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Chemical Composition Analysis of *Citrus×limon* (L) Osbeck Leaf Essential Oil and Its Activity as an Anti-Cervical Cancer Agent

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Abstract
Cervical cancer is a life-threatening disease. The use of synthetic drugs for its treatment can have serious adverse effects on the body. Therefore, it is necessary to search for natural drugs that are safe for the body. One of the secondary metabolites contained in plants is essential oils (EO), EO are known to have activity as anti-cervical cancer. Genus *Citrus* is known for containing EO compounds, one of the species of the genus is *Citrus×lemon* (L) Osbeck. The purpose of this study is to identify the chemical components of the EO isolated from the leaves of *C×limon* and to evaluate its cytotoxic activity against cervical cancer. EO was isolated from *C×limon* leaves collected in Padang City, Indonesia using the hydrodistillation method. EO was then analyzed by GC-MS. To determine its cytotoxicity, BSLT was conducted, followed by molecular docking and MTT assays. The experiment yielded a yellow liquid with a density of 0,8684 g/mL and a yield of 0,181%. GC-MS analysis of the EO identified 56 chemical components, with the main compounds are (-)-β-pinene (7.32%), (-)-limonene (28.40%), geranial (5.54%), caryophyllene (5.22%). The EO showed high toxicity against *Artemia salina* L larvae, with an LC_{50} value of 3,697 µg/mL. Through molecular docking, it is known the (-)-limonene, geranial, (-)β-pinene and caryophyllene compounds as the main compounds in of EO are known to bind to form complexes with cervical cancer proteins (HPV18E6). MTT assay showed weak cytotoxic activity of EO against HeLa cells, with an IC_{50} of 218.9 µg/mL.

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INTRODUCTION

Cervical cancer is a deadly disease often referred to as a “silent killer” due to its difficulty in detecting in early stages. Symptoms of cervical cancer may appear 15-20 years after infection (Hadisiwi & Arifin, 2022). Based on WHO data, cervical cancer is the second most common cancer in women, with a death rate of 21,003 cases (9.0%) out of 234,511 cancer deaths in Indonesia in 2017 (WHO, 2020). Human Papillomavirus (HPV) causes both squamous cell cervical cancer and cervical adenocarcinoma. The HPV Early 6 (E6) protein promotes carcinogenesis E6 binds to wild-type protein 53 (p53) and degrades it. Inactivation of p53 disrupts apoptosis regulatory mechanisms (Alia et al., 2016).

The high rate of cervical cancer cases and related deaths has led to the development of numerous synthetic drugs for treatment, but the use of synthetic drugs often results in harmful side effects. Therefore, it is necessary to search for drugs derived from natural ingredients that have the potential to be more effective and efficient anti-cervical cancer drugs for human health (Bernardini et al., 2018). Plants contain many secondary metabolites that can be

utilized as anticancer agents, including essential oil compounds. The essential oils are dominated by terpene group compounds, many of which have high toxicity properties (Prakash, 2018). Essential oils are commonly present in aromatic plants, such as species of the genus Citrus. The *Citrus* genus has been known to be antiproliferative against cervical cancer cells (Nguyen et al., 2017).

According to Plant Of The Word Online (POWO), *Citrus* genus is commonly grown in the form of hybrids and cultivars, including the *Citrus* species *Citrus*×*limon* (L) Osbeck (*C*×*limon*) (*Plants of the World Online*, 2023). The leaves of *C*×*limon* were found to contain compounds such as flavonoids, phenols, saponins, alkaloids, tannins and steroids/triterpenoids (Ehiobu et al., 2021; Grace et al., 2020; Mayasari & Laoli, 2018; Nsangou et al., 2021). The leaves of *C*×*limon* have been found to have antioxidant, has antimicrobial potential and cytotoxicity potential (Ehiobu et al., 2021; Riaz et al., 2023).

C×*limon* leaves contain numerous secondary metabolites that can be utilized. However, there is limited information available on essential oil content isolated from these leaves and their potential as cervical anticancer agents. Therefore, this study aims to isolate essential oils from *C*×*limon* leaves for potential use as a safe cervical anticancer drug, as an alternative to synthetic drugs. In this study, The hydrodistillation method was used to isolate *C*×*limon* leaves, hydrodistillation is a simple and cost-effective method for isolating essential oils, using water as a medium, it is the preferred method due to its ease of use and affordability compared to other methods (Khan et al., 2023).

The chemical components of the EO were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). Additionally, a cytotoxic test was conducted using the Brine Shrimp Lethality Test (BSLT) method to determine the EO's cytotoxic activity against *Artemia salina* shrimp larvae, expressed as the value of Lethal Concentration 50 (LC_{50}), this method is considered to have a positive correlation with the cytotoxic power of anticancer compounds. The cytotoxic ability of the main compound of the EO against cervical cancer cell protein receptors (HPV18E6) was determined through molecular docking, the molecular docking is in silico method, this computational-based method is used to predict interactions between compounds and target proteins (Lee et al., 2018). Further cytotoxic tests in-vitro against cervical cancer cells (HeLa cells) using the Microculture Tetrazolium (MTT) method. The anticancer activity of the EO compounds against HeLa cells was determined through the Inhibition Concentration (IC_{50}) value.

METHOD

Apparatus

The equipment included a set of hydrodistillation equipment for essential oil isolation and GC-MS equipment (GC-MS-QP-2010, Shimadzu, Tokyo, Japan) for essential oil component analysis. The BSLT cytotoxic test requires the use of filter paper, micropipettes, and glassware commonly found in chemical laboratories, as well as glass boxes for shrimp breeding containers. For the cytotoxic test using the MTT method, you will need a T-75 flask, conical tube, Eppendorf tube, micropipettes, serological pipette, 96-well plate, automated hemocytometer plate, automated cell counter hemocytometer TC-10, refrigerator, 37°C incubator with 5% CO_2, inverted microscope, centrifuge, safety cabinet laminar airflow, and Elisa reader. Molecular docking was required using the MOE 2015.10 software and the Worldwide Protein Data Bank (PDB) online database accessible through the link https://www.rcsb.org/ and the PubChem database accessible through the link https://pubchem.ncbi.nlm.nih.gov/3.

Materials

The materials used in this study include young and fresh *Citrus×limon* (L) Osbeck leaf, distilled water, and anhydrous copper sulfate. Materials for toxicity tests using the BSLT method are seawater, *Artemia salina* L shrimp larvae, and tween-80, while materials used for cytotoxic tests using the MTT method require HeLa cancer cells, Roswell Park Memorial Institute (RPMI) 1640 medium, Fetal Serum Bovine (FBS), antibiotics (1% penicillin-streptomycin), Trypsin-EDTA, Phosphate Buffer Saline (PBS) and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide).

Isolation of Essential Oil of *Citrus×limon* (L) Osbeck Leaf

A total of 600 g of fresh and young leaves of *C×limon* were cut into small pieces and hydrodistilled using a Clevenger apparatus for approximately 7 hours at 100°C. The essential oil (EO) was isolated and the water content was removed using anhydrous copper(II) sulfate (Suryati et al., 2021).

Essential Oil Analysis of *Citrus×limon* (L) Osbeck Leaves

The GC-MS-QP2010 (Shimadzu, Tokyo, Japan) was used to analyze the isolated essential oil of *C×limon* leaves. The samples were injected as 0.1 µL with a split ratio of 1:250 without the use of solvents, and the injector and detector temperatures were set to 200-230°C. Helium was used as the carrier gas, flowing at a speed of 45.5 mL/min. The column temperature was set to 60°C for 1 minute, followed by an increase of 10°C/min up to 210°C, and then held constant for 1 minute. The mass-to-charge ratio (m/z) was measured by MS in the range of 45-500 AMU with an ionization energy of 70 eV and a scan time of 3 seconds. The GC-MS Shimadzu software version 4 was used for parameter settings, data recording, and processing. The compound from the analyzed EO was identified by comparing it with the data from the National Institute of Standards and Technology (NIST) (Hüsnü Can Başer & Buchbauer, 2015).

Cytotoxic Test with Brine Shrimp Lethality Test (BSLT) Method

A stock solution of *C×limon* leaf essential oil was prepared with a concentration of 1000 µg/mL by dissolving 10 mg of EO, 10 µL of Tween 80 (0.1% v/v), and seawater in a 10 mL volumetric flask. The concentration of the test sample was then varied to 80, 40, 20, and 10 µg/mL. Each test solution was then used to expose 20 *Artemia salina* L shrimp larvae. After 24 hours, the number of dead *Artemia salina* L shrimp larvae in each test solution was recorded. Probit analysis and regression equations were used to determine the LC_{50} values for the negative control solution (Delnavazi et al., 2018).

Molecular Docking against Cervical Cancer Cell Proteins

In this study, the main compound found in the essential oil of the leaves of *C×limon* was used as the ligand and the doxorubicin compound was used as the control. The ligand structure obtained from the PubChem database using Canonical Smiles code was entered into MOE software. Ligand preparation involved 3D structure conversion and energy minimization using the Energy Minimize menu, and the prepared ligand was saved as a database with *.mdb extension. The protein structure, specifically the HPV18E6 protein, was obtained from Worldwide PBD with the protein code 6SJV in .pdb format. Water molecules and native ligands (Zn and sugar groups) were removed from the sequence and the 3D structure was displayed in the MOE 2015.10 window. The protein structure was then prepared using the QuickPrep button and active sites were found using the Site Finder menu. Docking simulation was performed using the Dock menu with Triangle Matcher placement, London dG score, and 30 poses, refinement using the Rigid Receptor method with GBVI/WSA dG score and 5 poses. Docking results are displayed in the Database Viewer

window as Docking Score (DS) and Root Mean Square Deviation (RMSD) tables. The Ligand Interactions menu allows viewing 2D images of ligand interactions with amino acid residues, which can be saved in either *.png or *.jpg format. The Surfaces and Maps menu displays the molecular surface shape of the docked complex based on the amino acid residues' atomic composition and polarity

Cytotoxic Test Using the Microculture Tetrazolium (MTT) Method

The isolated essential oil was dissolved in DMSO to obtain a liquid with a concentration of 1000 µg/mL. The variation concentrations of 1000, 500, 250, and 125 µg/mL were prepared by diluting the liquid with RPMI medium. HeLa cervical cancer cells were retrieved from storage and cultured in RPMI medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin). The cells were then incubated for 24 hours at 37°C, 95% humidity, and 5% CO_2. Cell growth was monitored using an inverted microscope. Cells that reached ≥ 80% confluency were harvested and suspended. The cells were then plated in 96-well plates and incubated for 24 hours at 37°C, 95% humidity, and 5% CO_2. Next, 20 µL of each test solution was added, and the cells were re-incubated for 72 hours under the same conditions. Afterward, the cell media was removed, and the cells were washed with 100 µL FBS. MTT reagent (0.5 mg/mL) was added to each well and incubated for an additional 4 hours. The MTT solution was discarded, and the formazan salt crystals that formed were dissolved in 100 µL of DMSO. The absorbance was measured at a wavelength of 550 nm using an Elisa reader plate. The obtained absorbance data was converted into percent cell viability, and the IC_{50} value was determined using GraphPad Prism 9.0 software (Suryati et al., 2021).

RESULTS AND DISCUSSION

Essential Oil of *Citrus×limon* (L) Osbeck Leaf Isolation Result

The essential oil obtained from 600 g of *C×limon* leaves was 1.25 mL with a mass of 1,0856 g, density of 0,8684 g/mL, and yield of 0,181%. The produced EO has a distinct aroma and clear yellow color. The GC-MS data analysis of the *C×limon* leaf EO, by comparing it with the National Institute of Standards and Technologies (NIST) database, revealed 60 chemical components. Refer to Table 1 for details.

Table 1. The chemical components of the essential oil of *C×limon* leaves.

Peak	RT[a] (Menit)	Compound Name	Molecular Formula	Area (%)	SI[b] (%)
1.	4.445	α-thujene	$C_{10}H_{16}$	0.26	96
2.	4.579	(-)-α-Pinene	$C_{10}H_{16}$	1.99	96
3.	5.080	β-Phellandrene	$C_{10}H_{16}$	3.60	93
4.	5.206	(-)-β-Pinene	$C_{10}H_{16}$	7.32	96
5.	5.690	Terpinene	$C_{10}H_{16}$	0.38	96
6.	6.075	(-)-Limonene	$C_{10}H_{16}$	28.4	93
7.	6.394	α-Terpinene	$C_{10}H_{16}$	0.67	97
8.	6.590	α-Thujene	$C_{10}H_{18}O$	0.39	94
9.	6.923	Terpinolene	$C_{10}H_{16}$	0.30	97
10.	7.051	β-Linalool	$C_{10}H_{18}O$	2.36	97
11.	7.185	4-Thujanol	$C_{10}H_{18}O$	0.27	92
12.	7.286	(5e)-3,3-Dimethyl-1,5-Heptadiene	C_9H_{16}	0.20	84
13.	8.123	β-Citronellal	$C_{10}H_{18}O$	3.94	95
14.	8.190	Isopulegol 2	$C_{10}H_{18}O$	0.56	93
15.	8.320	Verbenol	$C_{10}H_{16}O$	0.16	85

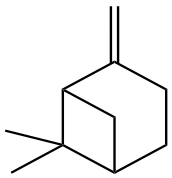
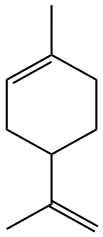
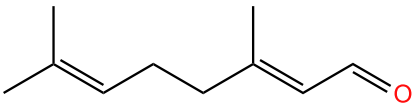
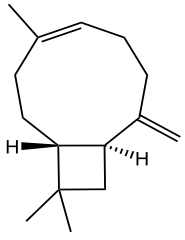
Peak	RT[a] (Menit)	Compound Name	Molecular Formula	Area (%)	SI[b] (%)
16.	8.403	Isopulegol 1	$C_{10}H_{18}O$	0.45	97
17.	8.681	Carane, 4,5-Epoxy-(e)	$C_{10}H_{16}O$	0.12	86
18.	8.820	(-)-4-Terpineol	$C_{10}H_{18}O$	0.97	97
19.	9.119	Linalyl Propanoate	$C_{13}H_{22}O_2$	2.84	94
20.	9.822	Vernol	$C_{10}H_{18}O$	3.45	94
21.	10.154	β–Citral	$C_{10}H_{16}O$	4.26	95
22.	10.838	Geranial	$C_{10}H_{16}O$	5.54	97
23.	11.493	Undecanal	$C_{11}H_{22}O$	0.34	97
24.	11.660	Methyl Citronellate	$C_{11}H_{20}O_2$	0.16	86
25.	11.908	Varamol	$C_9H_{10}O_2$	0.29	86
26.	12.408	(3r,4r)-1-Isopropyl-4-Methyl-3-(Prop-1-En-2-Yl)-4-Vinylcyclohex-1-Ene	$C_{15}H_{24}$	0.46	94
27.	12.521	6-Octen-1-Ol, 3,7-Dimethyl-Acetat	$C_{12}H_{22}O_2$	0.85	94
28.	12.820	Neryl Acetate	$C_{12}H_{20}O_2$	1.97	97
29.	13.273	Neryl Acetate	$C_{12}H_{20}O_2$	1.84	96
30.	13.744	2,4-Diisopropenyl-1-Methyl-1-Vinylcyclohexane	$C_{15}H_{24}$	0.33	96
31.	13.980	Tetradecanal	$C_{14}H_{28}O$	0.21	95
32.	14.340	Methyl Methylanthranilate	$C_9H_{11}NO_2$	4.39	96
33.	14.636	Caryophyllene	$C_{15}H_{24}$	5.22	95
34.	14.725	alpha.-Bergamotene	$C_{15}H_{24}$	0.77	96
35.	15.368	alpha.-Humulene	$C_{15}H_{24}$	1.23	96
36.	15.982	8-Isopropyl-1-Methyl-5-Methylene-1,6-Cyclodecadiene	$C_{15}H_{24}$	1.24	96
37.	16.273	1,3,6,10-Dodecatetraene, 3,7,11-Trimethyl	$C_{15}H_{24}$	1.38	94
38.	16.410	beta.-Bisabolene	$C_{15}H_{24}$	1.56	94
39.	16.850	delta.-Cadinene	$C_{15}H_{24}$	0.29	93
40.	17.500	2-(4,8-Dimethyl-3,7-Cyclodecadien-1-yl)-2-Propanol	$C_{15}H_{26}O$	0.24	89
41.	17.614	(6e)-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	$C_{15}H_{26}O$	0.69	98
42.	17.790	β-Germacrene	$C_{15}H_{24}$	0.25	89
43.	17.877	Squalen	$C_{30}H_{50}$	0.32	89
44.	18.439	(-)-5-Oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene-[1r-(1r,4r,6r,10s)]-	$C_{15}H_{24}O$	0.42	94
45.	19.650	Muurolol	$C_{15}H_{26}O$	0.41	84
46.	19.971	Ledol	$C_{15}H_{26}O$	0.52	83
47.	20.510	Bisabolol	$C_{15}H_{26}O$	0.47	96
48.	23.460	Bicyclo[2.2.1]Heptane, 2-[9-Borabi Cyclo[3.3.1]Non-9-Yloxy]-, 1,7,7-Trimethyl-	$C_{18}H_{31}BO$	0.26	88
49.	23.873	1,7,7-Trimethylbicyclo[2.2.1]Hept-2-Ylphosphonous Dichloride	$C_{10}H_{17}Cl_2P$	0.22	87
50.	24.403	Bicyclo[2.2.1]Heptane, 2-[9-Borabi Cyclo[3.3.1]Non-9-Yloxy]-, 1,7,7-Trimethyl-	$C_{18}H_{31}BO$	0.31	85
51.	24.710	(6e,10e,14e,18e)-3-Bromo-2,6,10,15,19,23-Hexamethyl-6,10,14,18,22-Tetracosapentaen-2-Ol	$C_{30}H_{51}BrO$	0.29	85
52.	24.871	Thiogeraniol	$C_{10}H_{18}S$	0.14	84
53.	25.324	(6e,10e,14e,18e)-2,6,10,15,19,23-Hexamethyl-1,6,10,14,18,22-	$C_{30}H_{50}O$	0.21	84

Peak	RT[a] (Menit)	Compound Name	Molecular Formula	Area (%)	SI[b] (%)
		Tetracosahexaen-3-Ol			
54.	25.504	5-(1-Bromo-1-Methyl-Ethyl)-2-Methyl-Cyclohexanol	$C_{10}H_{19}BrO$	0.48	78
55.	25.774	(7e,10e)-2,6,11,15-Tetramethyl-2,7,10,14-Hexadecatetraen-6-Ol	$C_{20}H_{34}O$	0.25	89
56.	25.986	2,2-Dimethyl-3-(3,7,16,20-Tetramethyl-Heneicosa-3,7,11,15,19-Pentaenyl)-Oxirane	$C_{30}H_{50}O$	0.48	82
57.	26.452	2-Decyldecahydronaphthalene	$C_{20}H_{38}$	0.80	80
58.	27.058	Naphthalene, 2-Decyldecahydro-	$C_{20}H_{38}$	1.35	81
59.	27.393	Geranyl Linalool Isomer B	$C_{20}H_{34}O$	0.62	84
60.	27.668	Thiogeraniol	$C_{20}H_{36}O$	0.31	80

[a] Retention time
[b] Similarity index

Table 1. shows that EO is composed of various groups of compounds, including oxidized monoterpenes (30,86%), hydrocarbon monoterpenes (43,83%), hydrocarbon sesquiterpenes (11,96%), oxygenated sesquiterpenes (3,32%), oxygenated diterpenes (0,62%), triterpenes (0,32%) and non-terpene compounds (9,09%) consisting of aldehyde group compounds, primary alcohols, benzoic acid esters, and long-chain fatty acids. The dominant compounds in this EO are those with percent area values ≥ 5%, as presented in Table 2.

Table 2. Structure of the main compounds of the essential oil of *C×limon* leaves.

No	Compounds Name	Percent Area (%)	Structure of compound
1.	(-)-β-Pinene	7.32	
2.	(-)-Limonene	28.40	
3.	Geranial	5.54	
4.	Caryophyllene	5.22	

The bioactivity of EO is determined not only by the content of major compounds but also by the presence of minor compounds.

Toxicity of Essential Oil of *Citrus×limon* (L) Osbeck Leaves *to Artemia salina* L. Larvae

The BSLT method, which uses *Artemia salina* L as a test animal, is one of the bioassay methods used to determine the toxicity of plant extracts. It is also used as a basis for preliminary toxicity tests on cell lines, as well as for antitumor and anticancer activity. The parameter used to measure the toxicity of the compound is the Lethal Concentration 50 (LC_{50}) value, which represents the minimum concentration that can cause 50% mortality of the test organism. According to Figure 1., the concentration of the solution is directly proportional to the number of dead shrimp larvae. A higher concentration of the test solution indicates a greater amount of active compound composition, resulting in more *Artemia salina* L shrimp larvae dying. The data in Figure 1. shows that the EO isolated from the leaves of *C×limon* has a high toxic cytotoxic activity, with an LC_{50} value of 3,697 µg/mL. This refers to the grouping of categories of toxicity levels of a compound, where a compound is said to be non-toxic if it has an LC_{50} value >1000 µg/mL, low toxicity if the LC_{50} value is 500-1000 µg/mL, moderate toxicity if the LC_{50} value is 100-500 µg/mL, and high toxicity if the LC_{50} value is 0-100 µg/mL (Hamidi et al., 2014).

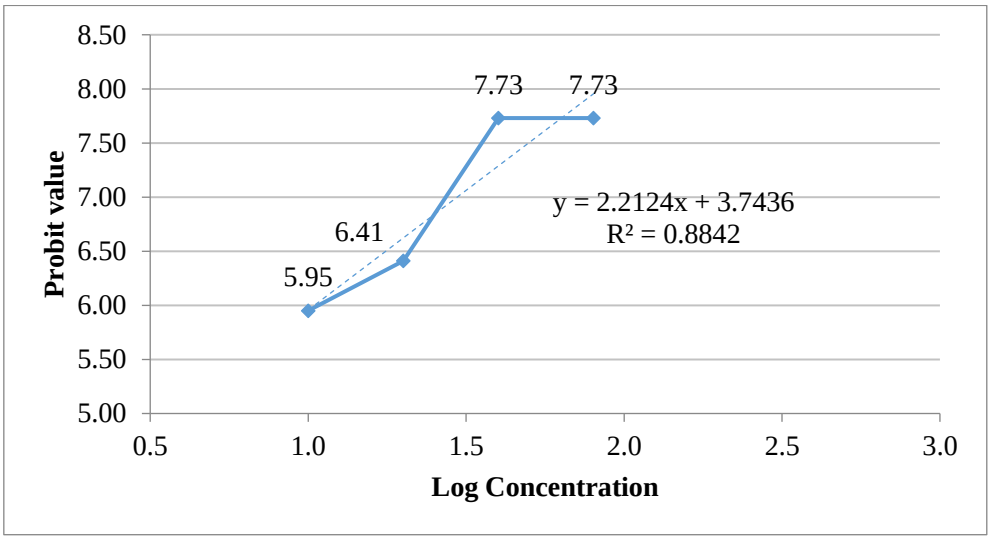


Figure 1. Log concentration and probit value in cytotoxic test with BSLT method.

The toxic compounds in the sample inhibit the growth of shrimp larvae by entering the digestive tract of *Artemia salina* L larvae through the mouth of shrimp larvae and being absorbed through the cell membrane into the digestive tract (Ningdyah et al., 2015).

Molecular Docking of Essential Oil main Compounds against Cervical Cancer Cell Proteins

In this study, four major compounds of *C×limon* leaf essential oil, namely geranial, (-)-limonene, caryophyllene, and (-)β-pinen were docked with protein HPV18E6 as a potential target for cervical cancer treatment. The four major compounds of *C×limon* leaf EO were shown to fulfill Lipinski's rule with 0 violation for the three compounds and one violation (MLOGP>4.15) for the caryophyllene compound (Table 3) (*SwissADME*, 2024).

Table 3. Bioavailability Test Results According to Lipinski's Rule

Compounds	LogP ≤5	Hydrogen Donor <5	Hydrogen Acceptor <10	Molecular Weight Da	Free Rotation ≤10	Fulfilling Lipinski's Rule
Geranial	2.49	0	1	152.23	4	Yes
(-) β- pinene	4.29	0	0	136.23	0	Yes
Caryophyllene	4.63	0	0	204.35	0	Yes
(-)-Limonene	3.27	0	0	136.23	1	Yes

In this study, molecular docking was performed on the HPV18E6 protein with the doxorubicin compound ligand, which is an anthracycline drug used to treat various types of cancer, including cervical cancer (X. Liu et al., 2017). The doxorubicin compound was used as a control in this molecular docking method.

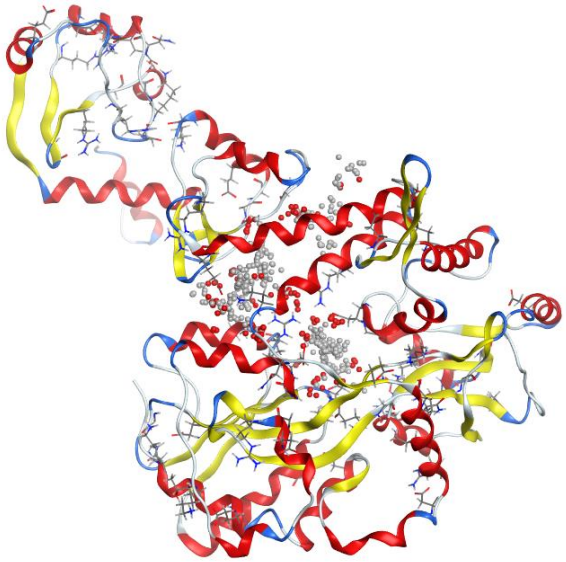


Figure 2. HPV18E6 protein with active side

Figure 2. shows the cervical cancer cell protein HPV18E6, which is a potential target for therapy due to its oncoprotein function in HeLa cell lines. The protein causes ubiquitin-mediated degradation of the p53 protein. Figure 2. indicates an active binding site on the protein, which is where ligands and proteins can bind. Table 3. and Figure 3. display the docking score value and ligand compound binding with HPV18E6.

Tabel 3: Docking Score and binding of essential oil and doxorubicin lead compounds with HPV18E6

Compounds	***Docking Score* (kcal.mol^{-1})**	**RMSD**	**Bond Distance (Å)**	**Bond Energy (kcal/mol)**	**Bond Type**	**Amino Acids**
Geranial	-5.2415	1.6574	4.41	-0.9	H-π	Trp 341
Caryophyllene	-5.9714	1.6932	4.15	-0.6	H-π	Tyr 156
			4.17	-0.8	H-π	Trp 63
(-) β- pinen	-4.5313	1.3259	4.07	-0.7	H-π	Trp 341
			4.12	-0.8	H-π	Trp 341
(-)-Limonene	-4.9753	1.6203	4.10	-0.7	H-π	Trp 63
Doxorubicin	-8.3309	1.6805	3.22	-0.9	H-donor	Asp 1058

The main compound of lime leaf essential oil can form hydrogen bonds by binding to the HPV18E6 protein. These bonds can enhance protein stability and structure, and also affect biological functions such as ligand binding and enzymatic reaction catalysis (Tripathi & Misra, 2017). The doxorubicin compound showed the strongest interaction with HPV18E6 protein with a docking score of 8.3309 Å. The binding energy analysis shows that geranial and doxorubicin compounds have the greatest binding energy of -0. The RMSD values for the EO of *C×limon* leaves and doxorubicin compounds fall into the 'good' category. The four main compounds of EO oil and doxorubicin compounds also have RMSD values in the 'good' category. According to the RMSD qualification, values are considered 'good' when RMSD ≤ 2.0 Å, 'between 2.0 to 3.0 Å' when RMSD is between 2.0 to 3.0 Å, and 'poor' when RMSD ≥ 3.0 Å (Ramírez & Caballero, 2018).

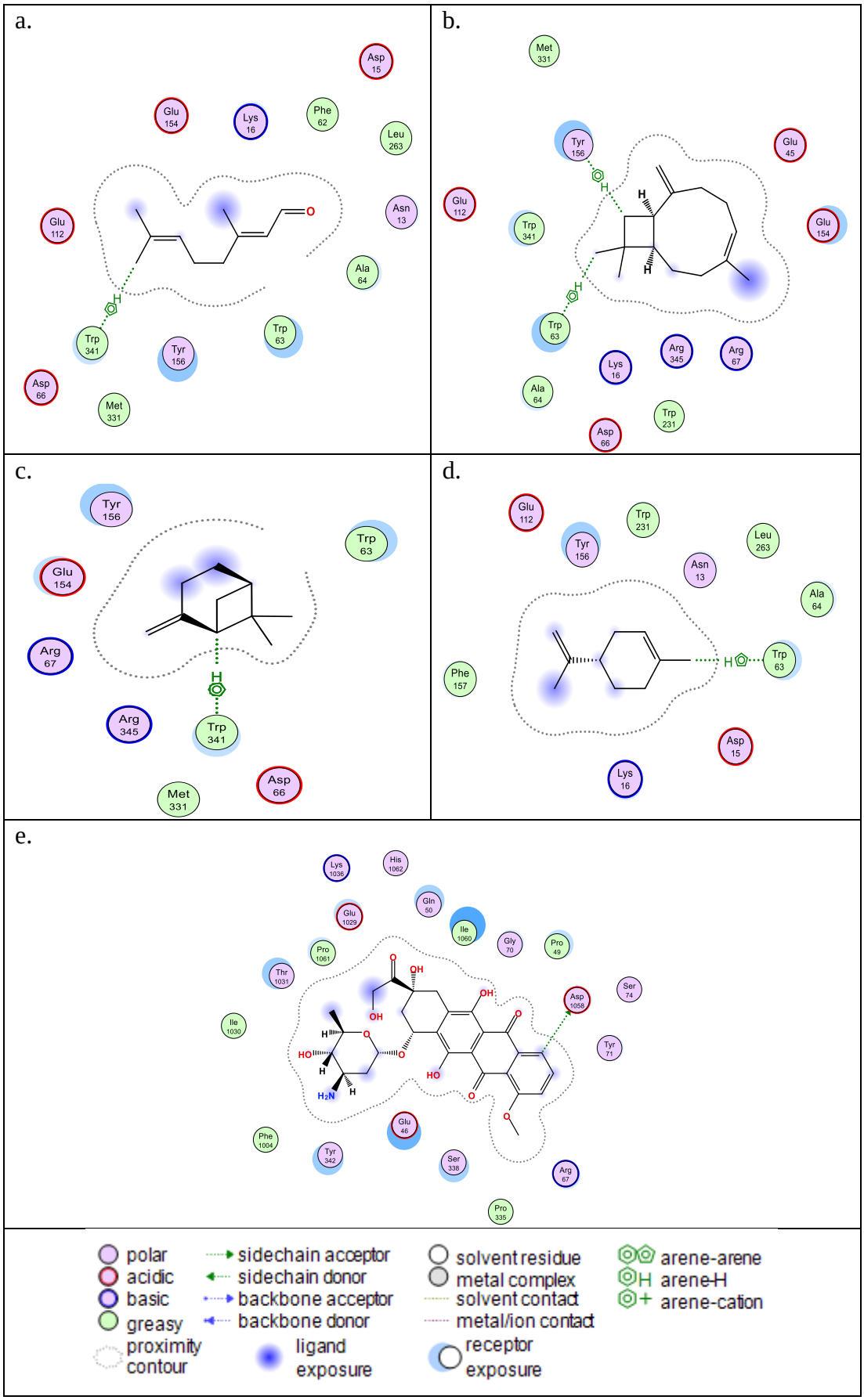


Figure 3. 2D interaction of ligand compound with HPV18E6 (Geranial (a); Caryophyllene (b); (-)-β-Pinene (c); (-)-Limonene (d); Doxorubicin (e))

According to the docking score, the doxorubicin compound has a score of -8.3309 kcal/mol. The compounds with docking scores close to doxorubicin are Caryophyllene (-5.9714 kcal/mol), geranial (-5.2415 kcal/mol), limonene (-4.9753 kcal/mol), and β-pinene (-4.5313 kcal/mol). A smaller docking score indicates a more stable complex and greater potential as an inhibitor. The configuration with the lowest docking score is considered the best (Thuy et al., 2020).

Cytotoxic Potential of *Citrus×limon* (L) Osbeck Leaf Essential Oil Against Cervical Cancer Cells (HeLa Cells)

The MTT method cytotoxic assay is used to determine the level of toxicity of a compound based on cell viability values. The test is based on the ability of cervical cancer cells (HeLa) to survive when treated with a compound; in this case, the cells used are cervical cancer cells and the compound tested is the isolated *C×limon* leaf essential oil compound.

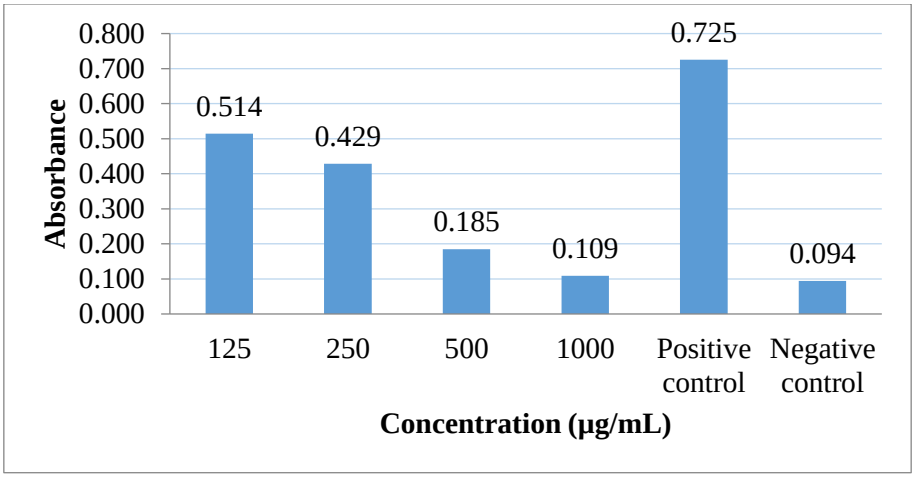


Figure 4. Absorbance results of *C×limon* leaf essential oil in the MTT assay

Figure 4. shows that as the concentration of EO increases, the absorbance decreases. This indicates that fewer formazan crystals are formed. Higher concentrations of toxic compounds in the sample lead to increased cell death caused by the enzyme succinate dehydrogenase, which reduces tetrazolium salt into formazan crystals. Figure 4. shows that the positive control, consisting of cells without the test sample, produced the highest absorbance of succinate dehydrogenase enzyme. Conversely, the negative control, which did not contain cells, had the smallest absorbance, indicating no production of succinate dehydrogenase enzyme to reduce tetrazolium salt into formazan crystals. The absorbance value can be used to determine the % viability of HeLa cells.

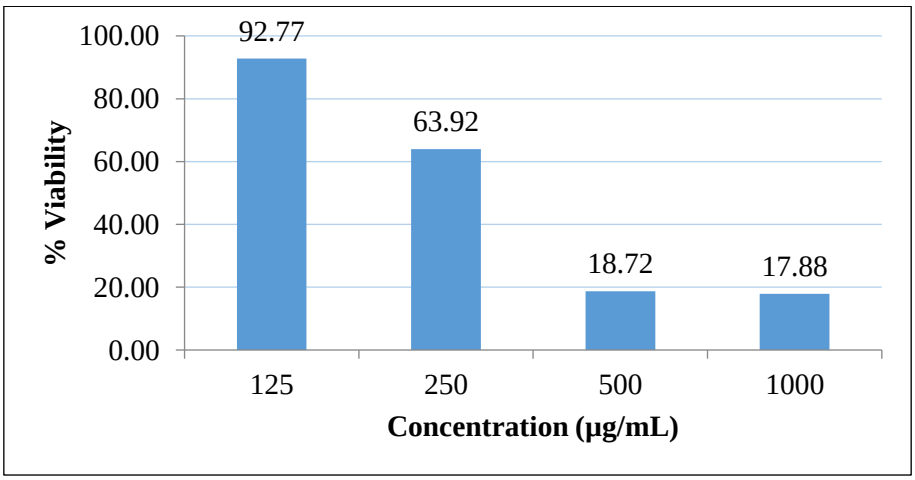


Figure 5. Relationship between essential oil concentration variation and % viability

Figure 5. shows the relationship between test sample concentration and % cell viability, while Figure 5. shows the relationship between the log of test sample concentration and % cell viability.

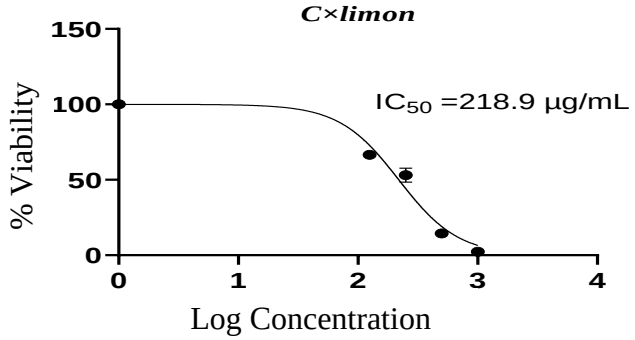


Figure 6. Relationship between log concentration variation and % viability

As the concentration of the test compound increases, the absorbance value of the wells decreases, resulting in a lower % viability value. This suggests that cell growth is increasingly inhibited with higher concentrations of the test sample. The positive control is expected to have a higher % viability value compared to the other wells due to the absence of the test sample. Figure 4 shows that at a concentration of 1000 µg/mL, the cell viability was 2,32%, indicating that approximately 97,68% of cells were unable to survive. At the lowest concentration of 125 µg/mL, the cell viability was 92,92 %, indicating that only about 7,08% of cells were unable to survive. Therefore, at this concentration, the test sample provided almost no inhibition on cancer cell growth.

Cytotoxicity tests against cancer cells are expressed as IC_{50} (inhibitory concentration) values. The IC_{50} value represents the concentration of test samples that causes 50% inhibition of cell growth in a specific time and under controlled conditions. The smaller the IC_{50} value, the more toxic the test compound. In this study, EO exhibited weak cytotoxic activity with an IC_{50} value of 218,9 µg/ml. This fragment describes the cytotoxic categories of a compound according to the National Cancer Institute (NCI). The categories are very cytotoxic ($IC_{50} \leq 20$ µg/ml), moderate cytotoxic (IC_{50} 21-200 µg/ml), weak cytotoxic (IC_{50} 201-500 µg/ml), and not cytotoxic ($IC_{50} > 501$ µg/ml). A compound may have potential as an anticancer drug if its IC_{50} value is less than 100 µg/ml (Sajjadi et al., 2015). Therefore, it can be inferred that the isolated essential oil from *C×limon* leaves has a weak cytotoxic effect on cervical cancer cells (HeLa). The literature search reveals that the main compounds of EO, in general, have the potential to act as anticancer agents against HeLa cells, as demonstrated in Table 3.

Table 3. Cytotoxic potential of the main compounds of essential oils against cervical cancer cells (HeLa)

Compound Name	Plant Names	Percentage (%)	IC_{50} (µg/mL)	Reference
Geranial	*Elsholtzia communis* (Collett & Hemsl.) Diels	24.1	8.09 ± 2.67	(Nath et al., 2021)
Caryophyllene	*Clibadium Surinamese* L	30.4	30.14	(Ulia & Santoni, 2023)
(-)-Limonene	*Citrus volkameriana*	68.5	0.093	(Said et al., 2019)

Based on the literature, the main compound of *C×limon* leaf EO has strong cytotoxicity against HeLa cells. Molecular docking results indicate that the main compound of EO has a good interaction with cervical cancer proteins. The LC_{50} value in the BSLT test suggests that EO has strong cytotoxic properties against cervical cancer cells. However, this study shows

that EO compounds have weak cytotoxicity against HeLa cells. The low toxicity ability of the constituents of EO is thought to be caused by the small percentage area of the chemical compounds it contains.

The small percentage area of the chemical compounds in the EO is thought to be the cause of this. It is known that the EO has the largest percentage area, specifically 28.40% for the limonene compound, and there are four other main compounds with an area percentage of no more than 7.00%. It is evident that the chemical compounds comprising the EO of *C×limon* leaves with a small area percentage can reduce the toxicity of the EO's compounds. The main compounds' composition in an EO is crucial in determining its cytotoxic properties. Minor compounds with an area percentage below 5% have a minimal impact on the EO's cytotoxic properties. The cytotoxic activity of isolated EO was also shown in the morphological form of cervical cancer cells (HeLa) after exposure to different concentrations of the test sample and before treatment with the EO (Figure 6).

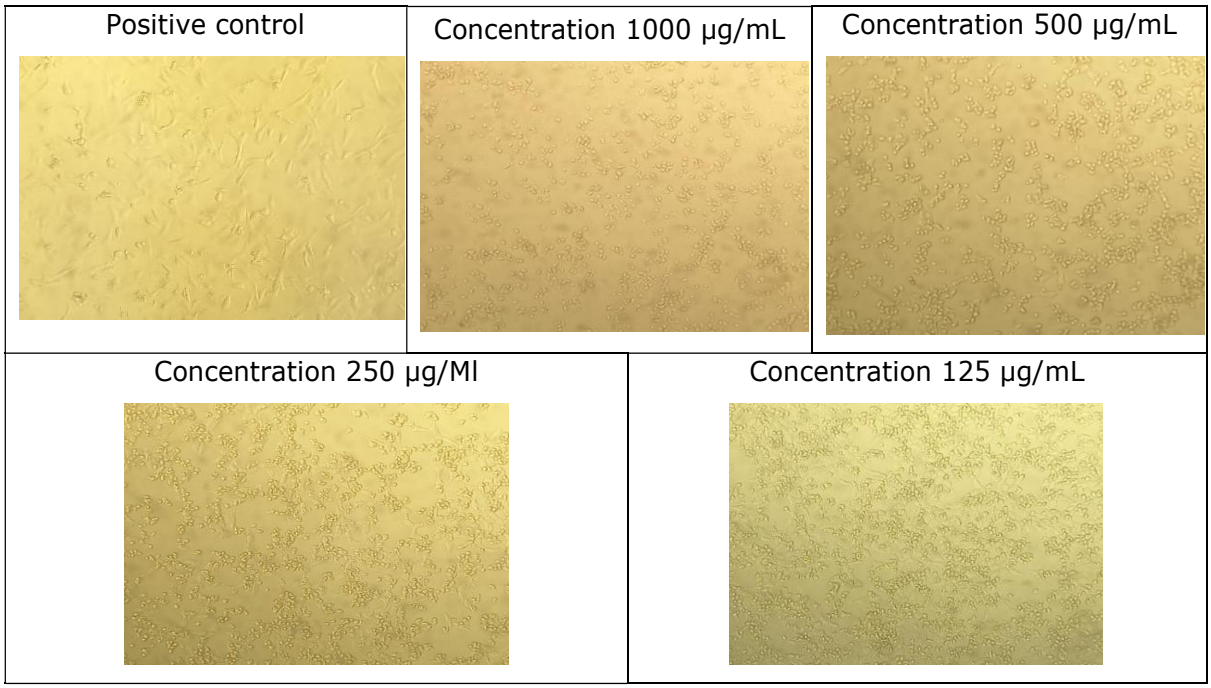


Figure 6. Morphology of cervical cancer cell (HeLa) growth.

Based on Figure 6 show the HeLa cells after incubation with EO at different concentrations after 72 hours showed the disintegration/fragmentation of cancer cells compared to positive control cells, the higher the concentration, the more cell division occurs compared to the positive control. Based on Figure 5 found, in the control group HeLa cells were polygonal with clearly visible nuclei. In HeLa cells, after incubation with EO at different concentrations, there is a change in cell morphology, treated cells experience changes in cell morphology to become rounder and cell density becomes lower than the positive control (Figure 6).

This cytotoxic activity is influenced by the lipophilic nature and low molecular weight of EO, which makes it easier for the chemical components in EO to cross the cell membrane, thus changing the composition and fluidity of the membrane. These changes result in leakage of ions and cytoplasmic molecules. These membrane changes can also lead to reduced ATP production and loss of mitochondrial function, resulting in cell death. In addition, EO also acts as a pro-oxidant that can cause oxidation-reduction reactions in cells that can also affect cell survival. These cytotoxic properties are also due to the complex interactions of different types of compound classes in EO such as phenols, aldehydes, ketones, alcohols, esters, ethers, and hydrocarbons (Sharifi-Rad et al., 2017). Based on the in-vitro cytotoxic test using

the MTT method, it is known that the EO compound of *C×limon* leaves has no potential as an anticancer drug for cervical cancer, so this EO cannot be used as a substitute for synthetic drugs for cervical cancer. Based on the in-vitro cytotoxic test using the MTT method, it is known that the EO compound of *C×limon* leaves does not have the potential as an anticancer drug for cervical cancer, so this EO cannot be used as a substitute for synthetic drugs for cervical cancer.

CONCLUSION

The research conducted indicates that the essential oil isolated from *C×limon* leaves has 60 chemical compound components with the main compounds being (-)-β-pinene (7.32%), (-)-limonene (28.40%), geranial (5.54%), and caryophyllene (5.22%). The toxicity tests carried out using the BSLT method showed that the essential oil of *C×limon* leaves was highly toxic to shrimp larvae (*Artemia salina* L.), with an LC_{50} value of 3.697 µg/ml. Based on molecular docking, the main compounds from the essential oil of *C×limon* leaves, including (-)-limonene, geranial, caryophyllene, and (-)-β-pinene, are known to form bonds with the HPV18E6 protein receptor with RMSD values in the good category. The order of docking score values (kcal) was also considered. It is known that the order of compounds with docking score values close to the control compound doxorubicin (-8.3309) is caryophyllene (-5.9714), geranial (-5.2415), (-)-limonene (-4.9753), and (-)β-pinene (-4.5313) per mol-1. According to the cytotoxic test using the MTT method, *C×limon* leaf essential oil exhibits weak cytotoxic activity against HeLa cells with an IC_{50} value of 218.9 µg/mL. Therefore, based on the MTT *Citrus* cytotoxic test, this *Citrus* does not have potential as an anticancer drug. As a result, essential oils of *Citrus* cannot replace synthetic anticancer drugs.

According to the BSLT results, C Limon essential oil shows potential as an anticancer agent. Further research is hopefully carried out to determine the cytotoxicity of C Limon essential oil against other cancer cell lines, such as breast cancer.

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