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Antioxidant and anticancer activity of *Canarium ovatum Engl.* (Pili) ethanolic leaf extracts

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ABSTRACT

The Pili (*Canarium ovatum* Engl.) tree, native to the Philippines, has been reported to have medicinal properties because of the biological and chemical properties it exhibits. This paper aimed to investigate the presence of phytochemicals, antioxidant, and anticancer activities of the ethanolic leaf extract of *C. ovatum*. To determine the phytochemicals, present in the extract, standard procedures for qualitative phytochemical screening were performed. The antioxidant activity of the extract was assessed in vitro using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The anticancer activity of the extract was assessed in vitro using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay against the HCT116 cancer cell line. Phytochemical screening of *C. ovatum* ethanolic leaf extract detected alkaloids, steroids, flavonoids, saponins, and tannins. The extract had extremely high antioxidant activity (IC_{50}=11.44 mg/L). The MTT assay revealed moderate cytotoxic activity of the leaf extract to HCT116 cancer cell line (IC_{50}=94.43 mg/L). These findings suggest that the *C. ovatum* ethanolic leaf extract has therapeutic potential because of the presence of beneficial phytochemicals, strong antioxidant activity, and anticancer capacity. Futher research is recommended to comprehensively evaluate the medicinal potential of Pili leaf extracts, including exploring other biological activities using various assays and employing different solvents for leaf extraction.

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1. INTRODUCTION

There is increasing global interest in using medicinal plants to treat diseases [1]. The world health organization (WHO) reports that 80% of people in developing nations rely on traditional medicine, much of which comes from plants [2]. As a result, numerous researchers are currently doing extensive study on medicinal plants in order to meet the growing global demand for natural products [3]. A wide of variety of secondary metabolites are produced by plants, and these compounds serve as the foundation of many commercially available pharmaceuticals and herbal remedies. The different chemicals found in medicinal plants have biological properties that can improve human health [4]. Herbal medicine is deeply rooted in Filipino

culture and traditions. As a result, Filipinos have a long history of utilizing plants to create medicines for treating various ailments [5]. The Pili tree, scientifically known as *Canarium ovatum* Engl., is a tropical species native to the Philippines. It is commonly grown for its edible fruit. The nut kernel is highly sought after in the global confectionery market, making it the most economically valuable part of the fruit [6]. There have been few reports of the medicinal uses of the different plant parts of *C. ovatum* in the Philippines. Local traditional healers from Claver, Surigao del Norte used the bark for fever [7]. Local inhabitants in selected barangays of the municipality of Malinao, Albay used the seeds for indigestion, insect bites, and skin allergies by creating a paste and applied it on the affected area [8]. The Manobo tribe of the municipalities of Carrascal and Cantilan, Surigao del Sur used the fruit and burned as incense for dizziness and fainting [9]. Different plant parts of *C. ovatum* have been shown to possess different biological and chemical properties. The pulp has been reported to have immunomodulatory properties wherein stimulatory effects on cellular and humoral responses in Balb/C mice in vivo were manifested, indicating its potential as an immunostimulatory agent [10]. The pulp also possesses antioxidant and anticancer activities [11]. Screening of phytochemicals of the crude pulp extract revealed sterols, triterpenes, flavonoids, alkaloids, saponins, glycosides, and tannins [10]. Meanwhile, screening of phytochemicals of the oil and ethanolic pulp extracts revealed alkaloids, glycosides, saponins, sterols, tannins and triterpenes [11]. The bark has been shown to have antiproliferative activity when its ethanolic extract has been evaluated for cytotoxicity and genotoxicity. Screening of phytochemicals of the crude bark extract revealed flavonoids, phenols, tannins, and traces of saponins [12].

The ethnomedicinal uses of its leaves have not yet been reported, but some studies suggest it has therapeutic potential because of the biological and chemical properties it exhibits. Screening of phytochemicals of the crude methanolic leaf extracts of *C. ovatum* revealed condensed tannins, saponins, terpenoids, flavonoids, cardiac glycosides, and phenolic compounds [13], while a chemical investigation of the dichloromethane leaf extracts through silica gel chromatography revealed β-amyrin, α-amyrin, epi-β-amyrin, epi-α-amyrin, epi-lupeol, β-carotene, and lutein [14]. The aqueous leaf extracts of *C. ovatum* have been used for the biosynthesis of silver nanoparticles for investigation of its antibacterial potential against Pseudomonas aeruginosa, wherein results suggest that *C. ovatum* leaves extract efficiently synthesizes silver nanoparticles and can be good candidates for therapeutics [15]. A subfraction of the methanolic extract of *C. ovatum* leaves, when its antimutagenic properties were evaluated via in vivo micronucleus test using Institute of Cancer Research (ICR) mice, showed the most promising activity against other medicinal plants [13]. The crude ethanolic leaf extracts of *C. ovatum* was subjected to an evaluation of its antihyperuricemic activity, in which results show in vitro xanthise oxidase inhibition and in vivo antihyperuricemic activity [16]. Despite the growing scientific literature on the medicinal potential of *C. ovatum* leaves, there remains a need for further research to strengthen the existing evidence base. The findings of this study can provide data that can be used to establish new possibilities for pharmacological research such as drug formulation to enhance healthcare, and implement conservation measures for the protection and preservation of the medicinal plant species. This paper aims to investigate the presence of phytochemicals, antioxidant activity, and anticancer activity of the ethanolic leaf extract of *C. ovatum*.

2. METHOD

2.1. In vitro plant collection and identification

Mature leaves of *C. ovatum* were collected at Barangay Fili, Bayugan City, Agusan del Sur, Philippines (46.7 m, 8°43' N, 125°46' E). Plant samples were preliminarily identified using the "Key descriptors for Pili nut (*Canarium ovatum* Engl.)" [17] and further verified by an expert at the Flora Terrestrial Biodiversity of Premier Research Institute of Science and Mathematics (PRISM) of Mindanao State University-Iligan Institute of Technology (MSU-IIT). The herbarium specimen (No. NSM-4854) was deposited at the MSU-IIT Natural Science Museum, Iligan City, Lanao del Norte, Philippines.

2.2. Ethical clearance

A request letter was given to the barangay captain of Barangay Fili, Bayugan City, Agusan del Sur, Philippines to provide the researcher authority in collecting *C. ovatum* samples in the study area. This was followed by seeking of a gratituous permit from the office of the Department of Environment and Natural Resources (DENR) Caraga at Barangay Ambago, Butuan City, Agusan del Norte, Philippines. A certification (No. 2022-11-78) from the barangay, and gratituous permit (No. R13-2022-37) from DENR Caraga were granted prior to the collection of plant samples.

2.3. Plant material preparation and extraction

Collected *C. ovatum* leaves were cleaned with tap water, rinsed using distilled water, and then left to air dry at room temperature for 30 days. After drying, the leaves were ground into a powder using a blender.

This powder was then stored in an airtight container until needed for extraction. To create the extract, a total of 250 g pulverized *C. ovatum* leaves were soaked in 1.5 L absolute ethanol (Scharlab Ethanol absolute, for analysis, ExpertQ®, ACS, ISO) for one week. After soaking, the mixture was filtered using a Whatman™ filter paper No. 1 to separate the liquid extract from the solid plant material. A rotary evaporator was used to concentrate the filtered extract in vacuo at 45 °C [1]. The concentrated ethanolic leaf extract was then transferred to storage vials and labeled as *Canarium ovatum* Ethanolic leaf (COEL) for further analysis.

2.4. Phytochemical screening

Phytochemical screening of the COEL extract was performed at the Department of Chemistry, MSU-IIT, following the phytochemistry section of the book "A guidebook to plant screening: phytochemical and biological" [18]. The leaf extract was subjected to a qualitative evaluation of phytochemicals including alkaloids, steroids, anthraquinones, flavonoids, saponins, tannins, and cyanogenic glycosides. The findings were recorded using a 3-point scale (+ light, ++ moderate, +++ heavy) in scoring following the "Handbook of Philippine Medicinal Plants" [19].

To detect the presence of alkaloids, a 2 g sample of the extract was first evaporated on a steam bath until nearly dry and then allowed to cool to room temperature. The residue was mixed with 5 mL of 2 M HCl and heated for 5 mins. After cooling, 0.5 g of NaCl were added, and the solution was stirred, filtered, and then adjusted to a final volume of 5 mL. A 1 mL portion of this mixture was then divided equally into two test tubes. The first test tube was treated with 2–3 drops of Dragendorff's reagent. The appearance of an orange or orange-red precipitate signaled a positive result. The second test tube was treated with 2–3 drops of Mayer's reagent. The formation of a cloudy or white precipitate indicated the presence of alkaloids.

To detect the presence of steroids, a 10 mL sample of the extract was evaporated on a steam bath until nearly dry and then cooled to room temperature. The extract was then repeatedly defatted using hexane. The defatted aqueous layer was heated on a steam bath to remove any remaining hexane. Finally, 3 mL of FeCl3 reagent was added to the sample, followed by the gradual addition of 1 mL H2SO4. Appearance of a reddish-brown color indicated positive results.

To detect the presence of anthraquinones, 1g leaf extract was mixed with 15 mL chloroform and then filtered. Next, 2 mL of the filtrate was transferred to a test tube and 1 mL 10% diluted ammonia was added. The mixture was then shaken. A pink-red color in the lower ammoniacal layer indicated positive results.

The presence of flavonoids was determined using the Bate-Smith and Metcalf method. Ten (10) g leaf extracts were defatted with 9 mL hexane. The defatted aqueous layer was mixed with 10 mL 80% ethanol, filtered, and divided into two test tubes. One test tube served as a control, while the other portion of the filtrate was treated with 0.5 mL concentrated 12 M HCl, and any color change was noted. The Bate-Smith and Metcalf method was used to test for the presence of flavonoids. The extract was warmed for 5 mins in a water bath was observed after one hour for color changes. Appearance of a strong red to violet color indicated positive results.

The presence of saponins was determined using the froth test. The 2 g powdered sample were added to a test tube containing 10 mL distilled water. The tube was then covered and shaken vigorously for 30 sec to create a stable, long-lasting froth. The tubes were allowed to stand for 10 mins, and formation of a honeycomb-like froth was observed. Froths that were greater than 2 cm in height from the liquid surface after 10 mins indicated positive results.

The presence of tannins was determined using the gelatin test. The 3 g leaf extracts were placed in an evaporating dish and allowed to evaporate under a boiling bath. After evaporation, the extract was cooled. Next, the extract was mixed with 20 mL boiling water, followed by three drops 10% NaCl solution. The solution was filtered, and the residue was rinsed with distilled water. The mixed filtrates were collected, rinsed and divided into three equal portions. The first portion contained an additional five drops 1% $FeCl_3$. The second portion contained an additional five drops gelatin-salt reagent. The third part acted as the control. A production of a black or blackish-blue precipitate with ferric chloride and white precipitate with gelatin-salt reagent indicated positive results.

To detect the presence of cyanogenic glycosides, 3 g leaf extracts were placed in a test tube. To enhance enzyme activity, the extract was moistened with water and a few drops of chloroform. To ensure the hydrolysis of the glycoside, 1 mL 1% emulsion solution was also added. A piece of picrate paper was attached to the cork stopper, and was suspended inside the test tube without touching the inner sides. The tube was firmly closed and warmed at 35 °C. Appearance of various shades of red within 15 mins indicated the presence of cyanogenic glycosides. If no color change was observed after three hours, absence of glycosides was indicated.

2.5. In vitro DPPH radical scavenging assay

Evaluation of the antioxidant activity of the COEL extract was performed at the Department of Chemistry, MSU-IIT, using in vitro 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [20]. This assay involved preparing varying concentrations of the extract, ranging from 10 to 100 mg/L, and a 0.004% DPPH solution, both using ethanol as the solvent. A 1 mL aliquot of each solution was then combined. These

mixtures were incubated in the dark for 30 mins at room temperature. Afterward, the absorbance of each mixture was measured at a wavelength of 517 nm, using pure ethanol as a blank for calibration. Ascorbic served as the positive control. The degree to which the DPPH solution changed color from purple to yellow provided a visual indication of the scavenging efficiency of the extract. A lower absorbance reading indicates greater free radical scavenging activity. The scavenging activity was calculated using (1):

$$DPPH\ scavenging\ activity\ (\%) = \frac{Ac - As}{Ac} \times 100 \quad (1)$$

where *Ac* is the absorbance of the control reaction or DPPH alone and *As* is the absorbance of the sample reaction or DPPH solution with the different concentrations of the leaf extract. The experiment was performed in three replicates. The IC_{50} value, which is the concentration of the sample needed to inhibit 50% of the DPPH free radical, was determined through linear regression analysis.

2.6. In vitro MTT cell viability assay

Evaluation of the anticancer activity of the COEL extract was performed at the cell culture and cell-based assay laboratory of PRISM, MSU-IIT, using in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay [21]. This assay determines the activity of metabolically active cells by measuring the reduction of tetrazolium salts by mitochondrial dehydrogenase enzymes. A stock solution of the extract was prepared by dissolving 3 mg of the leaf extract in 300 µL dimethyl sulfoxide (DMSO) to achieve a concentration of 10,000 mg/L. This stock solution was then diluted with cell culture medium to obtain the desired concentrations for testing. HCT116 human colorectal carcinoma cells (ATCC CCL-247, Manassas, VA, USA) were used for the assay. The cells were cultured and then seeded in 96-well plates at a density of 2×105 cells per well. After 24 hrs of incubation, cell viability was assessed using the CellTiter 96® non-radioactive cell proliferation assay (Promega, Madison, WI, USA) following the instructions of the manufacturer. The experiments were performed in two trials, with each trial including three replicates. The results were presented as mean ± standard deviation (SD), representing the percentage of viable cells in the treated groups compared to the untreated control group. Digitonin served as the positive control. The IC_{50} value, which represents the concentration of the extract needed to reduce cell viability by 50%, was determined through linear curve fitting of percent (%) cell viability plotted against the log concentration.

2.7. Statistical analysis

The data was first checked for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. To determine statistically significant differences between the control group and the treatment group, a two-sample independent t-test was used. IBM® SPSS® Statistics Version 25 was used for this analysis, with a p-value less than 0.05 being considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Phytochemical screening

Results of the phytochemical screening of the COEL extract are shown in Table 1. The screening of the extract revealed the presence of trace levels of alkaloids, and high levels of steroids, flavonoids, and tannins. Anthraquinones and cyanogenic glycosides were not detected in the extract. Medicinal plants are characterized by the presence of specific chemical compounds within one or more of their organs. These compounds can either be used directly for therapeutic purposes or serve as raw materials in the synthesis of important pharmaceuticals [22]. Phytochemicals are bioactive compounds produced by plants for their protection. Different phytochemicals possess numerous biological activities, and they also help in regulating gene transcription, enhancing cell communication, boosting immunity, and provide protection against certain cancers [23]. Alkaloids have been shown to exhibit numerous biological activities such as anticancer, antioxidant, anti-inflammatory, analgesic, immunomodulatory, anti-hypertensive, antibacterial, and antifungal activities [24]. Steroids have been shown to exhibit anti-inflammatory, antitumor, antimicrobial, antioxidant, immunomodulatory, cardiotonic, and hepatoprotective activities [25]. Flavonoids have been shown to exhibit anti-inflammatory, anticancer, anti-aging, cardioprotective, neuroprotective, immunomodulatory, antidiabetic, antibacterial, antiparasitic, antiviral activities [26]. Saponins have been shown to exhibit antitumor, antioxidant, antimicrobial [27], anti-inflammatory, anti-carcinogenic, hemolytic, and cytotoxic activities [28]. Tannins have been shown to exhibit antioxidant, anti-inflammatory, antidiabetic, cardioprotective, immunomodulatory, antimicrobial, and antitumor activities [29]. The presence of these phytochemicals in the screening suggests that COEL extract has the potential of possessing the aforementioned broad spectrum of biological activities. A previous study reported similar results, in which condensed tannins, saponins, terpenoids, flavonoids, cardiac glycosides, and phenolic compounds were found

in the crude methanolic leaf extract of *C. ovatum* [13]. Anthraquinones have also been shown to exhibit several biological activities including purgation, anti-inflammation, immunoregulation, antihyperlipidemia, and anticancer [30]. However, anthraquinones were not detected in the screening. The concentration of anthraquinones in the extract can be below the detection limit of the method used for the screening. Lastly, cyanogenic glycosides were also not detected in the screening. Cyanogenic glycosides are known to release toxic hydrogen cyanide [31]. Its undetected presence in the leaf extract suggests that COEL extract is safe and have fewer chances to produce toxic effects to the human body.

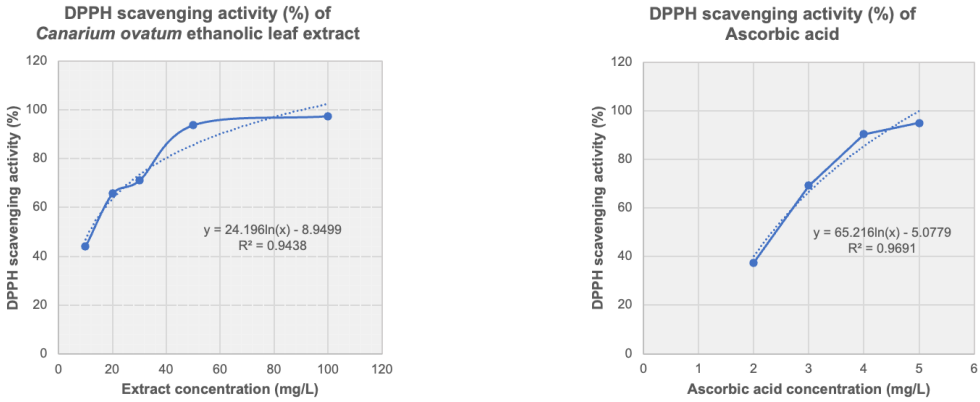
Table 1. Phytochemical screening of *Canarium ovatum* ethanolic leaf extract

Phytochemicals	Result
Alkaloids	+
Steroids	+++
Anthraquinones	-
Flavonoids	+++
Saponins	+++
Tannins	+++
Cyanogenic glycosides	-

(+) indicates presence: + light, ++ moderate, +++ heavy; (-) indicates absence

3.2. Antioxidant activity of COEL extract

Results of the DPPH assay are shown in Figure 1, with Figure 1(a) showing the DPPH scavenging activity of the COEL extract and Figure 1(b) showing the DPPH scavenging activity of ascorbic acid. The IC_{50} values of the COEL extract and ascorbic acid were 11.44 mg/L and 2.33 mg/L, respectively, calculated using the linear regression analysis formula also shown in Figure 1.



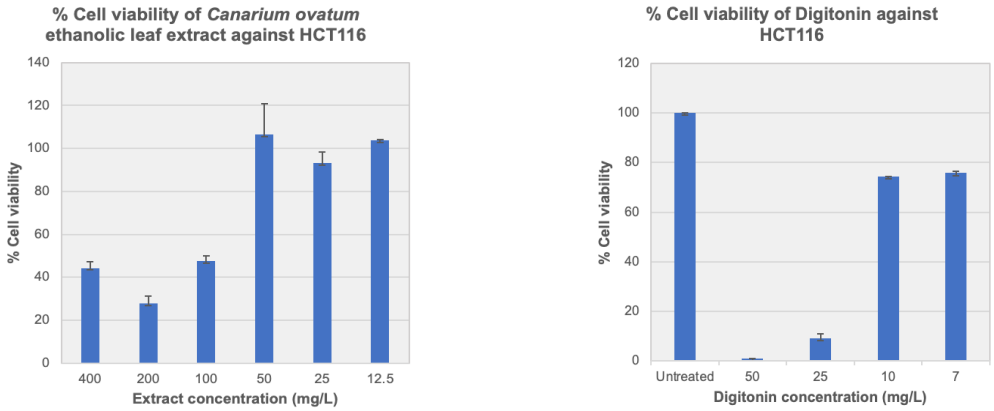
(a) (b)

Figure 1. Graph of DPPH scavenging activity (%) of (a) COEL extract (mg/L) and (b) Ascorbic acid (mg/L)

Antioxidants function in decreasing harmful reactive oxygen species (ROS) produced by the environment in living organisms. Damaging effects such as oxidative stress may happen within the cells in cases where high levels of ROS are reached. Oxidative stress may contribute to the development of many diseases including Alzheimer's, Parkinson's, cardiovascular disorders, and even cancer [32]. DPPH is a stable free radical that is reduced to α,α-diphenyl-β-picrylhydrazine when it interacts with an antioxidant. Antioxidants interrupt the chain oxidation process of free radicals by donating hydrogen from hydroxyl groups, resulting in the formation of a stable final product that does not initiate or propagate further oxidation [33]. Based from the data, the DPPH scavenging activity of COEL extract increases as their extract concentration increases. The results suggests that the higher the concentration of the extract, the more DPPH is being scavenged. The antioxidant activity is considered extremely high if the IC50 value is <50 mg/L, high if between 50–100 mg/L, moderate if 101–150 mg/L, and low if 151–200 mg/L [34]. The findings of the study suggest that COEL extract has an extremely high antioxidant activity. Statistical analysis reveals there were no statistically significant differences between the DPPH scavenging activity of the samples (t(7) 0.087, p 0.933). This finding is consistent with the observation that both COEL extract and ascorbic acid are very strong antioxidants, despite the positive control showing a lower IC50 value. The presence of antioxidant activity can be attributed to the phytochemicals present in screening of the leaf extract such as alkaloids, steroids, saponins and tannins, which have reported to have antioxidant properties [24], [25], [28], [29].

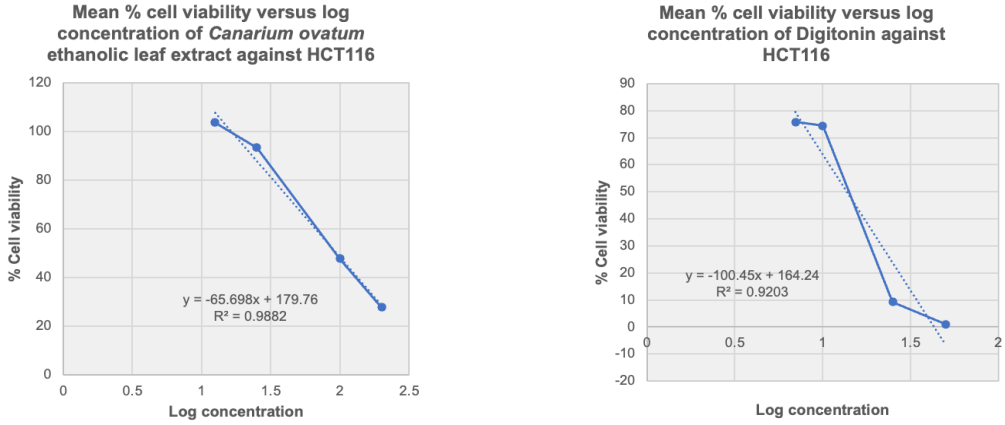
3.3. Anticancer activity of COEL extract

Results of the MTT assay are shown in Figure 2, with Figure 2(a) showing the % cell viability of the COEL extract and Figure 2(b) showing the % cell viability of digitonin. Figure 3 shows the IC_{50} values of the COEL extract and digitonin were 94.43 mg/L and 13.83 mg/L, respectively, calculated from the linear curve fitting of % cell viability versus log concentration as shown in Figures 3(a) and 3(b), respectively.



(a) (b)

Figure 2. Graph of % cell viability against HCT116 (mean±SD) of (a) COEL extract (mg/L), and (b) Digitonin (mg/L)



(a) (b)

Figure 3. Graph of % cell viability against HCT116 versus log concentration of (a) COEL extract (mg/L) and (b) Digitonin (mg/L)

Cancer is one of the global public health issues with its prevalence increasing substantially. It is a complex disease that features the abnormal growth of cells with the capacity to invade, which are the result of genetic mutations that alter gene expression and bring about genetic, physiological, and morphological modifications. This gives rise to uncontrolled cell growth and an ability to resist cell death. One of the primary treatments employed for cancer is chemotherapy, which employs chemotherapeutic drugs [35], [36]. Recently, there has been important emphasis on discovering natural substances such as from plants with a cytotoxic capacity, through mechanisms that impede the survival of tumor cells [37]. As observed from the data, varying % cell viabilities can be observed at different concentrations of the leaf extract. As per ISO 10993-5, the lower the % cell viability value, the higher the cytotoxic potential test samples have [38]. The findings of the study suggests that at slightly higher concentrations, COEL extract showed higher cytotoxic activity to the cancer cell lines. Furthermore, the IC50 is computed and refers to the concentration of the extract required to inhibit 50% of the viability of HCT116. According to the guidelines of National Cancer Institute (NCI), the cytotoxic activity is considered high if the IC_{50} value is ≤20 mg/L, moderate if between 21–200 mg/L, weak if 201–500 mg/L, and inactive if >501 mg/L [39]. Based from the data, COEL extract showed moderate cytotoxic activity to HCT116. Statistical analysis reveals there were no statistically significant differences between the % cell

viability of the samples ($t(8)$ 1.297, p 0.237). This finding is consistent with the observation that both COEL extract and digitonin have cytotoxic activities to the cancer cell lines, despite the positive control showing a lower IC_{50} value. In an in vivo study, a subfraction of the methanolic leaf extract of *C. ovatum* showed promising antimutagenic properties via micronucleus test using ICR mice [13]. The anticancer potential exhibited by the leaf extract can be attributed to the phytochemicals present in the screening of leaf extract such as steroids [25], flavonoids [26], saponins [27], [28], and tannins [29].

4. CONCLUSION

The findings of this study showed the medicinal potential of Pili (*Canarium ovatum* Engl.) leaves. Phytochemical screening of the ethanolic leaf extract detected the presence of several secondary metabolites, which exhibit numerous biological activities that have therapeutic benefits. The DPPH assay showed that the plant may be a good source of antioxidants because of the extremely high antioxidant activity presented by the leaf extract. The MTT assay showed that the leaf extract has anticancer potential because of the moderate cytotoxic activity to HCT116 cancer cell line. Use of other solvents for extraction, further isolation and quantification of bioactive compounds, confirming its biological activities with other assays, utilization of normal cells and other cancer cell lines, and conducting different toxicity studies to determine the safe dose range of the extracts are recommended to fully understand the effects of the leaf extracts before it can be used as therapeutics.

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