Biosaintifika 16 (3) (2024): 525-534 p-ISSN 2085-191X | e-ISSN 2338-7610
Journal of Biology & Biology Education https://journal.unnes.ac.id/nju/index.php/biosaintifika

A Cytotoxic Activity of *Clitoria ternatea* Flower Tea with the Cinnamon and Lemongrass Oil Vapor against T47D Breast Cancer Cells

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Submitted: 2024-07-13. Revised: 2024-10-07. Accepted: 2024-12-11.

Abstract. Breast cancer is a deadly disease for women. It is crucial to take preventive measures, one of which involves using natural ingredients as chemopreventive agents. *Clitoria ternatea* flowers, cinnamon, and lemongrass oil have been proven to have cytotoxic activity in several studies. This research aims to analyze the effect of adding cinnamon and lemongrass oil vapor to *C. ternatea* flower tea on the cytotoxic activity of T47D breast cancer cells. Cytotoxic activity testing was carried out colorimetrically using the MTT (3- (4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) assay method. The results of the research showed that there was a decrease in the IC_{50} value of *C. ternatea* flower tea with the addition of cinnamon and lemongrass oil vapor. The IC_{50} value of *C. ternatea* flower tea without additional ingredients was the most potent with 14159 μg/mL, compared to 15 g addition of cinnamon with 1164 μg/mL; meanwhile, for 15 seconds of evaporation time for lemongrass, it was at 331 μg/mL, respectively This research shows the potential of *C. ternatea* flower tea with the addition of cinnamon and lemongrass oil vapor as a chemopreventive agent against breast cancer cells.

Keywords: *C. ternatea*; chemopreventive; cinnamon; flower tea; lemongrass oil; T47D cells.

How to cite : Cahyono, E., Handayani, T., Nugrahaningsih, W. H., Azid, S. B. M. A., Alighiri, D., Zaky, A. M., Aisyah, S., & Permatasari, B. (2024). A Cytotoxic Activity of *Clitoria ternatea* Flower Tea with the Cinnamon and Lemongrass Oil Vapor against T47D Breast Cancer Cells. *Biosaintifika: Journal of Biology & Biology Education, 16(3)*, 525-534.

DOI: http://dx.doi.org/10.15294/biosaintifika.v16i3.8480

INTRODUCTION

Cancer is a disease with a high prevalence rate. Based on Globocan data, the International Agency for Research on Cancer (IARC) recorded that in 2020 there were 19 million cancer cases with 9.9 million deaths. Breast cancer is one type of cancer that causes the most deaths Breast cancer cases in 2022 globally have reached 2.3 million cases out of 19.9 million total cancer cases (Globocan, 2023). Risk factors that can increase a person's risk of developing breast cancer are genetic factors, age, hormones, obesity, and an unhealthy lifestyle. Genetic changes cause uncontrolled cell proliferation in the body, leading to the growth of cancer cells (Feng et al., 2018).

Current treatments to treat breast cancer include surgery, radiotherapy, and chemotherapy. This treatment method is not effective in removing cancer cells, and there are negative side effects for the body (Yusuf *et al*., 2020). Early prevention in treating and reducing cases of breast cancer is very important. One way to prevent this is to suppress the growth and development of cancer cells using chemopreventive methods. Chemopreventive methods use natural ingredients to prevent and inhibit cancer cell growth. Chemopreventive substances can inhibit the development of cancer by limiting exposure to carcinogens (Alotaibi, 2024; Gu & Li., 2020). Efforts to reduce the risk of cancer can be made by consuming tea, fruit, and vegetables.

The *C. ternatea* flower or butterfly pea flower (BPF) is a plant that is commonly used as a drink and natural dye. The anthocyanin compound content in BPF gives it a distinctive purplish-blue color. Biologically, BPFs have anti-stress, antidepressant, anti-inflammatory, analgesic, anti-diabetic, and anti-cancer activities (Gollen *et al*., 2018; Jeyaraj *et al*., 2021; Audina *et al*., 2023).

Research from Arsianti *et al*., (2022) shows that an extract of BPF has strong cytotoxic activity against T47D breast cancer cells with the content of flavonoid, tannin, and triterpenoid compounds. The flavonoid compounds in BPF can prevent tumors and inactivate carcinogenic compounds (Ren *et al*., 2003). The cytotoxic activity of BPF demonstrates its potential as an herbal tea for breast cancer prevention.

Cinnamon (*Cinnamomum burmannii*) is one of the herbal plants that has cytotoxic activity against cancer cells. Cinnamon has anticancer activity by activating the cell apoptosis process. The main compound in cinnamon, namely cinnamaldehyde, can induce cell apoptosis through deoxyribonucleic acid (DNA) fragmentation (Sadeghi *et al*., 2019). *Cymbopogon* or lemongrass plant also has cytotoxic activity against breast cancer cells (Piaru *et al.*, 2012). In several studies, lemongrass oil has shown cytotoxic activity with the mechanism of inducing cell apoptosis. Lemongrass oil is selective against cancer cells, so it does not cause cytotoxicity in normal cells (Alotaibi, 2024; Pan *et al*., 2021).

In this study, butterfly pea flower, cinnamon, and lemongrass oil were combined as herbal tea, which was tested for cytotoxic activity against T47D breast cancer cells. The addition of cinnamon and lemongrass oil vapor has the potential to increase the cytotoxic activity of *C. ternatea* flower tea. This study aims to analyze the effect of adding cinnamon and lemongrass oil vapor on the cytotoxic activity of *C.ternatea* flower tea. The breast cancer cell culture used is T47D cells, which have similar proteins to tumors (Dimarti *et al*., 2020).

This study provides scientific information about the potential of *C. ternatea* flowers which have been widely consumed by the public as a drink. Research on cancer prevention efforts has been widely carried out through various approaches. The use of *C. ternatea* flowers as a tested cancer prevention tea drink will provide data that encourages further research on its use and exploration of other natural resources to prevent cancer.

METHODS

Sample Preparation

C, ternatea flowers are used in dry powder preparations. The *C ternatea* flowers were dried using an oven at 42°C for 5 hours. The oven-dried *C. ternatea* flowers are then ground using a blender to produce powder. The additional ingredient is dry cinnamon bark, ground using a grinder until it becomes powder and sifted with a tea strainer. Cinnamon extract and lemongrass oil were characterized using GC-MS.

Phytochemical Analysis of *Clitoria ternatea* Flower Extract

Extraction of *C. ternatea* flowers was carried out using the infusion method (Purwanto *et al*., 2022). Firstly, 30 g of *C, ternatea* flower powder was added to 600 mL of distilled water, and the solution was heated on a hotplate at 100℃ for 30 minutes. Afterwards, the solution is cooled and filtered. The resulting filtrate was concentrated using a water bath at a temperature of 80℃. The next analysis; qualitative compound identification, was carried out using the following procedure (Noraida *et al*., 2019). The flavonoid test was carried out by adding 3 drops of 10% hydrocloric acid and a spatula of magnesium powder. Samples positive for flavonoid compounds are characterized by a color change to red, orange, or yellow. The alkaloid test uses two reagents, namely Mayer and Dragendorff. Mayer/Dragendorff reagent was added to a mixture of concentrated *C. ternatea* flower extract and 10%. The formation of a white precipitate when Mayer's reagent is added, indicates a positive alkaloid, while when Dragendorff's reagent is added an orange precipitate forms. The triterpenoids/steroids were tested using Liebermann-Burchard reagent, which is made from a mixture of acetic acid anhydride and concentrated sulfuric acid in a 1:1 ratio. Red or purple samples indicate that they are positive for triterpenoids. Meanwhile, if a bluish-green color appears, it is positive for steroids. To conduct the tannin test, 1 drop of $FeCl_3$ solution was added into *C. ternatea* flower extract. Meanwhile, for the saponin test, the concentrated extract is diluted with 2.5 mL of hot distilled water and shaken until it foams. Then, 1 drop of hydrochloric acid p.a. was added and the resistance of the foam was observed for 5 minutes.

Production of *Clitoria ternatea* Flower Tea with the addition of Cinnamon and Lemongrass Oil Vapor

The production of *C. ternatea* flower tea using a stirred boiler tank was adopted from Cahyono *et al*., (2021). Production begins by weighing 125 g of *C. ternatea* flower powder and cinnamon (Table 1), respectively, pouring it into tube 2 of the stirred boiler tank, and stirring for 15

minutes. A total of 300 mL of lemongrass oil was added into tube 1 of a stirred boiler tank and heated it to 80°C while maintaining a pressure of 2 bar (1500.12 mmHg). Once the pressure in the tube reaches 2 bar (1500.12 mmHg), the connecting tap on the stirred boiler tank was opened to allow the lemongrass oil vapor to flow into tube 2, following variations in evaporation time of 0, 5, 10, and 15 seconds. Then stir for another 15 minutes.

Table 1. Formulation of Clitoria ternatea Flower Tea with the addition of Cinnamon and Lemongrass Oil Steam

Formulation	Cinnamon Weight (g)	Evaporation Time of Lemongrass Oil (seconds)
F1	0	0
F2	0	15
F3	10	10
F4	10	15
F5	15	0
F6	15	5

Cytotoxic Activity Test

Cytotoxic activity testing on T47D breast cancer cells was carried out using the MTT assay method (CCRC, 2009). *Clitoria ternatea* flower tea without additional ingredients acted as a negative control, and blank media acted as a media control. T47D cells that had been added with RPMI media were transferred at 100 μL/well on a microplate and incubated in a carbon dioxyde (CO_2) incubator for 24 hours. The test sample of *C, ternatea* flower tea was weighed to 20 mg and dissolved in 200 μL dimethyl sulfoxide (DMSO). The sample concentration series solutions were prepared DMSO stock and Roswell Park Memorial Institute Roswell Park Memorial Institute (RPMI) media at concentrations of 1000,; 500,; 250,; 125,; and 62.5 μg/mL. The sample concentration series solution was transferred to a microplate containing T47D cells, and incubated for 24 hours at 100 μL/well in triplicate. The microplate was incubated again in a carbon dioxide (CO_2) incubator for 24 hours. After 24 hours, the condition of the cells was observed using an inverted microscope. The MTT reagent was prepared by mixing 1 mL of MTT stock in PBS (5 mg/mL) with 10 mL of RPMI media. MTT reagent was added to each microplate well in the amount of 100 μL and incubated for 4 hours at 37°C in a CO_2 incubator The formation of formazon crystals in the cells was observed using an inverted microscope. The formation of formazan crystals was stopped by adding 100 μL of 10% sodium dodecyl sulphate (SDS) stopper reagent in 0.01 N HCl to each well. Microplates were stored in the dark and incubated for 24 hours. Absorbance was measured using an ELISA reader at a wavelength of 595 nm. Calculation of the percentage of live cells and the IC_{50} value were calculated using a software Microsoft Excel. The cell percentage is calculated using the following formula:

$$\% \text{ live cells} = \frac{Absorbance\ of\ treatment - Absorbance\ of\ media\ control}{Absorbance\ of\ control\ cells - Absorbance\ of\ control\ media} \times 100\%$$

The percentage of cell viability was calculated for the IC_{50} value using probit analysis in the SPSS application. The IC_{50} value for each formula was compared to find the lowest value. Based on the IC50 value, cytotoxic activity is divided into three categories: very strong cytotoxic if the IC_{50} value is <10 µg/mL, strong cytotoxic if the IC_{50} 10-100 µg/mL, and moderate cytotoxic if the IC_{50} value is between 100 – 500 µg/m (Tunjung & Sayekti., 2019).

RESULTS AND DISCUSSION

Sample Preparation

The process of drying and grinding of *C. ternatea* flowers aims to extend the shelf life, and the smaller size can make mixing easier in the production process. Cinnamon additives and lemongrass oil vapor were characterized by GC-MS. GC-MS characterization of cinnamon extract showed the presence of the identified cinnamaldehyde compound. The results of GC-MS characterization of cinnamon extract are presented in the chromatogram shown in Figure 1.

The results of GC-MS characterization of lemongrass oil are presented in the chromatogram shown in Figure 2.

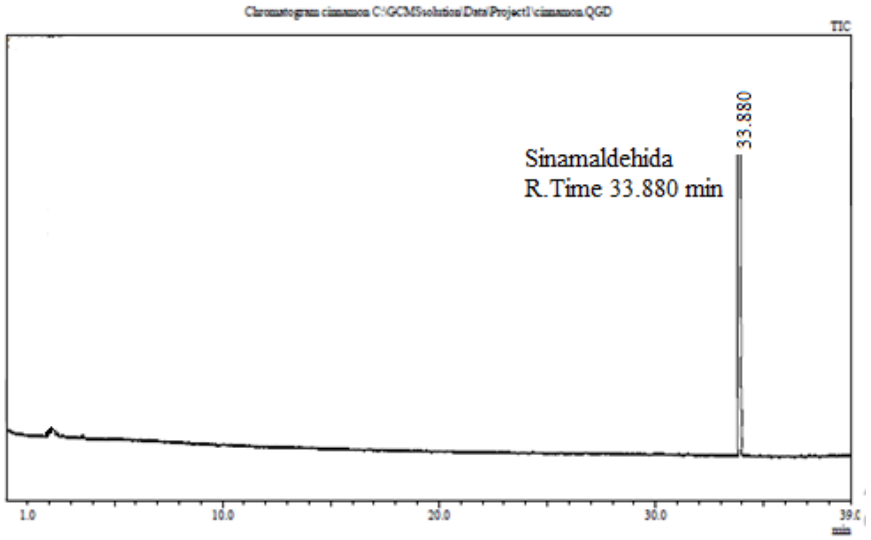


Figure 1. Chromatogram of Cinnamon Extract

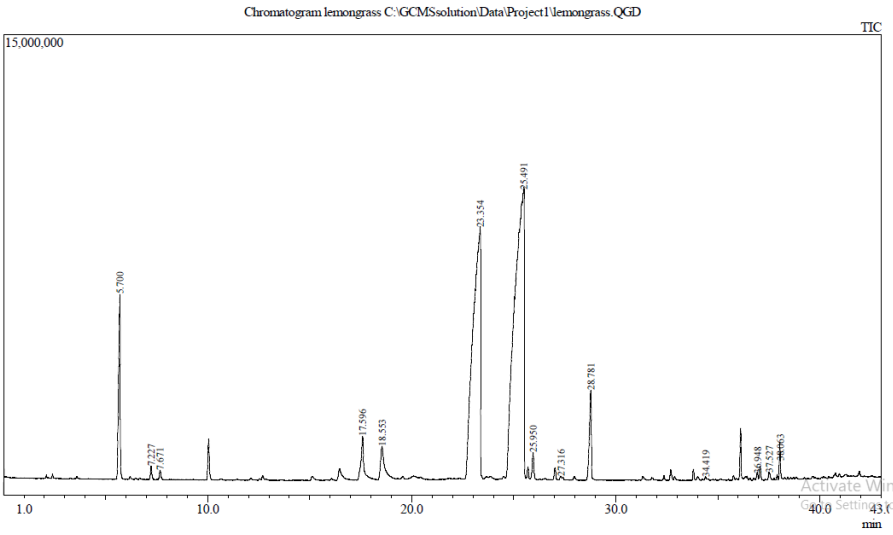


Figure 2. Chromatogram of Lemongrass Oil

Table 2. Results of Lemongrass Oil Chromatogram Analysis

Peak	R. Time	Area (%)	Height (%)	Name Compound
1	5.700	5.38	18.91	β-Myrcene
2	7.227	0.35	1.33	α-Pinene (-)-
3	7.671	0.25	0.94	1,3,6-Octatriene, 3,7-dimethyl-, (Z)- (CAS)
4	17.596	2.03	4.30	Linalool
5	18.553	2.20	3.35	Trans-Caran, 4,5-Epoxi-
6	23.354	33.58	25.31	*Z*-Citral
7	25.491	50.15	29.70	*E*-Citral
8	25.950	0.78	2.32	β −Citronellol
9	27.316	0.17	0.36	Isogeraniol
10	28.781	3.29	8.81	Geraniol
11	34.419	0.15	0.31	Epiglobulol
12	36.948	0.64	0.78	β −Bisabolene
13	37.527	0.28	0.73	(-)-Lavandulol
14	37.063	0.76	2.85	Geranic acid

From the characterization results of lemongrass oil, 14 compounds were identified. The three main compounds in lemongrass oil are citral (83.73%), *β*-myrcene (5.38%), and geraniol (3.29%). Citral (83.73%) is a combination of *Z*-citral and *E*-citral. These results are from Sayed *et al*., (2022) research, which showed GC-MS results for lemongrass oil with citral as the main compound (56.4%). Citral is a monoterpenoid compound that plays a role in producing a strong odor or ~~/~~aroma and is found in many essential plants.

Phytochemical Analysis of *Clitoria ternatea* Flower Extract

Phytochemical testing was carried out on concentrated extracts from the infusion of *C ternatea* flower powder with distilled water as a solvent. The compounds tested were flavonoids, alkaloids, triterpenoids/steroids, tannins, and saponins. The results of phytochemical testing of *C. ternatea* flower extract are presented in Table 3.

Table 3. Phytochemical Test Results for *Clitoria ternatea* Flower Extract

Active Compounds	Result
Flavonoid	+
Alkaloid	
- Dragendorff	-
- Mayer	
	-
Triterpenoid	+
Steroid	-
Tannin	+
Saponin	-

Description: (+) = detected; (-) = not detected

The phytochemical analysis of *C. ternatea* flower extract reveals that it contains flavonoid, triterpenoid, and tannin compounds. These results are aligned by research from Arsianti *et al*., (2022), which shows that *C. ternatea* flower extract contains flavonoid, triterpenoid, and tannin compounds. Flavonoid compounds have cytotoxic activity against cancer cells through the mechanism of inducing cell apoptosis (Widyanto *et al*., 2020).

Cytotoxic Activity of *Clitoria ternatea* Flower Tea with the addition of Cinnamon and Lemongrass Oil Vapor

The cytotoxic activity of *C.- ternatea* flower tea with cinnamon and lemongrass oil vapor against T47D breast cancer cells using the MTT Assay method over a 24-hour incubation period. The IC_{50} value from the cytotoxic activity test results is presented in Table 4.

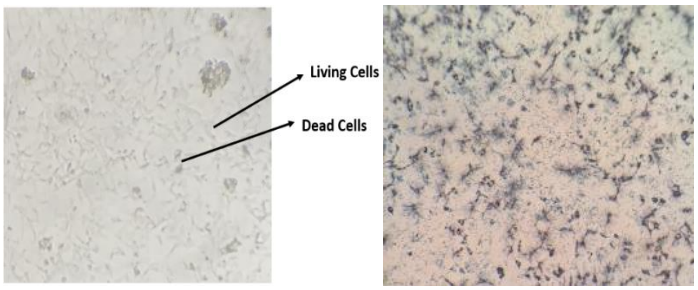
Table 4. IC_{50} Value of *Clitoria ternatea* Flower Tea Formulation

Formulation	IC_{50} ($\mu g/mL$)
F1 (0.00)	14159.048
F2 (0.15)	331.266
F3 (10.10)	871.862
F4 (10.15)	860.386
F5 (15.00)	1164.24
F6 (15.55)	2171.19

Formulation Description (a,b):
a = weight of cinnamon (grams)
b = Length of time for evaporation of lemongrass oil (seconds)

The results showed that the lowest IC_{50} value was 331.266 μg/mL for the *C. ternatea* flower tea formulation with 15 seconds of evaporation time for lemongrass oil without the addition of cinnamon (F2), while the highest IC_{50} value was 14159.048 μg/mL for the formulation without additional ingredients (F1). Based on the results of the IC_{50} value, they indicate that the formulation with additional ingredients has a lower IC_{50} value compared to the formulation without additional ingredients (F1).

A decrease in the IC_{50} value indicates that the number of living cells is decreasing~~,~~ due to inhibition of glucose absorption in the development of ATP and cell proliferation. The condition of the cells after sample treatment and the formation of formazan crystals is shown in Figure 3.



(A) (B)

Figure 3. (A) Cell conditions after adding *Clitoria ternatea* Flower Tea (F2) (B) Conditions for formation of formazan crystals.Figure 3 shows the condition of cells after being treated with *C. ternatea* flower tea with 15 seconds of evaporation time for lemongrass oil (F2) and the formation of formazan crystals. The condition of the living cells is oval in shape, attached to other cells, and appears shiny when attached to the bottom of the microplate. Meanwhile, dead cells are round, scattered, dark in color, and float. The formation of formazan crystals will be proportional to the number of living cells (Dimarti *et al*., 2020).

Previous studies showed flavonoid compounds exhibit cytotoxic activity against cancer cells by inhibiting the carcinogenesis process (Gu & Li, 2020). The inhibition of carcinogenesis happens at the starting, promoting, and progressing stages through molecular mechanisms such as blocking the activity of compounds that cause cancer, stopping cell growth, stopping the growth of new blood vessels, starting apoptosis, and antioxidant activity (Hasibuan & Sumaiyah., 2019; Yusuf et al., 2020; Nurani et al., 2023). Flavonoids inhibit cancer cell proliferation by inducing the activation of caspase-8, which in turn stimulates the Bid protein to stimulate the formation of Bax in cell mitochondria, which then stimulates the formation of Bax in cell mitochondria and, releases cytochrome C. The formed cytochrome C binds apoptosis activating factor 1 (Apaf-1) and forms apoptosomes, subsequently initiating the apoptosis process (Widyanto et al., 2020). Flavonoids can downregulate the p53 gene in humans which is capable of inducing cell apoptosis.

The compound content of *C. ternatea* flower tea with the Cinnamon and Lemongrass oil correlated with the death of T47D breast cancer cells after treatment. The decreasing of cancer cell viability may be caused by the citral contained in the formula of *C. ternatea* flower tea. Citral shows antioxidant effects and cytotoxic activity in suppressing various mutagens (Alotaibi, 2024; Pan *et al*., 2021). The citral has been proven to increase the apoptosis of breast cancer cells 4T1 (Zeng *et al*., 2015), and MDA MB-231(Nordin *et al.,* 2019). Citral induces caspase-3 activity, activates p53, and inhibits Bcl-2 which can trigger apoptosis. P53 is the gene most frequently mutated in cancer cells (Soleimani & Sajedi., 2020; Gaffar *et al*., 2019). The usual apoptosis pathway involves activating caspase-3, which activates other caspases for the apoptosis process (Tunjung & Sayekti., 2019). Apart from having an effect on the apoptosis pathway, citral also has an effect on proliferation pathways. Citral affected to reduce the phosphorylation of STAT3 and Src with influence to inhibit cell proliferation (Maruoka et al., 2018)

Another bioactive compound contained in the *C. ternatea* flower tea with Cinnamon and Lemongrass oil is Myrcene. Myrcene acts as an anticancer by inhibiting proliferation and increasing apoptosis (Wu, 2022). Myrcene induced DNA damage of HeLa cancer cells which caused cell cycle arrest (Pincigher et.al., 2023). In the pathway of apoptosis, myrcene increases cytochrome C and Caspase 8 (Bai et.al,2020).

The third main bioactive compound contained in the *C. ternatea* flower tea with Cinnamon and Lemongrass oil is geraniol. Geraniol elevated caspase 3 activity and lead to apoptosis (Galle et.al, 2014). Inhibition of the antiapoptotic protein Bcl-xl also occurs when geraniol is added to cancer cells (Kim et.al, 2012). Inhibition of Bcl-xl causes induction of apoptosis via cytochrome C and caspase 9. Geraniol inhibits cell proliferation by reducing the expression levels of membrane-bound Ras in A549 cell-bearing mice (Galle et.al, 2014). Geraniol also elevated the expression levels of p21Cip1 and p27Kip1 but reduced those of cyclin A, cyclin B1, and CDK2. As a result, the cell cycle will arrest in the G1 phase (Wiseman et al, 2007). In the MAPK pathway, geraniol inhibits AKT-mTOR signaling without affecting MAPK activity (Kim et.al, 2012).

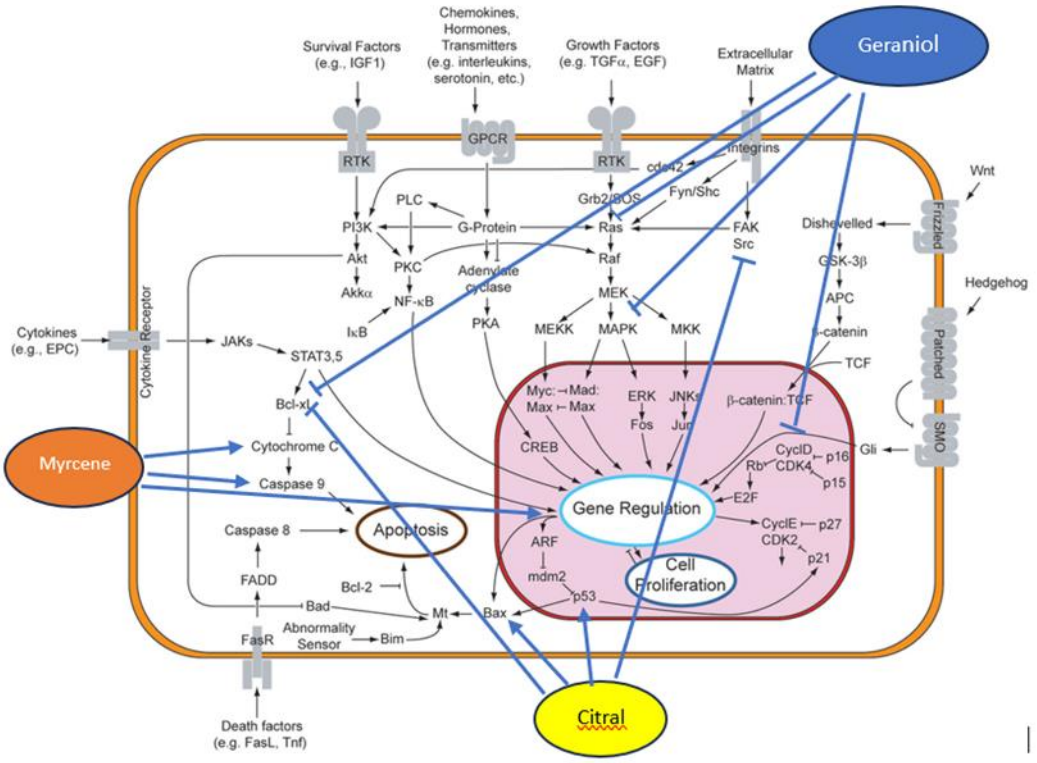


Figure 4. The target of C. ternatea flower tea with Cinnamon and Lemongrass oil as anticancer base on proliferation and apoptosis pathway (https://en.wikipedia.org/wiki/Apoptosis)

The findings of our experiment are shown in Figure 4. Figure 4 shows the mechanism of *C. ternatea* flower tea with Cinnamon and Lemongrass oil as an anticancer. Anticancer agents are represented by citral, myrcene, and geraniol. These three agents inhibit cancer growth by reducing proliferation and increasing apoptosis. Proliferation and apoptosis are vital pathways in the development of normal cells into cancer cells.

Based on the IC50 value, the formula C. ternatea flower tea and Lemongrass oil (F2) has a chemopreventive role. A compound's antioxidant activity also plays a role in warding off free radicals to prevent cancer cell development. Antioxidant activity can help chemopreventive agents form prooxidants so that cancer cell death will increase and cell proliferation can be inhibited (Widyanto *et al.*, 2020). *C, ternatea* flowers contain strong anthocyanins and have antioxidant activity (Jeyaraj *et al.*, 2022). Future development is to disseminate *C. ternatea* flower tea and Lemongrass oil (F2) as a chemopreventive formula. This formula can be developed in the form of healthy drinks or others.

CONCLUSION

This study shows that adding cinnamon and lemongrass oil vapor to *C.-ernatea* flower tea has an effect on T47D breast cancer cells' cytotoxic activity. The best formulation with an IC50 value of 331 μg/mL is *C. ternatea* flower tea with 15 seconds of evaporation time for lemongrass oil without cinnamon (F2). These results suggest that the addition of lemongrass oil vapor to *C.Iternatea* flower tea could potentially serve as a chemopreventive agent for breast cancer. This research is limited to in vitro studies. Further research is needed both in vivo and formula optimization to determine the compound of *C. ternatea* flower tea and Lemongrass oil vapor as a chemopreventive agent.

ACKNOWLEDGEMENT

We would like to acknowledge the support received from Lembaga Penelitian dan Pengabdian Masyarakat (LP2M) Universitas Negeri Semarang for research findings in 2023.

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