

# The Chemopreventive Effects of The Combination Between Tea Leaf and Mandarin Peel Extract on Breast Cancer Cell

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## Abstract

The number of breast cancer patients is increasing high in the world. This study aims to determine the chemopreventive effect of a combination of ethanolic extracts of tea leaves and mandarin peel in silico and in vitro on T47D breast cancer cells. Extraction by the maceration method used ethanol solvent 70%. The research is an in silico molecular docking utilized software of Autodock Vina to determine the binding affinity of tangeretin compounds and Epigallocatechin gallate (EGCG) on HER-2 protein. The antioxidant test used the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method to determine the antioxidant activity of the combination of tea leaf and mandarin peel (CTM) extract. The in vitro test used the method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to determine the value of IC<sub>50</sub> from CTM on T47D breast cancer cells. The result of this study showed that CTM had a vigorous antioxidant activity with an IC<sub>50</sub> value of 83 µg/ml. CTM had a weak cytotoxic activity with an IC<sub>50</sub> value of 1889 µg/ml. Molecular tethering results of tangeretin and EGCG compounds produced a docking score of -6.6 and -5.0 kcal/mol with docking score proportion consisting of -4.9 kcal/mol of original ligand, -6.1 kcal/mol of doxorubicin and -4.5 kcal/mol of 5-fluorouracil. CTM had potential as a chemopreventive agent based on the robust antioxidant activity data on T47D breast cancer cells and molecular docking on the HER-2 protein.

**Keywords:** Antioxidant test; Camellia sinensis; Citrus reticulata; MTT Assay; Molecular docking; T47D breast cancer cells

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## INTRODUCTION

Breast cancer is a deadly cancer attacking women with a high prevalence rate. In 2012, there were 26 cases per 100,000 women.<sup>1</sup> One of the proteins that mainly causes breast cancer cell growth is HER-2. In the majority of breast cancer patients, HER-2 protein experiences uncontrolled expression through gene amplification that increases HER-2 protein expression up to 40-100 times.<sup>2-5</sup> Breast cancer can grow and spread into its surrounding tissue, such as lymph nodes, and then enter the blood vessels and even to other organs (lungs, bones, liver, and brain).<sup>6</sup> Common treatments for breast cancer patients are such as chemotherapy, surgery, and radiation.<sup>7</sup> However, these treatments are expensive and also often cause adverse side effects, which can kill the healthy surrounding tissue and even the effects of immunosuppression. It is necessary to innovate in defeating cancer, for example, using safer alternative treatments, such as plant extracts.<sup>8</sup>

Natural ingredients that have the potential as a chemopreventive agent are tea leaves and mandarin (mandarin orange) peels. Tea leaves contain a flavonoid-polyphenol compound, epigallocatechin gallate (EGCG), which is proven to have a substantial antioxidant property. Mandarin (*Citrus reticulata*) peel contains a compound called 'tangeretin' that can potentially inhibit tumor cell growth, which is proven to be able to induce cell-cycle G<sub>1</sub> arrest processes.<sup>9</sup> Considering all those benefits, this study aims to determine the chemopreventive effect of a combination of ethanolic extracts of tea leaves and mandarine peels in silico and in vitro. The combination of both extracts is expected to have a good synergy effect that can

inhibit the development of cancer cells from being more significant.

## METHODS

### Plant Determination

Tea leaves (*Camellia sinensis*) were obtained from the Kaliurang area, Yogyakarta, and mandarin peel (*Citrus reticulata*) were obtained from the Bantul region, Yogyakarta. The determination of both plants was carried out in the Laboratory of Pharmacy Biology, Faculty of Pharmacy, UGM Yogyakarta.

### Macerating Extraction

The selection of the extraction method was based on the natures and compositions to be isolated.<sup>10</sup> Extraction was conducted using ethanol solvent 70% and stirred routinely every day for five days. After that, the pulp was re-macerated for two days. After two days, the maceration was filtered again and was then evaporated using a Rotary Evaporator until a thick extract was obtained.<sup>11</sup>

### Antioxidant Test of DPPH

The DPPH method was based on the free-radical reduction of DPPH with maximum absorption at a wavelength of 517 nm, which produced a purple color in ethanol.<sup>12</sup> After that, 2 mL of vitamin C solution was taken, which was the sample solution at various concentration series made into a 10-mL volumetric flask. The solution was added with 2 mL of DPPH and a methanol solution into the limit mark. The solution was vortexed for 30 seconds and left alone within the operating time. Uptake was read at  $\lambda$  maximum and replicated three times.

### Cytotoxic Test of MTT Assay

This method was based on the breakdown of tetrazolium salt or yellow

MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) by dehydrogenase succinate enzyme contained in the cell mitochondria to turn into purplish blue formazan crystals.<sup>13</sup> Cells with a density of 70-80% (confluent) were distributed into 96 well plates, observed under an inverted microscope to identify the cells' distribution, and then incubated for 48 hours so that the cells could adapt and attach to the bottom of the wells. On the following day, the media in the wells were dumped by turning the well plates over the dump, then drained with a tissue. The wells were washed using 100  $\mu$ L PBS which was added to all wells filled with cells, and then they were discarded. Furthermore, a series of concentrations of the test sample and culture media containing 0.2% DMSO (control) was added and then re-incubated for 24 hours. At the end of the incubation, culture media containing the sample were removed and washed with 100  $\mu$ L PBS, then added with the MTT reagent. In this case, living cells would react with MTT to form purple formazan crystals. After 4 hours of incubation, the condition of the cells was examined under an inverted microscope. When formazan was created, a 200  $\mu$ L SDS stopper solution was added into 0.1% HCl to dissolve the formazan crystals. The well plates were then covered with aluminum foil and incubated in a dark place at room temperature for 3 hours. Upon that process, the aluminum foil was opened and shaken for 10 minutes. The absorption was read with an ELISA reader at a wavelength of 595 nm. Based on the absorbance data, the percentage of living cells and IC<sub>50</sub> values could be calculated.

### Molecular Docking Test

Molecular docking is a computational method used to predict the interaction

between ligands and receptors or proteins to obtain the value which describes their total bond energy. The protein structure selected as the target of molecular docking in this study was HER-2 (PDB ID: 3PP0). The structure was downloaded from the Protein Data Bank (PDB) through [www.rcsb.org](http://www.rcsb.org); thus, the protein's PDB ID could be identified. The application used for protein and ligand preparation in this study was DS Visualizer. RMSD value was determined by filling in the Windows Command Prompt according to the code; therefore, some conformations would appear. Each conformation showed its RMSD affinity value, then the conformation, which had an RMSD value of less than 2 Å, was selected. The visualization process of docking results used the DS Visualizer app. The visualization was to determine the position and the image of the bond between proteins and ligands in 3D.

## RESULTS AND DISCUSSION

### Plant Determination

The results of the determination showed that the simplisia used in this study was tea leaf (*Camellia sinensis*) and mandarin orange peel (*Citrus reticulata*).

### Macerating Extraction

The maceration method in this study used ethanol solvent 70%, 10 L for 1 kg of dried tea leaf powder, and 1 kg of dried mandarin peel powder. Based on the filtering result, 2,450 ml of liquid extract was obtained. It was then dried using a rotary evaporator and produced 27.3 grams of thick tea leaf extract (TLE) with a dark green color and 26.4 grams of a concentrated mandarin peel extract (MPE) with a dark yellowish-green color. Both tea leaf extract and mandarin peel extract were mixed to get a combined extract.

### Antioxidant Test of DPPH

Absorbance data of DPPH antioxidant test results can be seen in Tables 1 and 2, and the graphic is shown in Figures 1 and 2. Also, the  $IC_{50}$  value of the antioxidant test is shown in Table 3.

**Table 1.** Data of comparative inhibition percentage for vitamin C

Concentration ( $\mu\text{g/ml}$ )	Average absorbance	Blank absorbance	% Inhibition
0.5	0.641	0.702	8.73
1.0	0.638	0.702	9.11
2.0	0.625	0.702	11.01
5.0	0.557	0.702	20.76
10.0	0.402	0.702	42.76
20.0	0.189	0.702	73.04
30.0	0.055	0.702	92.22

**Table 2.** Data of inhibition percentage for CTM extract

Concentration ( $\mu\text{g/ml}$ )	Average absorbance	Blank absorbance	% Inhibition
5.0	0.721	0.702	-2.61
7.5	0.703	0.702	-0.09
10.0	0.664	0.702	5.46
15.0	0.633	0.702	9.87
20.0	0.472	0.702	32.87
30.0	0.381	0.702	45.80
40.0	0.346	0.702	50.78

**Table 3.**  $IC_{50}$  value of antioxidant test for vitamin C and CTM extract

Compounds	Linear regression equation	$IC_{50}$ value ( $\mu\text{g/ml}$ )	Description
Vitamin C	$y=2.9913x+7.5319$ $R^2=0.9859$	14.19	Very strong
CTM	$y=0.6427x-3.3458$ $R^2=0.9698$	83.00	Strong

**Table 4.** The result of the molecular docking between ligands and HER-2 receptors

Compounds	RMSD value	Docking Score	Conformation
Native ligand	1.957	-4.9	7
EGCG	1.343	-6.6	2
Tangeretin	1.197	-5.0	4
Doxorubicin	1.542	-6.1	4
5-Fluorouracil	1.576	-4.5	3

### Molecular Docking with Autodock Vina

Molecular docking was conducted using the Autodock Vina application. Open label, and the shape of the structure were then visualized in 2D and 3D with the DS Visualizer. The data on the molecular docking results can be seen in Table 4.

### Cytotoxic Test of MTT Assay

The cytotoxic test was performed to determine MTT Assay activity towards T47D breast cancer cells by giving CTM extract treatment shown in Table 5.

**Table 5.** Data of living cell after CTM extract treatment (%)

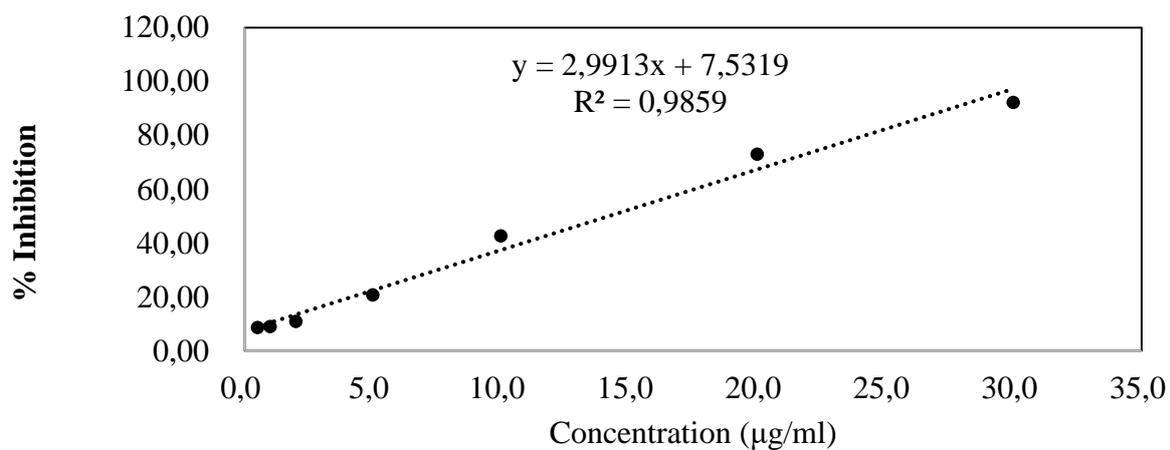
Content ( $\mu\text{g/ml}$ )	Average Sample Absorbance	Standard Deviation	% Living Cells
100	0.130	0.014	4.308
400	0.128	0.003	3.798
800	0.124	0.002	3.122
1000	0.118	0.001	2.009
Average Absorbance	Cell Control	0.663	
	Media	0.103	
Equation	$y = -0.0024x + 4.6713$ $R^2 = 0.9233$ $IC_{50}=1889 \mu\text{g/ml}$		

### Cytotoxic Test of MTT Assay

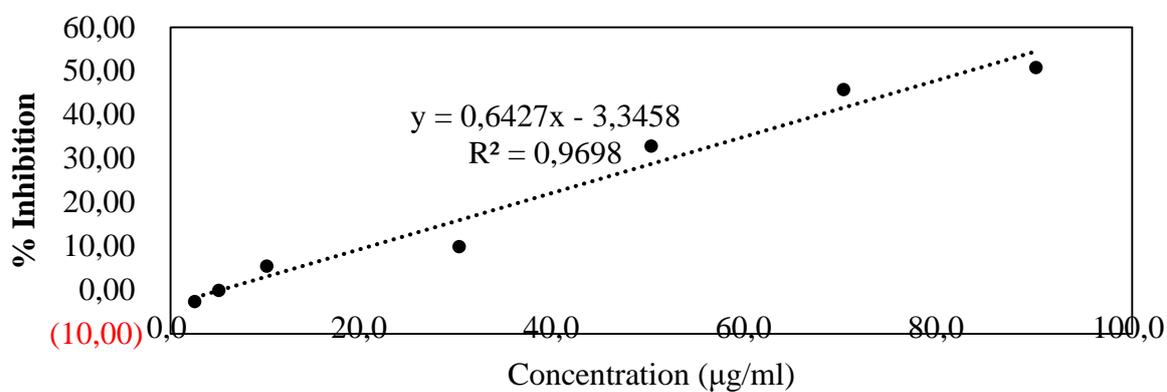
The cytotoxic test was performed to determine MTT Assay activity towards T47D breast cancer cells by giving CTM extract treatment, as shown in Table 6.

**Table 6.** Data of living cell after CTM extract treatment (%)

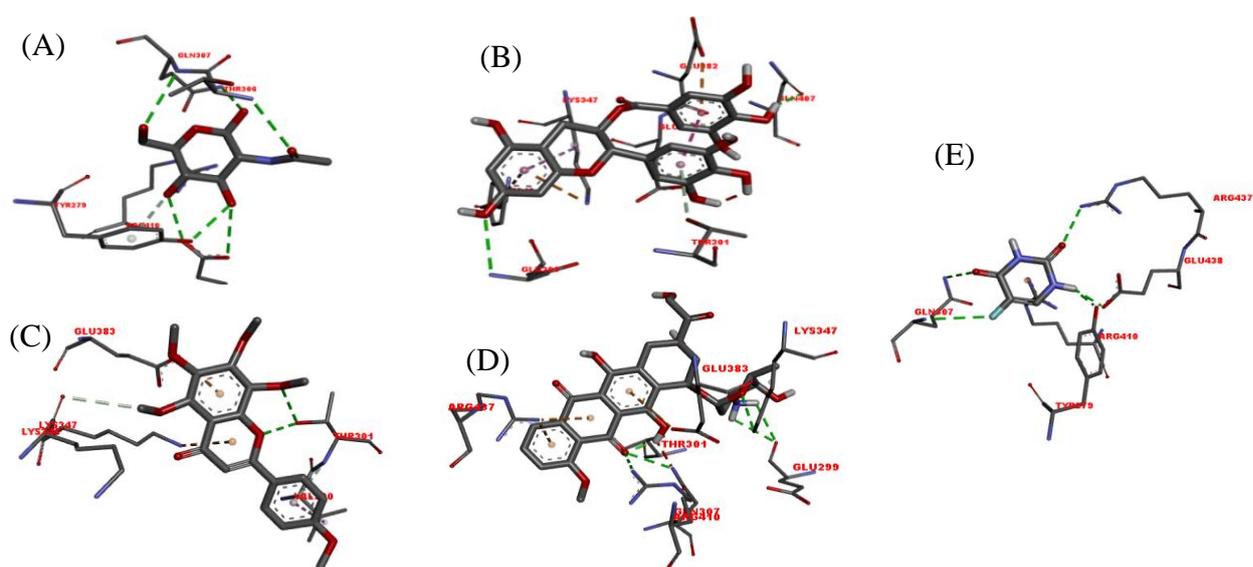
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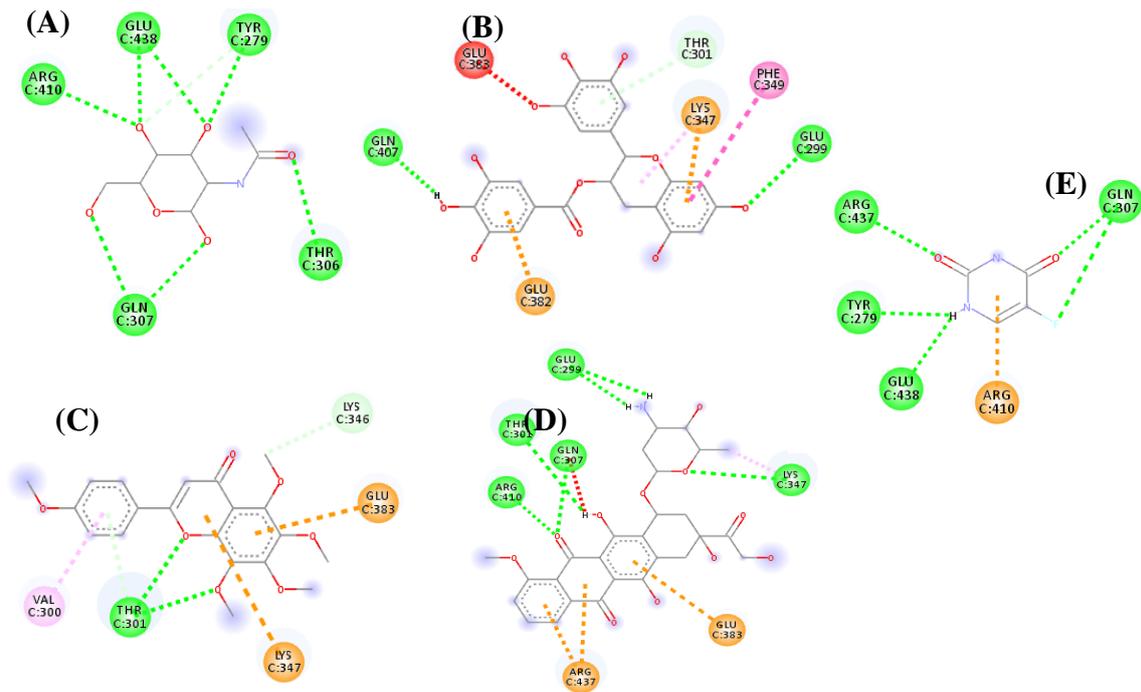
**Figure 1.** Chart of Vitamin C Inhibition



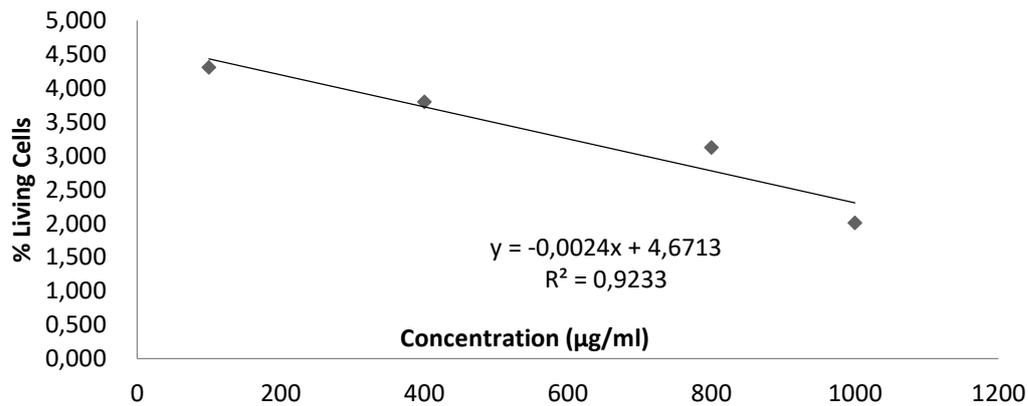
**Figure 2.** Chart of CTM Extract Inhibition



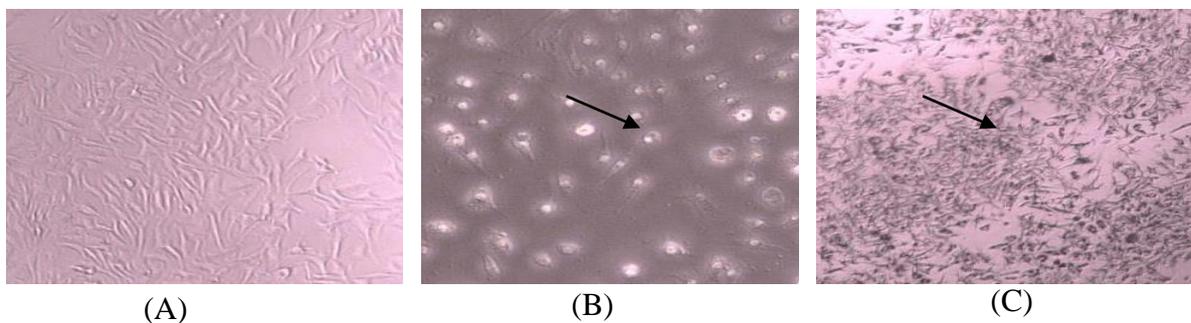
**Figure 3.** 3D structure of geometry optimization results: (A) Native ligand (B) EGCG (C)Tangeretin (D) Doxorubicin (E) 5-Fluorouracil



**Figure 4.** 2D structure of interaction of Test Compounds on HER-2 in atomic pockets: (A) Native Ligand (B) EGCG (C) Tangeretin (D) Doxorubicin (E) 5-Fluorouracil



**Figure 5.** Graphic of Living Cells T47D with CTM Extract Treatment (%)



**Figure 6.** Changes in T47D cell morphology (A) Before the extract treatment (B) Shortly after the extract treatment (C) After the extract treatment (sign '→' indicates damaged or dead cells)

Antioxidant test results showed an  $IC_{50}$  value of vitamin C compounds was 14.87  $\mu\text{g/ml}$ . Whereas, the  $IC_{50}$  value of TLE and MPE combination was 83.00  $\mu\text{g/ml}$ , which was weaker in capturing radicals compared to vitamin C. It occurred as the combination sample (CTM) was still in the form of crude extracts; thus, the number of active compounds that could inhibit free radical activity was likely to be smaller. However,  $IC_{50} < 100 \mu\text{g/ml}$  was obtained and classified as antioxidants with vigorous activity. Therefore, the combination of CTM extract had strong potential in the activity of capturing free radical compounds.

Referring to a similar study, an antioxidant test of *Kemuning* extract (*Murraya paniculata* (L) Jack) produced an  $IC_{50}$  value of 126.17  $\mu\text{g/ml}$ , and antioxidant analysis of mangosteen peel extract (*Garcinia mangostana* L.) resulted in an  $IC_{50}$  value of 44.49  $\mu\text{g/ml}$ .<sup>15</sup> Compared to this, CTM extract had more substantial antioxidant potential than *Kemuning* extract. However, it was weaker compared to mangosteen peel extract.<sup>16</sup>

The results of molecular docking showed that the EGCG compound had the most vigorous inhibitory activity on the HER-2 protein with a docking score of -6.6 kcal/mol, compared to other comparative compounds. Visualization of the docking results showed that EGCG compounds were comparable to doxorubicin which had 7 bounds interacting with amino acid glutamine at position 407 (GLN 407), glutamic acid at position 299 (GLU 299), glutamic acid at position 383 (GLU 383), threonine at position 301 (THR 301), glutamic acid at position 382 (GLU 382), lysine at position 347 (LYS 347), and phenylalanine at position 349 (PHE 349).

It was higher than the original ligand, which only had five bonds.

The tangeretin compound interacted by binding to 6 bonds interacting with the amino acid threonine at position 301 (THR 301), lysine at position 346 (LYS 346), lysine at position 347 (LYS 347), glutamine at position 383 (GLU 383), and valine at position 300 (VAL 300).<sup>17-20</sup> The 5-FU ligand and tangeretin interacted with five bonds of amino acids, as many as the original ligand. The more the bonds are formed between the ligand and the target protein, the stronger the potential activity of a compound will be. It indicates that the EGCG compound in silico has a strong potential to inhibit HER-2 protein.<sup>21</sup>

Based on the cytotoxic test results, CTM extract had a weak potential in inhibiting the viability of T47D breast cancer cells with  $IC_{50}$  values of 1889  $\mu\text{g/ml}$ . It was probably due to the small amount of tangeretin compound content in CTM extracts, so the cytotoxic effect was not reliable. The tangeretin flavonoid compound, which was suspected of having an effect as a chemopreventive agent, had a mechanism to induce cell cycle G1 arrest when the cell was in the restriction point phase.<sup>22-24</sup> A restriction point is a point where hyperphosphorylation occurs that causes the p27 inhibitor, a p53 expression gene (an apoptosis gene), to be degraded. At this point, the reparation of damaged DNA occurs. If DNA damage is severe and cannot be repaired, it will be immediately eliminated to the Go phase. The mechanism of the cell cycle starts with the entry of it from the Go phase to the G1 phase due to the growth factor stimuli. It then causes retinoblastoma protein (pRb) hyperphosphorylation and DNA to become loose. Furthermore, it later enters the restriction point and the S

phase to replicate DNA. If there is damage during the synthesis, it can be repaired before it finally enters the M phase. In cancer cells, the p53 gene is mutated.

## CONCLUSION

It can be concluded that the combination of ethanolic extract of tea leaves and mandarin orange peel (CTM) had strong potential as an antioxidant agent in chemopreventive activity based on DPPH and molecular docking tests. However, the results of the cytotoxic test showed a weak potential to inhibit the development of T47D breast cancer cells. Some compounds that were considered to play an essential role in the chemopreventive mechanism were tangeretin and EGCG compounds.

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## CONFLICT OF INTEREST

There is no potential for conflict of interest.

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