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Hybridization Synthesis and Anticancer Activity Test of New Carboxylic Acid Derivatives by In-Silico and In-Vitro

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Abstract: Design of new compounds as active ingredients of drugs must be selective and efficient to achieve therapeutic efficacy and minimize resulting side effects. Hybridization synthesis approaches using major components of essential oils can reliably generate new molecules that are superior as active ingredients of anti-cancer drugs due to their cytotoxic properties. This study synthesized a hybrid molecule of citronellyl salicylate by an esterification reaction using Steglich and Fisher method under ultrasonic assistance. The Steglich esterification is more efficient in producing citronellyl salicylate during 30 minutes reaction, yield 12.45%. Analysis of the synthesized product by FTIR is characterized by the presence of typical absorptions of the ester group at $\bar{\upsilon}$ 1650 cm^{-1} and 1270 cm^{-1}, while LC ESI-MS shows m/z 294 is indicated $[M+NH_4]^+$. Anticancer activity was tested in-silico for protein receptors MMP-9, MMP-2, Cyclin-A, p53, and BAK using Molecular Docking Pyrx 9.0 and the highest activity was shown binding affinity value -8.4 kcal/mol for the MMP-9 protein receptor. Similarly, the results of the in vitro activity assay of citronellyl salicylate to 4T1 breast cancer cells showed that the morphology of cancer cells was damaged and the viability of cancer cells was lower than that of normal cells.

Keywords: Anticancer; Citronellyl Salicylate; Hybridization synthesis; Molecular Docking

Introduction

Along with the times, cancer is still a disease with the highest percentage compared to other diseases. The cause of mortality in cancer patients is due to cancer metastases from one tissue to another, which complicates the healing process with chemotherapy, surgery or radiotherapy (Geiger & Peeper, 2009). The use of synthetic drugs today is regarded as less effective because of the many side effects caused by Altun and Sonkaya (2018), like doxorubicin, a drug used to treat cancer. The presence of quinones units in large doxorubicin molecules contributes to the formation of radicals and can lead to abnormal thickening of the walls and heart muscle (cardiomyopathy) (Ajaykumar, 2021), causes irritation of the subcutaneous tissue which can cause pain, necrosis (death of other tissues) of the skin and nerve tissue (Lao et al., 2013).

Efforts to reduce the side effects caused by the active ingredients of drugs that are more selective and effective in cancer treatment are needed (Fatmawati et al., 2022). Modern computational chemistry approaches using molecular docking methods can simulate how compounds interact with target proteins and predict the chemical characteristics required for drug activity (Widiyarti, Firdayani, et al., 2019). To obtain accurate results, docking is explicitly done through active sites on proteins. There are at least several proteins that have biological functions in cancer cell development, including MMP-9, MMP-2 (Cancemi et al., 2020) (Webb et al., 2017), Cyclin-A(Ma et al., 2009), p53 (Marei et al., 2021), and BAK (A'yun et al., 2022).

In addition, the design of cancer drugs can be done by hybridization design with a fused hybridization synthesis drug (FHSD) approach using basic materials whose molecules have known cytotoxic activity. Hybrid drug molecules with multiple active sides to interact with multiple receptors will overcome drug resistance, minimize drug side effects and improve the interaction of multiple drug pharmacological sites (Li et al., 2021; Panda et al., 2022).

Essential oil molecules that have been known to have activity as anticancer agents include citronellal (Oliveira Filho et al., 2017) (Crespo-Ortiz & Wei, 2012) (Ho et al., 2020), limonene (Jia et al., 2013) (Araújo-Filho et al., 2021), eugenol (Zari et al., 2021), and methyl

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salicylate (Yeasmin & Choi, 2020) (Karalis et al., 2020) as major components that have the potential to be combined into hybrid molecules. One method of synthesis that involves combining two essential molecules through an esterification reaction. In esterification reactions, homogeneous catalysts are generally used either from inorganic compounds such as HCl, NaOH (Fischer Esterification) or from organic compounds such as DCC-DMAP (Steglich Esterification). Citronellyl carboxylate esters obtained by the Fisher Esterification method have been reported to have anticancer activity (Widiyarti, Megawati, et al., 2019). While other alcohol derivatives obtained by Steglich Esterification method has antibacterial and antimicrobial activity (da Silva et al., 2018).

Recent developments for the purpose of improving the efficiency of the synthesis process are carried out using ultrasonic assistance. Ultrasonic-assisted synthesis is considered efficient due to its easier procedure, shorter reaction time and milder conditions. In synthesis, the role of ultrasonic wave radiation is to improve the interfacial mixture of reactants by lowering the activation energy, thereby increasing the reaction rate (Ramachandran et al., 2013; Majhi, 2021). This study aims to develop a new anticancer drug hybrid molecule through esterification reactions using an essential oil molecule as base material and molecular docking approach to determine its activity.

Method

Material and Equipment

Citronellal (grade: 85%) and methyl salicylate (grade: 72%) were obtained from Essentials Institute, UB, NaBH4 was obtained from Organic Laboratory, UB, DCC and DMAP catalysts were obtained from Sigma Aldrich, all chemicals and reagents were purchased from MERCK, Ultrasonic cleaner krisbow DSA100-GLI-2.8L model KW1801033. Aluminium Plates TLC (Thin Layer Chromatography) (Silicagel 60 F254, Merck). Analysis instruments are FTIR SHIMADZU 8400s, GCMS (GC, Agilent 7890B, MS, Agilent 5977B), HP-5 capillary column (meter), LC C8 column. Molecular Docking with Pyrx 9.0 software using MMP-9, MMP-2, Cyclin A-2, P53 and BAK receptor proteins with PDB ID 4H1Q, 3AYU, 2V22, 2VUK and 6UXM.

Synthesis of Citronellyl Salicylate by Steglich Esterification Method

Salicylic acid (366 mg, 3 mmol) and DCC (118 mg, 3 mmol) in DCM (CH_2Cl_2) (5 ml) were reacted at 40 °C for 30 min. After citronellol (312 mg, 2 mmol), DMAP (50 mg, 0.4 mmol) was added, the reaction was continued for up to 8 hours. The reaction mixture was filtered off and the liquid phase was washed successively with 5% (m/v) HCl (2×5 mL), 5% (b/v) $NaHCO_3$ (3×5 mL) and H_2O (3×5 mL). Anhydrous Na_2SO4 was added to the product, and the organic phase was evaporated under nitrogen gas flow. During the reaction, monitoring was done using TLC. The same procedure was carried out for 16 hours and 24 hours as well as using ultrasonics with varying reaction times of 30, 60 and 90 minutes. The crude compound was columnized with hexane:ethylacetate (9:1) solvent to obtain the pure compound.

Synthesis of Citronellyl Salicylate by Fisher Esterification Method

Citronellol (156 mg, 1 mmol) and salicylic acid (138 mg, 1 mmol) were reacted with 5% (w/w) NaOH catalyst. At 60 °C, the mixture was reacted and refluxed for 3, 6, and 8 hours. The mixture was neutralized with 1% HCl. The mixture was extracted with EtOAc and the organic phase was washed with distilled water to pH 7. The organic phase was dried with anhydrous Na_2SO_4 and the filtrate was evaporated under nitrogen gas flow. During the reaction, monitoring was carried out using KLT (Silicagel 60 F254; hex:EtOAc). The same procedure was carried out using ultrasonics with varying reaction times of 30, 60 and 90 min. To obtain the pure compound, the crude compound was columnized with hexane:ethylacetate (9:1) solvent.

Characterization of citronellyl salicylate

Citronellyl salicylate was characterized using FTIR by applying the product on a diamond widow, scanning at ῡ 500-4000 cm-1. The synthesis products were also analyzed by LC ESI-MS using an Acquity UPLC BEH C18 column in formic acid solvent in acetonitrile and in water with a gradient elution system. The column flow rate was set to 0.3 mL/min.

Molecular Docking Analysis

Molecular docking was performed by creating 3D structures of target compound ligands using Marvin Sketch software and target receptor proteins MMP-9 (PDB ID: 4H1Q), MMP-2 (PDB ID: 3AYU), Cyclin A-2 (PDB ID: 2V22), P53 (PDB ID: 2VUK), BAK (PDB ID: 6UXM), downloaded via (https://www.rcsb.org/). These receptors are separated by their water molecules and native ligands. The receptors were docked in Pyrx 9.0 software with native ligand, then citronellyl salicylate ligand, doxorubicin control ligand, citronellol ligand, and salicylic acid using the Open Babel tab. Energy minimization, conformation, and grid box adjustment for each receptor were performed, and docking was performed until the affinity energy number for molecular docking between the target protein and ligand appeared. The analysis results display the Root Mean Square Distances (RMSD) and binding affinity values of the docking results connections. Interactions between target receptor amino acid residues and ligands can be observed with Biovia Discovery Studio software or LigPlot.

In-Vitro Test of Anticancer Activity

The in-vitro anticancer activity test used 4T1 breast cancer cells from Ma-Chung University laboratory. Cancer cells were dissected and collected in 96-well plates and added to RPMI medium, and the bottom 12 wells were left to be used as solvent control, cell control and media control. Samples were made in graded concentrations of 1000, 500, 250; 125; 62.25; 31.25; 15.625; and 7.813 µg/mL were added to the wells and incubated for 24 hours. This procedure was repeated three times for each concentration. After the test method using MTT, the absorbance was measured at a wavelength of 595 nm using an ELISA reader to determine the IC50 value of the sample. Percent cell viability was calculated using the absorbance data obtained.

Result and Discussion

Synthesis of Citronellyl Salicylate

The citronellyl salicylate product synthesized according to the reaction in Figure 1 gives a pale-yellow liquid. The highest yield of citronellyl salicylate by Fischer's method of 95.51% was obtained using reflux aid for 8 hours and by Steglich's method for 24 hours. The longer reaction time under reflux, the larger the product obtained. However, due to the length of the reaction process, the use of ultrasonic-assisted can be used to overcome these weaknesses.

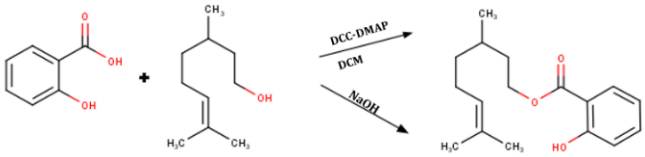


Figure 1. Ultrasonic and Conventional Synthesis of New Salicylate Ester Derivatives

According to Figure 2A. that the use of ultrasonic in the synthesis of citronellyl salicylate by both Steglich and Fischer methods tends to reduce the yield over time. Longer synthesis times increase the frequency of cavitation energy oscillations. This condition causes the ultrasonic to reach high pressure and re-breaking the citronellyl salicylate molecular structure that has been formed (Karthikesh & Yang, 2021). Comparing the synthesis from time to time, the Steglich method always gives higher yields than the Fischer method. This indicates that the DCC-DMAP catalyst works more effectively than his NaOH catalyst. The effectiveness of the Steglich catalyst derives from the ability of DMAP to donate amine substituents with excellent nucleophilic properties, and its stabilization of the pyridinium ion enhances reaction kinetics. On the other hand, DCC and carboxylic acids, can form an O-acylisourea intermediate that reacts with DMAP (Jordan et al., 2021), thereby accelerating and facilitating the binding of alcohols to produce esters. The phenomenon of the role of DCC-DMAP catalysis was also discovered in a study done by (Thuy Giang et al., 2021). While the Fischer reaction, the resulting ester can undergo a reversible reaction, ensuing in low yields. The synthesis by ultrasonic-assisted described above has advantages in terms of reaction time when compared to the citronellyl salicylate reaction using the reflux method, whether performed by the Steglich method or the Fischer method, as shown in Figure 2B.

Maximization of the heating conditions in the ultrasound-assisted synthesis of citronellyl salicylate using the Steglich and Fischer methods yielded maximum crude product yields of 63.56% and 65.8%, respectively, during a reaction time of 30 min. Data from each of the optimal crude oil yields can be used as a basis for analyzing the abundance of citronellyl salicylate using LC-ESI-MS.

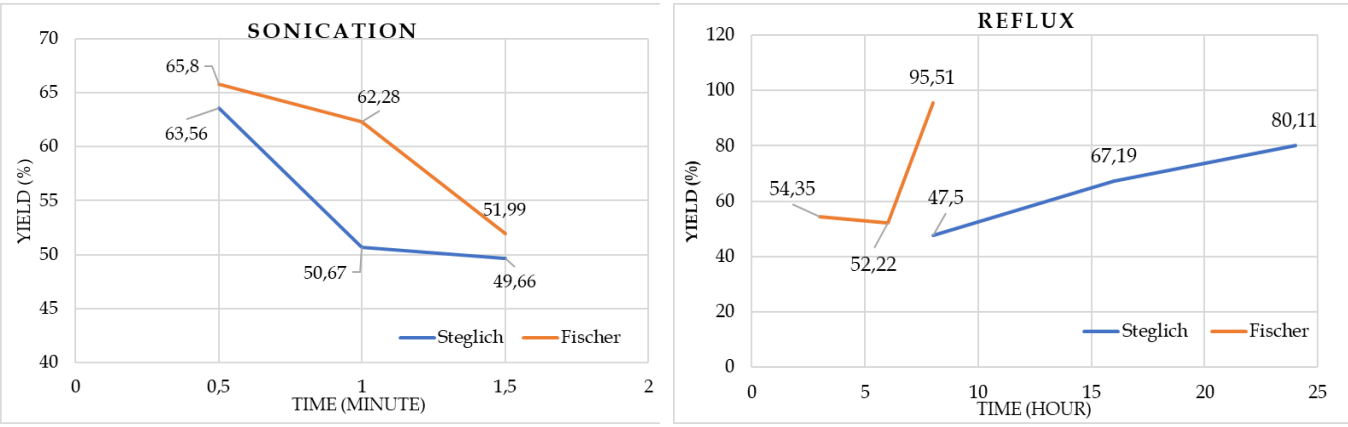


Figure 2. Effect of Sonication and Reflux Time on Percent Yield

Characteristics of Product Synthesis

The spectrum of citronellyl salicylate analysis by FTIR is shown in Figure 3. The appearance of absorption bands at $\bar{\upsilon}$ 1650 - 1710 cm^{-1}, indicating the presence of C=O ester groups, and bands at $\bar{\upsilon}$ 1230 - 1270 cm^{-1}, indicating the presence of C-O ester groups, are two functional groups that characterize this ester compound.

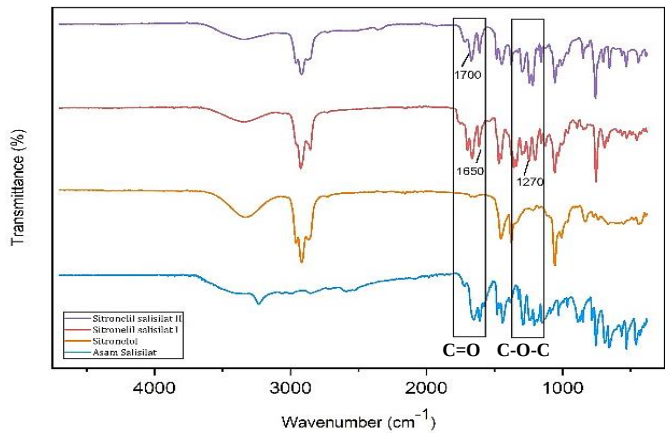


Figure 3. FTIR Spectrum of citronellyl salicylate I (Steglich method) and citronellyl salicylate II (Fischer method)

Figure 4 shows the mass chromatogram of the synthesized citronellyl salicylate. In accordance with the crude yield presented in Figure 2. Also, the abundance of the citronellyl salicylate compound synthesized by the Steglich method (Figure 4A) was 19.58%, which is higher than the abundance of the compound synthesized by the Fischer method (Figure 4B) of 11.06%. The citronellyl salicylate synthesis product appears in the mass spectrum as the citronellyl salicylate bound NH4+ ion or the molecular weight of $[M+NH4-H]^+$ as a base peak at m/z 293 and m/z 294 $[M+NH4]^+$.The possibility of the formation of $[M+NH_4]+$ as shown in spectra of ester compounds from the reaction can be influenced by the presence of ammonium formate in the acetonitrile-water solvent mixture when using the ESI technique (Steckel & Schlosser, 2019).

Molecular Docking Analysis

Molecular docking of each receptor in this study was based on an RMSD of 0 Å. Binding affinity (ΔG) values for the receptor proteins used in this study are shown in Table 1. The docking results of compound S1 (citronellyl salicylate) against MMP9, MMP2, and CyclinA2 receptors with continuous values of -8.4, -8.1, and -6.8 kcal/mol were compared to the reactants (citronellol and salicylic acid) and the positive control doxorubicin. This means that the bound compound S1 is more stable and better inhibits the performance of these receptors. On the other hand, the binding affinity values for the P53 and BAK receptors responsible for cell death are -5.3, and -5.6 kcal/mol which are greater than the binding to the positive controls (native ligand and doxorubicin). This fact suggests that compound S1 can also induce apoptotic in cancer cells.

A visualization of the hydrogen interactions of compound S1 with each receptor tested is shown in Figure 5. Among the interactions of compound S1 with different receptors, the interaction with the MMP-9 receptor appears to exhibit the most hydrogen and non-hydrogen bonds indicating that compound S1 bonds more strongly in inhibiting the performance of proteins as cancer-causing target receptors.

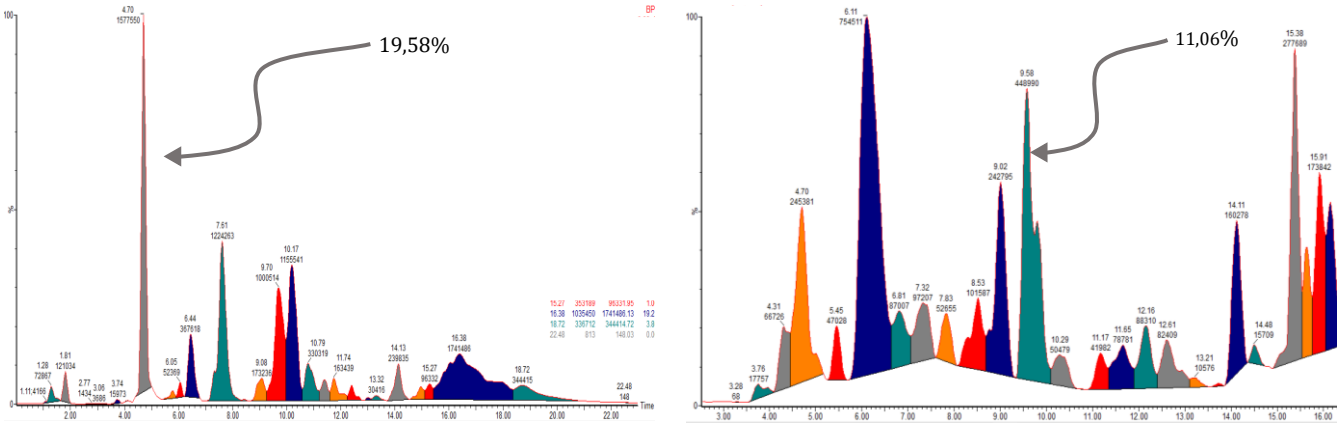
Compound S1 has log P = 4.52, hydrogen bond donor ≤ 5, hydrogen bond acceptor ≤ 10, and molecular weight ≤ 500 Da, suggesting that compound S1 has potential as a new anticancer drug candidate. The suitable of compound S1 to Lipinski's law follows the guidelines given (Daina et al., 2017).

Table 1. Molecular docking results for control doxorubicin, reactants and citronellyl salicylate compounds

	Compound	ΔG (kkal/mol)	Bond Interaction	Amino Acid Residue
MMP-9	OXX*	-8.8	Hydrogend	Ala189, Leu188, Ala191
			Hydrophobic	Met247 His226, Pro246, Tyr179, Pro193
	DXB	-7.6	Hydrogend	Gln227
			Hydrophobic	Met247, His226, Pro246, Tyr179, Ala189.
	S22	-6.5	Hydrogend	Ala242, Tyr245, His226, Leu243
			Hydrophobic	Leu222, Met247, Arg249, Tyr248
	S0	-6.5	Hydrogend	Ala242, Tyr245, Met247, Leu2 43
			Hydrophobic	Leu222, Val223, Arg249, Tyr248
	S1	-8.4	Hydrogend	Ala242, Tyr245
			Hydrophobic	Leu222, Met247, Arg249, Tyr248
MMP-2	DXB	-7.9	Hydrogend	Ala85
			Hydrophobic	Tyr73, His84, Leu81, Ala83
	S22	-6.8	Hydrogend	Ala139, Ala136, Leu137, Ile141
			Hydrophobic	Tyr142, Leu116, Thr143, Phe148
	S0	-6.1	Hydrogend	Ala136, Leu137, Ile141
			Hydrophobic	Thr143, Leu116, His120, Val117
	S1	-8.1	Hydrogend	Ala83, Leu82
			Hydrophobic	Leu81, Tyr142, His124, Gly180
Cyclin A2	C35*	-7.6	Hydrogend	Ile281, Gln254, Tyr286, Arg250
			Hydrophobic	Leu251, Met210, Ile213, Tyr280
	DXB	-6.7	Hydrogend	Gln254, Thr282, Trp217
			Hydrophobic	Thr285, Leu214, Ile213, Gln406
	S22	-4.8	Hydrogend	Arg250
			Hydrophobic	Leu214, Ile213, Leu253, Met210

	Compound	ΔG (kkal/mol)	Bond Interaction	Amino Acid Residue
MMP-9	OXX*	-8.8	Hydrogend	Ala189, Leu188, Ala191
			Hydrophobic	Met247 His226, Pro246, Tyr179, Pro193
	DXB	-7.6	Hydrogend	Gln227
			Hydrophobic	Met247, His226, Pro246, Tyr179, Ala189.
	S22	-6.5	Hydrogend	Ala242, Tyr245, His226, Leu243
			Hydrophobic	Leu222, Met247, Arg249, Tyr248
	S0	-6.5	Hydrogend	Ala242, Tyr245, Met247, Leu2 43
			Hydrophobic	Leu222, Val223, Arg249, Tyr248
	S1	-8.4	Hydrogend	Ala242, Tyr245
			Hydrophobic	Leu222, Met247, Arg249, Tyr248
P53	S0	-4.8	Hydrogend	Arg250
			Hydrophobic	Leu214, Ile213, Trp217, Gln254
	S1	-6.8	Hydrogend	Asp216
			Hydrophobic	Arg250, Gln254, Met210, Ile213
	P83*	-7.4	Hydrogend	Asp228
			Hydrophobic	Glu221, Pro227, Leu145, Thr230
	DXB	-7.7	Hydrogend	Thr150, Asp228, Asp148
			Hydrophobic	Pro151, Cys220, Leu145, Val147
	S22	-5.2	Hydrogend	Val147, Thr230, Leu145, Glu221
			Hydrophobic	Pro222, Trp146, Thr150
	S0	-4.5	Hydrogend	Leu145, Asp228
			Hydrophobic	Trp146, Pro222, Thr230, Glu221
	S1	-5.3	Hydrogend	Thr230, Val147
			Hydrophobic	Thr150, Asp228, Glu221, Pro222
BAK	PEE*	-5.4	Hydrogend	Asn86, Val129
			Hydrophobic	Gly133, Ala130, Ile85, Leu132
	DXB	-7.3	Hydrogend	Asn86, Arg137
			Hydrophobic	Gly133, Ala107, Ile85, Leu132
	S22	-5.4	Hydrogend	Asn86, Ile85, Arg137
			Hydrophobic	Asn90, Phe93, Tyr89
	S0	-4.8	Hydrogend	Asn86, Asp90, Arg137
			Hydrophobic	Gly82, Ile85, Phe93, Tyr89
	S1	-5.6	Hydrogend	Arg137
			Hydrophobic	Gly82, Ile85, Phe93, Tyr89, Leu132, Asn86, Val129

* Native ligand
**DXB: doxorubicin; S22: salicylic acid; S0: citronellol; S1: citronellyl salicylate



(A) (B)

Figure 4. LC ESI-MS Chromatogram (formic acid:acetonitrile) of citronellyl salicylate by Steglich (A) and Fisher (B) methods.

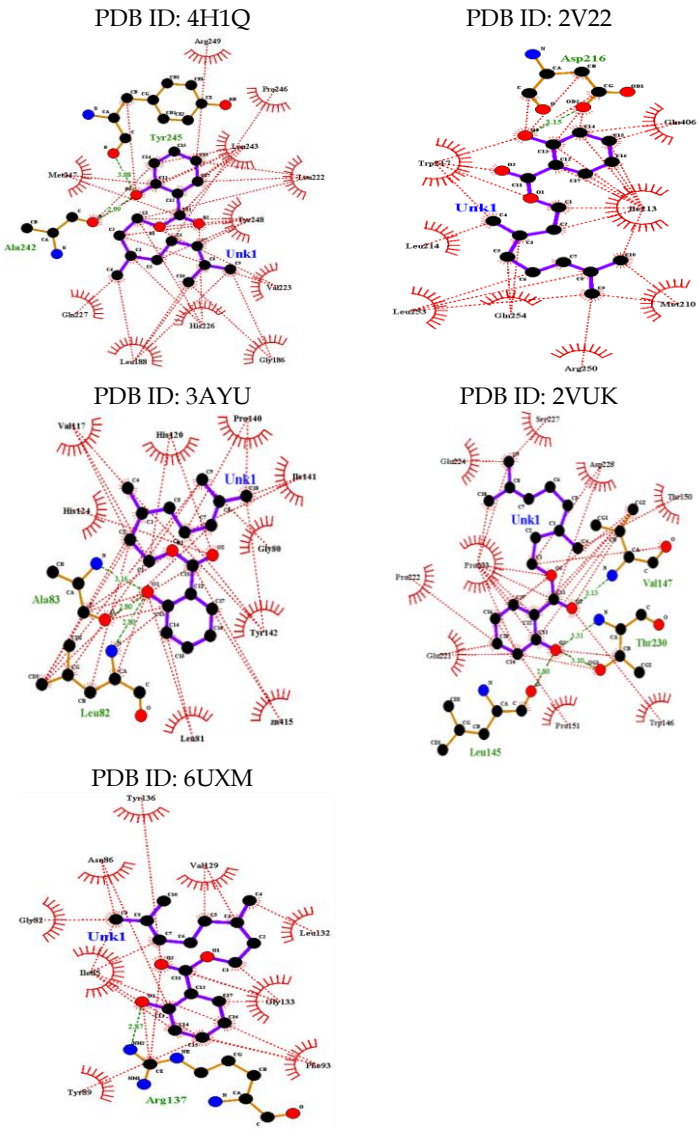


Figure 5. Visualization of Citronellyl Salicylate Interaction with Target Receptors Using LigPlot

In-Vitro Test of Anticancer Activity

As shown in Figure 6., pure isolates of citronellyl salicylate from isolation using chromatography columns were tested against 4T1 breast cancer cells using Vero cells as a control. Figure 6. shows that the morphology of 4T1 cells changed to an irregular shape, the cells were destroyed, the cell nucleus were damaged, and the cells died. This indicates that citronellyl salicylate has the ability to damage cancer cells. The absence of morphological changes in Vero cells indicates that the drug candidate, citronellyl salicylate, acts selectively, attacking only cancer cells and didn't significantly affect normal cells. In addition, various concentrations of the citronellyl salicylate isolate were tested for viability of her 4T1 cancer cells. Vero cell viability is higher than cancer cell viability when treated with a citronellyl salicylate compound.

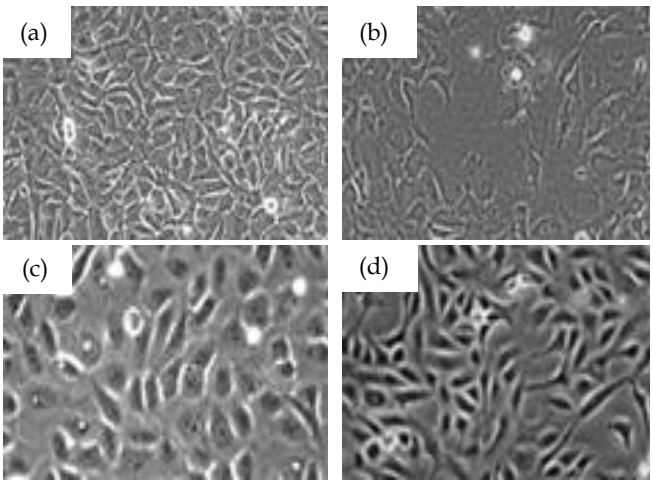


Figure 6. Results of 4T1 cancer cells (a) before and (b) after treatment with citronellyl salicylate. Results of Vero cells before (c) and (d) after treatment with citronellyl salicylate; 100x magnification

Conclusion

Synthesis of citronellyl salicylate by the Steglich method with ultrasonic assistance gives a yield of 12.69% with a time of 30 minutes which is better than the Fischer method. The interaction strength of citronellyl salicylate compounds in inhibiting protein receptors is on the order of MMP-9 (-8.4 kcal/mol), MMP-2 (-8.1 kcal/mol), and cyclin-A (-6.8 kcal/mol), apoptotic events in cancer cells are induced against receptors on the scale of BAK (−5.6 kcal/mol) and P53 (−5.3 kcal/mol). Based on Lipinski's Law predictions, citronellyl salicylate compounds can also be declared as new compounds with potential as anticancer agents. Results from in vitro activity tests against 4T1 cancer cells show that the drug candidate, citronellyl salicylate, acts selectively, attacking only cancer cells and didn't interfere significantly with normal cells as indicated by cell morphology and percent cell viability.

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Author Contributions

Authors listed in this article contributed to the research and development of the article.

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Conflicts of Interest

In writing this article, we sincerely declare that there are no conflicts of interest that may affect the objectivity and integrity of the results.

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