

## The effect of ethanolic leaves extract of soursop (*Annona muricata* L.) on human colorectal cancer cell line: cell viability and in silico study to cyclin D1 protein

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Murdani Abdullah<sup>1</sup>, Debby Desmarini<sup>2</sup>, Sofy Meilaini<sup>3</sup>, Puji Sari<sup>4</sup>, Luluk Yunaini<sup>4</sup>, Fadilah Fadilah<sup>5</sup>

<sup>1</sup>Departement of Internal Medicine, Faculty of Medicine, Universitas Indonesia, Ciptomangunkusumo National Hospital, Jakarta, Indonesia

<sup>2</sup>Master's Program of Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

<sup>3</sup>Virology and Cancer Pathobiology Research Centre, Faculty of Medicine, Universitas Indonesia, Ciptomangunkusumo National Hospital, Jakarta, Indonesia

<sup>4</sup>Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

<sup>5</sup>Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

Corresponding author: Murdani Abdullah

Email: murdani08@gmail.com

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### Abstrak

**Latar Belakang:** Kanker kolorektal merupakan transformasi patologis dari epitel kolon dan rektum normal menjadi massa jaringan abnormal, perubahan ini terjadi karena ekspresi berlebih dari protein cyclin D1 yang menginduksi proliferasi sel kolorektal secara berlebihan. Pengobatan dan pencegahan kanker kolorektal dapat dilakukan secara alami dengan mengonsumsi ekstrak daun *Annona muricata* L. (sirsak). Sirsak dikenal karena banyak komponen fitokimia yang berfungsi sebagai anti kanker.

**Metode:** Penelitian ini menggunakan sel kanker kolorektal HT-29 yang diberi ekstrak etanol daun sirsak dan 5-Fluorourasil (5-FU). Tujuannya untuk menemukan konsentrasi sitotoksitas yang dapat menghambat 50% populasi sel HT-29 (CC<sub>50</sub>) dan konsentrasi yang didapat sebelumnya akan diuji dengan metode uji MTT. Analisis docking molekuler dilakukan antara molekul-molekul dari ekstrak etanol daun sirsak terhadap protein Cyclin D1 menggunakan perangkat lunak molecular operating environment (MOE) 2013.08.

**Hasil:** CC<sub>50</sub> ekstrak etanol daun sirsak adalah 278 µg / mL dan 5-FU adalah 88 µg / mL. Persentase terendah sel HT-29 yang layak adalah 2 x CC<sub>50</sub> setelah perlakuan ekstrak etanol daun sirsak (40,4 ± 1,3%) dibandingkan dengan 5-FU (52,8 ± 4,3%), kontrol pelarut (97,2 ± 1,4%), dan kontrol sel (100%). Analisis docking molekuler untuk protein cyclin D1 diperoleh asam N-hexadecanoic dan molekul phytol sebagai kandidat yang baik untuk menghambat protein cyclin D1.

**Kesimpulan:** Ekstrak etanol daun sirsak dapat menurunkan viabilitas sel kultur kanker kolon HT-29 dan berdasarkan analisis molekuler docking dilihat dari energi bebas gibbs (ΔG) dan afinitas tertinggi (pKi) diperoleh N-hexadecanoic dan molekul phytol sebagai penghambat protein cyclin D1. (*Health Science Journal of Indonesia* 2019;10(2):96-102)

**Kata Kunci:** Kanker kolorektal HT-29, ekstrak etanol daun sirsak, viabilitas sel, molecular docking, cyclin D1

### Abstract

**Introduction:** Colorectal cancer is a pathological transformation of normal colon and rectum epithelial that becomes an abnormal tissue mass, due to the overexpression of cyclin D1 protein that inducing excessive proliferation of colorectal cell. The treatment and prevention of colorectal cancer could be done naturally by consuming leaves extract of *Annona muricata* L. (soursop). Soursop is known for many phytochemical components that serve as an anti-cancer.

**Methods:** This study was used HT-29 colorectal cancer cell that treated with ethanolic leaves extract of soursop and 5-Fluorourasil (5-FU) to find the cytotoxicity concentration that can inhibit 50% of HT-29 cell population (CC<sub>50</sub>) and the next concentrations of them were treated for next treatment with MTT assay. Molecular docking analysis of the compounds of ethanolic leaves extract of soursop to cyclin D1 protein used molecular operating environment (MOE) 2013.08 software.

**Results:** CC<sub>50</sub> of ethanolic leaves extracts of soursop was 278 µg/mL dan 5-FU was 88 µg/mL. The lowest percentage of viable HT-29 cell was 2 x CC<sub>50</sub> after ethanolic leaves extract of soursop treatment (40,4±1,3%) was compared to 5-FU (52,8±4,3%), solvent control (97,2±1,4%), and cells control (100%). Analysis of molecular docking to cyclin D1 protein was obtained N-hexadecanoic acid and phytol molecules as good candidates to inhibit cyclin D1 protein.

**Conclusions:** The ethanolic leaves extract of soursop could be a good alternative treatment for colorectal cancer and its compounds had ability to inhibit cyclin D1 protein (the highest gibbs free energy (ΔG) and affinity (pKi)). (*Health Science Journal of Indonesia* 2019;10(2):96-102)

**Keywords:** Colorectal cancer, ethanolic leaves extract of soursop, cell viability, molecular docking, cyclin D1

Colorectal cancer is a pathological change in the normal colon and rectal tissue to an abnormal tissue caused by genetic and environmental changes. The 'rise' of colorectal cancer can be attributed to the increasingly aging population, modern dietary habits and an increase in risk factors such as smoking, low physical exercise and obesity.<sup>1</sup> According to the International Agency for Research on Cancer (IARC), the incidence of male colorectal cancer in the world is the third largest case (21%) after lung cancer and prostate cancer, while the incidence of colorectal cancer in women in the world is the second largest case (14%) after breast cancer.<sup>2</sup> Colorectal cancer therapy used is surgery, radiotherapy and chemotherapy.<sup>3</sup> This is considered to be less effective because of side effects, so the alternative therapy is needed, such as consuming *Annona muricata* L. (soursop).<sup>4,5</sup>

*Annona muricata* L. is a type of tropical plant known for containing many phytochemical components such as alkaloids, annonaceous acetogenin, megastigman, flavonol triglycosides, phenolics, and cyclopeptides found in leaves, fruits, seeds, and roots that can act as anti-inflammatory, anti-inflammatory infection and anti-cancer.<sup>6,7</sup> Soursop leaves extract can produce cytotoxic effects on colorectal cancer cell cultures such as HT-29, HCT-116,<sup>5</sup> COLO-205,<sup>8</sup> and DLD-1.<sup>9</sup> Soursop leaves extract is also known to reduce the expression of cyclin D1 protein in phase G1/S.<sup>5,10</sup>

Cyclin D1 is a protein encoded by CCND1 gene and controls cell cycle especially at the G1 phase. In this process, the expression of cyclin D1 protein increases and binds to cyclin dependent kinase 4 or 6 (CDK4 / 6) protein to form active kinase. That complex can phosphorylate or inactivate the retinoblastoma (Rb) protein. Phosphorylated Rb causes the transcription factor E2 factor (E2F) to promote the transcription of genes needed for cell division.<sup>11</sup> In colorectal cancer, cyclin D1 can be a significant marker. Meta analysis of 21 studies on the prognostic value of cyclin D1 expression showed that high cyclin D1 levels were associated with poor overall survivor and cell line especially HT-29.<sup>12,13</sup> High regulation of cyclin D1 was detected at 527 of 557 (94,6%) tumor cases.<sup>14</sup> High expression of cyclin D1 causes abnormal cell cycle.<sup>15</sup> The aim of the study is to investigate the effect of ethanolic leaves extract of soursop on HT-29 colorectal cancer cell viability and molecular docking of its active composition on the cyclin D1 protein.

## METHODS

### Plant materials

Soursop leaves were extracted 96% ethanol and obtained from Indrawati, et al.<sup>9</sup> *Annona muricata* extract used

in this study is a standardized vacuum dried extract produced by Javaplant, Central Java, Indonesia.

### Cell culture

HT-29 (human colon cancer cells) were obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). The cells were maintained in high glucose-DMEM (Gibco™), 1% penicillin-streptomycin (Gibco™), 1% amphotericin B (Gibco™), and 10% fetal bovine serum (Gibco™) in a humidified atmosphere with 5% CO<sub>2</sub> in the air at 37°C.

### Cell viability assay

Cell viability was evaluated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay as previously described by Bahaguna A et al.<sup>16</sup> In brief, cells (1x10<sup>4</sup> cells/mL) were treated with ethanolic leaves extract of soursop, 5-Fluorouracil (5-FU) (Curacil®) as a standard anticancer drug was used as a positive control, dimethyl sulfoxide (DMSO) 100% (Sigma Aldrich) as a solvent control, and only complete medium as a negative control at different concentrations in 96-well plate and incubated for 24 h. The concentration of ethanolic leaves extract of soursop was 12,5-400 µg/mL, 5-FU was 25-100 µg/mL, and 0,1% DMSO. MTT assay was measured at 570 nm absorbance using a microplate reader (Promega™ Glomax™).

The anti-proliferative potential of the treatment is expressed as the value of the cytotoxicity concentration (CC<sub>50</sub>), i.e. the concentrations that causes inhibition of 50% cell growth and is calculated based on the percentage of cell viability. The percentage of cell viability is  $\frac{1}{4}$  (absorbance of treated cells / absorbance of untreated cells) x 100%. The CC<sub>50</sub> of ethanolic leaves extract of soursop and 5-FU were obtained by the first MTT assay and followed by using concentrations of  $\frac{1}{2}$  x CC<sub>50</sub>, 1 x CC<sub>50</sub>, and 2 x CC<sub>50</sub>.

### Molecular docking with cyclin D1 protein

Docking simulation of extract *Annona muricata* L. to cyclin D1 was started by ligand (compounds) and receptor (protein) preparation. The compounds were obtained from Gavamukulya Y, et al,<sup>17</sup> i.e 2-pentadecanol; oleyl alcohol; 1,2-benzenedicarboxylic acid, butyl octyl ester; 3,7,11,15-tetramethyl-2-hexadecen-1-ol; N-hexadecanoic acid; hexadecanoic acid, ethyl ester; phytol; 1,E-11,Z-13-octadecatriene; 7-tetradecenal, (Z); 9,12-octadecadienoic acid, ethyl ester; cis, cis, cis-7,10,13-hexadecatrienal; and

1,2-benzenedicarboxylic acid, diisooctyl ester by using GC-MS Analysis. These specific compounds have been analyzed from ethanolic leaves extract of soursop. The structure of the compounds was obtained from chemspider database ([www.pubchem.com](http://www.pubchem.com)). The fasta sequences of cyclin D1 protein were obtained from database national center for biotechnology information (NCBI) and the 3-D structure was from <http://swissmodel.expasy.org> (Fig 1).

The ligand and protein were optimized by molecular operating environment (MOE) 2013.08 software. Geometry optimization and energy minimization of cyclin protein were carried out using the MOE software with PDB format. The structure of cyclin D1 protein was added to parameters such as hydrogen atoms, partial charges, and gas phases. The addition of hydrogen atoms and protonation was performed on cyclin D1 protein. Partial charges were regulated by using a partial charge. Protein energy was minimized by the Merck Molecular Forcefield 94x (MMFF94x) force field. The protein was performed on gas phase solvation with a fixed charge and optimized with a mean square root gradient (RMS) of 0.05 kcal/Åmol. The overall optimization file was obtained in the .moe format.

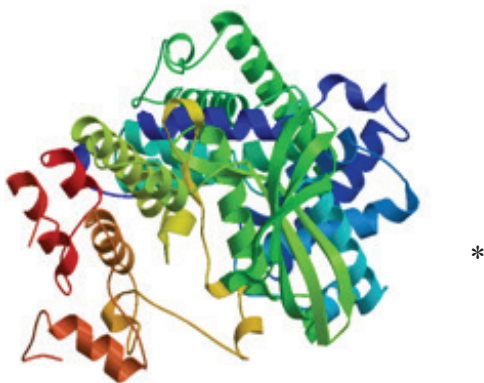


Figure 1. 3-D Cyclin D1 protein structure (2w96.1.A)

This protein has 100% homology with the template.

Geometry optimization and energy minimization of ligand structures using MOE software with the .mdb format. Ligand candidates were kept with the .mol format in the database viewer of MOE software. Ligands were washed with a computational program, the partial charge was adjusted and optimized using the MMFF94x force field. In addition, ligand energy was minimized using minimal energy with an RMS gradient of 0.001 kcal/ Åmol and the resulting file was saved in the .mdb format. Molecular docking of ligand molecules with cyclin D1 protein was carried out by a dock program simulation in MOE.

Molecular docking used triangular matching by repeating energy readings for each position on cyclin D1 protein 100 times (retain: 100). The assessment function used London dG and refinement force. The last retained of refinement products were the most suitable conformation of each ligand molecules.

Molecular docking calculations were seen in the output format of the viewer.mdb. Several parameters of protein-ligand interactions can be analyzed, including bond free energy ( $\Delta G$ ) and affinity (pKi). The protein-ligand complex selected was the smallest bond energy value and the greatest bond affinity.

#### Statistical analysis

Cell viability values were presented as mean  $\pm$  SEM from four different experiments. One-way variance analysis (ANOVA) was performed using SPSS v.21. Differences were considered significant at  $p < 0.05$ .

#### Ethical Clearance

This study does not require ethics approval because it does not use animal or human subjects.

## RESULTS AND DISCUSSION

#### Cell Viability Assay

One-way ANOVA statistical test and post-hoc LSD test shows the effect of exposure time of soursop leaves extract and positive control (5-FU) on HT-29 cell viability. The sign (\*) shows a significant difference ( $p < 0,05$ ) and the sign (\*\*) shows a significant difference ( $p < 0,01$ ).

The linear regression curve between the concentrations of the two test compounds obtained an equation for ethanolic leaves extract of soursop ( $y = -0,0363x + 60,078$  and  $R^2 = 0.8802$ ) and 5-FU ( $y = -0.3067x + 77.052$  and  $R^2 = 0.9782$ ). Equation showed  $CC_{50}$  was used to determine the concentration of ethanolic leaves extract of soursop and 5-FU which can inhibit half of the cell population. The  $CC_{50}$  value was obtained from the x variable by entering a value of 50 (the standard value was taken from 50% inhibition) to the y variable in the equations of the two tests. Based on the cytotoxic test of ethanolic leaves extract of soursop and 5-FU, showed that 5-FU had cytotoxic activity three times lower than ethanolic leaves extract of soursop, i.e.  $CC_{50}$  of soursop leaves extract was 278  $\mu\text{g/mL}$  and  $CC_{50}$  of 5-FU was 88  $\mu\text{g/mL}$ .  $R^2$  showed the correlation of the concentration with a decrease in viable cell.

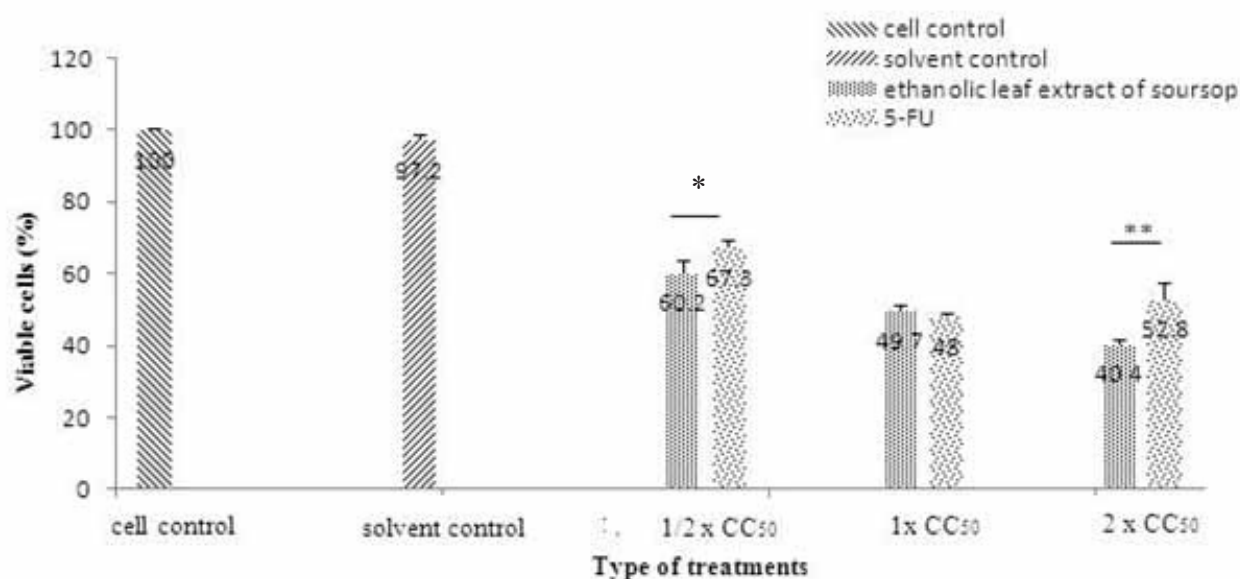


Figure 2. Cell viability of HT-29 after treatment with different CC<sub>50</sub> concentration of ethanolic leaves extract of soursop and control. The sign (\*) shows a significant difference ( $p < 0,05$ ) and the sign (\*\*) shows a significant difference ( $p < 0,01$ )

The treatment group between ethanolic leaves extract of soursop compared with 5-FU for a concentration of  $\frac{1}{2} \times CC_{50}$  was statistically significantly different with a value of  $p = 0,001$  ( $p < 0,05$ ). Soursop leaves ethanol extract showed cell viability of 7,1% lower compared to 5-FU. The ethanolic leaves extract of soursop compared to 5-FU for a concentration of  $1 \times CC_{50}$  did not have a statistically significant difference ( $p > 0,05$ ) with a value of  $p = 0,509$ . The difference in viability of the two was only 1,7%, namely the 5-FU viability was lower than the ethanolic leaves extract of soursop. The ethanolic leaves extract of soursop compared to 5-FU for a concentration of  $2 \times CC_{50}$  had a very statistically significant difference with a  $p$  value  $< 0,01$ . Soursop leaves ethanol extract had a cell viability of 12,4% lower compared to 5-FU (Fig 2).

#### Molecular Docking with Cyclin D1 Protein

Molecular docking analysis with MOE obtained two molecules that have the lowest  $\Delta G$  and the highest  $pKi$ , namely n-hexadecanoic acid ( $\Delta G = -9.7755$  kcal/mol,  $pKi = 7,219$ ) and phytol ( $\Delta G = -7.2147$  kcal/mol,  $pKi = 5,975$ ) (Table 1, Fig 3).

N-hexadecanoic acid binds to cyclin D1 in glutamine, lysine and threonine. The strength of glutamine with this ligand is 25% and 2.57 Å of the distance, lysine is 63% and 2.47 Å of the distance, threonine is 62% and 2.58 Å of the distance. Phytol binds to cyclin D1 in threonine, glutamic acid, and glutamine. The strength of threonine with this ligand is 96% and 2.57 Å of the distance, glutamic acid is 27% and 1.58 Å of the distance. Glutamine is 62% and 2.58 Å of the distance (Fig 4).

Table 1. Analysis of molecular docking of ethanolic leaves extract of soursop with cyclin D1 protein

No	Compounds	$\Delta G$ (Kcal/mol)	$pKi$	HBond
1	3,7,11,15-Tetramethyl-2- hexadecen-1-ol	-5,9512	3,289	Gln250, Asp253
2	1,E-11,Z-13- Octadecatriene	-4,8927	3,663	-
3	7-Tetradecenal, (Z)	-5,9102	3,915	Met252
4	cis, cis, cis-7,10,13- Hexadecatrienal	-6,0333	4,023	Met252
5	2-Pentadecanol	-5,5910	3,655	Asn251
6	Oleyl Alcohol	-6,1321	3,692	Asp253
7	1,2-Benzenedicarboxylic acid, butyl octyl ester	-5,4549	3,159	-
8	<b>n-Hexadecanoic acid</b>	<b>-9,7755</b>	<b>7,219</b>	<b>Lys44, Thr48, Gln247</b>
9	Hexadecanoic acid, ethyl ester	-6,1965	4,199	Met252
10	<b>Phytol</b>	<b>-7,2147</b>	<b>5,975</b>	<b>Thr48, Glu60, Gln247</b>
11	1,2-Benzenedicarboxylic acid, diisooctyl ester	-6,4074	5,322	Thr48
12	9,12-Octadecadienoic acid, ethyl ester	-4,9061	3,635	-

$\Delta G$  = Gibbs free energy (kcal/mol);  $pKi$  = affinity; glutamine (Gln), aspartic acid (Asp), methionine (Met), asparagine (Asn), lysine (Lys), threonine (Thr), glutamate acid (Glu). The number which is next to amino acid shows amino acid position of cyclin D1 sequences.



Figure 3. 2D-ligand structure with the lowest value of  $\Delta G$  and the strongest value of  $pK_i$ . N-hexadecanoic acid (left), Phytol (right).

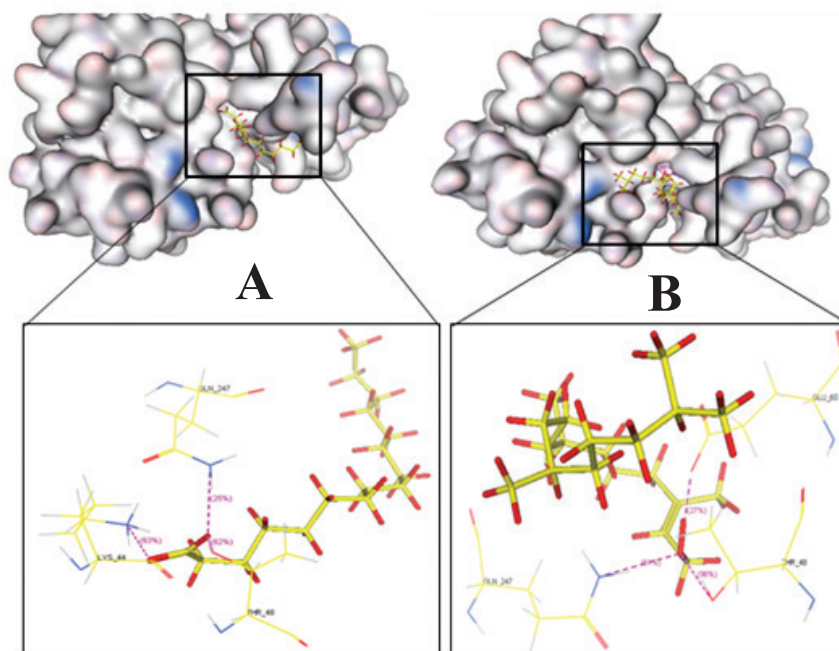


Figure 4. Molecular docking of n-hexadecanoic acid and phytol with moe software. (A) N-hexadecanoic acid binds to Gln247 (25%), Lys44 (63%), and Thr 48 (62%). (B) Phytol binds to Thr48 (96%), Glu52 (27%), and Gln247 (61%). The stick is ligand and the line is cyclin D1 protein. Yellow is carbon (C), double red line of the ligand is hydrogen (H), double red stick of protein is oxygen (O), gray is oxygen (H), and blue is nitrogen (N).

Previous study showed that soursop leaves extract can produce cytotoxic effects on colorectal cancer cell cultures such as HT-29, HCT-116,<sup>5</sup> COLO-205,<sup>8</sup> and DLD-1.<sup>9</sup> Soursop leaves extract is also known to reduce the expression of cyclin D1 protein in phase G1/S.<sup>5,10</sup> Another study showed that ethyl acetate leaves extract of soursop can induce apoptosis in rat azoxymethane-induced colonic aberrant crypt foci in rats and HT-29 cells.<sup>18</sup> In this study, a lower  $CC_{50}$  value indicates that the compound has greater cytotoxicity activity.<sup>19</sup> The  $CC_{50}$  value of 5-FU is lower than the ethanolic leaves extract of soursop because 5-FU is the gold standard therapy for colorectal cancer despite combination therapy is better.<sup>20</sup>

The optimal concentration of ethanolic leaves extract of soursop against colorectal cancer culture cells was 148  $\mu\text{g/mL}$ <sup>9</sup> and the optimal concentration of 5-FU against colorectal cancer culture cells was 37.4  $\mu\text{g/mL}$ . The effective concentration of 5-FU that can inhibit half the population of cancer cells is

30-120  $\mu\text{g/mL}$ ,<sup>21</sup> comparable to our study that 5-FU is more cytotoxic in colorectal cancer culture cells, especially in HT-29 cells with appropriate incubation time and concentration.

Our study showed that cell viability increased with 5-FU at concentrations above 100  $\mu\text{g/mL}$  whereas previous study showed 5-FU concentrations above 100  $\mu\text{g/mL}$  (770  $\mu\text{M}$ ) were non-cytotoxic for colorectal cancer cell due to a decrease in incorporation of 5-FU.<sup>22</sup> Other study showed that 5-FU had limitations for incorporation with DNA or RNA, namely at a concentration of 127 pmol/  $\mu\text{g}$  DNA and 1,0 pmol/  $\mu\text{g}$  RNA, and causing that at such high concentrations, 5-FU could not incorporate with DNA.<sup>23</sup>

N-hexadecanoic acid binds to lysine44, threonine48, and glutamine247. The phytol binds to threonine 48, glutamic acid 60 and glutamine 247. The amino acid position of 31 ... 153 (44, 48, 60) is N-terminal region

of cyclin D1 protein, while the amino acid position of 156 ... 269 (247) is C-terminal region of cyclin protein D1.<sup>24</sup> N-terminal domain is known as cyclin box (56-145). Cyclin box is a domain that regulates binding with cyclin dependent kinase (CDK) and CDK-inhibitors.<sup>25</sup> Cyclin box which is inhibited by other molecules, may cause the cell cannot go to the next phase of the cell cycle. Phytol binds to the cyclin box domain, while n-hexadecanoic acid does not. Phytol that causes complex of cyclin D1-CDK4/6 cannot be formed. N-hexadecanoic acid and phytol bind to glutamine 247, that is a C-terminal region called the PEST motif (241-290). The phosphorylation of cyclin D1 degradation is threonine 286 in the PEST motif (Fig 5).<sup>26</sup> In this study, N-hexadecanoic acid and phytol can trigger cyclin D1 degradation.

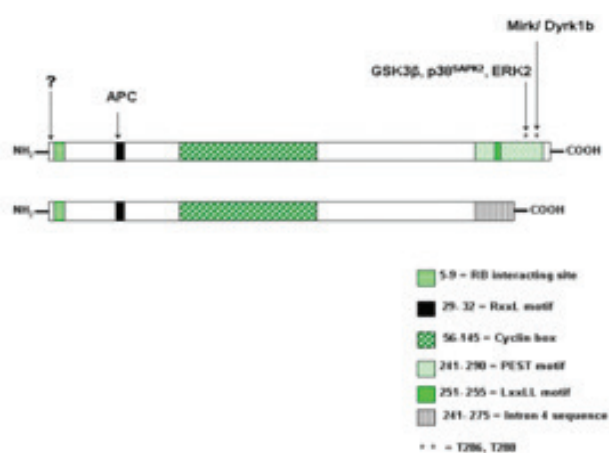


Figure 5. Cyclin D1 protein domain<sup>26</sup>

Molecular docking analysis with MOE obtained two molecules that have the highest  $\Delta G$  and pKi, namely n-hexadecanoic acid ( $\Delta G = -9.7755$  kcal/mol, pKi = 7,219) and phytol ( $\Delta G = -7.2147$  kcal/mol, pKi = 5,975) are good categories ( $< -6,9$  kcal/mol) because the bonds between of them are more stable. The bond only needs a little of energy and the affinity is stronger.<sup>27</sup>

There are three classifications of root mean square deviation (RMSD), i.e good category (RMSD  $\leq 2.0$  Å), category can be accepted (RMSD is between 2.0 and 3.0 Å), and bad category (RMSD  $\geq 3.0$  Å). RMSD is a parameter used to evaluate the similarity of two structures based on the distance between two structures. The stronger bond is the closest distance between them.<sup>28</sup> In this study, the best RMSD is glutamic acid (1.58 Å) in phytol because it has RMSD  $\leq 2.0$  Å and other RMSD of amino acids are acceptable because they have RMSD between

2.0 and 3.0 Å. The two ligands have a strong bond with cyclin D1 protein, n-hexadecanoic acid can be potential as a CDK inhibitor and cyclin D1 inhibition.

In conclusion, the  $CC_{50}$  of the soursop leaves extract is higher than 5-FU and cell viability with a 2 x  $CC_{50}$  concentration of ethanolic leaves extract of soursop is lower than from 5-FU. Molecules (phytochemical components) contained in the ethanolic leaves extract of soursop inhibit the active side of the cyclin D1 protein as shown by molecular docking.

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