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In Vitro Cytotoxicity and Antioxidant Study of *Leea manillensis* (Abang-Abang) Leaf Methanolic Extract against MCF-7 Breast Adenocarcinoma Cell

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Abstract

Cancer remains a significant health threat to humanity, as it continues to develop multidrug resistance to treatments and makes anti-cancer drugs ineffective. The study sought to use methanolic extract of *Leea manillensis* (locally known as Abang-Abang) against MCF-7 breast adenocarcinoma cell line. The antioxidant activity of *L. manillensis* was measured by DPPH Free radical scavenging assay, while the cytotoxicity was observed using PrestoBlue Assay on MCF-7 cells. The Inhibitory Concentration (IC50) values of *L. manillensis* and Doxorubicin were 2724 µg/mL and 0.7644 µg/mL respectively. Among the five concentrations of *L. manillensis* extract, the 60 µg/mL concentration displayed a high effectivity rate of 38.99% in DPPH Assay, while the ascorbic acid had 99.52%. Results showed that both positive controls from the tests performed obtained higher effectivity than the plant extract. The methanolic extract of *Leea manillensis* is ineffective in DPPH scavenging assay as well as in inhibiting viability of MCF-7 cells using the PrestoBlue Assay.

Keywords: *Leea manillensis*; MCF-7 Breast Adenocarcinoma Cell line; Cytotoxicity; Antioxidant

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Introduction

Breast cancer is the most common cancer diagnosed in women, accounting for more than 1 in 10 new cancer diagnoses each year. It is a disease in which abnormal breast cells grow out of control and form tumors that can spread throughout the body and become fatal. According to the World Health Organization (WHO), approximately two million breast cancer cases and 685,000 deaths were reported globally in 2020. In 2019, the Philippines ranked ninth globally and top in Asia for breast cancer rates, which are frequently detected only when the illness has progressed. The International Agency for Research on Cancer (IARC) estimates that 1 in every 13 Filipino women will experience breast cancer at some point in their lives, placing them at relatively higher risk of the disease (American Cancer Society, 2021). Moreover, it is estimated that 70% of breast cancer cases affect indigent women, making it more difficult for them to fight off the dreaded disease.

Treatment for breast cancer faces significant challenges in the present decade due to multidrug resistance, which develops from repeated exposure to chemotherapeutic agents and makes anti-cancer drugs less effective. Although chemotherapy has been a cornerstone in the prevention of cancer, its limitations such as side effects, toxicity, resistance, limited efficacy, and quality of life emphasize the need for further research and development of safer, more precise, and more effective treatments (American Cancer Society, 2021). Additionally, in low and middle-income countries, cancer patients typically have a poor prognosis due to a lack of awareness about the disease, delayed diagnosis, and anticancer agents and compounds derived from plants is crucial in discovering natural and safe alternatives that can reduce the side effects caused by chemotherapy and affordable cancer treatment strategy [1].

For centuries, plants have been utilized to treat various ailments. In many regions across the world, numerous plants are used for their health benefits as part of traditional folk medicine practices [2]. Plant extracts are often chosen among natural products because they are environmentally

friendly and cost-effective [3]. *Leea* species is a small tree or shrub belonging to the family of Leeaceae that grows up to 450 cm to 610 cm and is widely distributed throughout Northern and Eastern Australia, New Guinea, South and Southeast Asia, and parts of Africa [4]. Compounds present are flavonoids, tannins, and quercetin that can help against MCF-7 breast cancer cell line.

Numerous studies have shown that flavonoids are useful in the treatment of a variety of illnesses, such as immunological disorders, cancer, and cardiovascular diseases. These typically cause apoptosis, autophagic cell death, and inhibit cellular proliferation. In addition, these may also play parts in the reversal or inhibition of chemoresistance by modifying the activities of ROS-scavenging enzymes or by causing necrosis, cell cycle arrest, and inhibition of cellular migration, invasion, and tumor angiogenesis [5]. Tannins are diverse groups of naturally occurring polyphenolic products that also show promising cancer chemo preventive and therapeutic potentials. Research conducted in the past three (3) decades has exhibited their ability to specifically target numerous cellular pathways and molecules that play crucial roles in the development of cancer. Quercetin, on the other hand, is a flavonoid that exhibits a range of potential health benefits, including anti-inflammatory, anti-cancer, and antioxidant effects. Furthermore, it demonstrated anti-tumor effects on cancerous cells through the modulation of several molecular components of signaling pathways. It activates caspase-3, which reduces bioenergy and targets mitochondria to cause cancer cells to undergo apoptosis and promotes autophagy and apoptosis in cancer cells.

Overall, cancer remains a serious threat to public health as it continues to develop multidrug resistance to treatments over the years. Properties of *Leea manillensis* (Abang-Abang) were proven to have phytochemical capabilities but it appears to have limited studies on. Hence, the current research was conceived to determine the level of cytotoxic and antioxidant activity of *Leea manillensis* (Abang-Abang) against MCF-7 breast adenocarcinoma cell line in pursuit of assessing its prospect as an alternative treatment for breast cancer.

Research Methodology

Research design

The research used an experimental approach. It employed a scientific approach that initiated measures, which gave the researchers the capability to produce and gather the necessary results to test their specific hypotheses. The variables used in the study included the following: Positive control group (Doxorubicin), Experimental group (*Leea manillensis* Leaf Extract), and Negative control group (Dimethyl sulfoxide). This study aims to measure the cytotoxic efficacy of the methanolic extract derived from the leaves of *L. manillensis* against the MCF-7 breast cancer cell line.

Research locale

Leea manillensis (Abang-Abang) leaves were procured in Apayao, Philippines and authenticated at the Far Eastern University Herbarium. The leaves were prepared for methanolic extraction and subjected to quantitative phytochemical screening at the Adamson University Technology Research and Development Foundation, Inc., (AUTRDFI) located in Manila.

Doxorubicin with concentrations of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL, 1600 µg/mL and dimethyl sulfoxide that will serve as positive and negative controls for the PrestoBlue Assay, together with Ascorbic Acid that served as positive control for the DPPH Assay, were procured at St. Lukes Medical Center and University of Santo Tomas respectively.

Together with the cell line, the gathered plant extract was transported to the St. Luke's Medical Center located in Quezon City, where PrestoBlue Assay was conducted, and UST Research Center for the Natural and Applied Science located in Manila for the experimentation of DPPH assay.

Sample and Population

Research sample: The research sample in this study is the *L. manillensis* leaves. Four kilograms of *Leea manillensis* leaves were utilized for the study and procured in Apayao, Luzon. These were sent for phytochemical analysis and for methanolic extraction including the different sample concentrations needed for the tests. The *Leea manillensis* methanolic leaf extract was used for PrestoBlue Assay and DPPH Assay.

Research Population: The research population is the MCF-7 adenocarcinoma cell line. The cell line was acquired from the St. Lukes Medical Center in Quezon City.

Research ethics

The experiments were conducted upon the approval of the protocols. The plant species was identified by the Far Eastern University Herbarium (FEUH). The researchers secured certification that the study withheld any use of model organisms as a test subject, as per the guidelines of the Ethics Review Committee of Our Lady of Fatima University. The researchers only used the MCF-7 adenocarcinoma cell line in the experimentation.

Research instrument

Research Materials:

***Leea manillensis* leaves:** *Leea manillensis* is a tree or shrub that grows up to one meter tall. Its branches are glabrous and soft-wooded, with leaves that are bipinnate or tripinnate at the base. The leaves of *L. Manillensis* were used as the primary research instrument, and a research sample was tested for cytotoxic and antioxidant agents against the MCF-7 cancer cell line.

MCF-7 adenocarcinoma cell line: MCF-7, a breast cancer cell line that has a receptor for estrogen and progesterone and belongs to the luminal. A molecular subtype was used as the specimen and model to identify the cytotoxic effect of *L. manillensis* methanolic leaf extract.

Doxorubicin and Ascorbic acid: In identifying the cytotoxicity of *Leea manillensis* using PrestBlue Assay, it used Doxorubicin as the positive control which has already been proven to be an effective cytotoxic agent against cancer cell lines including MCF-7 cell line. On the other hand, the DPPH Assay used Ascorbic Acid as the positive control that was also proven to have high efficacy as an antioxidant.

Dimethyl sulfoxide (DMSO): Dimethyl Sulfoxide (DMSO) was utilized as a negative control in DPPH and PrestoBlue Assay since it is an aprotic polar solvent without any antibacterial qualities and may dissolve test substances such as polyeugenol and eugenol compounds.

Methanol: Methanol was frequently used as a solvent in the extraction of *L. manillensis* leaves. Substances such as phenolic compounds, alkaloids, flavonoids, and tannins are able to dissolve effectively in methanol.

Phosphate Buffered Saline (PBS): PBS, a balanced salt solution, was utilized to preserve the osmotic equilibrium and integrity of cells. It is frequently used to clean cells and get them ready for certain tests, such as PrestoBlue Assay.

Microplate: Numerous sample testing may be done at once by using microplates with numerous wells. A 96-well microplate is frequently used in the DPPH and PrestoBlue test setting because it facilitates quick and easy sample processing and analysis.

Cell culture medium: Cells in culture are maintained and nourished with the help of the cell culture media. Cells are usually grown in the medium for the PrestoBlue Assay, and different quantities of the test chemicals can be added to see how they affect the vitality of the cells.

Centrifugal solvent evaporator: In chemical and biological laboratories, a centrifugal evaporator is a tool used to gently and efficiently evaporate solvents from many samples at once as well as samples in microtiter plates.

UV-Vis Spectrophotometer: A spectrophotometer is a device that measures a sample's transmittance or absorbance in relation to the electromagnetic radiation's wavelength. This is mostly utilized for quantitative examination of ionic or molecular species in solutions.

Soxhlet extractor: This was used to effectively remove solvents through dissolving mixture in another soluble solvent. It performs best in terms of extraction and distillation.

Research procedure:

Authentication of *Leea manillensis* leaves: The leaves of *L. manillensis* were secured first. The leaves were brought to the FEU Herbarium, where they were identified and authenticated based on comparison with available accessions and/or botanical literature of the said facility.

Preparation of *Leea manillensis* crude leaf extract: Four kilograms of *Leea manillensis* were air-dried for three days. The *L. manillensis* leaves were soaked in a methanol solution and incubated for up to three days at room temperature. The substances were filtered using a 25 μm pore size Whatman filter paper, and the resulting clear supernatant was concentrated under reduced pressure with the help of a Soxhlet extractor at 40°C. In the end, the resulting residue was set aside for the following experiments, with the extract stored at 4°C. The method used in this is based on the protocol of the procedure of Adamson University Technology Research and Development Foundation, Inc. (2024). Maceration and distillation of the *L. manillensis* leaves yielded 175.27 g of methanolic extract.

Ultra-performance liquid chromatography protocols: The phytochemical analysis and specific subtype identification of *Leea manillensis*' flavonoids were assessed using UPLC. The method was based on Chen et al. [6], wherein the leaves of the *L. manillensis* were dried in a place that was dry, airy, and out of direct sunlight, and pulverized to powder. Twenty grams of this powder were placed into a single-neck round-bottomed flask made of glass with a volume of 500 mL. Then, 200 mL of different solvents (including water, methanol, ethanol, acetone, ethyl acetate, ethyl ether, dichloromethane, or hexane) were added, and the mixture was refluxed using a hotplate magnetic stirrer with methyl silicone oil as the heating medium for 6 h at the boiling points of the respective solvents. Whatman No.1 filter paper and Soxhlet extractor was used to filter and evaporate the extracts under reduced pressure at <50°C until dry. All dried extracts were weighed and stored at − 20°C until use. Yield was calculated as % yield = (weight of dry extract/initial weight of dry sample) × 100.

UV-Vis Spectrophotometry: UV-visible spectroscopy was conducted to qualitatively analyze and identify specific classes of chemical compounds in both pure samples and biological mixtures. This approach enabled the identification of active compounds present in different plant parts, as well as the qualitative and quantitative analysis of phytochemicals within plant extracts using a UV spectrophotometer. This instrument is used to separate and measure spectral components of physical phenomena [7].

The experimentation of this started with centrifugation of the extract at 10 min at 300 rpm, after that the extract was filtered using Whatman No. 1 filter paper. The same solvent was diluted at the ratio of 1:10. The extract was scanned using a Perkin Elmer Spectrophotometer and it was scanned at wavelengths between 200 and 1100 nm. Distinct peaks were found, and it was recorded. Following the UV-VIS analysis, it was determined that the sample contained tannins at a concentration of 1720 mg/L, equivalent to 0.172%. Conversely, the presence of saponins and alkaloids in *L. manillensis* were not detected. The quantification of these three components was conducted using spectrophotometric methods and this was based on the protocol of Adamson University Technology Research and Development Foundation, Inc. (2024).

PrestoBlue assay protocols: The cytotoxic activity of *Leea manillensis* leaf methanolic extract was assessed using the PrestoBlue Assay. The preparation done for experimentation, the MCF-7 cells were maintained in a complete medium, consisting of the following: Minimum Essential Medium (Gibco, 11700077) supplemented with 10% Fetal Bovine Serum (Gibco, 10438034), Penicillin-Streptomycin (Gibco, 15140122), and 0.01 mg/mL human recombinant insulin (Gibco, 12585014) at a 37°C incubator with 5% CO_2. Culture medium was removed from the flask and the cells were washed with IX Phosphate-Buffered Saline (PBS) to remove debris and traces of serum. To detach the cells from the flask, 0.1% (w/v) Trypsin-EDTA solution was added and incubated at 37°C incubator with 5% CO_2 for 5 min. An equal amount of complete medium was added to the cells to stop the action of trypsin-EDTA solution. The cell suspension was transferred to a conical tube and centrifuged at 130 x g for 5 min at 25°C. The supernatant was discarded, and the conical tube was then gently tapped to loosen the pellet. The cell pellet was then resuspended with a complete medium and dispensed into a cell culture flask. The cells were incubated in a 37°C incubator with 5% CO_2.

The MCF-7 cells were seeded at 1.5 × 10* cells per well on a 96-well flat clear bottom black plate (Corning, 3603) in triplicates and incubated 24 h. Cell density and viability were determined using a Trypan blue dye exclusion method. The following formulas were used:

$$Cell\ viability(\%) = \frac{Number\ of\ live\ cells}{Total\ number\ of\ cells} \times 100$$

$$Cell\ viability(\%) = \frac{Number\ of\ live\ cells}{Total\ number\ of\ cells} \times 10^4 \times\ dilution\ factor$$

For the preparation of extract and controls, 1.0 mL aliquots of the methanolic extract of *L. manillensis* was dried using a centrifugal solvent evaporator. The resulting dry weight of 98.3

mg was reconstituted with 1.0 mL of complete media to obtain a stock concentration of 98.3 mg/mL. The reconstituted extract was filtered through a 0.2 µM syringe filter (Pall, 4652) and diluted to different concentrations. The positive control, Doxorubicin, was reconstituted with 5.0 mL of Dimethyl Sulfoxide (DMSO) to obtain a stock concentration of 10 mg/mL. The positive control, Doxorubicin (Admac LifeSciences, DXL.J23B21-A), was reconstituted with 5.0 mL of Dimethyl Sulfoxide (DMSO) (Sigma, D8418) to obtain a stock concentration of 10 mg/mL. A series of dilutions for the extract and the positive control were prepared for the assay.

The IC_{50} of the positive control (Doxorubicin) was determined. The MCF-7 cells were treated with 10 uL of each extract and controls per well in triplicate. The controls used were cells without treatment (no treatment control), cells with DMSO (vehicle control), medium alone (blank), and Doxorubicin (positive control). The plate was then incubated in a 37°C incubator with 5% CO_2 for 72 h. The results of IC_{50} values are interpreted of based on the Table 1.

A *in vitro* cytotoxicity assay was performed using PrestoBlue cell viability reagent. A 7.5 µL of the PrestoBlue TM reagent was added to each well in the dark. The plate was then incubated in a 37°C incubator with 5% CO_2 for one hour. The fluorescence intensity was measured at a 540/25 nm excitation and 620/40 nm emission using Synergy H4 Hybrid Multi-Mode Microplate reader (GenS software, Lab Tech, USA).

The average fluorescence readings of blank wells were subtracted from all wells. The resulting values were then used for determination of cytotoxicity index (%). The computed cytotoxicity index (%) was used for data representation.

Percent cytotoxicity was calculated as follows:

$$Cytotoxicity\ index(\%) = \frac{F_c - F_T}{F_C} \times 100$$

Wherein, F_T = fluorescence of treated cells control

F_C = average fluorescence of no treatment

2,2′-Diphenyl-1-Picrylhydrazyl (DDPH) free radical scavenging assay protocols: The plant extract's free radical scavenging assay was measured by using 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) assay. The method used was a modified version of Clarke et al., twenty microliters (20 µL) of the sample diluted appropriately in Dimethyl Sulfoxide (DMSO) was mixed with 180 µL of DPPH in methanol (40 µg/mL) in wells of a 96-well plate. The plate was kept in the dark for 15 min after which the absorbance of the solution was measured at 540 nm in a plate reader. DMSO served as a blank and Ascorbic Acid (4 mg/mL) served as the standard. Samples were tested at a single concentration of 4 mg/mL to determine the antioxidant activity. The data was reported as percent DPPH scavenging effect using the following equation.

$$\text{DPPH } scavenging\ effect\ (\%)\ or\ percent\ inhibition = \frac{Absorbance\ of\ DPPH\ -\ Absorbance\ of\ sample}{Absorbance\ of\ DPPH} \times 100$$

Data analysis

All data were expressed as mean ± Standard Deviation (SD). The researchers performed statistical analysis using one-way Analysis of Variance (ANOVA) or Kruskal-Wallis test, to assess and compare the cytotoxic effects and scavenging activity of different concentrations of *Leea manillensis* leaf methanolic extract on MCF-7 breast adenocarcinoma cells. The significant level was set at $p<0.05$ for analysis of percent inhibition and cytotoxicity index of cell growth. In addition, IC_{50} was utilized to determine how much is needed to inhibit biological processes by half and its values were determined using GraphPad Prism Software. This statistical analysis helped the researchers by identifying concentration-dependent effects and contributed to the overall understanding of the extract's potential as an anti-cancer agent.

Tukey's HSD post-hoc test was also used to determine specific differences between the positive control, negative control, and experimental control group in the case that ANOVA shows significant differences. This will reveal which concentration works best to inhibit the MCF-7 breast adenocarcinoma cell line.

In addition, the t-test was utilized to determine the significant difference between the mean 50% inhibitory concentration of the two control groups, the positive and experimental, which are the doxorubicin and the methanolic extract of *L. manillensis*.

Result

Plant extract

About 175.27 grams of leaf methanolic extract of *L. manillensis* was obtained and subjected to UPLC, UV-VIS, DPPH Assay and PrestoBlue Assay (Table 2).

The quantitative analysis of phytochemicals in the table above showed tannins had the highest amount, with a result of 0.172%, while the flavonoids, specifically quercetin, had 0.017%. No saponins or alkaloids were detected in the leaf methanolic extract of *Leea manillensis*.

Cytotoxic activity of *L. manillensis* using PrestoBlue assay

Table 3 presents the mean fluorescence intensity readings at different concentrations of the three controls. *L. manillensis*' absorbance at 1600 ug/mL suggested a wide range of fluorescence intensities, while Doxorubicin expressed low fluorescence intensities. As for DMSO, its highest concentration showed lower intensities compared to the two control groups, which demonstrates a cytotoxic effect because lower fluorescence signals cytotoxicity.

In accordance with Table 3, the lower the fluorescence intensity, the higher the cytotoxicity. Based on the average in their respective highest concentration shown in Table 3.1, Doxorubicin (94.17) appeared to have a higher cytotoxicity, compared to *L. manillensis* extract (55.33) and DMSO (97.93).

Table 1: Interpretation of IC_{50} based on American National Cancer Institute.

VALUES	INTERPRETATION
<20 ug/mL	Very cytotoxic
100-500 ug/mL	Strongly cytotoxic
>500 ug/mL	Not cytotoxic

Table 2: Quantitative analysis of phytochemicals in *L. manillensis* leaf methanol extract using UPLC and UV-Visible spectrophotometer.

Analytes/Parameters	Result/s	METHOD
Flavonoids as Quercetin	0.017%	UPLC
Tannins	0.172%	Spectrophotometric
Saponins	(-)	Spectrophotometric
Alkaloids	(-)	Spectrophotometric

As shown in Table 3.2, the IC_{50} value of *Leea manillensis* is 2724 µg/mL, indicating that it is not cytotoxic to MCF-7 cells based on the standard given by the American National Cancer Institute. In contrast, the Doxorubicin (positive control), has an IC_{50} value of 0.7644 µg/mL, classifying it as an exceptionally potent cytotoxic agent.

$$t = \frac{(x_{-1} - x_{-2}) - (\mu_{-1} - \mu_{-2})}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

$$t = \frac{(2724\,ug/mL - 0.7644\,ug/mL) - (0-0)}{\sqrt{\frac{1}{8} + \frac{1}{9}}}$$

$t = 5604.3726$

The observed value (t=5604.3726) was greater than the critical value (-1.96), the researchers reject the null hypothesis. Therefore, there was enough evidence to support that the cytotoxicity level of Doxorubicin (positive control) is greater than the cytotoxicity level of *L. manillensis* when comparing their IC_{50}.

From the results of Table 4, *L manillensis* didn't pass the normality test (P<0.05), indicating the data is not normally distributed while both Doxorubicin and DMSO passed the normality test (P>0.05), signifying their data is normally distributed. Since *L. manillensis* didn't pass the normality test, the Kruskal-Walli's test or One-Way ANOVA is appropriate for comparing the cytotoxicity of the three variables.

Table 3: Raw fluorescence intensity readings of the *L. manillensis* extracts and controls.

	Concentration	Mean Fluorescence intensity (540 nm, 620/40 nm)		
L. manillensis	1600 ug/mL	286	639	564
	800 ug/mL	702	702	731
	400 ug/mL	698	688	687
	200 ug/mL	589	639	679
	100 ug/mL	692	636	692
	50 ug/mL	673	639	658
	25 ug/mL	568	712	553
	12.5 ug/mL	702	665	703
	6.25 ug/mL	687	509	699
Doxorubicin	1600 ug/mL	326	335	291
	800 ug/mL	328	335	321
	400 ug/mL	271	339	337
	200 ug/mL	317	332	338
	100 ug/mL	355	326	369
	50 ug/mL	259	385	381
	25 ug/mL	291	395	406
	12.5 ug/mL	418	403	424
	6.25 ug/mL	402	405	427
DMSO	50.00%	336	269	328
	0.02%	860	834	795
	0.01%	728	691	818
	0.01%	636	612	360
	0.00%	731	663	340
	0.00%	722	599	794
No Treatment Control		736	741	734
Blank		290	293	323

Table 3.1: Computed cytotoxicity indices (%) of the *L. manillensis* extracts and controls.

	Concentration	Mean Fluorescence intensity (540 nm, 620/40 nm)			Average
L. manillensis	1600 ug/mL	103.68*	22.53	39.77	55.33
	800 ug/mL	8.05*	8.05*	1.38*	5.83
	400 ug/mL	8.97*	11.26	11.49	10.57
	200 ug/mL	34.02*	22.53	13.33	23.29
	100 ug/mL	23.22*	10.34	10.34	14.63
	50 ug/mL	22.53*	14.71	18.16	18.47
	25 ug/mL	42.30*	5.75	38.85	28.97
	12.5 ug/mL	16.55*	8.05	7.82	10.81
	6.25 ug/mL	52.41*	11.49	8.74	24.21
Doxorubicin	1600 ug/mL	94.48	92.41	95.63	94.17
	800 ug/mL	94.02	92.41	102.53	96.32
	400 ug/mL	107.13	91.49	91.95	96.86
	200 ug/mL	96.55	93.1	91.72	93.79
	100 ug/mL	87.82	94.48	84.6	88.97
	50 ug/mL	109.89	80.92	81.84	90.88
	25 ug/mL	102.53	78.62	76.09	85.75
	12.5 ug/mL	73.33	76.78	71.95	74.02
	6.25 ug/mL	77.01	76.32	71.26	74.86
DMSO	50.00000%	92.18	107.59	94.02	97.93
	0.02000%	-28.28	-22.3	-13.33	-21.3
	0.01000%	2.07	10.57	-18.62`	6.32
	0.00500%	23.22	28.74	86.67	46.21
	0.00250%	1.38	17.01	91.26	36.55
	0.00125%	3.45	31.72	-13.1	7.36

*Values were excluded from the data representation

Table 3.2: 50% inhibitory concentration of the three variables.

	DMSO	Doxorubicin	*L. manillensis*
IC50 value (ug/mL)	-	0.7644	2724

As can be seen in Table 4.1, a high cytotoxic index indicates higher efficiency as a treatment for the MCF-7 adenocarcinoma cell line, making Doxorubicin (82.896 ± 11.361) an incomparable cytotoxic agent to *L. manillensis* and DMSO.

Table 4.2 shows the substantial disparity in mean ranks between the three control groups, suggesting significant differences in the data distributions among these groups. The results likely indicate that Doxorubicin exhibits a significantly different (higher mean rank) effect or measurement compared to *L. manillensis* and DMSO.

As illustrated in Table 4.3, the significance level was below 0.001. Given that the p-value was less than 0.05, there was sufficient data to infer that the ways in which *L. manillensis*, Doxorubicin, and DMSO inhibit the MCF-7 cell line differ significantly from each other. Therefore, we reject the null hypothesis.

Table 4.4 delineated the comparison between control groups based on p-values and mean rank difference. It indicates significantly higher ranks for Doxorubicin. These findings underscore the distinct effects or measurements observed across the different groups analyzed. A p-value of <0.001 is shown above indicating a significant difference

Table 4: Kruskal-Wallis test for comparison of the cytotoxicity of the three variables.

	L. manillensis	Doxorubicin	DMSO
Mean	12.425	82.896	22.03083
Standard Deviation	10.34484	11.36143	34.80409
Sample Size	10	15	12
Std. error of mean	3.271327	2.933508	10.04708
Lower 95% conf. limit	5.024744	76.60425	-0.08263
Upper 95% conf. limit	19.82526	89.18775	44.1443
Minimum	1.49	71.26	-18.62
Median (50th percentile)	9.54	78.62	13.79
Maximum	38.85	109.89	91.26
Normality Test KS	0.280	0.250	0.221
Normality Test P-value	<0.05	>0.05	>0.05
Passed the normality test?	No	Yes	Yes

Table 4.1: Cytotoxicity mean score of the three variables.

L manillensis	Doxorubicin	DMSO
12.425 ± 10.345	82.896 ± 11.361	22.031 ± 34.804

Table 4.2: Kruskal-Wallis statistic.

Group	Number of Points	Mean of Ranks
L manillensis	10	11.1
Doxorubicin	15	28.46667
DMSO	12	13.75

Kruskal-Wallis Statistic: KW=19.625 (corrected for ties)

Table 4.3: Significant difference between the *L manillensis*, Doxorubicin, and DMSO.

KW value	P-value	Analysis	Decision
19.625	<0.001	There is a significant difference	Reject null hypothesis

Table 4.4: Post hoc analysis. Dunnet's multiple comparisons test.

Comparison	Mean Rank Difference	P-value
***L manillensis vs.* Doxorubicin**	-70.471	<0.001
***L manillensis vs.* DMSO**	-9.606	0.745
Doxorubicin *vs.* DMSO	60.865	<0.001

between the Doxorubicin and the two groups, reflecting the higher cytotoxic effect of Doxorubicin. However, in the comparison of *L. manillensis* and DMSO, the p-value was found to be greater than <0.001, suggesting no significant difference between these groups. This implies that both *L. manillensis* and DMSO exhibit a negligible cytotoxic effect, with little to no discernible distinction between them.

Scavenging activity of *L. manillensis* using DPPH assay

In accordance with Table 5, the Ascorbic Acid (positive control) has 99.52% free radical inhibition. DMSO, the negative control, exhibited 0% free radical inhibition. On the other hand, *L. manillensis* leaf methanolic extract expressed a mean of 25.42% free radical inhibition as presented in Table 5.1. This signifies that ascorbic acid was more effective in scavenging free radicals than *L. manillensis* leaf methanolic extract. Among the five concentrations of *L. manillensis* methanolic extract, the 60 µg/mL concentration displayed a high effectivity rate of 38.99%. In contrast, the concentration that showed a low effectivity rate was 6.25 µg/mL with a result of 13.87%.

Based on Table 6, both DPPH and Ascorbic Acid passed the normality test (P>0.005), suggesting that their data are normally distributed while *L. manillensis* did not pass the normality test (P<0.005), indicating its data are not normally distributed. Hence, the use of Kruskal-Walli's test is necessary in order to compare their cytotoxicity effectively.

Table 6.1 shows the absorbance values with ascorbic acid as the standard absorbance value. Lower absorbance indicates higher free radical scavenging activity, making ascorbic acid (0.002 ± 0.002) more effective than *L manillensis*, which has a value of 0.359 ± 0.003.

Table 6.2 posits the analysis revealing the distinct performance levels among the groups examined. Ascorbic acid achieved the most favorable outcome with the lowest mean rank (2.00), indicating superior effectiveness. It is followed by *L. manillensis* with a mean rank of 5.00. Lastly, DPPH exhibited a mean rank of 8.00 that suggested lower efficacy among the controls.

Table 6.3 reveals that the significance level is <0.05 which implies that there was sufficient data to infer that the ways in which DPPH, ascorbic acid, and *L manillensis* inhibit the free radicals differ significantly from each other. Therefore, reject the null hypothesis.

In Table 7, it suggests that there is a significant difference between the three groups based on the p value of <0.001. Ascorbic acid has the prevailing antioxidant activity compared to *L manillensis* and DPPH. However, *L manillensis* has the greater effect compared to the DPPH when compared.

Discussion

Phytochemical analysis of *Leea manillensis*

Phytochemicals are naturally occurring biologically active compounds that have significant antitumor effects which have potential to improve treatment and reduce adverse effects [8].

Phytochemical analysis of *Leea manillensis* methanolic leaf extract shows the presence of flavonoids and tannins while there is the absence of saponins and alkaloids. Flavonoids as reported by Park et al. [9], are a naturally occurring substance with phenolic structure that has a phenolic ring structure. This has antitumor and antioxidant properties. Flavonoids can also inhibit tumor growth that causes cell death therefore it is possible to treat cancer using flavonoids. Additionally, quercetin, a significant flavonoid that exists in some vegetables and beverages, has anti-cancer and antioxidant effects [10].

Foods high in tannins may be antioxidants, fight cancer, and lower the risk of cardiovascular disease. Research has been done on the anticancer properties of tannins against various cancer cells. Tannins showed dose-dependent cytotoxic action in the majority of the cell lines, including those from Hepatocellular Carcinoma (HCC), breast cancer, lymphocytic leukemia, lung cancer, and epidermoid carcinoma. Tannins' phenolic hydroxyl groups allow them to react with oxygen radicals easily and produce a lot of free hydrogen as a result. Tannins can lower the risk of aging and illnesses associated with it, such as cardiovascular disease, aging, and cataracts, since they are potent free radical scavengers.

Stereoisomers of saponins may exhibit stereospecific pharmacological actions that include anti-inflammatory, antibacterial, anti-photoaging, anti-tumor, antioxidative, antidiabetic, and neuroprotective properties. Since saponins have demonstrated anticancer potential, using them in cancer treatment is seen to be

Table 5: Raw absorbance readings of the *L. manillensis* extracts and controls.

Absorbance	DPPH	Ascorbic Acid	*Leea manillensis* leaf extract				
			6.25 µg/mL	12.5 µg/mL	20 µg/mL	40 µg/mL	60 µg/mL
Trial 1	0.4800	0.0006667	0.4197	0.3757	0.3707	0.3327	0.2907
Trial 2	0.4840	0.001667	0.4027	0.3807	0.3717	0.3357	0.2987
Trial 3	0.4850	0.004667	0.4257	0.3807	0.3677	0.3427	0.2947
AVE	0.4830	0.002333	0.4160	0.3790	0.3700	0.3370	0.2947
SD	0.002646	0.002082	0.01193	0.002887	0.002082	0.005132	0.004000
% Inhibition		99.52	13.87	21.53	23.40	30.23	38.99

Table 5.1: Computed percent inhibition of *L. Manillensis* leaf methanolic extract using DPPH assay.

1	2	3	4	5	MEAN	SD
13.87%	21.53%	23.40%	30.23%	38.99%	25.42%	12.68124

Table 6.0: Kruskal-Wallis test for comparison of the cytotoxicity of the three variables.

	DPPH	Ascorbic Acid	*L manillensis*
Mean	0.483	0.002334	0.359367
Standard Deviation	0.002646	0.002082	0.00254
Sample Size	3	3	3
Std. error of mean	0.001528	0.001202	0.001467
Lower 95% conf. limit	0.476428	-0.00284	0.353056
Upper 95% conf. limit	0.489572	0.007505	0.365677
Minimum	0.480	0.000667	0.3579
Median (50th percentile)	0.484	0.001667	0.3579
Maximum	0.480	0.000667	0.3579
Normality Test SW	0.485	0.004667	0.3623
Normality Test P-value	>0.05	>0.05	<0.05
Passed the normality test?	Yes	Yes	No

Table 6.1: Absorbance mean score of the three variables.

DPPH	Ascorbic Acid	*L manillensis*
0.483 ± 0.003	0.002 ± 0.002	0.359 ± 0.003

Table 6.2: Kruskal-Wallis statistic.

Group	Number of Points	Mean of Ranks
DPPH	3	8.00
Ascorbic Acid	3	2.00
L. manillensis	3	5.00

Kruskal-Wallis Statistic: KW=7.261

Table 6.3: Significant difference between the *L manillensis*, Ascorbic acid, and DMSO.

KW value	P-value	Analysis	Decision
7.261	<0.05	There is a significant difference	Reject null hypothesis

promising [11].

Alkaloids have a wide range of biological activity and are present in many plant species. Certain cancer cells are severely affected by certain alkaloids [12].

IC_{50} of *Leea manillensis* and Doxorubicin

The researchers have conducted a cytotoxicity test of *Leea manillensis* against MCF-7 adenocarcinoma cells using PrestoBlue Assay. Half maximal inhibitory concentration was used in this study to show inhibitory effects of the extract to the MCF-7 cell line. This IC_{50} also tells how much extract is required to inhibit a biological activity in 50%. This provides information on the measure of potency of the *L. manillensis* extract against the MCF-7 adenocarcinoma cell line. In this assay, nine concentrations were used to identify the IC_{50}.

Table 7: Post hoc analysis. Dunnet's multiple comparisons test.

Comparison	Mean Rank Difference	P-value
Ascorbic Acid vs. DPPH	0.4807	<0.001
Ascorbic Acid vs. *L manillensis*	-0.3570	<0.001
DPPH vs. *L manillensis*	0.1236	<0.001

The 50% inhibitory concentration of the *L. manillensis* is 2724 µg/mL. In contrast, the IC_{50} of the doxorubicin is 0.7644 µg/mL. According to the study of Berrouet et al., low IC_{50} means that the drug is potent at low concentrations. It was also stated in the study of Maqsood in 2017 [13], the US NCI stated that if the plant extract inhibits 50% of the cell at 20 µg/mL, it is generally considered to have cytotoxicity effects. According to the interpretation guidelines provided of the IC_{50} values by the American National Cancer Institute, doxorubicin exhibits a very cytotoxic effect on MCF-7 cancer cells. In contrast, the methanolic extract of *L. manillensis* is deemed to be not cytotoxic against the said cancer cell line.

In the statistical analysis of the *L. manillensis* and Doxorubicin, it was evident that the null hypothesis should be accepted since the observed value (5604.3726) was greater than the critical value (-1.96). This supported the claim that Doxorubicin has higher cytotoxicity than the Leea manillensis.

Significant difference of *Leea manillensis* and Doxorubicin

Doxorubicin is an anthracycline drug derived from *Streptomyces peucetius var. caesius* as its secondary metabolite. It is widely used as an efficient antineoplastic for various cancers such as breast cancer. According to the study of Dapar in 2021, *Leea manillensis* contains phytochemicals including alkaloids, flavonoids, tannins, and quercetins. These compounds were determined by using methanolic leaf extracts.

The *Leea manillensis* extract and doxorubicin produced a result of significant difference after the experimentation. In accordance with the study of Fohlen et al. [14], a higher cytotoxicity index indicates a greater likelihood of discovering an agent suitable for locoregional therapy. For the comparison of Doxorubicin and *L. manillensis*

extract, the doxorubicin had a cytotoxicity index of 95.63% while the *L. manillensis* had 39.77% at their highest concentrations. Doxorubicin yields a lower absorbance than the extract of *L. manillensis* which results in doxorubicin having a higher cytotoxic effect compared to *L. manillensis*. In the statistical analysis, the P-value of *L. manillensis* and doxorubicin was less than 0.001 which indicated that there existed a significant difference between the two groups and *L. manillensis* cytotoxicity was significantly lower than doxorubicin.

Mean absorbance in DPPH assay

Antioxidant activity of methanolic extract of *L. manillensis* in scavenging free radical was determined using DPPH assay. This test involves mixing the plant extract with a stable free radical (DPPH) and measuring the degree of color change. Increased antioxidant activity is indicated by more discoloration [15].

A percentage of inhibition can vary from 0% to 100% and is dependent on various factors such as the concentration of the oxidant (radicals), the concentration of the antioxidant, the solvent employed, the ratios of the reagents used, the temperature, the incubation duration, and whether metal, hydrogen, or water are present in the measurement systems [16]. In terms of raw absorbance readings of *Leea manillensis* leaf methanolic extract and controls it showed that ascorbic acid, the positive control, has 99.52% free radical inhibition; DMSO, the negative control, exhibited 0% free radical inhibition; and the leaf methanolic extract of *L. manillensis* expressed a mean of 25.42%.

Olszowy-Tomczyk [16] also stated that greater antioxidant activity is indicated by a higher inhibition % value. Thus, with the result stated above, the ascorbic acid was more effective in scavenging free radicals than *L. manillensis* leaf methanolic extract.

Significant difference of Ascorbic acid and *Leea manillensis*

Ascorbic acid is a crucial micronutrient antioxidant with strong reducing properties, essential for many physiological functions in the human body. It is a water-soluble antioxidant and enzyme co-factor and is produced by plants and some animals [17]. *Leea* spp. are known as important sources of natural antioxidants due to the presence of secondary metabolites such as phenols and flavonoids that serve as sources of antioxidants and perform scavenging activities [4].

The *Leea manillensis* extract and ascorbic acid produced a result of significant difference after the experimentation. A higher concentration of extracts and ascorbic acid resulted in lower absorbance values. This was due to more compounds in the solution to capture free radicals (DPPH) at higher concentration, leading to a decrease in the purple color of the DPPH solution. For the comparison of ascorbic acid and *L. manillensis* extract, the ascorbic acid had a percentage of inhibition of 99.52% while the *L. manillensis* had 38.99% at their highest concentrations. In the statistical analysis, the P-value of *L. manillensis* and ascorbic acid was less than 0.05 which means that there's a significant difference between the two groups and *L. manillensis* antioxidant activity was significantly lower than ascorbic acid. The percentage inhibition of the ascorbic acid proved to be capable of greatly neutralizing the free radicals compared to the extract of *L. manillensis*.

Leea manillensis as antioxidant and cytotoxicity agent

The cytotoxicity value for *Leea manillensis* was 12.425 ± 10.345, compared to 82.896 ± 11.361 for Doxorubicin. Additionally, the half-maximal inhibitory concentration (IC_{50}) of *L. manillensis* was higher at 2724 µg/mL, as compared to 0.7644 µg/mL for Doxorubicin. According to the American National Cancer Institute, IC_{50} values are considered very cytotoxic if they are <20 µg/mL, indicating that Doxorubicin exhibited higher cytotoxic activity than *L. manillensis*.

Moreover, the absorbance of *Leea manillensis* in the DPPH assay was much higher at 0.359 ± 0.003, compared to Ascorbic acid at 0.002 ± 0.002. Lower absorbance indicates higher free radical scavenging activity, thus making Ascorbic acid more effective than *L. manillensis*. In terms of DPPH scavenging effect, *L. manillensis* exhibits 38.99% inhibition at its highest concentration of 60 µg/mL, while Doxorubicin shows 99.52% inhibition. This means that Ascorbic acid demonstrated higher antioxidant activity than *Leea manillensis*.

Doxorubicin and ascorbic acid both showed higher antioxidant and cytotoxic activity than the methanolic extract of *Leea manillensis*. Both the DPPH assay and PrestoBlue assay revealed a significant difference between the positive controls and the methanolic extract of *L. manillensis*, indicating that the methanolic extract was less effective than the positive controls. In a study conducted by Vibala et al. [18], isolated compounds from the plant extract demonstrated superior anticancer activity compared to the crude extract. While the methanolic extract at higher concentrations can reduce cell viability, the fractionated forms of the extract exhibited the highest activity at significantly lower concentrations compared to the methanolic fraction [19].

Conclusion

To conclude, the study meticulously analyzed the cytotoxic and antioxidant properties of *Leea manillensis* methanolic leaf extract against the MCF-7 breast cancer cell line. Results revealed significant findings regarding the extract's efficacy and potential therapeutic benefits. Notably, quantitative phytochemical analysis identified substantial concentrations of tannins (0.172%) and flavonoids (0.017%) in the extract, indicative of its pharmacological relevance. However, the IC_{50} values underscored the superiority of Doxorubicin, with an IC_{50} of 0.7644 µg/mL compared to *L. manillensis'* 2724 µg/mL, indicating its greater efficacy in inhibiting cancer cell proliferation. It was clearly stated that less than 20 ug/mL was considered cytotoxic indicating that Doxorubicin has exceptional cytotoxic effect compared to the plant extract which has low to no cytotoxic effect. The PrestoBlue assay corroborated these findings, showing a mean cytotoxicity score of 12.425 ± 10.345 for *Leea manillensis*, significantly lower than Doxorubicin's cytotoxicity of 82.896 ± 11.361.

Furthermore, the DPPH assay provided insights into the extract's antioxidant activity, with *Leea manillensis* demonstrating low free radical inhibition compared to the positive control, ascorbic acid. Absorbance means scores highlighted the extract's effectiveness in scavenging free radicals, albeit less than ascorbic acid. Statistical analysis, including ANOVA and Tukey's post-hoc test, confirmed significant differences in cytotoxic and antioxidant activities among the experimental and control groups, with p-values below 0.05 indicating statistical significance. Post-hoc analysis further elucidated these differences, emphasizing Doxorubicin's superior cytotoxicity compared to both *L. manillensis* and Dimethyl Sulfoxide (DMSO). Additionally, the percent inhibition rate provided a quantitative measure of the extract's efficacy, further supporting its potential as an anticancer and antioxidant agent.

The researchers therefore concluded that methanolic leaf extract

of *Leea manillensis* is ineffective in scavenging the radicals using DPPH Assay as well as inhibiting viability of breast cancer cells using PrestoBlue Assay.

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