ORIGINAL ARTICLE

In Silico Study and *In-Vitro* Activity of Buds Cloves (*Syzygium aromaticum* L.) of Nonvolatile Compounds as Anticancer by Inhibiting Cell Cycle Regulators

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Abstract

Aim: In order to investigate the potential biological activities, we simulated a series of cyclin-dependent kinases (CDKs) (CDK1, CDK2, and CDK6 series) with molecular docking approach experiments to identify the bioactive compounds and evaluated the *in vitro* antitumor activity of ethanol extract of buds cloves (EEBC). In addition, we conducted a series of experiments to identify the potential biological mechanisms of the EEBC. **Material and Methods:** In this study, we investigated *in silico* molecular docking to know about their molecular mechanisms of EEBC and the active components for cytotoxic activity. This research was carried out using AutoDock4 software. To prove the *in silico* docking result, we also investigated the cytotoxic effects of EEBC using cell lines of human cancer. **Results:** Based on all the docking result, Myricetin and Quercetin in EEBC played a main role as CDKs inhibitor because they showed very good free energy binding against CDK2, CDK6, and CDK1. IC_{50} values of EEBC (IC_{50} of 24.45 µg/ml) on HCT-116. **Conclusion:** EEBC could be potential sources from natural products that have cytotoxic properties against colorectal cancer through CDKs inhibition mechanism.

Key words: Anticancer, cell cycle inhibition, clove, *in silico*, *in vivo*, Syzygium aromaticum

INTRODUCTION

Cell cycle checkpoints, especially G1/S and G2/M, were controlled the cell-cycle activity, is involved in regulating and monitoring the progression.[1] The complex of cyclin-dependent kinases (CDK) show a linear progression that lead resting state activity from the cell (G0), growth phase (G1), DNA replication (S), and until the cell division (M). Complex of cyclins, associated CDKs, and assembly factors will affect the canonical roles regulation of cell cycle checkpoint.[2] CDK proteins are expressed in cells, that synthesized at specific stages of the cell cycle, based on the response of various molecular alert.[3,4]

Overexpression of Cyclin A linear with its responsibility of invasion and metastasis in prostate cancer and may in colorectal carcinogenesis. Increasing of Cyclin A was happened at S-phase onset. Cyclin A will bind to CDK1, CDK2, and phosphorylates targets that manage DNA replication.[5] The therapeutic target in cancer was Cyclin D,[6] that had main contribution in cell cycle progression with CDK4 and CDK6.[7] Cyclin D1 also had specific functions to manage gene expression of local chromatin, promote chromosomal instability, and cellular migration.[8] Therefore, inhibit the activity of CDKs potentially become a good method of cancers therapy, one of them was inserting small molecules into its ATP-binding pocket.[9]

Natural compounds from plants were often showed activity to inhibit tumor cell proliferation by *in vitro*. Compounds

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that have anti-proliferative activities also showed similar molecular mechanisms in downregulation of specific cyclins and CDKs, while upregulating inhibitors of CDKs.[10] Buds Cloves (Syzygium aromaticum L.) that one of biggest commodity herbs from Indonesia, are consist of a mixture of flavonol, glucosides, tannins, and phenolic acids. Volatile oils (eugenol, acetyl eugenol) of buds cloves showed the best anti-oxidant activity among other plants.[11,12] Besides that, cloves buds also show good activities, such as anti-inflammatory, antiproliferative, antibacterial anti-inflammatory, and antiseptic, which make this plant had potential activity for anticancer.[11-13]

In order to investigate the potential biological activities, we simulated a series of CDKs (CDK1, CDK2, and CDK6 series) with molecular docking approach experiments to identify the bioactive compounds and evaluated the *in vitro* antitumor activity of ethanol extract of buds cloves (EEBC). In addition, we conducted a series of experiments to identify the potential biological mechanisms of the EEBC.

MATERIAL AND METHODS

In silico experiment

MacBook with operating system MacOS High Sierra v10.13.3, RAM 8 GB, processor 1,8 GHz Intel i5, Graphics Intel HD 6000 1536MB, storage 1600 MHz DDR3.

Protein and ligand preparation

3D structure of CDK series (Protein Data Bank [PDB] ID: 1O86) was downloaded from the PDB.[14] The chemical structure of nonvolatile compounds of buds cloves were prepared using Marvin Sketch and saved in PDB format. The protein and ligands were converted to PDBQT file using AutoDocks tool to set the atomic coordinates.

Analysis of target active binding sites

The active sites are the coordinates of the ligand in the original target protein grids, and these active binding sites of target protein were analyzed using the Phyton Molecule Viewer.[15]

Molecular docking analysis

A computational ligand-target docking approach was used to analyze structural complexes of the CDK with nonvolatile compounds of bud clove (ligand) in order to understand the structural basis of this protein target specificity. Molecular docking of designed compounds was carried out using the Lamarckian genetic algorithm in AutoDock4.2 tools with default docking parameter. Protein-ligand attraction was investigated for hydrophobic/hydrophilic properties of these complexes by AutoDock. Docking result was carried out based on scoring functions. Docking interactions had been clustered to decide the Gibbs energy (ΔG), and optimum docking energy conformation was considered as the fine-docked pose.

Preparation of buds clove

The buds cloves were collected from Balai Penelitian Tanaman Rempahdan Obat (Balitro) in Bogor. Before making the extract, the clove should be cleaned, dried in the room temperature for 24 h, and then blended until they are smooth enough to be diluted with solvent.

Production of EEBC

The making of EEBC uses 500 g of buds clove that have been pounded and smoothed and undergo maceration with ethanol 70% for about 1000 ml. After 3 days, the solution is filtered to separate the filtrate and the precipitate. The filtrate is collected and it is done until the filtrate is colorless. After that, rotary evaporator is used with 50 rpm in the temperature of 30–40°C to conduct the evaporation, resulting more concentrated filtrate that can be used as the EEBC. Then, the extract is dried by using oven at the temperature of 40°C and keep it at the temperature of 4°C.

In vitro experiment

Human cancer cells, including breast cancer cells (MCF-7), cervical epithelial cells (HeLa), and colorectal cancer cells (HT-29), were got from Pathological Anatomic Department, Faculty of Medicine Universitas Indonesia. Cells were maintained in RPMI-1640 media (GIBCO, USA) that enriched with 10% fetal bovine serum (Invitrogen) in a humidified incubator with 5% CO_2 at temperature 37°C.

The inhibitions effect of samples on HeLa, HCT-116, and MCF7 cells line were tested using chemical 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) or MTT (Merck).[16] First, in 96-well plates, cells were seeded at 5000 cells/well and incubated for 24 h. Media become renewed and the cells had been added with several concentrations of the extract (6–100 µg/ml) and doxorubicin as positive control (0–16 µg/ml) incubated for a further 24 h. After 24 h, 20 µm of MTT solution (0.5 mg/ml MTT solution in media) had been added to each well and incubated for 4 h at 37°C. The supernatant was aspirated and the MTT-formazan crystals formed by way of metabolically viable cells were dissolved in 100 µl of dimethyl sulfoxide/DMSO (Merck). In the end, the absorbance was monitored by a microplate reader at a wavelength of 570 nm. The percentage of viable cells was plotted versus the concentration of the test compound. The IC_{50} value was determined using linear regression analysis.

Statistical analysis

The results are expressed as mean ± standard error of the mean of three replicate determinations. One-way analysis of variance and *post hoc* Tukey's test were used to determine the differences among the means. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

In silico docking

Natural compounds in EEBC were screening to know which compound that plays the main role as CDKs inhibitor. The result is shown in Table 1.

From Figure 1 and Table 2, input binding site residues, Ile19, Val27, Ala41, Lys 43, Val77, Phe98, His100, Val101, Gln103, Asp104, Gln149, Asp163, Leu152, Ala162, and Asp163 have the interactions with the ligands that used in this study. Fifteen residues of the protein showed have interactions with the ligand, 9 residues were hydrophobic, and 6 residues were polar. The residues Ile 19, Val 27, Lys43, Phe98, Val101, Asp104, Gln149, Asp163, Leu152, Ala162, and Asp163 have more interactions with the ligands. The residues Lys 43 and Val 101 have interaction in the crystal structure which means that residues were important binding site residues. From the result, many of the ligands interacting with these residues through Hydrogen bonds, thus emphasizing the importance of them as targets for inhibitors.[17] Myricetin, Quercetin, and Luteolin, were observed to have a ΔG of −10.29 kcal/mol, −10.04 kcal/mol, and −10.03 kcal/mol, respectively. The native ligand (inhibitor) of CDK6 showed good binding interaction to CDK6 protein with lower docking ΔG score (−10.24 kcal/mol) than Myricetin, but higher docking ΔG score of other Clove's compounds [Table 1].

From Figure 2 and Table 3, input binding site residues Lys33, Val64, Phe80, Phe82, His84, Asp86, and Asp145 have the interactions with the ligands that used in this study. Twenty-one of the protein showed have interactions with

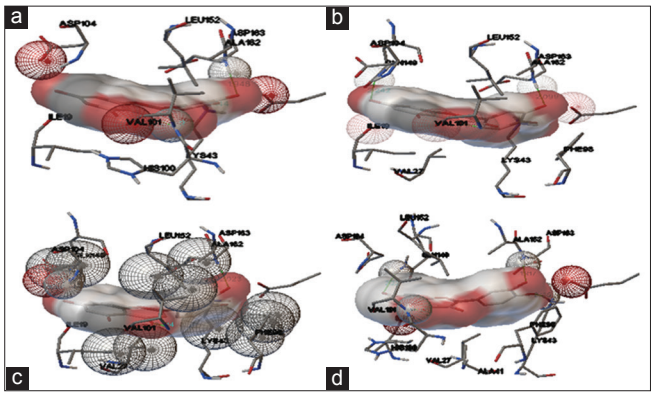

Figure 1: 3D interaction between ligand and cyclin-dependent kinases 6 receptor residue (a) Myricetin, (b) Quercetin, (c) Luteolin, (d) Native ligand, *green dots with red circle showed the hydrogen binding

Table 1: Docking result of unvolatile compounds of EEBC

Compound	CDK6 (1XO2)		CDK2 (3FZ1)		CDK1 (4Y72)	
	ΔG (Kcal/mol)	Ki (nM)	ΔG (Kcal/mol)	Ki (μm)	ΔG (Kcal/mol)	Ki (nM)
Biflorin	−8.87	312.41	−8.00	1.36	−8.50	583.52
Dimetilluteolin	−8.44	646.86	−7.69	2.30	−8.77	373.10
Ellagic acid	−8.93	286.26	−7.68	2.33	−8.42	671.20
Gallic acid	−5.20	154 μm	−4.93	242.22	−5.11	179.95 μm
Hydrogallic acid	−5.42	106.32 μm	−5.08	189.26	−5.94	44.54 μm
Isobiflorin	−9.37	136.49	−8.22	0.94	−9.18	187.26
Isorhamnetin	−9.89	56.47	−8.17	1.03	−9.50	107.93
Kaempferol	−9.42	124.32	−7.97	1.44	−9.22	174.10
Luteolin	−10.03	44.33	−8.22	0.94	−9.78	68.29
Myricetin	−10.29	28.48	−8.68	0.43	−10.22	32.06
Naringenin	−9.01	247.24	−7.60	2.70	−9.39	131.33
Oleanolic acid	−8.22	0.93	−9.87	0.058	−7.89	1.65 μm
Quercetin	−10.04	43.89	−8.27	0.86	−9.94	51.59
Quinic acid	−5.19	152.15 μm	−4.97	228.11	−5.22	148.06 μm
Rhamnetin	−9.98	48.32	−8.22	0.94	−10.02	45.21
Rhamnocitrin	−9.28	157.95	−7.91	1.58	−9.37	136.39
Native ligand	−10.24	31.26	−6.68	12.70	−9.25	166.83

EEBC: Ethanol extract of buds cloves, CDK: Cyclin-dependent kinases

Table 2: Residue CDK6 – ligand interaction analysis

Amino acid	Myricetin	Quercetin	Luteolin	Native ligand
Ile19	√	√	√	√
Val27	√	√	√	√
Ala41	√	-	-	√
Lys43	√*	√	√	√*
Glu61	√	-	-	-
Val77	√	-	-	-
Phe98	√	√	√	√
His100	√	-	-	√
Val101	√*	√*	√	√*
Gln103	√	-	-	-
Asp104	√	√	√	√
Gln149	-	√*	√	√*
Leu152	√	√	√	√
Ala162	-	√	√	√
Asp163	√*	√*	√	√*

CDK: Cyclin-dependent kinases

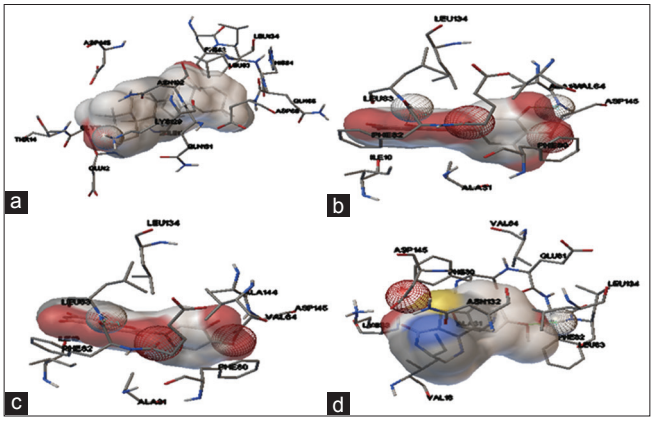

Figure 2: 3D interaction between ligand and cyclin-dependent kinases 2-receptor residue (a) Oleanolic acid, (b) Myricetin, (c) Quercetin, (d) Native ligand, *green dots with red circle showed the hydrogen binding

the ligand, 11 residues were hydrophobic, and 10 residues were polar. The residues Val64, Phe80, Phe82, and Asp145 have more interactions with the ligands. Honda *et al*.[18] stated that Asp145 is part of the active site of CDK2, which have responsibility in substrate recognition and Lys 33 may have responsibility in stabilizing the triphosphates moiety of ATP for catalysis. From the docking result, it showed that the ligands were binding to Asp145, this emphasize the ligands could be potential CDK2 inhibitors. Oleanolic acid, myricetin, and quercetin were observed to have a ΔG of −9.87 kcal/mol, −8.68 kcal/mol, and −8.27 kcal/mol, respectively. The native ligand (inhibitor) of CDK2 showed good binding interaction to CDK2 protein with lower docking ΔG score (−6.68 kcal/mol) than most of the clove's compounds [Table 1].

Table 3: Residue CDK2 – ligand interaction analysis

Amino acid	Oleanolic acid	Myricetin	Quercetin	Native ligand
Ile10	√	√	√	-
Glu12	√	-	-	-
Gly13	√	-	-	-
Thr14	√	-	-	-
Val18	-	-	-	√
Ala31	-	√	√	√
Lys33	-	-	-	√
Val64	-	√	√	√
Phe80	-	√	√	√
Glu81	-	-	-	√
Phe82	√	√	√	√
Leu83	√	√*	√*	√*
His84	√	-	-	-
Gln85	√	-	-	-
Asp86	√	-	-	-
Lys129	√*	-	-	-
Gln131	√	-	-	-
Asn132	√	-	-	√
Leu134	√	√	√	√
Ala144	-	√	√	
Asp145	√	√*	√	√

CDK: Cyclin-dependent kinases

From Figure 3 and Table 4, input binding site residues Lys33, Val64, Phe80, Ser84, Asp86, and Asp146 have the interactions with the ligands that used in this study. Fourteen residues of the protein showed have interactions with the ligand, 8 residues were hydrophobic, and 6 residues were polar and. The residues Val64, Phe80, and Asp146 have more interactions with the ligands. Honda *et al*.[18] stated that Asp146 is part of the active site of CDK1, which have responsibility in substrate recognition and Lys 33 may have responsibility in stabilizing the triphosphates moiety of ATP for catalysis. From the result, many of the ligands interacting with these residues through hydrogen bonds, thus emphasizing the importance of them as targets for CDK1 inhibitors. Myricetin, Rhamnetin, and Quercetin were observed to have a ΔG of −10.22 kcal/mol, −10.02 kcal/mol and −9.94 kcal/mol respectively. The native ligand (inhibitor) of CDK1 showed good binding interaction to CDK2 protein with lower docking ΔG score (−9.25 kcal/mol) than most of the clove's compounds [Table 1]. Based on all the docking result, Myricetin and Quercetin in EEBC play a main role as CDKs inhibitor because they showed very good free energy binding against CDK2, CDK6, and CDK1.

In-vitro activity

We analyzed the growth inhibitory effects of EEBC in MCF7, HCT-116, and Hela cell line. The IC_{50} concentration using

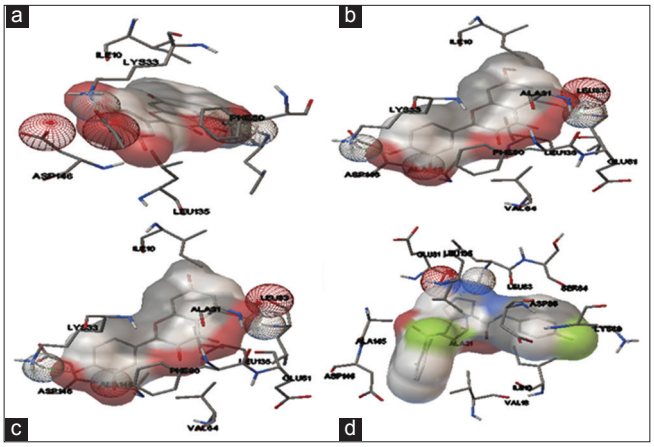

Figure 3: 3D interaction between ligand and cyclin-dependent kinases 1 receptor residue (a) Myricetin, (b) Rhamnetin, (c) Quercetin, (d) Native ligand, *green dots with red circle showed the hydrogen binding

Table 4: Residue CDK1 – ligand interaction analysis

Amino acid	Myricetin	Rhamnetin	Quercetin	Native ligand
Ile10	√	√	√	√
Val18	-	-	-	√
Ala31	-	√	√	√
Lys33	√	√*	√*	-
Val64	-	√	√	-
Phe80	√	√	√	-
Glu81	-	√	√	√
Leu83	√*	√*	√*	√*
Ser84	-	-	-	√
Asp86	-	-	-	√
Lys89	-	-	-	√
Leu135	√	√	√	√
Ala145	-	√	√	√
Asp146	√	√*	√*	√

CDK: Cyclin-dependent kinases

Table 5: Cytotoxic activity

Compound	MCF-7 IC_{50} (µg/ml)	HCT-116 IC_{50} (µg/ml)	HeLA IC_{50} (µg/ml)
EEBC	30.62±0.105	24.45±0.123	45.08±0.116
Doxorubicin	14.46±0.050**	20.91±0.082	29.70±0.080

**Statistically significance, $P<0.05$

the MTT assay was found from the regression line. From the MTT assay result, we got the IC_{50} value, that the smaller IC_{50} value, the higher cytotoxic activity. From the MTT assay result, EEBC showed the best cytotoxic activity in HCT-116 cell line with IC_{50} value 24.45 ± 0.123µg/ml. The IC_{50} value of EEBC was similar to IC_{50} value of doxorubicin as positive control. Cytotoxicity assay of gallic acid derivative are summarized in Table 5.

Serine/threonine protein kinases or CDK are essential kinases that mediate the cell cycle. There are nine CDKs that have been identified and each CDKs manages specific points of the cell cycle. CDK4, CDK6, and CDK2 mediate the G1 phase,CDK2 regulate the S phase, and CDK1 control the G2-M phase. Cyclin E-dependent kinase activity substantially higher in colorectal cancers and expression levels of CDK2 are also high in colorectal adenomas. Three colorectal cancer cell lines, HCT116, HCT15, and DLD-1, expression of CDK2 will dramatically increase.[19] As shown in Table 2, EEBC gave better inhibition activity in HCT-116 cell line than in MCF-7 and HeLa cell line. This result may because of high expression of CDKs in HCT116 cell line. This *in vitro* result are also in line with the result of *in silico* docking, that showed EEBC compounds can inhibit CDKs activity. Based on the criteria of the American National Cancer Institute, crude extract that promising for further purification based in the IC_{50} values is lower than 30 µg/mL.[20] Consider to this criteria, IC_{50} values of EEBC (IC_{50} of 24.45 µg/ml) on HCT-116 are well within the limit. Therefore, EEBC could be potential sources from natural products that have cytotoxic properties against colorectal cancer through CDKs inhibition mechanism.

CONCLUSION

EEBC could be potential sources from natural products that have cytotoxic properties against colorectal cancer through CDKs inhibition mechanism.

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