Bioinformatics Study: *Syzygium samarangense* Leaves Chalcone Extract as Lung Cancer Proliferation Inhibitor

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Abstract: Wax apple (*Syzygium samarangense*) is a plant commonly found in Indonesia with various health benefits, especially leaves. Anticancer effect in chalcone of the extract of *Syzygium samarangense*’s leaves has been proven. This study identifies chalcone extract potential, a flavonoid active compound group in *Syzygium samarangense*, as a proliferation inhibitor in lung cancer based on bioinformatics analysis. The extract of *Syzygium samarangense*’s leaves identification analysis shows that our extract contains flavonoids with a 3.63% w/w yield. We identified protein target from the main candidate of active compound from chalcone in *Syzygium samarangense*, Dimethyl Cardamonin (DMC). A total of 263 potential targets has been collected, and 21 of 263 genes were further analyzed, yielding five gene protein roles in cancer pathways in humans based on DAVID V6.8. Furthermore, expression level and survivabilities of 5 genes in lung cancer were analyzed, resulting in only HDAC1, a significant result. This result was further strengthened by molecular docking DMC with HDAC1 protein compared to the native ligand and inhibitor, showing no significant differences in binding energy between DMC and all comparator compounds. The compound interaction profile shows that DMC interacts similarly with zolinza. Thus, DMC was predicted to be potential lung cancer therapy by inhibiting HDAC1 hence cancer proliferation. However, additional research in the form of *in vitro* and *in vivo* proves our prediction.

Keywords: *Syzygium samarangense*; chalcone; proliferation; lung cancer; bioinformatics.

1. Introduction

Wax apple (*Syzygium samarangense*) is a plant from the Myrtaceae family that is commonly found in Asia-Pacific, especially in Indonesia [1,2]. Leaf extract from this plant has some bioactivities. Leaf extract from this plant has some bioactivities, such as antioxidant, lipolytic, antityrosinase, and anti-cellulite [3].

Chalcone is a derivate of the polyphenolic compound that belongs to the flavonoid group [4]. Chalcone is a biosynthetic product that consists of aromatic ketones and enones that have anticancer effects and are synthesized through the shikimate pathway [5].

Bioinformatics is an integrated system of biology, mathematics, medical science and medicine, statistics, information technology, and computer science to analyze and interpret biological data to predict, describe, and visualize data. Bioinformatics has great potential in analyzing various areas such as genomics, proteomics, medicines, protein structures, cells, molecular modelings, and gene expressions [6-8].
Lung cancer is the second-highest incidence and the highest mortality rate, with 2,206,771 new cases (11.4%) and 1,796,144 deaths (18.0%) from all types of cancer in the world in 2020 [9]. Case of lung cancer is closely associated with smoking behaviors, with more than 80% of the cases in the western world related to cigarette smoking [10]. Another risk factor is air pollution which is also highly associated with increased cancer cases, mostly in Lung Adenocarcinoma (LADC) [11].

Several biological activities of Syzygium samarangense extract have been investigated both in vitro and in vivo, including antibacterial, antifungal, antiviral, antihyperglycemic, analgesic, anti-inflammatory, cytotoxic, antioxidant, and anticancer [2,12-17]. The known mechanisms of Syzygium samarangense as an anticancer are terminating the proliferation of HCT116 cells, LOVO cells in the colon, and A549 cells in lung carcinoma and a cytotoxic effect in SW-480 colon cancer cells [18, 19]. In addition, Syzygium samarangense water extract is known to have chemopreventive potential by inhibiting Nrf2-mediated cellular defense in skin cancer [17]. Therefore, this study aims to identify the potential of the active compound of Syzygium samarangense chalcone extract as an inhibitor of lung cancer cell proliferation based on bioinformatics analysis.

2. Materials and Methods

2.1. Extraction.

2 kg of fresh leaves of Syzygium samarangense were air-dried in indirect sunlight for five days. Then, the leaves were sorted, weighed, and obtained a dry leaf weight of 1.5 kg. Crush the leaves into powder with a blender. Put the leaf powder into the jar for the maceration process. The sample was macerated with 70% ethanol as solvent. Samples were stored at room temperature for three days. Then the sample was filtered with sari paper. The filtrate obtained was then concentrated with a rotary evaporator at a temperature of 40°C to obtain a viscous extract.

2.2. Thin-layer chromatography.

10 mg of crude extract of Syzygium samarangense was dissolved in methanol and then vortexed. The supernatant was taken and applied to the TLC plate. Optimization of the mobile phase was carried out using variations in the concentration of methanol and chloroform, which are 100% methanol, methanol:chloroform (9:1), methanol:chloroform (7:3), and methanol:chloroform (1:1). The optimization from concentration variations will obtain the mobile phase that fits for elusion. Then, the mobile phase is used to elute the sample with the reference standard, quercetin, to ensure that the leaves of Syzygium samarangense contain chalcone.

2.3. Screening protein target prediction associated with DMC.

The data collection stage is carried out through the web-based tool SwissTargetPrediction (http://www.swisstargetprediction.ch/) [20], Similarity ensemble approach (SEA) (https://sea.bkslab.org/) [21], and TargetNet (http://targetnet.scbdd.com) [22]. Access is done by entering the SMILES DMC code CC1=C(C=C(C(=C1O)C(=O)C=CC=CC=CC=C2)OC)O. The cut-off used in each database is different. In SwissTargetPrediction and SEA, only the SMILES Code from the DMC and the selection of the Homo sapiens species is required. The TargetNet database used
cut-off MCC 0.7 and MACCS type fingerprint. The protein genes selected from SwissTargetPrediction, TargetNet, and SEA, were then entered into a Venn diagram using an automated web-based tool, Interactivenn (http://www.interactivenn.net/), to determine the protein genes that coincided in the three previous databases.

2.4. Screening protein target prediction associated with cancer pathway.

Prediction results of DMC target genes are then followed by screening the role of each gene in specific biological function pathways through the Database for Annotation, Visualization, and Integrated Discovery (DAVID) V6.8 (https://david.ncifcrf.gov/home.jsp) [23]. The gene codes from the previous screening were entered with the identifier OFFICIAL_GENE_SYMBOL and the species code 9606 for Homo sapiens. In the annotation section, select the pathway section and then KEGG_PATHWAY to determine the pathway of these genes based on the KEGG database with a p-value < 0.05.

2.5. Screening protein target prediction specific for lung cancer.

The protein genes associated with cancer were then analyzed for their expression levels and survivability in patients with lung cancer adenocarcinoma using the UALCAN web resource (http://ualcan.path.uab.edu/analysis.html) [24] based on The Cancer Genome Atlas (TCGA) database, especially the Lung dataset. Adenocarcinoma. Survivability curve analysis from using the webserver Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (http://gepia2.cancer-pku.cn/) [25] using the overall survival method, group cut-off median, with 95% confidence interval, and lung adenocarcinoma dataset.

2.6. Evaluating prediction of DMC potential as lung cancer therapy.

2.6.1. Protein preparation.

The molecular docking test in this study used HDAC1 protein as the target protein. The target protein structure (HDAC1) was obtained from the Protein Data Bank (PDB) (https://www.rcsb.org). The selected protein was in the form of an active dimer that was still bound to the native ligand with the code 6Z2J in the form of the HDAC1/MIDEAS/DNTTIP1 MiDAC deacetylase complex dimer with a resolution of 4.00. The native ligand ((2,3,4,5,6-pentaphosphonoxyxycyclohexyl) dihydrogen phosphate or IHP located on the A and F chains) was removed using the BIOVIA DS software to obtain a pocket that was used as the anchorage. HDAC1 protein has six chains: A, B, C, D, E, and F (Figure 1). The preparation of dimeric HDAC1 protein in this study used the A chain. HDAC1 was used in the dimer form because the dimer form is the active form of the protein [26].

Figure 1. Results of HDAC1 dimer protein preparation.
2.6.2. Sample preparation.

The structure of the DMC (PubChem CID: 10424762) was obtained from The National Center for Biotechnology Information (NCBI) (https://pubchem.ncbi.nlm.nih.gov). The structure was downloaded in .sdf format and converted to .mol2 using the BIOVIA DS program (Figure 2). Positive control tests were also performed for comparison using native ligands (PubChem CID: 86580100), MS-275 (PubChem CID: 4261), and zolinza (PubChem CID: 5311) with the same structural preparation as the test compound DMC (Figure 2). MS-275 and zolinza were used as comparison compounds because they have inhibitory activity on the HDAC1 protein [27].

![Figure 2. Results of Optimization of the Structure of: (a) DMC; (b) Native Ligand ((2,3,4,5,6-pentaphosphonoxyeclohexyl) dihydrogen phosphate); (c) Zolinza; (d) MS-275.](image)

2.6.3. Molecular docking module.

Molecular docking is done using iGEMDOCK (Version 2.1) software to bind compounds with HDAC1 protein. iGEMDOCK is a virtual screening environment that is integrated from preparation to post-screening analysis with pharmacological interactions. iGEMDOCK provides an interactive interface for preparing binding sites of target proteins and screening compound lists [28]. Each compound in the list is docked to the binding site using the in-house docking tool iGEMDOCK. iGEMDOCK produces a protein-compound interaction profile in the form of electrostatic forces (E), hydrogen bonds (H), and Van der Waals bonds (V). Based on the three interaction profiles and the structure of these compounds, iGEMDOCK concludes the pharmacological interactions and categorizes the screening results for post-screening analysis. Then, iGEMDOCK ranks and visualizes the screening results by combining pharmacological interactions and energy-based scoring [29].

2.6.4. Molecular docking mechanism.

Molecular docking was performed using the iGEMDOCK software. The protein used in .pdb format and the IHP binding site center is selected with a binding site radius of 8.0 Å. The algorithm used the accurate docking setting (population size: 800; generation: 80; the number of solutions: 10). The selected scoring function uses the GEMDOCK scoring function with a preference for hydrophobic and hydrostatic ligands of 1.00 Å. From molecular docking, the bond with the lowest energy is selected. If the generated energy is positive (due to clash or unfavorable electrostatic interactions), it is necessary to re-docking. The bonding is carried out between the compound and the protein, resulting in the binding affinity and the time of the molecular docking process [29]. The results of molecular docking are visualized in 3D. The visualization was performed using Chimera software (Version 1.13.1) to see the surface area and the best conformation of proteins and ligands [30].
2.7. Evaluation of DMC as a potential oral drug.

DMC compounds were predicted for the rules of five properties through a database based on the Biosig website (http://biosig.unimelb.edu.au) [31]. The analysis was carried out by entering the SMILES DMC code CC1=C(C(=C1O)C(=O)=C(=C2=CC=C(=C2)OC)O on the Biosig database website and selecting Prediction Mode ADMET to get the rules of five properties. The obtained properties are determined according to the cut off values obtained in the literature.

3. Results and Discussion

3.1. Test for chalcone content in Syzygium samarangense leaves.

The resulting extract was 54.35 g of crude extract obtained from a total of 1.5 kg leaves samples. The total yield obtained was 3.63% w/w. Optimization of the mobile phase was carried out to elute the extract with varying concentrations of methanol and chloroform, namely 100% methanol, methanol : chloroform (9:1), methanol : chloroform (7:3), methanol : chloroform (1:1) which were then sprayed using AlCl₃ reagent (Figure 3). Best elution results were obtained using 100% methanol and methanol : chloroform (9:1). The optimized mobile phases were used to elute samples and the standard flavonoid compound, namely quercetin on Silica gel 60 F₂₅₄ TLC plate and cellulose reverse phase TLC plate. The chromatography results were sprayed using AlCl₃, and the results are shown in Figure 3. The Rf value of the sample and quercetin in the different mobile phases is shown in Table 1. AlCl₃ spray reagent was used to confirm flavonoid compounds. Flavonoids will form stable complexes with C4 keto groups and C3 or C5 hydroxyl groups on flavonoids when reacted with AlCl₃. Positive results are evidenced by the formation of yellow or green fluorescence at UV light 254 and 366 nm [32].

![Figure 3. TLC Result: (a) Optimization; (b) with Silica gel 60 TLC Plate F₂₅₄; (c) with Cellulose Reverse Phase TLC Plate.](image)

| Rf | Normal Phase | Reverse Phase |
|----|--------------|---------------|
|    | Methanol 100% | 9 M : 1 C     |
| Sample | 0.68         | 0.62          |
| Quercetin | 0.56         | 0.56          |

3.2. DMC-related target protein prediction screening.

From the website databases, 43 protein genes were obtained from the SEA website, 109 from TargetNet, and 111 from the SwissTargetPrediction. Thus, the total protein genes obtained were 263 protein genes. The resulting data were then analyzed using the web-based tool Interactivenn (Figure 4). As a result, four protein genes intersect on three database websites, namely ABCG2, KCNA3, MMP1, and MMP9. From these results, it was also found that 17 protein genes intersect on two database websites totaling 21 protein genes combined.
namely ABCB1, ACHE, AKR1B1, ALOX5, APP, GRM4, HDAC1, MAOA, MAOB, MCL1, MMP13, PTGS1, PTGS2, PTPN1, TLR9, ABCG2, KCNA3, MMP1, MMP9, ESR1, MMP2. From these results, it can be predicted that the 21 proteins have potential as targets of DMC.

3.3. Screening predicted target proteins related to cancer pathways.

Pathway analysis of the biological function of 21 protein genes previously screened was carried out to determine protein genes associated with cancer according to KEGG Pathway analysis (Table 2). A total of 6 protein genes are included in the biological function pathway of serotonergic synapses with a p-value of $7.2 \times 10^{-6}$, namely APP, ALOX5, MAOA, MAOB, PTGS1, and PTGS2. Five protein genes are cancer-related with a p-value of $1.7 \times 10^{-2}$, namely HDAC1, MMP9, MMP2, MMP1, and PTGS2. There are also biological function pathways of MicroRNA in cancer in as many as four protein genes with a p-value of $3.7 \times 10^{-2}$, namely ABCB1, MCL1, MMP9, and PTGS2. Protein genes that play a role in pathways in cancer were selected for further analysis in the next part.

| Biological function pathway | p-value   | Protein Genes                          |
|-----------------------------|-----------|----------------------------------------|
| Serotonergic synapses       | $7.2 \times 10^{-6}$ | APP, ALOX5, MAOA, MAOB, PTGS1, PTGS2  |
| Pathways in cancer          | $1.7 \times 10^{-2}$ | HDAC1, MMP9, MMP2, MMP1, PTGS2        |
| MicroRNAs in cancer         | $3.7 \times 10^{-2}$ | ABCB1, MCL1, MMP9, PTGS2              |

3.4. Lung cancer-specific target protein prediction screening.

Protein genes in cancer-associated pathways were further analyzed by determining differences in protein gene expression levels in LUAD cancer and analyzing survivability curves in LUAD cancer patients. Of the five protein genes in the biological pathway, only HDAC1 had significant differences in expression levels between tumor cells and normal cells (Figure 5a). In normal cells, HDAC1 gene expression averaged 60,874 transcripts per million total transcripts. Whereas in tumor cells, the average HDAC1 gene expression was 98,796 transcripts per million total transcripts. The resulting difference was very significant, with a statistical significance value of $1.62 \times 10^{-1}$. From the analysis of the survivability curve of cancer patients, the LUAD cancer group with high expression of the HDAC1 protein gene had a lower survival percentage than the group with low expression of the HDAC1 protein gene with a p-value = 0.09 (Figure 5b). Therefore, the results can be predicted that HDAC1 is a potential therapeutic target in LUAD cancer.
Figure 5. Results of screening prediction of specifically targeted proteins in lung adenocarcinoma (LUAD) lung cancer: (a) HDAC1 expression level diagram in LUAD tumor cells and normal cells; (b) LUAD cancer survivability curve on HDAC1 protein expression level.

3.4. Evaluating prediction of DMC potential as lung cancer therapy.

3.4.1. Molecular docking to target protein.

The results obtained from the DMC molecular docking process with HDAC1 protein are in the form of bond energy and hydrogen bond interactions formed. Three-dimensional visualization of the interaction of DMC with HDAC1 dimers showed that the three compounds had different best conformations of HDAC1 protein (Figure 6). The best conformation is selected based on bond energy values, and the lowest bond energy value is selected from the bonding results.

Figure 6. 3D visualization results in the form of HDAC1 dimer protein interaction surface area with compounds. The compounds were colored green or yellow: (a) DMC; (b) native ligand; (c) zolinza; (d) MS-275.
Different colors indicate different values of the electrostatic potential on the surface. The red color represents areas with low values (maximum negative). This area is very favorable for electrophilic reactions. The blue color indicates areas with high values (maximum positivity). This area is very favorable for nucleophilic reactions [33]. Figure 6 also shows the electrostatic potential of HDAC1 based on the visualization of its surface area. The compound has molecular interactions with HDAC1 protein, both in the form of hydrogen bonds and hydrophobic interactions. The strength of the interaction between DMCs and the comparison compounds in detail can be seen in Table 2.

| Compound | Total Energy | VDW | HBond | Elec |
|----------|--------------|-----|-------|------|
| DMC      | -93.0709     | -78.0894 | -14.9815 | 0    |
| Native ligand | -135.773 | -77.4403 | -46.5193 | -11.8134 |
| MS-275   | -113.693     | -93.1842 | -20.5084 | 0    |
| Zolinza  | -96.958      | -74.3196 | -22.6384 | 0    |

The results obtained from the molecular docking process are in the form of bond energy and hydrogen bonding data from the ten best conformations. The bond energy shows the compound’s affinity with the target protein. The lower the bond energy, the more stable the bond between the test compound and the target protein [32]. The binding energies of DMC, native ligand, MS-275, and zolinza with HDAC1 protein were -93.07; -135.77; -113.69; and -96.96, respectively (Table 2). The descriptive statistical analysis results obtained a mean value of bond energy is 109.873725 with a standard deviation of 19.4466. The standard deviation value is smaller than the mean, so the variation of data is small. The slight variation in the data showed that the bond strength of the DMC compound was not significantly different from the comparison compound, so it was suspected that DMC had the same potential as the comparison compound as an HDAC1 inhibitor.

| Compound | HS-LYS-31 | H-M-304 | H-S-THR-305 | H-M-ILE-306 | H-M-ARG-306 | H-S-ARG-871 | H-S-TYR-306 |
|----------|---------|--------|------------|-----------|------------|------------|------------|
| DMC      | 0       | 0      | -3.32      | -6.44     | -4.83      | 0          |            |
| Native ligand | -10.5   | 7.40   | -6.20      | -2.42     | -1.61      | -7.7374    |            |
| MS-275   | 0       | -3.5   | -3.5       | -1.87     | -5.70      | 0          |            |
| Zolinza  | 0       | 0      | 0          | -7        | -6.93      | -8.71      | 0          |

The hydrogen bonds interaction in amino acids was the same in DMC and comparators in Ile-305 and Arg-306 with the target protein (Table 3). H indicates the interaction that occurs in a hydrogen bond, M indicates the interaction occurs in the main chain of amino acids, and S indicates the interaction occurs in the side chain of amino acids [29]. HDAC1 bond with the native ligand is stronger than DMC because there is more interaction within the HDAC1-native ligand than an HDAC1-DMC hydrogen bond, and energies in HDAC1-native ligand hydrogen bonds is smaller than HDAC1-DMC. DMC has a similar amino acids interaction with the zolinza, so it is suspected that DMC has the same potential activity as the zolinza, namely as an inhibitor of HDAC1 protein.

3.4.2. Evaluation of DMC as a potential oral drug.

Lipinski's rules of five parameters, commonly abbreviated as Ro5, evaluate whether chemical compounds with certain pharmacological activities have physical and chemical properties that make them acceptable as oral drugs in humans. A compound can be accepted as an oral drug if it does not violate more than one of the following rules; namely, the molecular
weight does not exceed 500 daltons, the log P is not more than five, not more than five hydrogen bond donors, and not more than ten hydrogen bond acceptors [34].

| Rules of five Properties                  | Value  | Cut off |
|-------------------------------------------|--------|---------|
| Molecular Weight                          | 298,338 Da | 500 Da |
| Log P                                     | 3.61934 | 5       |
| Hydrogen Bond Acceptor                    | 4      | 5       |
| Hydrogen Bond Donor                       | 2      | 10      |

Drug molecules’ weight affects the permeability of drugs to cross barriers in the body. The larger the molecular weight, the worse the permeability because normally, the size is also getting bigger. Log P is a logarithmic function of the ratio of the number of molecules distributed in the octanol phase to the amount of the aqueous phase. This parameter describes the lipophilicity of a molecule. The greater the log P value, the greater the lipophilicity of the molecule. There are too many hydrogen bonds that can be formed by a molecule, either as a donor or as an acceptor; it will reduce the permeability of the molecule when it passes through the bilayer membrane. This is because the molecule will be bound to the membrane through hydrogen bonds [35,36]. DMC does not violate any Ro5 parameters, so DMC can be predicted to be accepted as a potential oral drug.

3.5. Correlation between HDAC1 and lung cancer.

HDAC1 or Histone deacetylase 1 is an enzyme that plays a vital role in the histone deacetylation process. This process is one of the primary regulatory mechanisms of gene expression in humans. HDAC1 expression has been reported to be associated with the development and prognosis of several types of cancer. Several studies have shown that high HDAC1 expression has been associated with the incidence of ovarian cancer, liver cancer, breast cancer, gastric cancer, pancreas cancer, neuroblastoma, and lung cancer [37-43]. In addition, the expression level of HDAC1 correlates with tumor stage, differentiation level, and metastasis status [44,45].

The genes whose expression is regulated by HDAC1 play a vital role in the cell cycle such as p21 and p27 [44]. The two proteins are proteins inhibit the Cyclin Dependent Kinase (CDK), which plays an essential role in cell proliferation. By inhibiting HDAC1, p21 and p27 will inhibit CDK, thus inhibiting cell proliferation [46,47]. In addition to cell cycle gene regulation, HDAC1 inhibits the TGFβ signaling pathway. TGFβ, or Transforming Growth Factor β, is a protein that functions in signaling cell growth. If a cell receives a TGFβ signal, the TGFβ receptor activates the Smad2/3 protein to reduce the cell's sensitivity to anti-growth signals. HDAC1 can inhibit this signaling pathway by inhibiting Smad2/3. Conversely, the inhibition of Smad2/3 will cause cells to become sensitive to anti-growth signals (Figure 7) [48].

The effects of HDAC1 inhibitors have been studied in several types of cancer, such as liver cancer, gastric cancer, pancreas, neuroblastoma, breast cancer, and lung cancer [37-43]. These results are consistent with the data we got in Figure 5 that HDAC1 might be a suitable therapeutic target in lung cancer therapy.
4. Conclusions

Based on the research that has been done, it can be concluded that DMC from *Syzygium samarangense* leaves extract predicted to inhibit the proliferation of lung cancer cells through the inhibition of HDAC1 protein in the form of dimers. However, further research is needed regarding our results to examine the potential of *Syzygium samarangense* leaves extract in inhibiting the proliferation of lung cancer cells both *in vitro* and *in vivo*.

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**Conflicts of Interest**

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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