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Evaluation of the Anticarcinogenic Potential of Propolis Ethanolic Extract (PEE) from Philippine Stingless Bee (*Tetragonula biroi* Friese)

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***Abstract*— *With the increasing cancer morbidity and mortality, several treatments have been conducted to find alternative remedies for cancer. Natural products such as bee propolis derived from plant resins were investigated on their anticarcinogenic potential by determining their antioxidant, antimitotic, cytotoxic, and antiangiogenic activities. Propolis from Tetragonula biroi Friese was extracted with 96% ethanol to produce the propolis ethanolic extract (PEE), then was tested for the presence of phytochemicals. Total phenolic content (TPC) and total flavonoid content (TFC) of PEE was determined by calculating the standard curve from absorbance reading. The antioxidant activity was determined using the DPPH radical scavenging assay. Allium cepa test was utilized to determine the mitotic index of PEE for its antimitotic activity. The cytotoxic activity of PEE was tested using the MTT Assay using MCF-7 cells. Chorioallantoic membrane (CAM) assay using duck eggs was performed to determine the antiangiogenic activity of PEE. Results showed that PEE is dark brown in color with a sticky consistency with a percent yield of 38.064%. Qualitative analyses of PEE detected the presence of the following: flavonoids, phenolics, tannins, and triterpenes. Furthermore, determination of total phenolic content (TPC) and total flavonoid content (TFC) revealed 996.1755 mg GAE/mg and 50.1407 ug QE/mg, respectively. The antioxidant activity of PEE has 100% inhibition, with no significant difference with the positive control, gallic acid (P=0.274). Antimitotic activity was strongly correlated at higher PEE concentration, 1 mg/ml (r=-0.923). Cytotoxic activity of PEE was found to be the highest at 40.15% (100ug/ml) cell inhibition and with an IC50 of 39.34 ug/ml. The antiangiogenic activity of PEE was significant compared to negative control and not significant among treatment groups. The promising results obtained were indications of a strong potential of propolis to be a chemotherapeutic drug in inhibiting cancer progression.*

Keywords*— *Antiangiogenic, antimitotic, antioxidant, cytotoxic.

I. INTRODUCTION

The increased prevalence of cancer over the years can be attributed by the advancements and discoveries made in the field of cancer research. But despite of this, cancer morbidity and mortality are still at peak, ranking second as the leading cause of death globally accounting for 9.6 million deaths in 2018, with about 70% of deaths coming from low- and middle-income countries including the Philippines (WHO 2018). According to Department of Health (DOH), cancer is the third leading cause of morbidity and mortality in the Philippines affecting 189 out of 100,000 Filipinos in 2018. Research and technology allow development of different treatments for cancer. These includes chemotherapy, radiation therapy, immunotherapy, and stem cell transplants (NCI 2019). Although these methods are widely used and proven effective, they are often associated with severe side effects, in addition to their high-cost, low availability, and poor accessibility. According to a report of Philippine Statistics Authority (2016), about 26.3% of the Philippine population is beneath the poverty threshold and does not have the means to afford standard-of-care treatments for cancer. Because of this, the search for a more cost- effective cancer treatment alternative with fewer complications is emerging to provide patients a better quality of life.

Cancer can be caused by several factors, one of which is by the mutation of genes due to DNA alterations. This mutation causes abnormal expression of genes leading to rapid cell division (mitosis), tumor formation and development of new blood vessels or angiogenesis, both are crucial processes in the progression of cancer (Bayuran 2017). Although angiogenesis plays an important role in many physiological processes like embryogenesis, tissue repair, and organ regeneration, the excessive or insufficient angiogenesis contributes to the growth of cancer cells and the metastasis of tumors (Kim and Byzova 2014). This is controlled by chemical signals such as the vascular endothelial growth factor (VEGF), Fibroblast Growth Factor (FGF), and Epidermal Growth Factor (EGF). Angiogenesis Inhibitors (AIs) recognize and bind to these chemical signals, such as VEGF, blocking its receptors, and therefore inhibiting formation of new blood vessels. This causes nutrient and oxygen shortage leading to pan-hypoxia and pan-necrosis in the tumor tissues (Periyanayagam et al. 2009).

Several synthetic AI drugs have been developed and are available in the market such as Avastin and retinoic acid; however, these drugs are costly and have uncertain pathologic side effects (NCI 2019). In view of this, several natural products gained attention in cancer research for their pharmacologic potentials which are safer in long term exposures compared to synthetic drugs. Some of these natural products are commercially available as antioxidants or scavengers. Antioxidants counteract the presence of reactive oxygen species (ROS) in the cells. If not controlled, ROS can

damage DNA and other biomolecules in the cell which can lead to the development of cancer cells (Kim and Byzova 2014). It has been reported that antioxidant activity from natural products, such as plants, is also associated with its cytotoxic activity against cancer cells (Sammar et al. 2019). Cytotoxicity is the ability of compounds to destroy cells by inducing necrosis or apoptosis.

Many natural products have been studied with their ROS-scavenging property, which is also associated with antimitotic, cytotoxic, and antiangiogenic activity. These products can be utilized as preventive and therapeutic agents against cancer cells. Propolis is a natural product derived from plant resins collected by honeybees. It has been used in folk medicine for centuries for its antimicrobial, antioxidative, anti-ulcer and anti-tumor activities, making it a popular area of study in medicine (Lofty 2006).

The wide pharmaceutical potential of propolis can be associated with the synergistic contributions of the different chemical compounds such as vitamins B, C, and E, flavonoids, phenols, and aromatic compounds (Ahangari et al. 2018; Daleprane and Abdalla 2013). The extraction procedure is an essential step in obtaining the said bioactive constituents from propolis. Specifically, maceration of the propolis samples in alcohol, specifically ethanol is considered the easiest and simplest method of extraction in a small research setting and can result to high yield of desired phytochemicals (Azwanida, 2015).

According to Daleprane and Abdalla (2013), the common phytochemicals found in propolis from different plant & bee sources include polyphenols and flavonoids. These are found to have cardioprotective, vasoprotective, antioxidant, antiatherosclerotic, anti-inflammatory, and antiangiogenic. Thus, establishing the wide potential of propolis in medicine and pharmacy.

Although many studies have investigated the wide pharmaceutical potential of propolis, the resin's chemical composition varies depending on the plant from which the bees collect the materials to make it, and as well as the variety of bee species where the propolis was derived. Therefore, studies on bee propolis from different bee species collected in different geographical regions are of utmost significance since these factors affect the property of the specific propolis variant (Bonamigo et al. 2017).

In the Philippines, there are at least seven species of stingless bees. One of them is *Tetragonula biroi* Friese, or locally known as "lukot". It belongs to the tribe Meliponini, subfamily Apinae, family Apidar, under the order Hymenoptera. These species are more abundant than other bee species; thus, its management became cheaper and simpler. This has drawn interest from beekepers and researchers to explore its potential benefits (Belina-Aldemita et al. 2019).

It was reported that propolis from *T. biroi* contains phenols, flavonoids, artepillin C that have potential antioxidant, antimitotic, and antiangiogenic properties (Lamberte et al. 2011; Mendoza 2011; Fajardo 2014; Batac et al. 2020; Belina-Aldemita et al. 2020).

Despite the considerable evidence of the wide spectrum pharmaceutical property of propolis, the evaluation of the bioactive substance from Philippine stingless bee propolis remains poorly understood (Desamero et al. 2019).

In the Philippines, the anti-tumor property of Philippine stingless bee propolis against gastric cancer was examined, but other than that, no other studies were available, and thus the potential of propolis from Philippine stingless bee is poorly understood (Desamero et al. 2019). Therefore, further studies on the bioactive potential of propolis from local source can provide additional knowledge to maximize its use.

Thus, this study investigated the antioxidant, antimitotic, cytotoxic, and antiangiogenic activity of Philippine stingless bee propolis (*Tetragonula biroi* Friese) in cancer cells by its ability to inhibit mitosis and blood vessel formation.

To achieve this, the objectives were the following:

1. To characterize the propolis ethanolic extract (PEE) through phytochemical analyses;
   1.1. Qualitative Analysis
      a. Flavonoids
      b. Phenolics
      c. Tannins
      d. Triterpenes
   1.2. Total Phenolic Content (TPC)
   1.3. Total Flavonoid Content (TFC)
2. To determine the antioxidant activity of PEE through DPPH radical scavenging assay;
3. To determine the antimitotic activity of the PEE varying concentrations using the Allium cepa test;
4. To determine the cytotoxic activity of the PEE varying concentrations using MTT assay; and
5. To determine the antiangiogenic activity of the PEE varying concentrations using chorioallantoic membrane (CAM) assay.

II. METHODOLOGY

A. Propolis Collection and Storage

One (1) kilogram of propolis samples from stingless bee (*T. biroi* Friese) were acquired from a bee farm in Los Baños, Laguna. The propolis samples were certified and authenticated for their source. The collected samples were stored in a refrigerator at 4°C and kept until extraction to preserve the quality of the propolis (Mendoza 2011).

B. Propolis Preparation

The propolis samples were prepared by weighing one (1) kilogram of propolis at room temperature. The samples were cut into small pieces with an average dimension of 0.5mm by 0.5 mm. They were then washed twice with distilled water to remove dirt and sugars. After which, the samples were air dried to remove the excess moisture content (Mendoza 2011; Batac et al. 2020).

C. Preparation of Propolis Ethanolic Extract (PEE)

The extraction followed the method of Pratami et al. (2018) with modifications. The dried propolis samples were macerated with 96% ethanol with a ratio of 1:5 and were allowed to stand for 16 hours. After which, the filtrate and residue were separated through filtration. Distilled water was added to the extract until 70% ethanol-water v/v is obtained. The resulting solution was evaporated using a rotary

evaporator under vacuum at 40°C; then, the resulting dry constituent was stored at the refrigerator until further use.

The percentage yield was determined by using the dry weight of the extract (a) and the soaked sample material (b) using the equation below:

Percentage Yield (%) = a/b x 100

D. Phytochemical Analyses

The preliminary and qualitative phytochemical analyses were used to test the presence or absence of selected phytochemical constituents according to standard methods (Raheel et al. 2017).

To detect the presence of flavonoids, 1 ml of 5% aluminum chloride ($AlCl_3$) was added to 1 ml of reconstituted PEE. An appearance of yellow color solution confirmed the presence of flavonoids.

To detect the presence of phenolics, 2 ml of reconstituted PEE was mixed with equal volume of 5% ferric chloride ($FeCl_3$) solution. A bluish black color is an indication of the presence of phenolics.

To detect the presence of tannins, 50 mg of PEE was mixed with 20 ml distilled water and put into a boil. After which, a few drops of 0.1% $FeCl_3$ was added. The presence of blue-black or brownish green color in the solution is the indication of the presence of tannins.

Salkowski's test was used to detect the presence of triterpenes. Two (2) ml of reconstituted PEE was mixed with few drops of chloroform then filtered. Then, 3-4 drops of concentrated sulfuric acid ($H2SO_4$) were added to the filtrate. The resulting solution was shaken and allowed to stand for 5 minutes. The presence of a golden yellow color solution is an indication of the presence of triterpenes.

The total phenolic content (TPC) assay was performed at the Mammalian Cell Culture Laboratory, Institute of Biology – University of the Philippines Diliman. The method was adapted from Magalhaes (2020). Gallic acid was prepared in 1, 10, 100 and 1000 ppm solutions. 1 mg/ml of the sample was also prepared. In a 96-well plate, the following were added to each well in succession: 50 µl of gallic acid/sample, 50 µl 1:5 Folin-Ciocalteu reagent and 100 µl 0.35M NaOH. Absorbance was read at 760 nm. A standard curve was calculated from the absorbance readings. The total phenolic content of the sample was presented as µg gallic equivalent (GAE)/ml. Three trials were performed in triplicate.

The total flavonoid content (TFC) assay was performed at the Mammalian Cell Culture Laboratory, Institute of Biology – University of the Philippines Diliman. The method was adapted from Sanchez (2020). Quercetin was prepared in 1, 10, 100 and 1000 ppm solutions. 1 mg/ml of the sample was also prepared. In a 96-well plate, the following were added to each well: 50 µl of 6 g/l $NaNO_2$, 50 µl of quercetin/sample, 50 µl $AlCl_3$ (22 g/l) and 50 µl 0.8M NaOH. After a 3-minute incubation, absorbance was read at 510 nm. A standard curve was calculated from the absorbance readings. The total flavonoid content of the sample was presented as µg quercetin equivalent (QE)/ml. Three trials were performed in triplicate.

E. Antioxidant Activity of PEE using DPPH Radical Scavenging Activity

The DPPH radical scavenging assay was performed at the Mammalian Cell Culture Laboratory, Institute of Biology – University of the Philippines Diliman. The method was adapted from Molyneux (2004). Stock solution of DPPH was prepared by dissolving 1 mg of 2,2-diphenyl-1- (2,4,6-trinitrophenyl) hydrazyl (DPPH) in 10 ml ethanol. From the solution, 95 µl was dispensed to 96-well microtiter plates. Gallic acid serves as positive control while ethanol, the solvent of the sample, as negative control. Five microliters of the controls and test sample were dispensed to the wells making a final volume of 100 µl. The plate was incubated in ambient temperature and stored in the dark for 60 minutes. After incubation, absorbance was read at 517 nm. Based on the absorbance readings, free radical inhibition of the test sample was computed using the formula:

$$\%Inhibition=(Abs_{control}-Abs_{sample})/(Abs_{control}-Abs_{GA}) \times 100$$

F. Antmitotic Activity of PEE using Allium cepa Test

The *A. cepa* test method was adapted by the protocol of Raheel et al. (2017) with modifications. *A. cepa* (50 + 10 g individual weight) were acquired from a local market and were grown in containers at room temperature with distilled water without light until rooting. The water was changed daily. Varying concentrations (T1=0.125, T2=0.25, T3=0.50, T4=1mg/ml) of PEE were prepared in containers with tap water. The bulb roots measuring from 2-3 cm were transferred to the containers with PEE and were incubated at room temperature for 12 hours. Onion bulbs that were grown in water were used as control. The mitotic index of the control and treatment groups were recorded after 12 hours.

To determine the mitotic index, the root tips (2-3 cm) were cut and fixed in 45% acetic acid: 1N HCl (9:1). To soften the cell walls, the tips were transferred to 1N HCl for 5 minutes. Then, the root tips were squashed and stained with 0.1% methylene blue. For each root tip, hundred cells were counted in ten (10) fields of observation under high power objective (40X) using a light compound microscope. Cells showing different stages of mitosis were counted and the following formula were used to determine the mitotic index:

$$Mitotic\ Index\ (MI)=(P+M+A+T)/(Total\ cells) \times 100$$

where P is prophase, M is metaphase, A is anaphase, and T is telophase. The treatment groups were compared with root tips grown on water.

G. Cytotoxic Activity of PEE using MTT Assay

The cytotoxicity potential of PEE was determined by MTT assay to evaluate the percent cell inhibition and IC_{50} value in comparison with the standard chemotherapeutic drug, doxorubicin.

The assay was performed at the Mammalian Cell Culture Laboratory, Institute of Biology – University of the Philippines Diliman. The method was adapted from Mosmann (1983) using MCF-7 cells, a human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors. In detail, MCF-7 cells were seeded at 4 or 6 x 104 cells/ml (depending on the cell culture used) in sterile 96-well

microtiter plates. The plates were incubated overnight at 37°C and 5% CO_2. Eight two-fold dilutions of the sample were used as treatments starting from 100 µg/ml down to 0.78 µg/ml. Doxorubicin served as positive control while dimethyl sulfoxide (DMSO) served as negative control. Following incubation, cells were treated with each extract dilution. The treated cells were again incubated for 72 hours at 37°C and 5% CO_2.

After incubation, the media was removed and 3-(4,5-dimethylethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye at 0.5 mg/ml PBS was added. The cells were again incubated at 37°C and 5% CO_2 for 4 hours. After which, DMSO was used to dissolve the formazan crystals formed by the reduction of the dye by the live cells. Absorbance was read at 570 nm. The Inhibition Concentration 50 (IC_{50}) was computed using GraphPad Prism 6. GraphPad Prism 6 computed for the IC_{50} of the sample by employing non-linear regression curve fit on the computed percent inhibition per concentration of the sample. Samples with IC_{50} values less than 30 µg/ml were considered active (Jokhadze et al. 2007).

H. Antiangiogenic Activity of PEE using Chorioallantoic (CAM) Assay

To evaluate the angiogenic activity of PEE, chorioallantoic membrane (CAM) assay was performed. The blood vessel count of PEE varying concentrations was compared with a negative control (untreated setup).

The method for CAM assay was adapted by the protocol of Ribatti (2017) with modifications. Twenty (20) fertilized duck eggs (at day 0 incubation) were acquired from a poultry farm in Laguna. The eggs were incubated at 37 °C until its age reached the third day. On the third day of incubation, the eggs were removed from the incubator and swabbed with 70% ethanol for sterilization. Then, the air sac of each egg was determined using a flashlight. Without damaging the eggs, holes were punctured near the edge of the air sac using a sterile pithing needle. After which, 0.1ml of varying concentrations of PEE (T1=1, T2=3, T3=5mg/ml) were administered to the eggs using a 1-ml sterile syringe. The punctured holes were resealed using melted candle wax. Then, the eggs were incubated for another 48 hours.

After 48 hours of incubation, the duck eggs were removed from the incubator and the vascularization of the CAM were determined. To examine the CAM, the eggshells were removed then the embryos extracted and were individually placed in a sterile container. The image of each embryo was captured using a camera phone and the vessels were manually counted.

I. Data Gathering and Statistical Analysis

To characterize the phytochemicals in the PEE, qualitative tests for the presence of flavonoids, phenolics, tannins, and triterpenes were performed. The total phenolic content (TPC) and total flavonoid content (TFC) of PEE were generated using a standard curve equation.

The antioxidant property of PEE was determined through the percent inhibition (%) of its radical scavenging activity in DPPH. The number of dividing cells in A. cepa root tips in each specific stages of cell division (prophase, metaphase, anaphase, and telophase) determined the PEE's antimitotic activity through the mitotic index (MI). The percentage of cell viability and inhibitory concentration at 50% determined the PEE's cytotoxic activity in MCF-7 cell lines. Lastly, blood vessel count determined the PEE's antiangiogenic activity. The anticarcinogenic potential of PEE was determined from the obtained antioxidant, cytotoxic, antimitotic, and antiangiogenic activity values of PEE.

The data gathered from the antioxidant, cytotoxic, and antiangiogenic activity of PEE were analyzed using the following statistical tools using SPSS software at P value of 0.05 at 95% confidence level:

On the assumption that the results followed a normal distribution curve, one-way analysis of variance (ANOVA) were utilized to determine the significant difference between the treatment groups. For further analysis, post-hoc test was used to determine the specific difference values among the treatment groups. Then, t-test for independent samples was used to determine the significant difference between the control and treatment groups.

Meanwhile, the antimitotic activity of PEE was analyzed using Pearson correlation using the SPSS software.

III. Results and Discussion

A. Propolis Ethanolic Extract (PEE) Physical Characteristics

The PEE exhibited a brown color with a sticky consistency. The resulting consistency of the PEE can be attributed with the undissolved fat content of the wax from the propolis. As the propolis efficiently dissolves in ethanol with concentration higher than 80%, the beeswax remnants do not easily dissolve and settles down as sediments (Pujirahayu et al. 2014).

The calculated percentage yield of the PEE was 38.064%. This value comparable with the reported values of propolis extract yield by Pujirahayu el al. (2014) of 18.33 + 1.82%, Wahyuni and Riendriasari (2021) of 41.80%, as cited by Bankova et al. (2021) reports by Trusheva et al. (2007) of 55%, Zhao et al. (2012) of 38%, Woo et al. (2015) of 20-56%, Cunha et al. (2004) of 48.4%, and Biscaia and Ferreira (2009) of 43.3%.

It was reported that propolis extracted from alcohol, specifically methanol and ethanol have been found to show high antioxidant activities. This coincides with above-mentioned referenced values as these studies also reported high antioxidant activity as reflected in their high total phenolic content (TPC) and total flavonoid content (TFC) values (Bankova et al. 2021).

There was no reported standard value on the optimal extraction yield of the propolis extract as the values can differ due to several factors. The difference can be due to the difference in the concentration of the ethanol solvent, propolis origin, propolis content, type of bee, food resource, and harvest time (Devequi-Nunes et al. 2018, Mulyati et al. 2020). Thus, the yield of the propolis extract relies on these factors and can be optimized based on the target phytochemical and application.

B. Qualitative Phytochemical Analyses

Based on the several qualitative phytochemical tests done for the presence of select phytochemicals in the PEE, flavonoids, phenolics, tannins, triterpenes were detected in the PEE (Table 1).

TABLE 1. PEE Qualitative Phytochemical Tests.

Phytochemical Test	Color of the solution	Result (+/-)
Flavonoid	Yellow color	+
Phenolics	Bluish black	+
Tannins	Brownish green	+
Triterpenes	Golden yellow	+

The confirmed presence of the abovementioned phytochemicals was also reported in several studies compiled by Anum et al. (2019). Flavonoids, phenolics, and triterpenes have been correlated with the antioxidant activity of PEE, thus have pharmacological effects (Rosli et al. 2016). Since propolis are constituted from different plants for collection of resin, the phytochemicals present in plants were reflected in the phytochemical profile of the PEE (Georgieva et al. 2019).

The presence of polyphenols including tannins confirmed reports that caffeic acids, flavonoids, and phenolic esters were the predominant biological active components of propolis (Fatoni et al. 2008). In addition, the vegetation diversity in tropical regions like the Philippines, allows propolis from the said geographical location to have higher pharmacological activity (Badiazaman et al. 2019).

C. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

It was calculated that the total phenolic content (TPC) as represented by mean gallic acid equivalents (GAE) in µg/mg of PEE is 996.1755 µg/mg (Table 2). This value is very high in comparison to reports with Tetragonula spp. propolis with a TPC ranging from 262.36 to 348.64 µg/mg (Pratami et al., 2019) and with TPC values of 269.57 to 426.91 µg/mg (Kartika et al., 2017). However, the present results were lower than reported values ranging from 2119.070 to 2509.767 µg/mg (Christina et al. 2018).

TABLE 2. Total phenolic content (TPC) and total flavonoid content (TFC) of PEE.

Phytochemical	Mean	SD
TPC	996.1755 µg GAE/mg	16.0874
TFC	581.409 µg QE/mg	50.1407

The total flavonoid content (TFC) as represented by mean quercetin equivalents (QE) of PEE was 581.409 µg/mg. This value is comparable with the reported values of Pratami et al. (2018); 324.43 to 791.06 µg/mg, Asem et al. (2019) of 135.93 µg/mg, and Farida et al. (2021) of 15.89 µg/mg.

With the same principle of the propolis extract yield, the values of TPC and TFC varied depending on the source of the propolis, its geographical location, and extraction method. The higher value of TPC and TFC is an indication of its valuable amounts of bioactive substance with pharmacological potential as there is no gold standard value for these.

It was reported that Tetragonula spp. produced high amounts of total flavonoid and phenolic compounds compared to other bee species (Dos-Santos 2003), as represented in its high values of TPC and TFC. These compounds are vital in the PEE's antioxidant activity (Fikri et al. 2019). The difference in the reported values can be attributed to the type of extraction method done, origin in terms of phytogeographical location, season at which propolis was collected, diversity of the resin's plant parts, thus the chemical composition varied at significant values (Cumbao et al. 2016).

The high reported value of TPC and TFC in this study can be explained by the solvent used, ethanol, and its high concentration (96%). The semi-polar nature of ethanol allowed different active compounds with varied polarity be extracted efficiently (Kartika et al. 2017, Syed Salleh et al. 2021).

The polyphenols present in propolis is a result of plant resins collected by bees mixed with enzymes from the bees' mouth. These compounds have been reported to be linked with its pharmacological potential, including anticancer (Badiazaman 2019).

D. PEE Antioxidant Activity through DPPH Radical Scavenging Assay

The antioxidant activity of PEE is comparable with the positive control (Table 3), gallic acid with both 100% mean percent inhibition with no statistically significant difference (P=0.274). On the other hand, there was a significant difference between the mean percent inhibition on the antioxidant activity of PEE compared with the negative control, DMSO (P=0.000).

The calculated antioxidant activity of PEE (100%) was higher in comparison with various studies reporting the antioxidant activity of propolis extract from Tetragonula spp. For instance, the study of Sukemi et al. (2021) reported a scavenging activity value of 69.08 to 82.32%, while Kothai and Jayanthi (2014) reported 83% antioxidant potential, and Agus et al. (2019) reported value of 91.5% antioxidant activity.

TABLE 3. Antioxidant activity of PEE through DPPH radical scavenging assay compared to negative and positive controls.

Sample	Mean (%) Inhibition*
Gallic Acid (Positive)	100^a
DMSO (Negative)	0^b
PEE	100^a

** Similar letters means there is no significant difference between means at P<0.05*

DPPH radical scavenging assay is the most common method to determine a sample's ability to donate hydrogen to DPPH radicals (Hsu et al. 2006). The presence of secondary metabolites, specifically phenolics and flavonoids, is responsible for the PEE's antioxidant activity as it can neutralize the DPPH radicals when it donates a proton.

It is reported that propolis, among other bee products, has the highest antioxidant activity (Kartika et al. 2018). Thus, PEE can reduce or eliminate free radicals that can prevent lipid oxidation (Agus, 2019; Hamilton, 2017; Sukemi et al. 2021). This mechanism supports the concept that propolis' antioxidant potential can be used for diseases related to oxidative stress, such as cancer (Kothai and Jayanthi 2014).

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E. PEE Antioxidant Activity through DPPH Radical Scavenging Assay

The highest PEE concentration, 1 mg/ml, has the lowest mitotic index of 63%. Whereas, the lowest PEE concentration (0.125 mg/ml) has the highest mitotic index of 87% next to the negative control, water with 92% mitotic index (Table 4). Pearson correlation showed that a higher PEE concentration is significantly correlated with its antimitotic potential (r=-0.923). A negative value close to 1 is an indication of inverse correlation. This means that higher concentration of PEE exhibits significant antimitotic activity as it decreases the mitotic index.

It was also observed that the dominant stage of division of cells in the observation field is prophase, among the various stages of mitosis namely: prophase, metaphase, anaphase, and telophase.

TABLE 4. Mitotic index of PEE varying concentrations.

PEE (mg/ml)	Mitosis Stage				Mitotic Index (%)
	Prophase	Metaphase	Anaphase	Telophase	
0.125	87	0	0	0	87
0.250	76	0	0	0	76
0.500	69	0	0	0	69
1.000	63	0	0	0	63
Negative Control (Water)	90	0	0	2	92

Mitotic Index = No. of total dividing cells/100 cells in observation field x 100%

It can be observed that the cells in prophase stage is evident as reported in the previous table. The number of dividing cells decrease as the PEE concentration increases (Fig. 1). All other stages do not exhibit any activity.

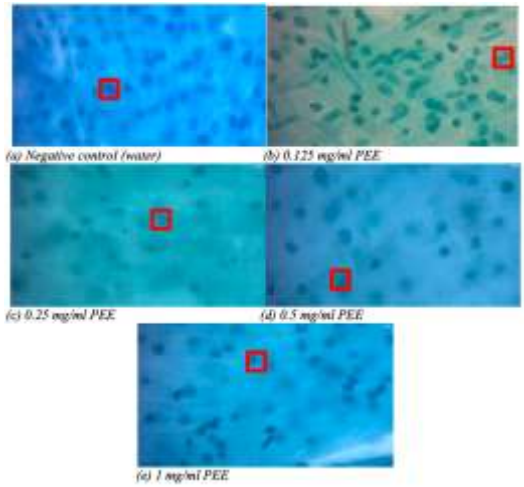

Fig. 1. Microscopic observations of A. cepa test of PEE varying concentrations (HPO, 400X).
Nucleus enlargement (sign of prophase) in red box: (a) Control and (b) 0.125 mg/ml PEE: High mitotic activity; (c) 0.25 mg/ml PEE, (d) 0.5 mg/ml, (e) 1 mg/ml: Low mitotic activity.

The prominent enlarged and condensed nucleus in the micrographs is an evidence of the predominating mitotic phase in all the microscopic observations, which is prophase. It can be observed that in micrograph (a) and (b), the number of actively dividing cells in prophase was numerous compared to micrographs (c), (d), and (e). The number of cells undergoing prophase also decreased as the PEE concentration increases.

The antimitotic activity of PEE showed a trend of decrease in mitotic index as the PEE concentrations decreased. This result indicate that higher concentrations of PEE can be potent as compound for antiproliferative associated human diseases. Specifically, the prevention of abnormal cell division can be a vital mechanism of therapeutic means of treating uncontrolled cell division in cancer patients. It is common among chemotherapeutic drugs such as cytotoxic drugs to inhibit cancer cell division making them antimitotic in nature (Raheel et al. 2017). Specifically, the PEE's ability to arrest mitosis at early phases (G0/G1) is a typical mechanism of action to several anti-tumor drugs (Popolo et al. 2009).

A. cepa test is a preliminary assay to determine the antimitotic potential of a substance. Thus, it is vital to perform other quantitative and qualitative tests to confirm the potential of the sample to inhibit cell division.

F. PEE Cytotoxic Activity through MTT Assay

Doxorubicin's peak percent inhibition was at 2.5 µg/ml at 67.35% and lowest at 0.15625 µg/ml with 15.62% cell inhibition (Fig. 2). On the other hand, PEE's peak percent inhibition was at 100µg/ml at 40.15% and lowest at 6.25 µg/ml with -9.30% cell inhibition. The negative percentage cell inhibitions indicates that the MCF-7 cells were proliferating instead of inhibited upon treatment. The PEE inhibited cell proliferation at higher concentrations compared to doxorubicin.

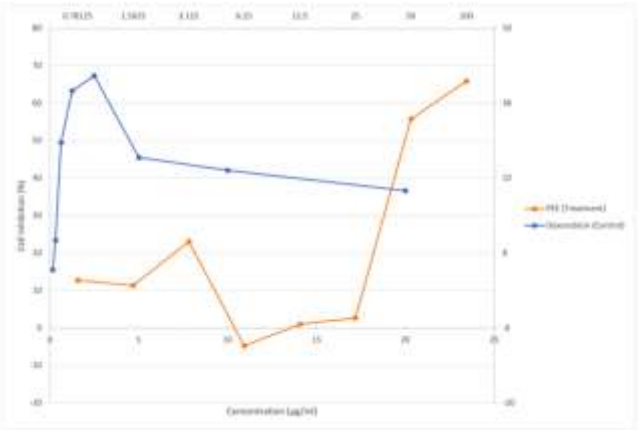

Fig. 2. Percent (%) cell inhibition of MCF-7 cells with doxorubicin (control) and PEE (treatment) after 72hrs incubation.

The cells are also observed under the microscope upon application of the treatments. It can be observed that doxorubicin decreased the number of MCF-7 cells in contrast when there was no treatment yet (pre-treatment) (Fig. 3). The application of PEE to MCF-7 cells also showed decrease in the number of MCF-7 cells but not as evident in the control group.

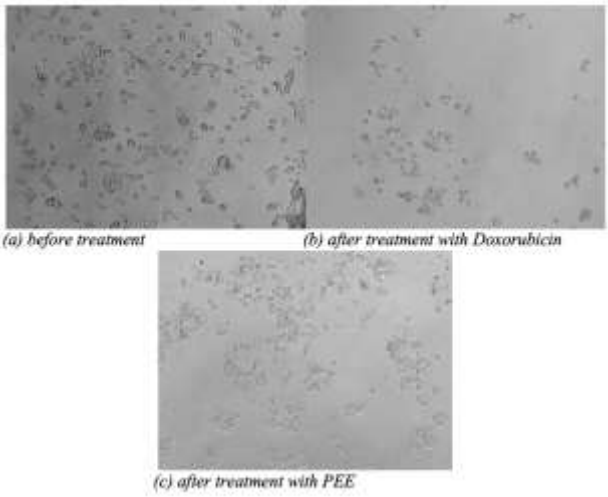

Fig. 3. Comparison of number of MCF-7 cells (a) before treatment (LPO, 40x), (b) after treatment with the highest concentration of Doxorubicin (LPO, 40x), and (c) after treatment with the highest concentration of PEE (LPO, 40x).

It can be observed in Fig. that the number of cells was highest at micrograph (a) and lowest at micrograph (b). Although the number of cells at micrograph (c) was lower compared to prior treatment (micrograph (a)), it is evident that the positive control, doxorubicin (micrograph (b)), still decreased the number of cells.

The half maximal inhibitory concentration or IC50 was the value of a substance's potency in inhibiting a certain biological function, in this instance, cell proliferation. Doxorubicin showed a lower IC50 of 0.4274 µg/ml as compared to PEE IC50 with a value of 39.34 µg/ml. The obtained PEE's IC50 value was comparable with the reported propolis' cytotoxicity values of Mohamed et al. (2020) of 32.70 µg/ml + 0.034, Teerasripreecha et al. (2012) of 41.3-53.5 µg/ml, Thirugnanasampandan et al. (2012) of 43.46 µg/ml, Delos Reyes et al. (2018) of 37.8 µg/ml.

A lower IC_{50} value means a small dosage or amount of a substance was potent enough to inhibit cell proliferation, as exhibited by the control, doxorubicin. According to Suffness and Pezzuto (1999), crude extracts with IC_{50} value of ≤ 100 mg/ml can be considered cytotoxic and can be considered potent to be evaluated for further studies. Thus, the calculated values were within the accepted range and can be considered a potential anticancer agent.

The study quantified the potential of PEE to be a chemotherapeutic agent, specifically a cytotoxic drug, that can inhibit progression of abnormal cancel cells. The differences in the reported values of the cytotoxic potential of propolis of different studies was due to the difference of the propolis' geographical origins.

According to Delos Reyes et al. (2018), the antitumor potential of propolis against MCF-7 cells can be attributed with its ability to induce apoptosis, regulate levels of cell division checkpoint regulators such as ANXA7, p53 and NF-κB p65, increase ROS, and decrease mitochondrial membrane potential. In addition, the phenolics found in propolis can induce necrosis causing cancer cell death by apoptosis via mitochondria-mediated or death signal-mediated mechanisms (Teerasripreecha et al. 2012). It was also reported that cancer cells are sensitive to cell death in the presence of flavonoids, one of the common bioactive constituents found in propolis (Vijayarathna and Sasidharan, 2012).

It can be noticed that there were inconsistencies of using MTT assay as the data sets of the reported cell inhibitions in the groups as well as the large deviation in the values of the IC_{50}, thus has low precision. This can be attributed with different factors that can be considered as a drawback for MTT assay as a preliminary test to determine a substance's potential cytotoxicity. There were reports that MTT assay can exhibit non-specific intracellular reduction of tetrazolium which led to inconsistent values (Jo et al. 2015). According to Stepanko and Dmitrenko (2015), metabolic and energy perturbations, inconsistent oxidoreductase activity, and intracellular trafficking due to continuous reprogramming of cells at a metabolic and mitochondrial level. This can significantly over and underestimate the cell viability value being reported. Thus, it is necessary to optimize parameters such as cell number, MTT concentration, incubation time for specific cell line and condition to avoid these inconsistencies (Ghasemi et al. 2021).

G. PEE Angiogenic Activity through Choriallantoic Membrane (CAM) Assay

The PEE concentrations significantly inhibited the blood vessel growth in comparison to the control group (Table 5). In addition, there was no significant difference between the blood vessel group among the treatment groups. This is an indication that all treatment groups are potential antiangiogenic agents regardless of its concentration. Thus, the antiangiogenic activity of PEE is already evident at its lowest concentration, 1mg/ml. This is evident in the macroscopic observations of the duck embryo as illustrated in Fig.4.

TABLE 5. Angiogenic Activity of PEE varying concentrations compared to control.

Sample		**Mean Blood Vessel Count***
Control (water)		7.4[a]
PEE	1 mg/ml	1.2[b]
	3 mg/ml	1.2[b]
	5 mg/ml	1.8[b]

**Similar letters means there is no significant difference between means at P<0.05*

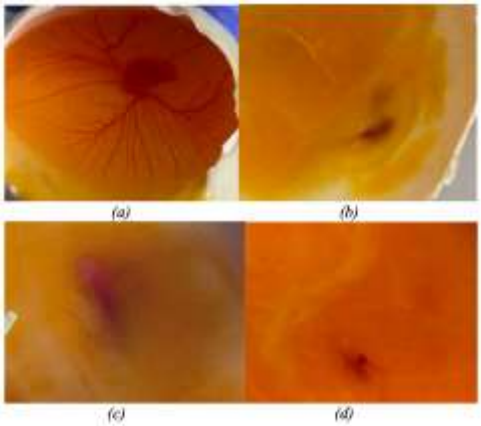

Fig. 4. Macroscopic observations of duck embryos blood vessel count (a) control (water), (b) 1 mg/ml PEE, (c) 3 mg/ml PEE, (d) 5 mg/ml PEE.

Studies have reported that propolis rich with flavonoids and phenolics can prevent the development of blood vessel vascularization, a key process in the progression of cancer and heart diseases (Park et al. 2014). These bioactive constituents act in synergistic manner and target certain cellular signaling pathways responsible for angiogenesis (Iqbal et al. 2019). The antioxidant nature of phenolics found in propolis is reported to target several steps in the angiogenesis and inhibit them (Varinska et al. 2010). Bioactive constituents of propolis were reported to inhibit tube formation and suppress tumor- induced angiogenesis.

There are various literatures reporting the possible mode of mechanism of the antiangiogenic nature of propolis. Some of which include the apoptosis inducing capacity of propolis in vascular cells causing increased production of apoptosis markers such as p53, ROS, and caspase-3 (Iqbal et al. 2019).

The antioxidant activity of PEE can also be directly attributed with its antiangiogenic activity. This is due to the ability of ROS production that can induce VEGF-induced angiogenesis and VEGF-induced tube formation (Izuta et al. 2009). Preventing VEGF activity is vital as it is responsible for endothelial cell proliferation in blood vessels which promote angiogenesis and increase vascular permeability (Eteraf-Oskouei et al. 2020).

IV. CONCLUSION

The anticarcinogenic potential of PEE from *T. biroi* Friese was determined by its antioxidant, antimitotic, cytotoxic, and antiangiogenic activity.

The PEE was confirmed to contain flavonoids, phenolics, tannins, and triterpenes as reflected in its qualitative phytochemical analyses. Furthermore, the flavonoid and phenolic content of PEE in comparison to gallic acid and quercetin as standards respectively were significant in amount which is an indication of its high pharmacological potential. The bioactive constituents present in the PEE can be attributed to the nature of the plant resins components and as well as the bee source's geographical location.

It was also determined that the antioxidant activity of PEE is comparable with the radical scavenging ability of gallic acid, the positive standard. This is an indication of the ability of PEE to suppress oxidative stress brought by ROS.

PEE has the potential to suppress tumor formation by arresting cell division at earlier phases specifically during prophase. In addition, the cytotoxic activity of PEE is promising as it fits in the acceptable range to be a potential agent in inhibiting cell proliferation of cancer cells.

Lastly, the PEE was found to have an antiangiogenic potential by inhibiting significant blood vessel growth. There is possible evidence of correlation between the antioxidant activity and antiangiogenic activity of PEE which makes it a good candidate as anticarcinogenic agent.

The results of the study indicate the strong anticarcinogenic activity of PEE from *T. biroi* Friese and have the potential to be further evaluated to be an alternative pharmaceutical drug that targets the antioxidant, antimitotic, cytotoxic, and antiangiogenic mechanism of inhibiting cancer progression.

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