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Effects of ethanolic leave extract of soursop (*Annona muricata* L.) on human colorectal cancer line: cell viability and in silico study to cyclin d1 protein

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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 the high proliferation of colorectal cell. Treatment and prevention of colorectal cancer could be done naturally by consuming leave extract of *Annona muricata* L. (soursop). Soursop is known for many phytochemical components such as alkaloid, annonaceous acetogenin, phenol, and flavonol that serve as an anti-cancer.
Method: The research was used HT-29 colorectal cell that given ethanolic leave extract of soursop with 278 µg/mL concentration, 5-Fluorourasil (5-FU) with 88 µg/mL concentration as positive control, solvent control, dan cells control as negative control. The parameters are cell viability with MTT Assay and analysis of molecular docking from ethanolic leave extract of soursop to cyclin D1 protein with molecular operating environment (MOE) 2013.08 software.
Result: Percentage of viable HT-29 cell line decrease in accordance with increasing concentration and the lowest percentage of viable cell is 2 x cytotoxicity concentration 50 (CC50) after ethanolic leave extract of soursop treatment (40,4±1,3%) was compared to 5-FU (30,68±0,93%), 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 that have the lowest free energy (ΔG), i.e 9,7755 kkal/mol and -7,2147 kkal/mol.
Conclusion: Ethanolic leave extract of soursop causes decreasing cell viability of HT-29 cell line on 2 x CC50 concentration was compared to 5-FU, solvent control, dan cells control. N-hexadecanoic acid and phytol molecules have ability to inhibit cyclin D1 protein.

Keywords: Colorectal cancer, ethanolic leave extract of soursop, cell proliferation, molecular docking, cyclin D1

Introduction
Colorectal cancer is a pathological changes in normal colon and rectal tissue to an abnormal tissue caused by genetic and environmental changes [1]. According to International Agency for Research on Cancer (IARC), the colorectal cancer incidence of men in the world is the third largest case (21%) after lung cancer and prostate cancer, while the colorectal cancer incidence of women in the world is the second largest case (14%) after breast cancer [2]. The therapy of colorectal cancer are used to surgery, radiotherapy and chemotherapy [3]. These are less effective because of side effects so the alternative therapy are needed, such as consuming *Annona muricata* L. (soursop) [4, 5].
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 and the find at the leaves, fruits, seeds, and roots that can act as anti-inflammatory, anti-inflammatory infection and anti-cancer [6, 7]. Soursop leave extract can produce cytotoxic effects on colorectal cancer cell cultures such as HT-29, HCT-116, [5] COLO-205, [8] and DLD-1 [9]. Soursop leave extract is also known to reduce the expression of cyclin D1 protein in phase G1/S [5, 10].
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.
The phosphorylated Rb causes a transcription factor E2 factor (E2F) to promote the transcription of genes that needed for cell division [11].

In colorectal cancer, cyclin D1 can be a significant marker. Amplification of the cyclin D1 gene was found in 47 colorectal cancer cell lines or colorectal cancer cell cultures, such as HT-29 cells [12, 13]. Bahnassy *et al*. denotes high regulation of cyclin D1 was detected at 68.3% carcinoma cases and compared with normal colorectal mucosa [14] High protein expression of cyclin D1 was detected at 47% stage I and II, 37% stage III and IV [15]. High expression of cyclin D1 causes abnormal cell cycle.

Material and Method Plant Materials

Soursop leave was extracted by 96% ethanol and obtained from Indrawati, *et al*. [9]

Extract has concentration of 0, 36% (b/b) annonaceous acetogenin, 46, 83% (b/b) phenol, and 0, 83% (b/b) flavonoid.

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 (GibcoTM), 1% penicillin-streptomycin (GibcoTM), 1% amphotericin B (GibcoTM), dan 10% fetal bovine serum (GibcoTM) in a humidified atmosphere with 5% CO_2 in the air at 37 °C. Cells in the exponential growth phase were collected for the next experiments.

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 Bahaguna A *et al*. [16] In brief, cells (1x104 cells/mL) were treated with ethanolic leave 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 negative control at different concentrations in 96-well plate and incubated for 24 h. Concentration of ethanolic leave extract of soursop is 12,5-400 µg/mL, 5-FU is 25-100 µg/mL, and 0,1% DMSO. The colorimetric assay was measured at the absorbance of 570 nm using a microplate reader (PromegaTM GlomaxTM). The antiproliferative potential of the treatments were expressed as cytotoxicity concentration (CC50) value, the concentrations that caused 50% inhibition of cell growth which was calculated based on the percentage of cell viability. The percentage of cell viability is ¼ (absorbance of treated cells/absorbance of untreated cells) x 100%. The CC50 of ethanolic leave extract of soursop and 5-FU were obtained by the first MTT assay and evaluated by next MTT assay. This assay was used concentration ½ x CC50, 1 x CC50, and 2 x CC50. The procedure of this MTT assay is as the same as procedure above.

Molecular Docking with Cyclin D1 Protein

Docking simulation of extract *Annona muricata* to cyclin D1 was started by ligand (compounds) and reseptor (protein) preparation. The compounds were obtained by Gavamukulya Y, *et al*, 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; dan 1,2-benzenedicarboxylic acid, diisooctyl ester. The structure of the compounds were from chemspider database (www.pubchem.com). Cylin D1 protein of fasta sequences were obtained from database national center for biotechnology information (NCBI) and the 3-D structure was from http://swissmodel.expasy.org (Figure 1).

The ligand and protein were optimized by molecular operating environment (MOE) 2013.08 software. Geometry optimization and minimization of cyclin protein energy were carried out using MOE software with the PDB format. The structure of cyclin protein D1 are added parameters such as hydrogen atoms, partial charge, and gas phase. The addition of hydrogen atoms and protonation were performed on cyclin D1 protein. Partial charges were regulated by using a partial charge and energy was minimized by the Merck Molecular Force field 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 is obtained in the moe format.

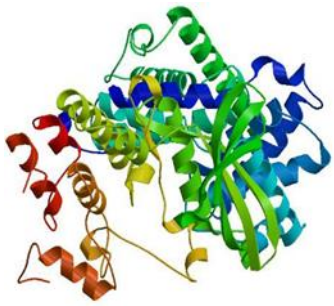

Fig 1: 3D-cyclin D1 protein structure (2w96.1.A)

Geometry optimization and energy minimization of ligand structures using MOE software with the format .mdb. Ligand candidates were kept with the .mol format in the database viewer of MOE software. The ligands was washed with the compute program, the partial charge was adjusted and optimized by using the MMFF94x force field. Furthermore, the energy of the ligands was minimized using energy minimize with the RMS 0.001 kcal/ Åmol gradient and the result file was kept with the format .mdb. Molecular docking of the ligand molecule with cyclin D1 protein was performed with the computer simulation of dock program in the MOE. Molecular docking used triangle matcher 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 retain of the refinement results was the most suitable conformation of each ligand molecules. The calculation of molecular docking were seen in the output of the viewer.mdb format. Some parameters of the protein-ligand interaction can be analyzed, including bond free energy (ΔG) and affinity (pKi). The selected protein-ligand complex was the smallest bond energy value and the greatest bond affinity.

Statically Analysis

Value of cell viability was presented as mean±SEM of four different experiments. A one-way analysis of variance (ANOVA) was performed using SPSS v.21. Differences were considered as being significant at $p<0.05$.

Results and Discussion

HT-29 culture cell before and after treatment

One of HT-29 characteristic is adherent or stick to the base of the flask or microplate. They are seen under microscope like oval and flat cells. The treated HT-29 cells with MTT assay shows viable and dead cells. Viable cells have formazan

crystals shaped like needles and purple, while dead cells do not have these formazan crystals (Figure 2).

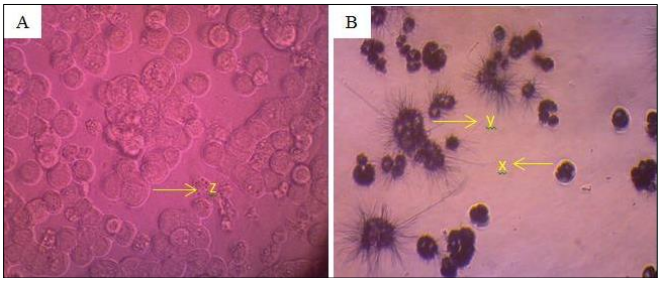

Fig 2: HT-29 cell before and after treatment (MTT assay). (A) HT-29 before treatment with 70% confluence (400x), (B) HT-29 after treatment (400x). (x: dead cell, y : viable cell with formazan crystals, z : viable cells before treatment).

Cell Viability Assay

The linear regression curve between the concentrations of the two test compounds, i.e ethanolic leave extract of soursop and 5-FU with the percentage of HT-29 cells (viable). The regression equation of ethanolic leave extract of soursop is y = -0.0363x + 60.078 and R2 = 0.8802, while the regression of 5-FU is y = -0.3067x + 77.052 and R2 = 0.9782 (Figure 3). The CC50 value was obtained from x variable by entering 50 (the standard value was taken from inhibition of 50%) to y variable in the regression equation of two compounds. CC50 is used to determine the concentration of ethanolic leave extract of soursop and 5-FU that can inhibit a half of cell population. The compound with the lowest CC50 value has the greatest citotoxicity activity [17].

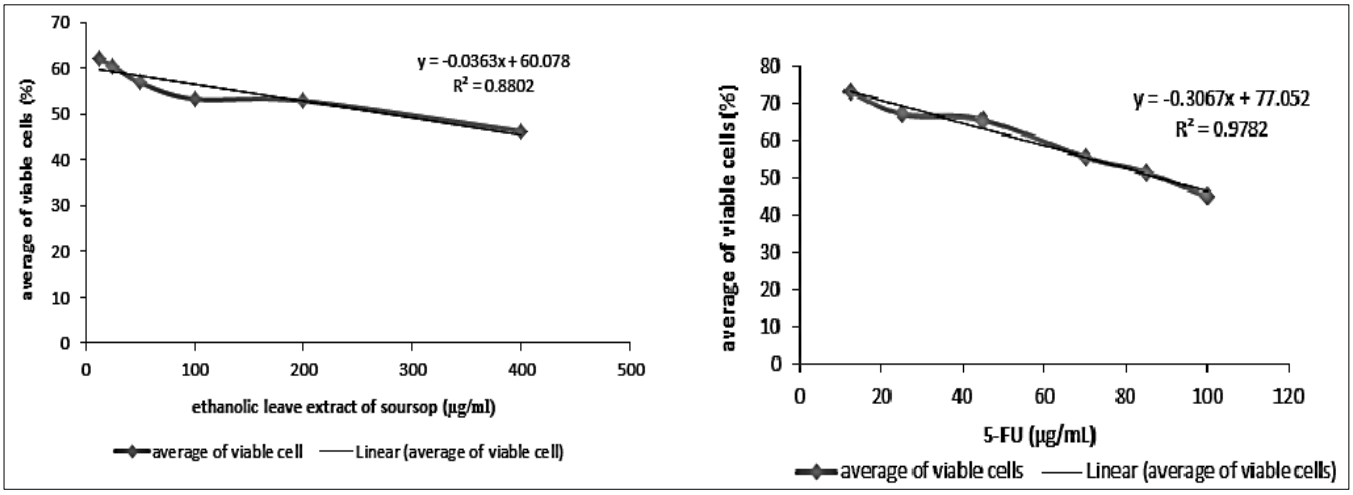

Fig 3: The standard curve of the cytotoxicity test of soursop leave extract and 5-FU. (A) CC50 of soursop leave extract is 278 µg/mL. (B) CC50 of 5-FU is 88 µg / mL.

Based on the cytotoxic test of ethanolic leave extract of soursop and 5-FU showed that 5-FU has cytotoxic activity three times lower than ethanolic leave extract of soursop, that is CC50 of soursop extract is 278 µg / mL and CC50 of 5-FU is 88 µg/mL. Indrawati, *et al*. [9] has investigated the optimal concentration of ethanolic leave extract of soursop against colorectal cancer culture cells was 148 µg/mL. Calabro-Jones, *et al*. [18] has investigated the optimal concentration of 5-FU against colorectal cancer culture cells was 37.4 µg/mL. 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.

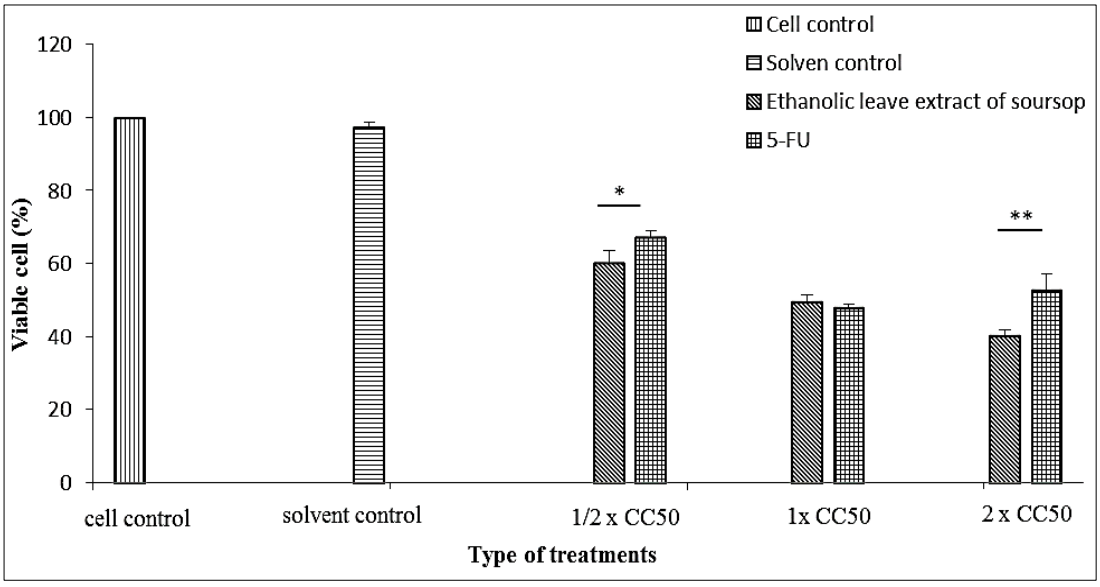

Fig 3: Cell viability of HT-29 after treatment with ethanolic leave extract of soursop and control. One-way ANOVA statistical test and post-hoc LSD test shows the effect of exposure time of soursop leave extract and positive control (5- FU) on HT-29 cell viability. The sign (*) shows a significant difference (p <0.05) and the sign (**) shows a significant difference (p <0.01).

The group of the soursop leave extract was compared to 5-FU for ½ x CC50 concentration and appeared to be significantly difference with a value of p = 0.001 (p <0.05). Ethanolic leave extract of soursop has 7.1% lower viability cell than compared to 5-FU. Ethanolic leave extract of soursop was compared to 5-FU for 1 x CC50 concentration have no significantly difference (p> 0.05) with a value of p= 0.509. The difference of this viability is only 1.7%, that is viability cell of 5-FU is lower than ethanolic leave extract of soursop. Ethanolic leave extract of soursop was compared to 5-FU for of 2 x CC50 concentration has a significantly difference with a value of p< 0.01. Cell viability of ethanolic leave extract of soursop is 12.4% lower than 5-FU (Figure 2). If we can see through ½ x CC50, 1 x CC50, dan 2 x CC50 concentration, cell viability of ethanolic leave extract of soursop is lower than 5-FU, but if we can see the value of concentration, soursop extract is 139, 278, dan 556 µg/mL and 5-FU is 44, 88, dan 176 µg/mL, 5-FU has lower concentration than soursop extract.

Calabro-Jones, *et al*.,18 showed effective concentration of 5-FU that can inhibit a half of population of colorectal cancer cells is 30-120 µg/mL and De Angelis *et al*. [19], showed concentration of 5-FU above 100 µg/mL (770 µm) can be noncytotoxic for colorectal cancer cell because of decreasing of 5-FU incorporation. Noordhuis *et al*. [20], showed 5-FU has a limit to incoporate with DNA or RNA, that is 127 pmol/ µg DNA and 1,0 pmol/ µg RNA and it causes high concentration 5-FU cannot incorporate to DNA.

Molecular Docking with Cyclin D1 Protein

Table 1: Analysis of molecular docking between molecules from ethanolic leave extract of soursop and cyclin D1 protein

No	Compounds	ΔG (Kcal/mol)	pKi	Residu Contact
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	N-Hexadecanoic acid	-9,7755	7,219	Lys44, Thr48, Gln247
9	Hexadecanoic acid, ethyl ester	-6,1965	4,199	Met252
10	Phytol	-7,2147	5,975	Thr48, Glu60, Gln247
11	1,2-Benzenedicarboxylic acid, diisooctyl ester	-6,4074	5,322	Thr48
12	9,12-Octadecadienoic acid, ethyl ester	-4,9061	3,635	-

Ket: ΔG = gibbs free energy (kcal/mol); pKi = affinity; glutamine (Gln), aspartic acid (Asp), methionine (Met), asparagin (Asn), lysine (Lys), threonine (Thr), asam glutamate acid (Glu). The number which is next to amino acid shows amino acid position of cyclin D1 sequences.

The analysis of molecular docking with MOE was obtained two molecules that have the lowest gibbs free energy (ΔG) and the strongest affinity (pKi), N- hexadecanoic acid (ΔG = -9.7755 kcal/mol, pKi = 7,219) and phytol (ΔG = - 7.2147 kcal/mol, pKi = 5,975) (Table 1, Figure 4). Value of ΔG is less than -6.9 kcal/mol is the stable bond between ligand and protein [21].

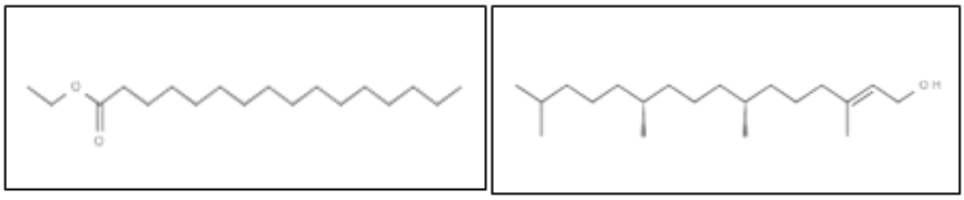

Fig 4: 2D-ligand structure with the lowest value of ΔG and the strongest value of pKi. N-hexadecanoic acid (left), Phytol (right).

N-hexadecanoic acid binds to lysine44, threonine48, and glutamine247. The phytol binds threonine 48, glutamic acid 60 and glutamine247. 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. [22] N-terminal domain is known as cyclin box (56-145). Cylin box is domain that regulate binding with cylin dependent kinase (CDK) and CDK-inhibitors [23]. Cyclin box which is inhibited by another molecule, 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 causes complex of cyclin D1-CDK4/6 cannot be formed. N-hecadecanoic acid and phytol bind to glutamine247, that is C- terminal region called the PEST motif (241-290). The phosphorylation of cyclin D1 degradation is threonine286 in the PEST motif (Figure 5) [23]. In this study, N- hecadecanoic acid and phytol can trigger cyclin D1 degradation.

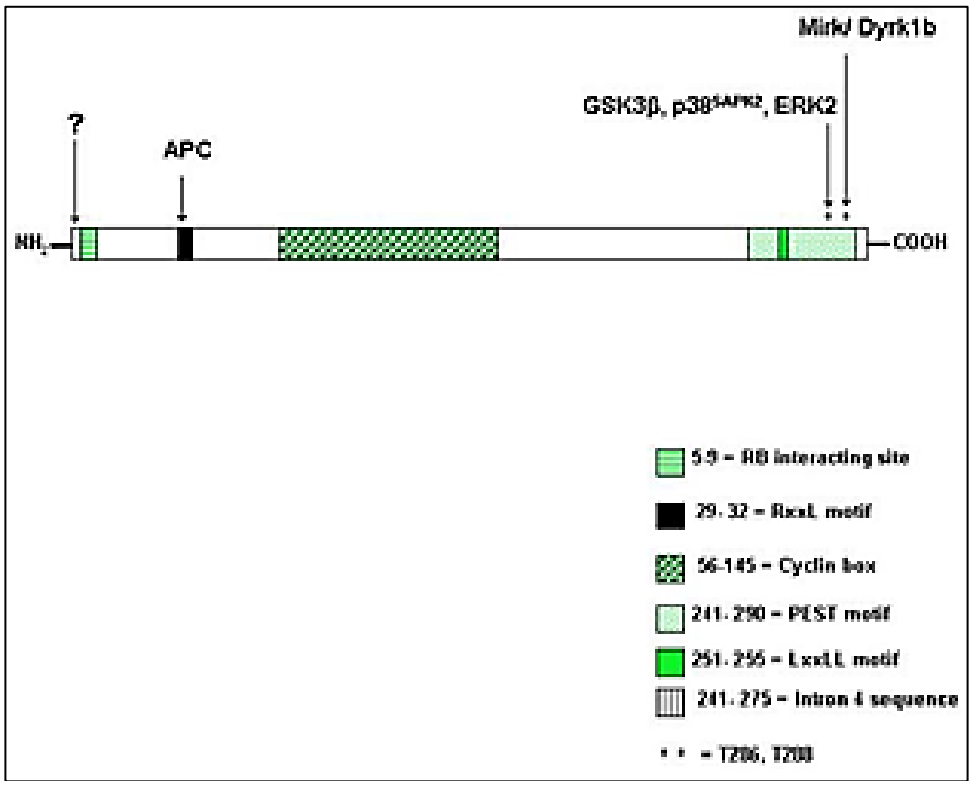

Fig 5: Cyclin D1 structure [23]. Cyclin D1 has some domains such as RB interacting site, RxxL motif, cyclin box, PEST motif, and LxxL motif.

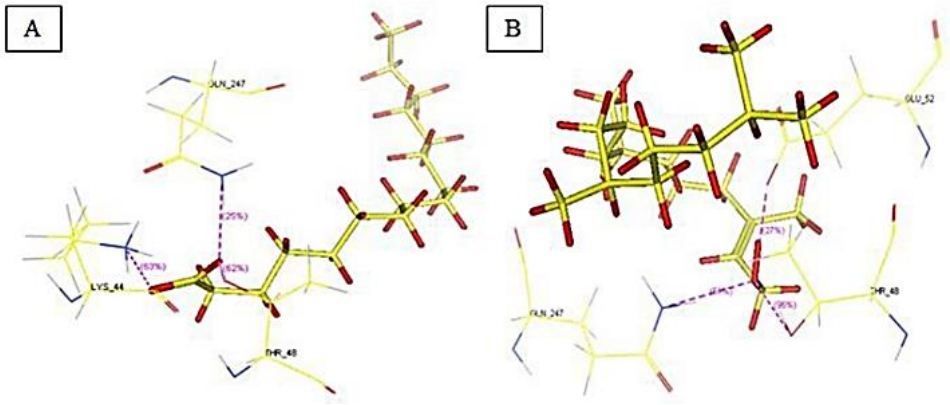

Fig 6: Molecular docking of N-hexadecanoic acid and phytol with MOE software. (A) N-hexadecanoic acid binds to Gln247 (25%), Lys44 (63%), and Thr 48 (62%). (B) Phytol binds to Thr48 (96%), Glu52 (27%), and Gln247 (61%). The stick is ligand and the line is cyclin D1 protein. Yellow is carbon (C), double 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).

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 threonin 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 (Figure 6).

There are three classification of root mean square deviation (RMSD), good category (RMSD ≤ 2.0 Å), category can be accepted (RMSD is between 2.0 and 3.0 Å), and bad category (RMSD≥ 3.0 Å). RMSD is a parameter used to evaluate the similarity of two structures based on the distance between two structures. The stronger the bond is the closest distance between them [24]. In this study, the best RMSD is glutamic acid (1.58 Å) in phytol because it has RMSD ≤ 2.0 Å and other RMSD of amino acids are acceptable because they have RMSD between 2.0 and 3.0 Å. Two ligands have a strong bond with cyclin D1 protein, n-hexadecanoic acid can be potential as a CDK inhibitor and cyclin D1 inhibition.

Acknowledgements

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