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Compounds Derived from Calamansi Orange Peel Exhibit Potency as HER2 Inhibitors in Breast Cancer

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ABSTRACT:

Breast cancer indicated uncontrollably proliferation and spread of cells that related to HER2 overexpression. In 2020, Indonesia showed the highest prevalence of breast cancer with 65.858 cases. Several therapeutic methods had been carried out, including chemotherapy, and radiation therapy. However, it induced various side effect during the treatment. Herbs are considered as an alternative therapy for treatment. The calamansi orange peel is considered as a waste, yet it known for containing various therapeutic properties. The therapeutic efficacy of calamansi orange peel in the treatment of breast cancer requires more investigation. Thus, the purpose of this study was to determine the potency of compounds from calamansi orange's peel against breast cancer. Five compounds of calamansi orange peel were collected as a sample from PubChem database. Those compounds were addressed for several analysis, such as analysis of biological activity by using PASS Online, toxicity analysis by using ProTox 3.0, and drug-likeness analysis by using SWISS ADME. Furthermore, HER2 protein was selected as a protein target. The interaction between sample compounds and protein target was simulated through molecular docking analysis by using PyRx software and continued with amino acid residue analysis. Moreover, further analysis toward HER2 protein was conducted through protein-protein network analysis using STRING database. Result showed that all sample compounds had biological activity related to anticancer activity, such as antineoplastic, apoptosis agonist, and anti-inflammatory with Pa score 0.5 – 0.7. Moreover, the drug-likeness analysis according to Lipinski's rule of five depicted that all sample compounds fulfil the required parameters. Those compounds also demonstrated low toxicity level with average toxicity class 4 and 5. Interaction of compounds toward protein target demonstrated all sample compound had comparable binding affinity score as compared to native ligand and reference ligand. In brief, 3-Isopropenyl-5,5-Dimethyl-Cyclopentene had binding affinity -6,5 kcal/mol. The native ligand and sample compound both shared amino acid residues in Ala751, Met774, Ser783, Leu785, and Thr862. Additionally, HER2 protein depicted a link to the immune system, and cell proliferation pathway. Thus, compounds derived from calamansi orange peel considered had potential therapeutic effect for breast cancer treatment.

KEYWORDS: *binding affinity, breast cancer, calamansi orange, HER2, molecular docking.*

INTRODUCTION :

Cancer is characterized as an abnormal condition of cells that have uncontrolled division throughout the body [1]. According to World Health Organization (WHO), cancer caused 8.9 million deaths in the world with 70% of instances originated from developing countries[2,3]. The breast cancer cases increased by the time which data from 2020 showed that it also responsible for 685.000 deaths in worldwide[4]. In Indonesia, the prevalence of breast cancer is increased with a total of 396,914 cases, and 234,511 deaths in 2020[5,6]. Several treatments of breast cancer therapy had been widely used, including chemotherapy, radiation therapy, and hormone therapy[7,8]. However, those treatments are known for causing various side effects, such as urination disorders, fatigue, vomiting, weight loss, anaemia, and others. Apart from that, all those treatments are also needing a high cost. Thus, an alternative treatment and therapy that affordable are needed[9].

Herbs is considered as an alternative therapy for disease. The herbs therapy utilized the plant's compound with research approach[10,11]. Oranges are well-known as a fruit with huge health benefit. Previous studies found that citrus-related plants had various bioactivity, including quercetin and flavonoid compound in lime[12], alkaloid, flavonoid, phenolic, and terpenoid compound in kaffir lime[13], tangeretin and kaempferol in tangerine[14,15], and terpenoid compound on lime berry[16]. In brief, calamansi orange (*Citrus microcarpa*) also known for containing various compounds, namely monoterpene, sesquiterpene, ester, ketone, alcohol, aldehyde, flavonoid, limonoid and phytosterol groups of compounds[17]. However, most of calamansi orange peel are not commonly utilized and become a waste.

HER2 or receptor tyrosine-protein kinase erbB-2, is a transmembrane glycoprotein measuring 185 kDa. HER2 bind with HER1 and/or HER3 to initiate phosphorylation process. It related to the cell division, survivability process, migration, adhesion, and angiogenesis. Simultaneously, it also influences the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), Phosphatidylinostiol-3-kinase (PI3K/AKT), and RAS/RAF/MEK/ERK pathways[18,19,20]. Heterodimers in HER receptor protein family had more signal potency compared to homodimers structure. However, there is also a possibility that HER2 homodimer formed when the HER2 is overexpressed. The overexpression of HER2 result in uncontrol cell division, migration, survivability, and signalling pathway activity[21,22,23]. Therefore, this study aimed to identify compounds derived from calamansi orange that had potential ability as an inhibitor of HER2 in breast cancer. The research analysis carried out through protein network and drug discovery analysis.

MATERIALS AND METHODS:

Ligand Preparation

Simplified Molecular Input Line Entry System (SMILE) of five compounds derived from calamansi orange peel (*Citrus microcarpa*), namely: D-Limonene, R-(+)-Citronellal, 3-Isopropenyl-5,5-Dimethyl-Cyclopentene, Gamma Terpinene, and Citronellol were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Lapatinib (CID: 20809) was chosen as reference ligand and 03Q was utilized as native ligand. The 3D structure of sample compounds, reference ligand, and native ligand were collected in .sdf format.

Protein Preparation

HER2 protein 3D Structure sample (PDB: 3PP0) was taken from PDB database (https://www.rcsb.org/) in .pdb format. Furthermore, the water molecule from the protein sample was eliminated through Discovery Studio software. The polar hydrogen was added by using PyMOL Software.

Biological Activity Analysis

The biological activity of sample compounds, Lapatinib (reference ligand), and 03Q (native ligand) were identified by using Prediction of Biologically Active Substances (PASS) Online (http://www.way2drug.com/passonline/). Several biological activities related to breast cancer mechanisms were selected, such as anti-inflammatory, apoptosis agonist, and antineoplastic in breast cancer. The results of biological activity determined based on the Probability of Activity (Pa) and Probability of Inactivity (Pi) score on a scale of 0-1. The Pa score in range 0.5 - 0.7 were addressed for further analysis[24].

Toxicity Analysis

The toxicity analysis of sample compounds and reference ligand were carried out by using ProTox 3.0 (https://tox.charite.de/protox3/). It aimed to classify the toxicity of compounds using Globally Harmonized System (GHS) and toxicity prediction based on the Lethal Dose (LD50) in mg/kg. Additionally, toxicity prediction as hepatoxicity, immunogenicity, and cytotoxicity were also measured in range of scale 0-1[25].

Drug-likeness Analysis

The drug-likeness analysis was carried out by using SWISS ADME (http://swissadme.ch). The analysis was addressed based on the Lipinski's rule of five parameters, including: molecular Weight (MW) in 500 g/mol, number of donor hydrogen (5), number of acceptor hydrogen (10), and lipophilicity (MlogP) in 4.15. The analysis aimed to determine by the absorption ability of compounds into the human body[26].

Molecular Docking Analysis

All compound samples were then addressed for simulation of interaction toward HER2 protein through molecular docking. The analysis was carried out utilizing specific docking method in PyRx software by selecting the active site of HER2 protein as the target with adjusted grid box coordinate (X = 16.94 Å; Y = 15.18 Å; Z = 21.62 Å) with the center coordinates at (X = 19.01 Å; Y = 18.34 Å; Z = 27.51 Å). The interaction of sample compounds and protein were measured as binding affinity score (kcal/mol) that compared to reference ligand, and native ligand. In brief, the binding affinity values were acquired from RMSD model 0, and the models were saved in .pdbqt format for visualization of ligand-protein interactions.

Analysis of Amino Acid Residues

The complex of sample compound and HER2 protein were addressed for amino acid residues analysis by using Discovery Studio software. The interaction type of amino acid residues was compared to the reference ligand and native ligand.

Protein-Protein Interaction Network (PPIN) Analysis

All sample compounds were analysed by using STITCH database (http://stitch.embl.de/) to explore its interaction towards various protein. Furthermore, those various protein were subjected for further analysis by using STRING database (https://string-db.org/). It aimed to identify the protein interaction related to cancer pathway through gene ontology (GO) and false discovery (FDR) rate.

RESULT:

All the sample compounds derived from calamansi orange peel possessed biological activity related to cancer, including anti-inflammatory, apoptosis agonist, and antineoplastic in breast cancer. In brief, D-Limonene and citronellol had high anti-inflammatory activity with Pa score more than 0.5. All sample compounds also demonstrated the apoptosis agonist activity with high Pa score, except gamma terpinene which depicted Pa score in 0.348. However, only D-limonene that showed high Pa score in antineoplastic activity. Whilst, lapatinib which served as a control is specifically demonstrated the bioactivity as antineoplastic in breast cancer (Figure 1).

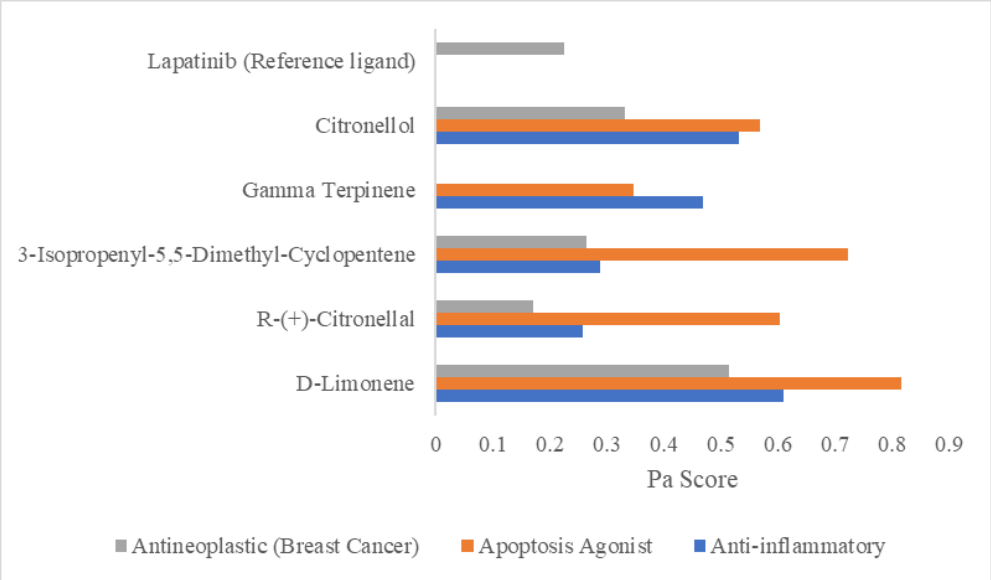

Figure 1. Predicted biological activity of compounds derived from of calamansi orange peel

Further identification in toxicity analysis showed that all sample compounds and reference ligand are classified into toxicity class 4 and 5. Moreover, further analysis demonstrated that all sample compound also possessed inactive probability for hepatotoxicity, immunotoxicity, and cytotoxicity. Therefore, the sample compounds are considered as safe (Table 1).

Table 1. Predicted toxicity properties of compounds derived from calamansi orange peel

Compounds	LD50 (mg/kg)	Toxicity Class	Accuracy (%)	Probability		
				Hepatotoxicity	Immunotoxicity	Cytotoxicity
D-Limonene	4400	5	100	0.76 (Inactive)	0.95 (Inactive)	0.82 (Inactive)
R-(+)-Citronellal	2420	5	100	0.70 (Inactive)	0.99 (Inactive)	0,82 (Inactive)
3-Isopropenyl-5,5-Dimethyl-Cyclopentene	3850	5	69.26	0.81 (Inactive)	0.96 (Inactive)	0.77 (Inactive)
Gamma Terpinene	2500	5	100	0.83 (Inactive)	0.98 (Inactive)	0.82 (Inactive)
Citronellol	3450	5	100	0.84 (Inactive)	0.99 (Inactive)	0.86 (Inactive)
Lapatinib (Reference ligand)	1500	4	23	0.80 (Active)	0.96 (Active)	0.76 (Active)

After all the sample compounds are demonstrated the biological activity related to anti-cancer mechanism and showed safety, further analysis to determine the drug-likeness properties are conducted. As compared to Lapatinib (reference ligand), result depicted that all selected compounds derived from calamansi orange peel fulfil the required parameters of Lipinski rule of five. All sample compounds did not demonstrate any violation towards molecular weight threshold, hydrogen donor number, hydrogen acceptor number, and lipophilicity properties (Table 2).

Table 2. The drug-likeness characteristics of calamansi orange peel derived compounds

Compounds	Lipinski Rule of Five				Violation
	H-Bond Donor < 5	H-Bond Acceptor < 10	Molecular Weight < 500 g/mol	MLOGP < 4.15	
D-Limonene	0	0	136.23	3.27	0
R-(+)-Citronellal	0	1	154.25	2.59	0
3-Isopropenyl-5,5-Dimethyl-Cyclopentene	0	0	136.23	3.27	0
Gamma Terpinene	0	0	136.23	3.27	0
Citronellol	1	1	156.27	2.70	0
Lapatinib (Reference ligand)	2	8	581.06	3.44	1
03Q (Native ligand)	2	9	493.87	2.46	0

All sample compounds and reference ligand are depicted bind into the same binding site location as native ligand (Figure 2). It also indicated by similar amino acid residues which shared between sample compounds, reference ligand, and native ligand. In specific, shared hydrophobic interaction and van der walls interaction, including Leu726, Ala751, Met774, Ser 783, Leu785, Leu852, and Thr862 (Table 3). However, the binding affinity score of sample compounds are still higher than native ligand. In brief, those binding affinity score are almost similar to reference ligand with score -6.9 kcal/mol (Table 4).

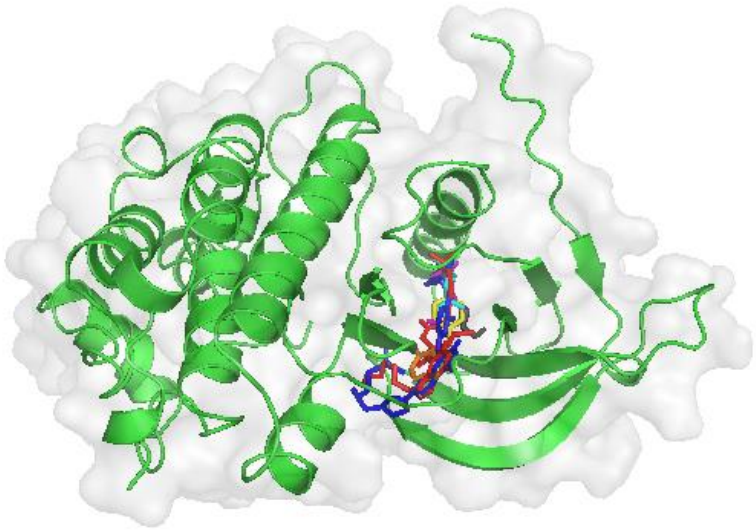

Figure 2. Complex of HER2 protein and compounds derived from calamansi orange peel: D-Limonene (Yellow), R-(+)-Citronellal (Pink), 3-Isopropenyl-5,5-Dimethyl-Cyclopentene (Cyan), Gamma Terpinene (Orange), dan Citronellol (Grey), 03Q (Red), and Lapatinib (Dark Blue)

Table 3. Amino acid residues and chemical bond of compounds derived from calamansi orange peel toward HER2 Protein

Compounds (Ligand)	*Hydrogen Bond*	*Hydrophobic Bond*	*Halogen Bond*	*Van Der Waals Bond*	*Unfavorable Bond*
D-Limonene	-	Val734, Ala751, Lys753, Met774, Leu785, Leu796, Phe864	-	Glu770, Ser783, Thr798, Thr862, Asp863	-
R-(+)-Citronellal	Asp863: 3.87 Å	Ala771, Met774, Leu785, Leu796, Phe864	-	Lys753, Glu770, Ser783, Thr798, Thr862	-
3-Isopropenyl-5,5-Dimethyl-Cyclopentene	-	Lys753, Ala771, Met774, Leu785, Leu796, Phe864	-	Glu770, Ser783, Thr798, Thr862, Asp863	-
Gamma Terpinene	-	Leu726, Val734, Ala751, Lys753, Leu852	-	Thr798, Leu800, Met801, Gly804, Thr862, Asp863	-
Citronellol	Ala751: 4.33 Å Lys753: 5.01 Å Leu796: 4.82 Å	Ala771, Met774, Leu785, Phe864	-	Val734, Ile752, Glu770, Ser783, Val797, Thr798, Thr862, Asp863	-
Lapatinib (Reference ligand)	Ser728: 5.10 Å Gln799: 6.41 Å Arg849: 4.72 Å	Leu726, Val734, Ala751, Lys753, Met774, Leu785, Leu796, Thr798, Cys805, Leu852	Glu770, Asp863	Gly727, Gly729, Ala771, Ser783, Leu800, Gly804, Asn850, Thr862, Phe864, Gly865	Met801
03Q (Native ligand)	Ser728: 4.90 Å Gln799: 6.96 Å	Leu726, Ala751, Met774, Leu785, Leu852, Phe864	Glu770, Leu796	Gly729, Val734, Ile752, Lys753, Ala771, Ser783, Val797, Thr798, Gln799, Gly804, Cys805, Asn850, Thr862, Asp863	Met801, Arg849

Table 4. Binding affinity score of compounds derived from calamansi orange peel toward HER2 Protein

Compounds	*Binding Affinity* (kcal/mol)
D-Limonene	-6.5
R-(+)-Citronellal	-6.2
3-Isopropenyl-5,5-Dimethyl-Cyclopentene	-6.6
Gamma Terpinene	-6.5
Citronellol	-6.4
Lapatinib (Reference ligand)	-6.9
03Q (Native ligand)	-11.1

HER2 protein as a target protein had essential role in breast cancer pathway. Protein interaction network demonstrated that HER2 protein closely related to regulation of cell population proliferation. HER2 protein connected with most protein in the network and low FDR score about 1.13e-18. Furthermore, protein interaction network also depicted the relation with regulation of apoptotic process (Figure 3, Table 5).

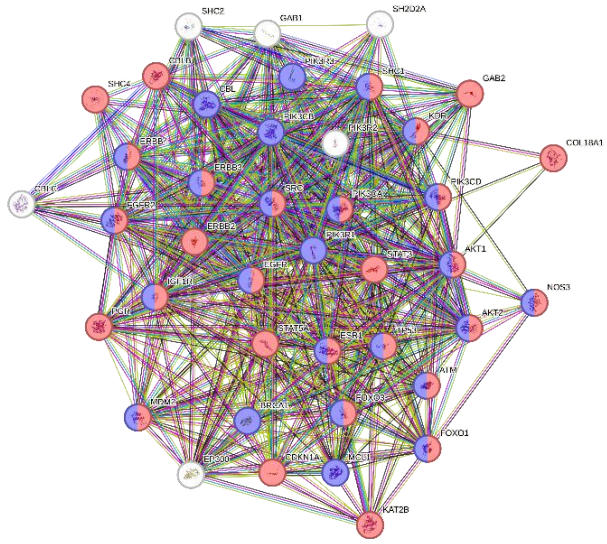

Figure 3. Protein-protein interaction pathway related to breast cancer: Regulation of cell population proliferation (red) and Regulation of Apoptotic Process (Blue)

Table 5. Biological process of proteins related to breast cancer

GO-term	Biological Process	Proteins	False Discovery Rate (FDR)
GO:0042127	Regulation of cell population proliferation	SHC4, CBLB, SHC1, GAB2, ERBB4, KDR, FGFR2, ERBB3, SRC, PIK3CA, PIK3CD, PGR, IGF1R, EGFR, STAT3, AKT1, COL18A1, NOS3, STAT5A, ESR1, TP53, AKT2, MDM2, FOXO3, ATM, CDKN1A, FOXO1, KAT2B	1.13e-18
GO:0042981	Regulation of apoptotic process	PIK3R3, CBL, SHC1, ERBB4, ERBB3, KDR, FGFR2, SRC, PIK3CA, PIK3CD, PIK3R1, IGF1R, EGFR, AKT1, NOS3, ESR1, TP53, AKT2, ATM, FOXO3, MDM2, MCL1, FOXO1	1.36e-15

DISCUSSION:

Human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor that belongs to epidermal growth factor receptor (EGFR) family. HER2 is expressed at low rate in normal tissues by starting the intracellular pathways that support cell survival and proliferation[27,28]. However, the overexpression of HER2 could lead to uncontrolled cell growth, such as in breast cancer. Approximately, about 20% of breast cancer had overexpression of HER2 and lead to disease progression[27]. Therefore, the use of targeted inhibitors is essential to manage the aggressive cancer and improve the patient outcomes. Lapatinib is small molecule that specifically targeting HER2 protein. By inhibiting the HER2 receptors, lapatinib blocks downstream signal pathway that promote cell proliferation and cell survival in cancer[29].

Through the simulation of the ligand-target protein interaction, the molecular docking simulation plays a critical role in the identification of potential inhibitors[30]. In this research, all sample compound derived from calamansi orange peel determined as a ligand and simulated the interaction toward HER2 protein. Most of sample compounds had binding affinity score that almost similar to Lapatinib as reference ligand supported by hydrogen bond and hydrophobic bond[31]. Nevertheless, it had significant score of binding affinity as compared to native ligand with -11.1 kcal/mol, shows that all ligands have stable and strong energy to bind towards HER2 protein[32]. Moreover, there are similarities amino acid residues in hydrophobic and Van der Waals bonds between the sample ligands, native ligands and reference ligands, including: Leu 726, Ala 751, Met 754, Ser 783, Leu 785, Leu 852, and Thr 862 which indicate that the sample ligand's have a similar opportunity to native ligand and reference ligand as HER2 inhibitor[33].

Sample compounds have been extensively studied as potential alternative therapies for breast cancer. Further investigations are necessary to fully validate the herbal compound efficacy and its activity. The results of PASS Online analysis determined based on Pa score and the Pi score (Figure 1). The Pa score showed the probability of compound for possessed certain biological activity, while Pi value shows the opportunity of a compound to be inactive to carry out certain biological activity. The Pa score that less than 0.5 indicated poor potential, 0.5< Pa < 0.7 suggested moderate potential, and Pa score greater than 0.7 indicated high potency of compound in certain biological activity[34]. All the sample compound derived from calamansi orange peel showed high potential biological activity as anti-inflammatory agent and apoptosis agonist. Whilst, only D-limonene showed high potential in antineoplastic activity. All those compounds possessed high potency as compared to Lapatinib. Those compounds also classified in low toxicity class and less harmful toward cells and liver organ compared to Lapatinib, which suggests all ligands were non-toxic and had no risk to cause cytotoxicity, hepatotoxicity, and immunotoxicity[35].

In the context of drug pharmacokinetics, the mechanisms of Absorption, Distribution, Metabolism, and Excretion (ADME) are strongly linked to Lipinski's Rule of Five[36,37]. Although the primary focus of ADME is pharmacokinetics—what the body does to a medication—it is crucial to comprehend the interaction of ADME with pharmacodynamics, which is termed as what the drug does to the body, including drug administration, circulation, chemical alteration and elimination mechanism in the body[38]. All the compound derived from calamansi orange peel fulfil the threshold of required parameters. It is essential for developing the medicine for oral administration. Furthermore, the STRING result demonstrates that the calamansi ligand, which interacts with the HER2 protein, is involved in the inhibition mechanism and influences the regulation of the ERBB pathway, protein kinase at the receptor, cell proliferation, apoptotic process, and immune system. The ligand which interacts with the HER2 protein will cause the entire signalling pathway under HER2 will be inhibited. As a result, the active number of HER2 will decrease because of the inhibition. Despite this, the compounds derived from calamansi oranges peel is still need to be updated and analysed. Moreover, *in vitro* and *in vivo* research must be conducted further to confirm the results[33].

CONCLUSION:

Various compounds derived from calamansi oranges peel (*Citrus microcarpa*) offer a promising alternative therapy for breast cancer due to their potency as an inhibitor of HER2 protein compared to Lapatinib.

Furthermore, the compounds demonstrated bioactivity related to anti-cancer, such as anti-inflammatory activity and apoptosis agonist activity. Additionally, it also showed safety and potency in drug pharmacokinetics.

CONFLICT OF INTEREST:

There are no disclosed conflicts of interest associated with this study by the authors.

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