B and DMEP-C differentially inhibited the proliferation of a metastatic HNSCC cell line.[25]

Propolis from stingless bees has a variety of mechanisms with anti-cancer potential. These mechanisms include apoptotic events; modulation of BAX, BAD, BCL2-L1 (BCL-2 like 1), and BCL-2; mitochondrial membrane depolarization; increased caspase-3 activity; poly (ADP-ribose) polymerase (PARP) cleavage; and necroptosis-induced cell death via receptor-interacting protein kinase 1 (RIPK1) activation. Furthermore, a link between substances with antioxidant and anti-inflammatory properties is revealed, which aids in preventing cancer growth.[26]

In another study conducted on stingless bee propolis from Indonesia, propolis isolated from *Trigona incisa* induced apoptosis in the SW620 human colorectal cancer cell line. Cardol, a critical bioactive ingredient in *Trigona incisa* propolis from Indonesia, has high in vitro antiproliferative action against the SW620 colorectal cancer cell line (IC_{50} of 4.51±0.76 μg/ml). Cardol causes G0/G1 cell cycle arrest and apoptotic cell death. Cardol-induced cellular death in SW620 cells was induced by increased oxidative stress and the mitochondrial apoptotic pathway, which could be a potential molecular explanation for Cardol's antiproliferative action.[27] Recently, a study found that products derived from the stingless bee *Trigona* spp. from Luwu Utara (South Sulawesi, Indonesia), particularly a water-soluble extract of propolis and bee pollen, inhibited the proliferation of the human breast cancer MCF-7 cell line. The results of the DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant experiment demonstrated that water-soluble propolis and bee pollen had high antioxidant activity, with half-maximal effective doses against DPPH radicals of 1.3 and 0.4 mg/ml, respectively. Water-soluble propolis and bee pollen had substantial antiproliferative activity in MCF-7 cells, with IC_{50} values of 10.8±0.06 and 18.6±0.03 mg/ml, respectively ($p<0.05$). The current findings suggested that water-soluble propolis and bee pollen could be progressively researched as breast anti-cancer therapies.[28]

At last, propolis and its components have been shown to suppress cancer signaling pathways, including PI3K/AKT/mTOR, NFκB, JAK-STAT, TLR4, VEGF, TGFβ, and apoptosis and autophagy. Propolis and its polyphenolic/flavonoid components may have anti-cancer effects through the following cellular and molecular mechanisms: (i) suppression of cancer/precancerous cell proliferation via direct cytotoxic effect or immunomodulatory effect; (ii) reduction in cancer stem cell populations; (iii) inhibition of specific oncogene signaling pathways; (iv) antiangiogenic effects; (v) modulation of the tumor microenvironment; (vi) inhibition of cellular glucose uptake and metabolism in the cancer cell; and finally, (vii) as a supplementary or complementary approach to conventional anti-cancer.[29]

Conclusions

The aqueous extract of *Geniotrigona thoracica* Sumatrans's propolis showed cancer-killing effects on the Colo-201 colon cancer cell line and senescence Colo-201 colon cancer cell line induced by low-dose doxorubicin. This finding suggested that the aqueous extract of *Geniotrigona thoracica* Sumatrans's propolis could be used as a complementary treatment for colon cancer.

Conflict of Interest

The authors stated that they have no conflicts of interest.

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