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Acetylation of Emodin and Cytotoxic Activity Effect Against HepG2 Cell Lines

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Abstract

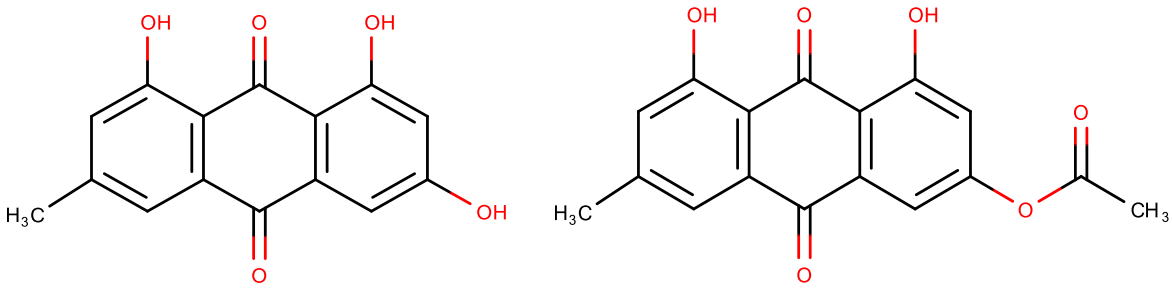
Emodin (1,3,8-trihydroxy-6-methyl-9,10-anthraquinone) is an anthraquinone bioactive compound used as a lead compound because it exhibits potential anticancer properties. Structural modifications were made at the C3 position and its relationship to cytotoxic activity against the HepG2 cell line to determine the pharmacophore group of this compound. The hydroxy group at C3 emodin is converted to an ester group to produce 3-acetyl emodin. In addition, docking simulations into the cancer target protein casein kinase-2 were also carried out to predict molecular interactions. Emodin was reacted with anhydrous acetate and confirmed the product confirmation using LCMS/MS, FTIR, ^{1}H-NMR, and ^{13}C-NMR. Emodin and 3-acetyl emodin were tested for cytotoxicity against HepG2 cells in vitro. Cytotoxic emodin and 3-acetyl emodin tests on HepG2 cells resulted in Cytotoxic concentrations 50 (CC_{50}) of 0.54 mM and 0.42 mM, respectively. The results showed that modifying the C3 hydroxyl group with acetyl can increase the cytotoxic effect more than emodin. This research is expected to provide information regarding the structure-activity relationship of emodin in cancer cells and the expansion of new drug applications for additional cancers.

***Keywords**: Acetylation, cytotoxic, docking simulation, Emodin, HepG2*

Background

Emodin (1,3,8-trihydroxy-6-methyl-9,10-anthraquinone) is an active compound that can be found in plant extracts including *Cassia multijuga* (Leguminosae) (Kristanti, 2010), *Rumex japonicus* (Guo *et al*., 2011), *Fallopia japonica* and *Fallopia sachalinensis* (Frantík *et al*., 2013), *Polygenum multiflorum, P. cuspidatum, Rumex patentia, Rhamnus catharticus, Rhamnus orbiculatus, Aloe vera, Acorus tatarinowii, Cassia obtusifolia, C. occidentalis, Rheum palmatum, R. officinale, Eriocaulon buergerianum, Dendrobium thyrsiflorum, Fibraurea tinctoria, Coptis chinensis, Scutellaria baicalensis, Isatis indigotica*, and *Rumex chalepensis* (Dong, 2016; Hsu, 2012). The structure of emodin has similarities with cancer drugs and doxorubicin and mitoxantrone, so many

studies have been conducted to develop new anticancer. Emodin shows the activity as anti-neoplastic, anti-inflammatory, anti-angiogenesis, and toxicological potential for use in pharmacology, both *in vitro* and *in vivo* (Dong, 2016). Emodin exhibits cytotoxic effects through cell cycle arrest and apoptosis induction in cancer cells. Overall emodin molecular mechanisms include cell cycle arrest, apoptosis, and promotion of the expression of hypoxia-inducible factor 1a, glutathione S-transferase P, N-acetyltransferase, and glutathione phase I, as well as detoxification enzymes II. In addition, emodin inhibits angiogenesis, invasion, migration, chemically induced carcinogen-DNA formation, HER2/neu, CKII kinase, and p34 cdc2 kinase in human cancer cells (Hsu, 2012; Teich, 2012).



A **B**

Figure 1. (A) Emodin and (B) 3-acetyl emodin

However, the activity of emodin is still relatively low, so it is necessary to optimize and modify the structure further to increase its activity. One of the methods used in this research is to perform semi synthesis on its derivatives, namely 3-acetyl emodin. In this study, the structural modification was carried out only at the C3 position of emodin because it was the easiest to modify by replacing the hydroxy with an acetyl group. To predict the interaction between emodin and 3-acetyl emodin with casein kinase-2 as a drug target, molecular modeling is carried out with a computational program. Emodin and 3-acetyl emodin act as ligands docked into the three-dimensional structure of casein kinase-2 as a receptor using the Molegro Virtual Docker 6.0 program.

Materials and Methods

Reagents

Emodin (CAS 518-82-1) was purchased from Shaanxi Pioneer Biotech Co., Ltd., China. Molecular weight was identified using a Water High-Resolution Mass Spectrometer-Time of Flight (MS-TOF) ES(+). Acetic anhydride, tetrahydrofuran, and pyridine were purchased from Merck.

Cell culture

HepG2 cells were obtained from the Center of Pharmaceutical and Medical Technologies, Agency for The Assessment and Application of Technology, Tangerang Selatan, Indonesia. Continuous culture of the cells was maintained in 75 cm^2 culture flakes in Dulbecco's modified Eagle's medium high glucose (DMEM HG; Gibco) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.1%fungizone. The cells were subcultured at seven-day intervals by dilution to 7.5 x 10^4 cells/ml.

Molecular docking simulation

Emodin and 3-acetyl emodin act as ligands docked into the 3D structure of casein kinase 2 (PDB ID 3BQC) as a therapeutic target in treatment of various human cancers to predict their interaction using the Molegro Virtual Docker 6.0 (Thomson & Christensen, 2006). Emodin is already coupled to this protein structure as a reference ligand. Docking analysis was performed to determine the

minimal energy and interactions, including the presence of hydrogen bonds resulting from the complex. After removing the reference ligand, the redocking procedure is used to validate the approach. If the RMSD number is less than 2, the docking method is claimed valid (Hevener *et al*., 2009). The 3-acetyl emodin was imported and aligned with the reference ligand position. Simulations of docking were run, and the results were examined.

Acetylation of emodin

A stirred solution of compound (1) (100 mg, 0.00037 moles) in THF (20 ml) was treated with pyridine (2 ml) and acetic anhydride (0.00185 moles) at room temperature for 4 hours. The reaction mixture was put into cold water for crystallization, then filtered and washed with dilute HCl. Recrystallization carried out with hot ethyl acetate.

Cytotoxicity assays

To determine the cytotoxicity of the compounds in HepG2 cells, the cells were seeded into 96-well plates at a density of 2.5 x 10^4 cells/ml per well and cultured in DMEM HG medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.1%fungizone. One day after seeding, cells were left untreated for control or treated with serial doses of testing compounds, ranging from 15.6 ppm to 500 ppm, for 1 day. The cell viability was measured by an MTT assay. After 24 hours of treatment with different concentrations, 5 mg/ml of MTT (in phosphate buffer solution) was added to each well and incubated at 37°C for 4 hours. Next, SDS 10% was added, and product was incubated for 24 hours at room temperature to dissolve formazan crystals. Absorbance values were recorded at 570 nm and 630 nm for reference by using an ELISA reader. The MTT values were counted using the regression analysis equation. The cytotoxicity of a compound was expressed as the compound's concentration (M) that reduced the viability of the cells by 50% (CC_{50}). Percent of cell viability in negative control was assumed 0% (Mosmann, 1984).

Result and Discussion

Modifications were made to the hydroxyl group at the position of carbon number 3 (C3) to see whether the OH group at C3 was a pharmacophore or a functional group that affected the cytotoxic activity of emodin. At this stage a reaction has been carried out to change the OH group at the C3 position from emodin to an ester group. The structure of emodin has three hydroxy groups, namely at positions C1, C3 and C8. In this study, it is hoped that only the hydroxyl group at the C3 position will be converted to an acetyl group. This can happen because the OH groups at C1 and C8 are side by side with the ketone groups, so it is possible that hydrogen bonds can occur between the H from the hydroxy group and the O from the ketone group. Thus, the OH group at the C1 and C8 positions will be more difficult to replace.

Before doing work in a wet laboratory, it's good to see the possibilities and predict the activity of the compounds we design by doing virtual simulations, such as predicting their physicochemical properties and molecular docking simulations. The molecular docking simulation results show that 3-acetyl emodin has a better interaction, indicated by a more negative Rerank score, than emodin. The interaction with the amino acids in the target protein also shows that more hydrogen bonds are formed. This indicates that 3-acetyl emodin is thought to have better activity as an anticancer. Casein kinase 2 is a therapeutic target in treating various human cancers (Janeczko *et al*., 2017; Sarno *et al*., 2002).

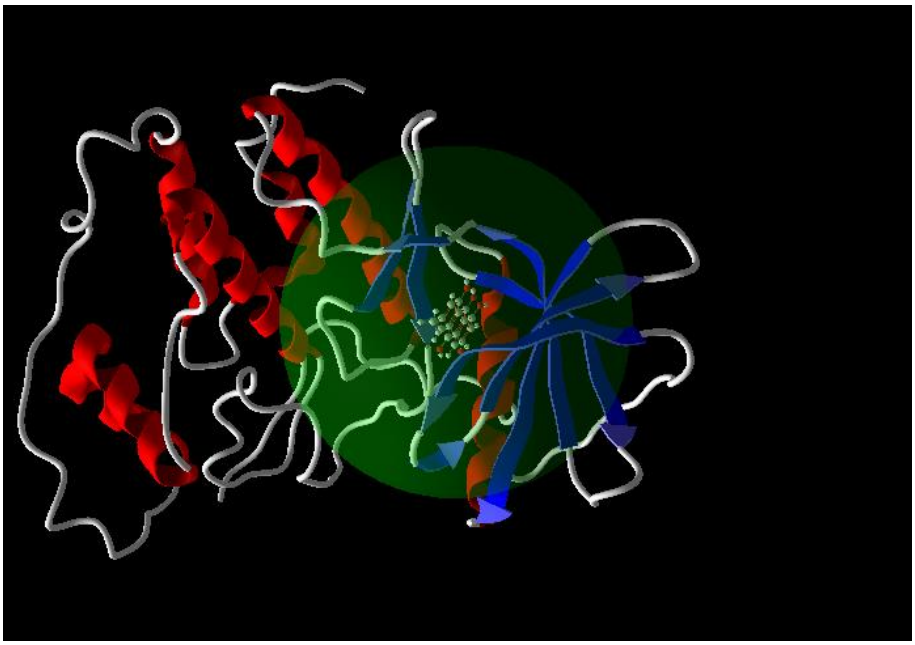
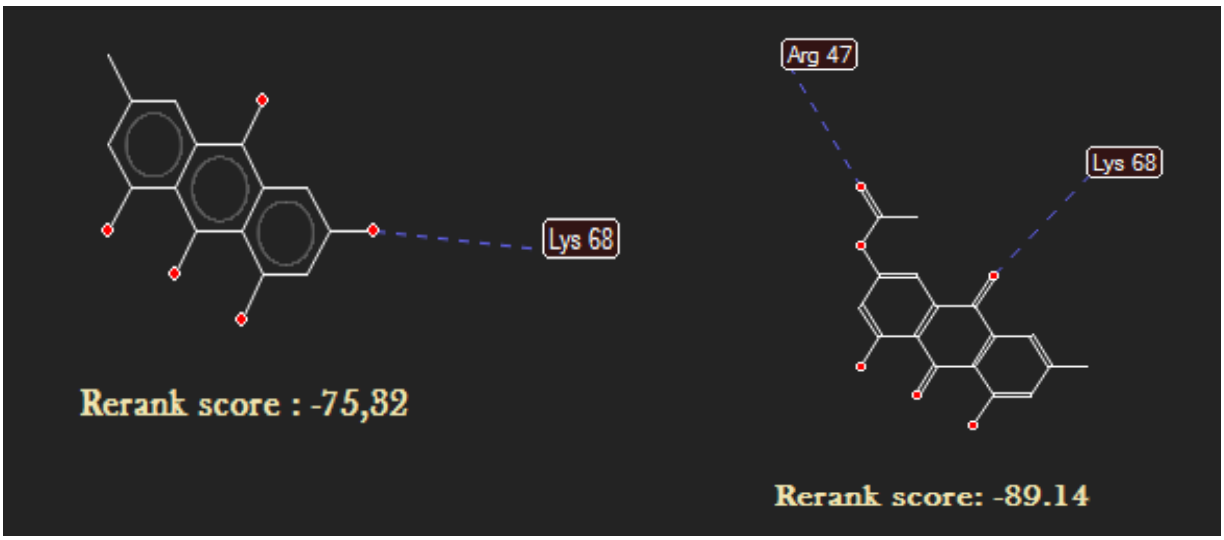


Figure 2. Molecular docking into casein kinase-2 (PDB ID 3BQC)



A **B**

Figure 3. Interaction emodin (A) and 3-acetyl emodin (B) with amino acid residue in casein kinase-2

A study of the physicochemical properties to check compliance with Lipinski's rule was carried out on the emodin ester derivatives with the results as shown in **Table 1**. Based on these data, it can be seen that the compounds designed do not violate the rules for oral administration designing small molecule drug candidates (Lipinski, 2004).

Table 1. Physicochemical properties of emodin and 3-acetyl emodin

No	Compound	LogP	Hydrogen acceptor	Hydrogen donor	MW
1	Emodin	1.74	5	3	270
2	3-acetyl emodin	1.71	6	2	312

The 3-acetyl emodin compound was synthesized by reacting emodin with acetic anhydride and pyridine. Acetic anhydride was chosen as a reagent compared to acetyl chloride so that the reaction proceeds more slowly and the yield is greater. Acetyl chloride is a very reactive reagent so it is possible to react quickly but the product yield is small because it can attack the hydroxy groups at

the C1 and C8 positions. One of the parameters that determine the high yield is the ratio of emodin substrate, anhydrous acetic reagent and pyridine base. Therefore, optimization of the synthesis conditions was carried out as shown in **Table 2** to obtain high yields.

Table 2. Acetylation condition

Emodin (mmol)	Acetic anhydrate (mmol)	Pyridine (mmol)	Yield (%)
1	10	3	48.7
1	10	6	49.7
1	5	6	Mixture
1	7	6	Mixture
1	3	6	Unform
1	1	6	Unform
1	10	1.5	Unform

Based on the product formation and yield obtained, it is known that the reaction of emodin with anhydrous acetate can take place at a ratio of 1 : 10. If less acetic anhydride is given, the reaction is not optimal because emodin does not react completely and side products may be formed. The addition of insufficient pyridine can cause the reaction not to occur because pyridine in addition to acting as a catalyst, it is also a carboxylate ion catcher that is formed. The use of excess pyridine is also not very beneficial because the yield produced is not significantly increased.

Acetylation of emodin resulted yellow orange solid that were very soluble in hot ethyl acetate and acetone, less soluble in hexane and CHCl3, and insoluble in water. The purity of product was confirmed by TLC which showed just one spot. It was identified using IR, MS, ^{1}H and ^{13}CNMR spectrophotometer : FTIR v: 1768 (carbonyl), 1616 and 1469 (aromatic), 1377 (methyl) cm^{-1}. ^{1}H-NMR (CDCl3) δ (ppm): 2.36 (3H, s, CH_3), 2.46 (CH_3), 7.05 (1H, d, J=1,95Hz), 7.11 (1H, s), 7.56 (1H, d, J=1.95Hz), 7.66 (1H, d, J=1.3 Hz), 11.98 (1H, s, OH) and 12.23 (1H, s, OH). ^{13}C-NMR ($CDCl_3$): 21.31(C-CH_3); 22.44(C-CH_3); 113.75; 114.13; 114.16; 116.93; 121.83; 124.85; 133.23; 135.28; 149.57; 157.30; 163.02; 164.23; 168.33; 181.43; and 191.78 ppm. HRMS (ESI) m/z: 313.1278 [M+H]+, calculated for $C_{17}H_{12}O_5$: 312,0634. Yield: 49%, m.p.: 180-182°C.

Cytotoxic test was carried out using the MTT method with an incubation time of 4 hours from MTT administration. The results of absorbance readings using ELISA Reader at a wavelength of 570 nm were then calculated the percentage inhibition of proliferation and analyzed to obtain a graph as shown in **Figures 4** and **5**.

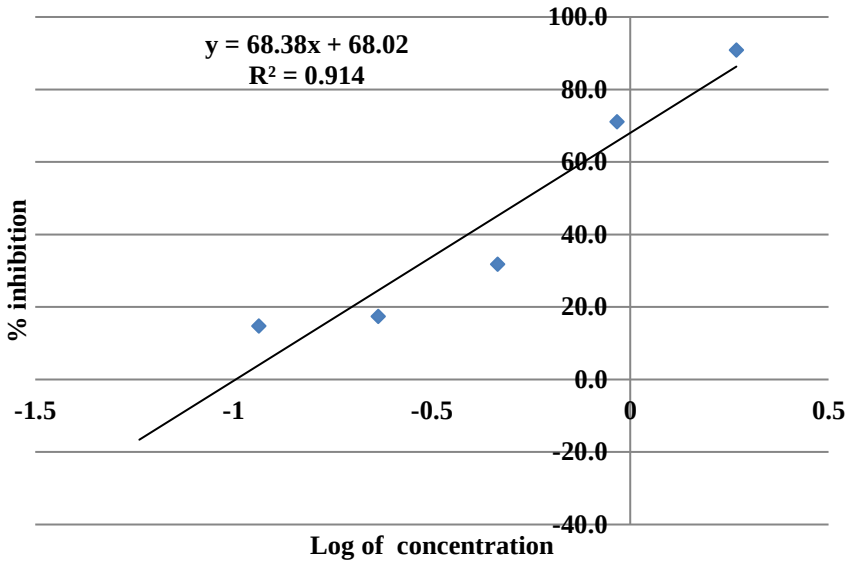


Figure 4. Dose-response curves of emodin against HepG2 cells

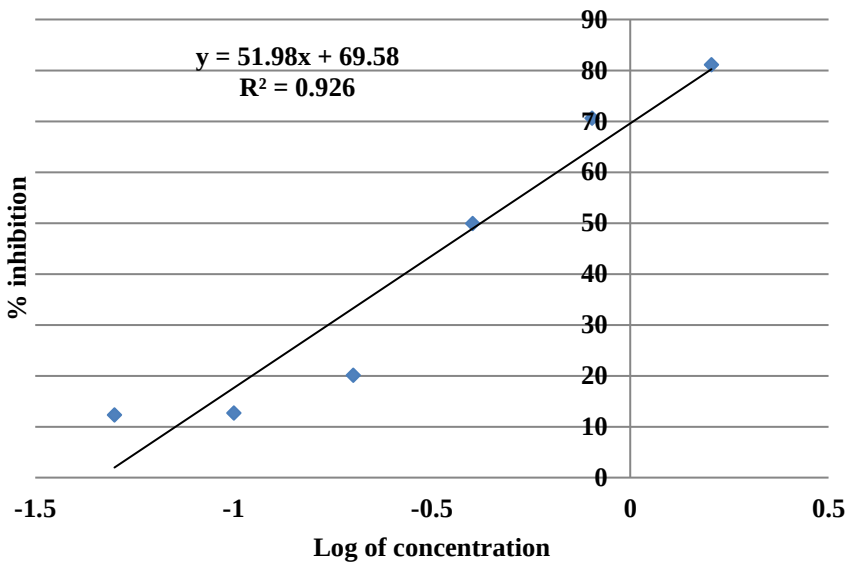


Figure 5. Dose-response curves of 3-acetyl emodin against HepG2 cells

Based on the obtained line equation, the IC_{50} value of each sample is calculated and it is determined that the IC_{50} of emodin is 0.54 while 3-acetyl emodin has a lower IC_{50} value of 0.42. It is suggested that structure modification of emodin that replaces hydroxy at C3 position becomes acetyl group will increase the cytotoxic effect against HepG2 cell lines. It was shown that the IC_{50} value of 3-acetyl emodin lower than the IC_{50} value of emodin. Thus, it is predicted that the hydroxy group at C3 is not one of the functional groups or pharmacophores because it has been proven that replacing this group could result in influencing the cytotoxic effect of emodin.

Conclusion

The 3-acetyl emodin is more cytotoxic again HepG2 cell lines than emodin. It is suggested that hydroxy group at Carbon position 3 (C3) is not one of pharmacophores. Further structure modification of emodin is possible at this position to obtain emodin derivatives that have an even higher activity to discover and develop novel anticancer candidates from emodin derivatives.

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