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Original Article

Cytotoxic Effects of *Clitoria Ternatea* L. Extract from Ternate Island on Brine Shrimp (*Artemia salina* L.) and MCF-7 Breast Cancer Cell Line

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Abstract
Cancer is a leading cause of death worldwide, with nearly 10 million fatalities reported in 2020, including 2.3 million new cases of breast cancer. Due to its high prevalence, effective treatment options remain a critical priority. *Clitoria ternatea* L., a medicinal plant widely found in Indonesia, is traditionally used for various therapeutic purposes and may possess anticancer properties. This study aimed to evaluate the cytotoxic effects of ethanol extracts of *Clitoria ternatea* L. collected from Ternate Island. Cytotoxicity was assessed using two methods: the Brine Shrimp Lethality Test (BSLT) to determine general toxicity, and the PrestoBlue assay to evaluate antiproliferative effects on MCF-7 human breast cancer cells. In the BSLT, *Artemia salina* larvae were exposed to various concentrations of the extract (7.81-1000 µg/mL) for 24 hours, and LC50 values were calculated using probit analysis. For the PrestoBlue assay, MCF-7 cells were treated with extract concentrations ranging from 7.81 to 1000 µg/mL for 48 hours, followed by measurement of cell viability via absorbance at 570 nm. Results showed that the LC50 for the BSLT was 105.681 µg/mL, indicating low toxicity of the extract. In contrast, the extract did not demonstrate significant cytotoxicity against MCF-7 cells, with an IC50 value exceeding 1000 µg/mL. In conclusion, the ethanol extract of *Clitoria ternatea* L. exhibits low general toxicity but lacks significant cytotoxic effects on MCF-7 breast cancer cells under the tested conditions. Further studies are warranted to isolate active compounds and explore their potential anticancer mechanisms.

Keywords: *BSLT; Clitoria ternatea L.; MCF-7; Prestoblue Assay.*

INTRODUCTION

Cancer is a major global public health issue and one of the leading causes of death worldwide (Siegel et al., 2022). According to the 2022 Global Cancer Statistics, nearly 10 million people died from cancer in 2020, with more than 19.3 million new cases diagnosed globally. Among these, approximately 2.3 million women were diagnosed with breast cancer, which caused around 685,000 deaths worldwide in the same year. By the end of 2020, breast cancer had become the most frequently diagnosed cancer globally, surpassing all other types (Sung et al., 2021). The International Agency for Research on Cancer (IARC) for 2020 provides an explanation based on data sources from the GLOBOCAN cancer statistics agency. According to the primary findings, the death rate for new instances of breast cancer is 11.7%, whereas the mortality rate for all other malignancies is 6.9% (Dhillon et al., 2018). High cases certainly require a treatment strategy, plants have been used to prevent and treat various diseases for

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thousands of years (Kidayi et al., 2023). Indonesia, known for its rich biodiversity, is home to numerous plant species with potential anticancer properties, among which is the butterfly pea flower (*Clitoria ternatea* L.) (Rahayu et al., 2023).

Clitoria ternatea L., a member of the Fabaceae family, is commonly found in tropical and lowland regions. Known as the butterfly pea, this plant is easily recognized by its vibrant dark blue flowers. Many civilizations throughout the world have long utilized this plant as food and medicine. Previous studies have indicated that *Clitoria ternatea* exhibits cytotoxic and anticancer activities in vitro, suggesting its promise as a source of novel anticancer agents (Madhumitha et al., 2023). Phytochemicals found in *Clitoria ternatea* L. include tannins, flavonoids, steroids, anthocyanins, resins, and more. In addition to giving plants different qualities, these substances may be employed as medications to target proteins and other molecules implicated in a number of illnesses. However, further study is required to confirm and create these solutions to treat different illnesses (Madhumitha et al., 2023). Toxicity screening, such as the Brine Shrimp Lethality Test (BSLT), serves as a convenient and cost-effective preliminary assay to detect bioactive compounds in natural products (Raihan & Dalimunthe, 2022). This test is widely used to evaluate general toxicity and potential anticancer properties before advancing to more specific cell-based assays.

The BSLT has been shown to correlate positively with cytotoxicity assays conducted on cancer cell cultures, so this method is often used to screen for anticancer compounds where the results show that there is a correlation between the cytotoxic assay on cancer cells and BSLT (Masuda et al., 2011). The BSLT test was carried out by observing the level of death caused after extract was given to *Artemia salina* leach shrimp larvae after incubation for 1x24 hours. The results obtained are then calculated as the LC50 (Lethal concentration) value of the extract, where the concentration of the extract that could lead to death of *Artemia salina* L. is 50% (Raihan & Dalimunthe, 2022). Although BSLT was although the study successfully identified significant anticancer activity in the tested compounds, its primary limitation was the difficulty in distinguishing between different levels of sensitivity anticancer candidates with strong, moderate, and weak potential. Clonogenic techniques, such the Presto Blue assay, can be used to assess extracts antiproliferation against the development of cancer cells in order to implement the anticancer activity approach (Masuda et al., 2011). Cell lines play a crucial role in molecular cancer research by serving as in vitro models for diagnosis and therapeutic testing. Among breast cancer models, MCF-7 cells are the most commonly used line to study estrogen receptor (ER)-positive breast cancer due to their well-characterized biology and responsiveness (Comşa et al., 2015).

Based on the problem description, the butterfly pea flower (*Clitoria ternatea* L.) extract demonstrates cytotoxic potential in the BSLT assay; however, variations in anticancer activity require further evaluation using cancer cell models. Therefore, this study aimed to assess the anticancer capacity of the ethanol extract of *Clitoria ternatea* L. by evaluating its toxicity against *Artemia salina* larvae and comparing the results with its cytotoxic effects on MCF-7 breast cancer cells.

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METHODS

Instrumentation

Biosafety Cabinet (BSC) (Thermo scientific 1300 series A2), Centrifuge (Thermo scientific microCL17), CO2 Incubator (Thermo scientific series 8000DH), Microscope (Thermo scientific EVOS XL Core), Multimode Reader (Tecan Infinite M200 PRO).

Material

Cisplatin (EDQM C2210000), Antibiotic (Sigma Aldrich P4333), and Dimethyl Sulfoxide (DMSO) (Merck D1435) are the selected compounds for this study. For buffering, we use Phosphate Buffered Saline (PBS) (Gibco 18912-014) was used to assess cell viability assessment, the PrestoBlue™ Cell Viability Reagent (Thermofisher A13262) is employed. Additionally, Roswell Park Memorial Institute Medium (RPMI), specifically Gibco product number 11875-093 is utilized in conjunction with Fetal Bovine Serum (FBS) (Gibco 10270-106) and Trypsin-EDTA (Gibco 25200-056). Trypan Blue solution (Sigma Aldrich T-8154) was used for cell viability counting. Shrimp larvae, ethanol, and distilled water were also utilized as required.

Sample collection

The flowers of the *Clitoria ternatea* L. used were taken from around the island of Ternate with advanced stages of processing into dried plant material at the Pharmacology and Toxicology Laboratory, Pharmacy Department, Faculty of Medicine, Khairun University, Ternate, North Maluku.

Preparation of extracts

The butterfly pea flower extract was prepared using the maceration method. Five hundred grams of dried *Clitoria ternatea* L. flower powder were placed in a maceration container and soaked with 96% ethanol. The mixture was stirred several times and allowed to stand at room temperature for three days. This process is repeated three times, after which the extract is filtered from the residue using filter paper. After that, evaporation is carried out at low pressure at a set temperature until a thick paste extract is obtained.

Research Stages

In vivo cytotoxic assay using the BSLT method

The cytotoxic assay treatment was carried out 5 times on 96% ethanol extract. Preparation of *Artemia salina L* larvae by hatching *Artemia salina L* eggs for 48 hours before testing. Then a stock solution was made by mixing 100 mg of 96% ethanol Dissolve in 100 mL of distilled water to create a stock solution with a concentration of 1000 ppm, then a series of dilutions at concentrations of 7.81, 15.63, 31.25, 62.5, 125, 250, 500 ppm were carried out. As a control, distilled water was used and then 10 *Artemia salina L* larvae were added. Observations were carried out for 24 hours by counting the number of larval deaths. Analysis was carried out on the percentage of deaths of shrimp larvae by looking for the LC50 value with probit analysis.

In vitro cytotoxic assay using the PrestoBlue assay method

Media preparation and controls

Roswell Park Memorial Institute Medium (RPMI), supplemented with 50 µL of antibiotics per 50 mL and 10% Fetal Bovine Serum (FBS), was prepared as the culture medium. Cisplatin was used as the positive control. The test samples were prepared as stock solutions at specific final concentrations using a solvent that is non-toxic to cells. The working solution for the antiproliferation assay was prepared using PrestoBlue™ Cell Viability Reagent.

Cell preparation

The cells are rinsed twice with 1 mL of PBS once the medium is disposed of on

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a dish and the cells are at least 70% confluent. To disperse the cell layer, add 1 mL of Trypsin-EDTA solution and incubate for 5 minutes. The cells will seem to float when seen under an inverted microscope. Place the cells in a media-filled tube. For five minutes, centrifuge the cells at 3000 rpm. After discarding the supernatant once more, dissolve the pellet in the media-containing tube.

Seeding cells into 96-well plates

After assessing the viability and quantity of cells using trypan blue exclusion, resuspend the cells in media to achieve a final density of 170,000 cells/mL, with a concentration of 17,000 cells per well. To perform cell counting, prepare 10 µL of trypan blue solution in a sterile microtube, then mix it thoroughly with 10 µL of the cell suspension. Clean the hemocytometer and cover slip with 70% ethanol and allow them to dry completely. Carefully load 10 µL of the trypan blue–cell mixture into one chamber of the hemocytometer. Count the number of viable (unstained) cells under the microscope to determine the cell concentration per milliliter. Once this step is complete, seed the cells into 96-well Place the plates placed in an incubator maintained at 37°C with 5% CO2 for a duration of 24 hours, or until they achieve a minimum confluence of 70%.

Treatment of cells with sample, positive control, and negative control

Eight 1.5 mL microtubes were prepared and labeled with the respective dilution concentrations. The stock sample was serially diluted with solvent media to obtain eight concentration levels. Cells were removed from the incubator and cultured in a 96-well plate. The layout indicating which rows were assigned to samples or controls was marked along the left edge of the plate. The culture medium in each well was then carefully aspirated. Using a micropipette, 100 µL of each sample dilution was added to the designated wells. The positive control, cisplatin, was also added to the appropriate wells from its respective microtube. Following treatment, the plate was incubated for an additional 48 hours at 37°C in a humidified atmosphere with 5% CO_2.

Administration of PrestoBlue reagent and absorbance measurement

The PrestoBlue working solution was prepared by mixing 9 mL of culture medium with 1 mL of PrestoBlue™ Cell Viability Reagent (1:10 dilution). Each well of a 96-well plate was supplemented with 100 µL of this mixture and incubated for 1–2 hours to allow color development. Resazurin in the reagent is reduced to resorufin by metabolically active cells. This conversion, proportional to the number of viable cells, was quantitatively measured using a microplate reader at 570 nm, with 600 nm as the reference wavelength to correct for background.

RESULTS

Extraction of butterfly pea flower (*Clitoria ternatea* L.)

A total of 500 grams of dried butterfly pea flower powder was extracted using the maceration method using 5 L of 96% ethanol solvent, then evaporated utilizing a rotary evaporator, the solution was concentrated to yield a viscous extract weighing 69.54 grams.

In vivo cytotoxic assay using the BSLT method

The cytotoxicity of the ethanol extract was evaluated using the BSLT. This bioassay determines the concentration of the extract required to cause mortality in 50% of the test organisms (*Artemia salina* larvae). Larval mortality induced by the extract reflects its toxic effects (Jelita et al.,

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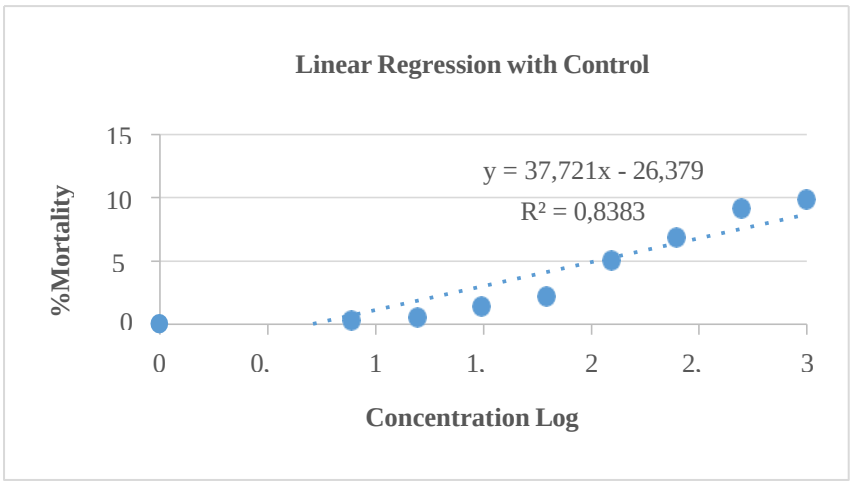
2020). The results of the cytotoxicity assay for butterfly pea flowers can be seen in Table 1.

In vitro cytotoxic assay using the PrestoBlue method

Resazurin, the active compound in the PrestoBlue reagent (a derivative of Alamar Blue), serves as an indicator of cellular metabolic activity through redox reactions involving reduction and oxidation processes (Puasa et al., 2018). The results of the cytotoxicity assay using the PrestoBlue method on MCF-7 cells can be seen in Table 2.

Table 1. Cytotoxicity of *Clitoria ternatea* L. ethanol extract against *Artemia salina* larvae using BSLT method

No	Concentration (µg/mL)	Treatment 1		Treatment 2		Treatment 3		Average % Mortality
		Dead Curve	% Mortality	Dead Curve	% Mortality	Dead Curve	% Mortality	
1	Control	0	0	0	0	0	0	0 %
2	7,81	1	10	2	20	0	0	2,12 %
3	15,63	0	0	1	10	2	20	5,12 %
4	31,25	2	20	3	30	2	20	13,40 %
5	62,5	1	10	1	10	2	20	21,79 %
6	125	4	40	7	70	7	70	50 %
7	250	5	50	2	20	7	70	68,05 %
8	500	8	80	9	90	8	80	91,35 %
9	1000	10	100	10	100	8	80	98,07 %



y=bx+a
y=37,721x-26,379
50=37,721x+26,379
37,721x=50+26,379/37,721
x=2,024
antilog x= 105,681 ppm

Figure 1. Standard curve of butterfly pea flower extract (*Clitoria ternatea* L.)

PrestoBlue reagent is the cells have been supplemented, and the final outcome is an absorbance measurement obtained with a multimode reader. In this assay, dimethyl sulfoxide (DMSO) solution is employed both as a negative control and as the solvent for the sample, because DMSO has non-volatile solvents. Apart from that,

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DMSO is the strongest organic solvent which can dissolve various kinds of organic materials effectively (Dinnar, 2022). Cisplatin was employed as the positive control. This synthetic anticancer agent is widely used in clinical cancer treatment due to its cytotoxicity, which primarily arises from its ability to damage DNA in cancer cells, but the side effects of these drugs can cause resistance in patients (Sami, 2020).

Table 2. Absorbance values and cell viability percentages of MCF-7 cells following exposure to various concentrations of *Clitoria ternatea* L. Extract

	Media	Media + Cells	Cisplatin	Solvent	Sample Concentration (µg/mL)							
					1000,00	500,00	250,00	125,00	62,50	31,25	15,63	7,81
Absorbance	0,4511	0,7292	0,5627	0,7242	0,7244	0,7223	0,7257	0,7282	0,7262	0,7178	0,7322	0,7191
	0,4590	0,7248	0,5649	0,7322	0,7143	0,7261	0,7290	0,7349	0,7314	0,7428	0,7354	0,7403
% Cell Viability	0	100,81	39,58	98,97	99,04	98,27	99,52	100,44	99,71	96,62	101,91	97,10
	0	99,19	40,39	101,91	95,33	99,67	100,74	102,90	101,62	105,81	103,09	104,89
Average % Cell Viability	0	100,00	39,99	100,44	97,19	98,97	100,13	101,67	100,66	101,21	102,50	100,99
SEM	0	0,81	0,40	1,47	1,86	0,70	0,61	1,23	0,96	4,60	0,59	3,90
Normalized % Cell Viability	0	99,56	39,81	100,00	96,76	98,54	99,69	101,23	100,22	100,77	102,05	100,55

Media	Media + Cells	Cisplatin	Solvent	Sample Concentration (µg/mL)							
				500,00	500,00	250,00	125,00	62,50	31,25	15,63	7,81

Figure 2. PrestoBlue assay colorimetric results for MCF-7 cells treated with *Clitoria ternatea* L. extract and controls.

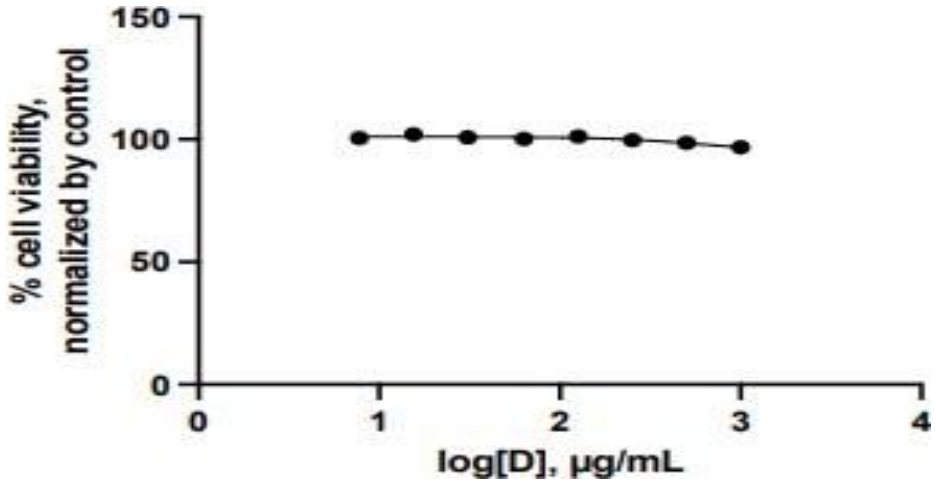

Figure 3. Cytotoxicity dose-response curve of *Clitoria ternatea* L. extract on MCF-7 cells

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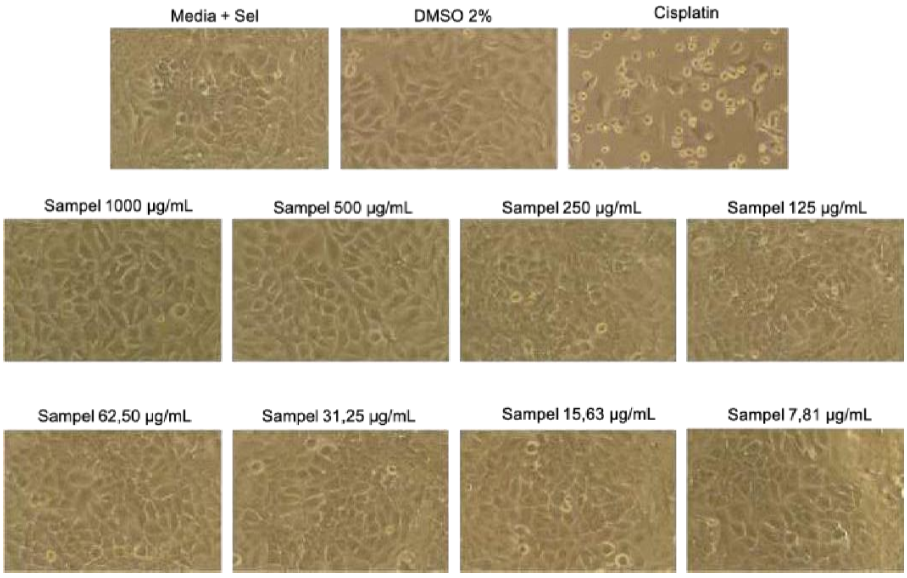

Figure 4. Morphology of MCF-7 cells following treatment with *Clitoria ternatea* L. extract and controls.

DISCUSSION

In vivo cytotoxic assay using the BSLT method

The observed larval mortality ranged from 0% to 100%, which allowed for the generation of a linear dose-response curve, enabling an accurate determination of the LC50 value in the BSLT assay. The concentration used for cytotoxicity is the percentage of larval death in the range between 0% and 100%, specifically at 500 to 1000 µg/mL.

Consistent with established toxicological principles, the results demonstrated that the higher the concentration of the extract, the greater the number of larvae that died. So, the percentage of larval death was also higher. This would increase the death of the larvae (Raihan & Dalimunthe, 2022). Larval death is related to the function of the content of secondary metabolite compounds in butterfly pea flowers, namely flavonoids and saponins, which can inhibit the eating ability of brine shrimp larvae (Slamet et al., 2020).

Flavonoid compounds can induce DNA fragmentation to become damaged, resulting in a cell apoptosis process which results in cell death (Raihan & Dalimunthe, 2022). *Artemia salina* L larvae died after administration of the extract because of the presence of phenolic compounds, namely flavonoids, in the extract which could cause the death of *Artemia salina L* larvae. Flavonoids and alkaloids enter the bodies of *Artemia salina L* larvae through the mouth and affect the digestive tract by disrupting the larvae's taste receptors, resulting in the larvae dying due to starvation (Kurniawan & Ropiqa, 2021) (Temarwut, 2022). Flavonoids inhibit larval growth by inhibiting signals to the cell nucleus because the activity of these receptors plays a role in increasing the malignant growth of cancer cells (Raihan & Dalimunthe, 2022). Flavonoid compounds also work as antioxidants that inhibit the formation of free radicals, where free radicals can trigger cell damage or the emergence of cancer cells. Saponins contribute to larval mortality by binding oxygen in the aquatic

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environment, reducing its availability and causing hypoxia in *Artemia salina* larvae (Nuralifah et al., 2021).

The data were analyzed using probit analysis to determine the LC50, which is defined as the concentration required to kill 50% of the test organisms (Saragih et al., 2022). The calculation is carried out by comparing the dead larvae to the total number, so that the percentage of deaths can be seen in the probit table value. From this data it will be known that the probit value is entered into the regression equation, so that the LC50 value can be obtained (Oktaviana et al., 2019). There is also another explanation where the LC50 value can be calculated by entering the value 5 (50% probit of test animal death) as y to get the x value as the log concentration value. The antilog value of x is the LC50 value (Fadli et al., 2019).

The parameter shown to determine the presence of biological activity in a compound on test animals is to count the number of larvae that died due to the effect of administering a compound with a predetermined concentration (Agustini, 2012). This shows an increase in the concentration of the extract followed by an increase in the probit value (larval death response) (Rani et al., 2022).

The test results are presented in Table 1. After carrying out the probit analysis, it can be seen that the graph of the straight-line equation is Y = 37.721x-26.379. In Figure 1 above, the concentration log shows the probit value obtained from the percentage of larval deaths. Then enter the Y value, namely the probit value for 50% of the test animals and obtain a value of A compound is toxic and has the potential to act as an anticancer in the BSLT test if it has an LC50 value less than 1000 µg/mL (Sugrani et al., 2023). A compound is said to have high toxicity if it has an LC50 value of 1000 ppm (Rohmawati et al., 2023). The LC50 obtained for the ethanol extract of the blue butterfly pea flower was 105.681 µg/mL, indicating low toxicity with potential anticancer propertie. The Brine Shrimp Lethality Test (BSLT) is widely used to assess the toxicity of compounds by calculating the LC50 based on mortality rates of *Artemia salina* larvae following exposure to the test substances (Rani et al., 2022).

In vitro cytotoxic assay using the PrestoBlue method

Eight concentrations of ethanol extract from butterfly pea flower (*Clitoria ternatea* L) were prepared, 1000.00, 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, and 7.81 µg/mL, to evaluate the correlation between extract concentration and MCF-7 breast cancer cell viability.

The BSLT indicated potential cytotoxicity with an LC50 value below 1000 µg/mL, suggesting biological activity of the extract (Abriyani et al., 2022). However, based on the data presented in Table 2, the PrestoBlue assay on MCF-7 cells revealed no significant cytotoxic effect, with an IC50 value equal to or greater than 1000 µg/mL, indicating weak or no cytotoxicity against these cancer cells.

Cytotoxic activity is typically classified by IC50 values as follows: values less than 100 µg/mL indicate strong cytotoxic potential; values between 100 and 1000 µg/mL indicate moderate cytotoxicity; and values exceeding 1000 µg/mL suggest lack of significant cytotoxic effect (Arifin et al., 2023). The color in Figure 2 shows the positive control (Cisplatin) produced, namely purple, indicating a cytotoxic effect on MCF-7 breast cancer cells. Meanwhile, the ethanol extract of butterfly pea flower (*Clitoria*

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ternatea L.) does not change color, meaning that there is no inhibition of cell growth. The decrease in cancer cell activity is influenced by increasing the concentration used. The following curve shows the relationship between concentration and cell uptake.

Figure 3 illustrates that the ethanol extract of *Clitoria ternatea* L. does not significantly reduce the viability of MCF-7 cells even at the highest tested concentration (1000 µg/mL). The dose-response curve appears relatively flat, with an IC50 value exceeding 1000 µg/mL. This dose-response profile indicates weak or negligible antiproliferative activity and minimal cytotoxic effects on this breast cancer cell line under the tested conditions

Cytotoxic activity is expressed by IC50 (concentration causing death of 50% of population cells) which is analyzed (Rahayu et al., 2023). The cytotoxicity of the synthesized complex was tested in vitro against MCF-7 cancer cells and compared with cisplatin; the most commonly used drug today known to be toxic to these cancer cells. From the results shown in According to criteria established by the US National Cancer Institute (NCI) and the Geran protocol, cytotoxicity is classified as follows: IC50 ≤ 20 µg/mL indicates very strong cytotoxicity; IC50 between 21 and 200 µg/mL indicates moderate cytotoxicity; IC50 between 201 and 500 µg/mL indicates weak cytotoxicity; and IC50 greater than 501 µg/mL indicates no cytotoxic effect (Rahmah et al., 2020). The IC50 value obtained for the ethanol extract of *Clitoria ternatea* L. in this study was 1000 µg/mL, indicating that the extract does not exhibit significant cytotoxicity against MCF-7 cells under the tested conditions.

The discrepancy between the positive BSLT results and the lack of cytotoxicity in MCF-7 cells may be explained by differences in the mechanisms underlying toxicity in the brine shrimp model versus human cancer cells. The BSLT is a general toxicity assay that reflects nonspecific cytotoxic effects, while the MCF-7 assay evaluates specific antiproliferative activity against breast cancer cells. This limitation of the BSLT in predicting anticancer efficacy highlights the need for further studies to isolate active compounds and investigate their mechanisms of action in relevant cancer models.

Morphological observations of MCF-7 cells were carried out in wells containing media with cells and treated with cisplatin (positive control), 2% DMSO (solvent control), and ethanol extract of butterfly pea flower (*Clitoria ternatea* L.). The difference in cell density produced in each sample when compared with the cell density produced by the addition of cisplatin shows that the ability to inhibit the growth of MCF-7 breast cancer cells by each sample is still less active (as shown in Figure 4).

MCF-7 cells typically exhibit an elongated, clustered morphology. Upon cisplatin treatment, the cells underwent morphological changes, becoming more rounded and less clustered, indicative of cytotoxic effects. In contrast, cells treated with the ethanol extract of *Clitoria ternatea* L. maintained their original elongated and clustered morphology, showing no comparable changes to those induced by cisplatin. These findings suggest that the ethanol extract of butterfly pea flower does not significantly inhibit MCF-7 cell proliferation. The high cell density and lack of morphological signs of apoptosis, such as cell shrinkage or detachment, indicate that cell death was minimal or absent under the treatment conditions.

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CONCLUSION

The ethanol extract of butterfly pea flowers (*Clitoria ternatea* L.) contains various phytochemicals such as tannins, flavonoids, steroids, anthocyanins, resins. In the BSLT using *Artemia salina* L., the LC50 value of the *Clitoria ternatea* L. extract was 105.681 µg/mL, classifying it as having low toxicity according to the standard range of 100-1000 µg/mL. However, based on the results of the study of cytotoxic activity against MCF-7 breast cancer cells, the ethanol extract is an inactive (not toxic), with an IC50 value exceeding 1000 µg/mL, indicating it does not possess significant cytotoxic activity against these cancer cells. Furthermore, morphological observations of MCF-7 cells treated with the extract showed no notable changes in cell shape or density compared to the control, supporting the conclusion that the extract does not induce cytotoxicity or apoptosis under the tested conditions.

CONFLICT OF INTEREST

There are no conflicts of interest to declare by the authors.

AUTHORS' DECLARATION

It is hereby declared that this article is an original work prepared in collaboration with the co-authors, and full responsibility for its content and any resulting claims is acknowledged.

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