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In silico test of brotowali (*Tinospora crispa*) as potential anticancer agent targeting mTOR on colorectal cancer

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Abstract

Colorectal cancer is one of the deadliest diseases in the world. Treatment to overcome colorectal cancer has been pursued in such a way. Still, the results are unsatisfactory, so treatment turns to herbal plants such as brotowali (*Tinospora crispa*) as an alternative colorectal anticancer. *T.crispa* is one of the herbal plants that contains a typical compound in the form of N-acetylnornuciferine and alkaloid-derived active compounds in the form of N-formylanonaine, N- trans-coumaroyltyramine, and Tyramine which are potential as colorectal anticancer agents. The study used the mTOR (Mammalian Target of Rapamycin) receptor with PDB code (4DRJ) with its native ligand, RAP (receptor-associated protein). This study aims to analyze the potential of colorectal anticancer targeted by active compounds of alkaloid derivatives in *T.crispa* against mTOR. The method used is to explore active compounds through the KnapSack web and 3D protein structures on NCBI, docking validation using PyMol, tethering compounds N-acetylnornuciferine, N-formylanonaine, N-trans-coumaroyltyramine, and Tyramine to the target ligand using Pyrx and then visualized using BIOVIA Discovery Studio Visualizer. Analysis of the in silico test results showed that the compounds of N-acetylnornuciferine, N-trans-coumaroyltyramine, and Tyramine in *T.crispa* have good potential as colorectal anticancer agents in low doses but are not recommended for people with heart disease.

[Keywords: Colorectal anticancer, molecular docking, *Tinospora crispa*]

Introduction

Colorectal cancer is the second deadliest cancer in the world that occurs frequently. According to the latest statistics in 2022, the incidence of new cases of cancer in men and women in Indonesia is 15.7 % and 8.9%, respectively, with a mortality rate of 6.6% per 100,000 population (Kemenkes, 2024). Colorectal cancer is a malignant tumor that arises from the epithelial tissue of the colon or rectum (Sayuti & Nouva, 2019). Common symptoms of this disease usually occur in the digestive organs, such as constipation or diarrhea and the presence of blood in the stool. Some of the risk factors that cause this disease are age and genetic factors, which play an essential role in the development of colorectal cancer. Other factors such as unhealthy lifestyle, consumption of processed meat, alcohol consumption, smoking, obesity, and stress can also aggravate cancer (Sung et al., 2020; Ahmad et al., 2023).

MTOR (Mammalian Target of Rapamycin) activity supports colorectal cancer in humans. This protein actively contributes to the growth, survival, and metabolism of colorectal cancer stem cells. Stimulation of mTOR is claimed to be one of the primary mechanisms to sustain colorectal cancer progression and metastasis. Thus, mTOR inhibitors are required to halt the progression of colorectal cancer stem cells. However, studies investigating the effects of mTOR inhibitors on cancer cells have so far been very limited. Therefore, mTOR is a commonly sought-after anticancer target in human cancers such as colorectal cancer (Francipane & Lagasse, 2014; Gari et al., 2023).

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Various treatments continue to be pursued to suppress the growth of colorectal cancer cells. These efforts can be done with physical therapy through surgery or synthetic treatment through chemotherapy. However, these efforts have not provided maximum results, so exploring the potential of natural herbs is necessary. One of the potential plants to be developed is brotowali (*Tinospora crispa*). *T.crispa* has been known to contain quaternary flavonoids and alkaloids, including apigenin, berberine, palmatin, borapetol a, borapetol b borapetosida a, borapetosida b, and pikroretin which have the most dominant pharmacological activity on cancer cells (Adnan et al., 2016). Compounds included in the alkaloid derivatives in *T.crispa* are N-acetylnornuciferine, N-formylanonaine, N-trans-coumaroyltyramine, and Tyramine.

Exploring *T.crispa*'s potential as an anticancer needs to be studied more broadly using a comprehensive approach to in silico prediction. The in silico method complements the in vitro and in vivo methods commonly used in drug discovery (Fadlan et al., 2020). Computational capabilities are increasing exponentially, and there is an opportunity to develop simulations and calculations in drug design. The current development of bioinformatics studies allows researchers to predict the mechanism of action of *T.crispa* constituents as colorectal anticancer. Therefore, this study conducted molecular docking to analyze the potential of colorectal anticancer targeted by some active compounds alkaloid derivatives on *T.crispa* against mTOR.

Materials & Methods

Material and method

This study conducted in silico tests on target compounds, including N-acetylnornuciferine as a typical compound in *T.crispa*, and three other alkaloid-derived compounds, namely N-formylanonaine, N-trans-coumaroyltyramine, and tyramine, as well as receptor-associated protein (RAP) as a control ligand. This research has three main stages: material preparation, drug-likeness and ADMET screening, and molecular docking.

Material preparation

Material preparation is the first step that must be done before molecular docking is performed. The preparation starts from the exploration of target compounds in *T.crispa* through the KNApSAcK website (http://www.knapsackfamily.com), while their potential can be searched through PASS online (http://way2drug.com/passonline). After obtaining typical compounds and active compounds of alkaloid derivatives and their potential, the next step is the preparation of two-dimensional (2D) structures of target compounds that can be downloaded on the PubChem website (https://pubchem.ncbi.nlm.nih.gov).

Screening drug-likeness and ADMET

After obtaining the 2D structure, the next step is drug-likeness and ADMET analysis. Drug-likeness is a screening of similarity properties with drugs, while ADMET analyzes the absorption, distribution, metabolism, excretion, and toxicity profiles of active compounds in *T.crispa*. Determination of physico-chemical properties parameters using the SwissADME website (http://www.swissadme.ch/) on the structure of the target compound. ADME analysis was carried out to know the molecular weight, hydrogen bond acceptor, hydrogen bond donor, octanol/water partition coefficient (MLogP and WLOGP), rotatable bonds, topological polar surface area (TPSA), blood-brain barrier (BBB), and violation of Lipinski's rule. After knowing the physical and chemical properties of the compound, the next step is to determine the pharmacological parameters through the Pre-ADMET website (http://preadmet.bmdrc.org/). This prediction provides information on oral bioavailability, cell permeability, metabolism, elimination, and toxicity that characterize the pharmacokinetics and pharmacodynamics of a drug molecule (Kalita et al., 2019).

Molecular docking

After the target compound in *T.crispa* meets the ADMET requirements, it is continued with molecular docking. This stage begins with the preparation of a three-dimensional (3D) structure of the mammalian target of rapamycin (mTOR) and RAP receptor that can be downloaded on the protein data bank website (https://www.rcsb.org/). The 3D structure was then saved in .pdb format and converted into .pdbqt format ready to be used in the molecular tethering process with the AutoDock Vina program in PyRx (5). Before molecular tethering, it is necessary to validate first using Pymol 2.5.5 software to obtain binding affinity (kcal mol^{-1}) and Root Mean Square Deviation (RMSD) values. This validation is carried out to re-bind the ligand to the receptor that has been separated first (Kalita et al., 2019).

Molecular tethering is done by placing the ligand on the binding site (grid box). The results of the addition can be analyzed using BIOVIA Discovery Studio Visualizer software. The analysis is carried out to see the conformational pose of the compound that has been successfully tethered and see its interaction with the mTOR receptor in 3D and 2D (Fadlan et al., 2020).

Results & Discussion

Colorectal anticancer activity prediction

The analysis provided Pa values, indicating the probability of a compound acting as a colorectal anticancer agent (Table 1). Compounds with a Pa value >0.3 were subjected to molecular docking. Using in silico analysis to research medication development has evolved rapidly. In silico research was created to find molecules that may be used as medications because of their quick, economical, and effective processes (Berdigaliyev & Aljofan, 2020).

The results showed those compounds with a value >0.3 had potential as in silico colorectal anticancer agents (Table 1). Compounds with a Pa score greater than 0.9 had potential and a high probability of being tested by in silico, meanwhile compounds with a Pa score between 0.3 and 0.7 had potential as in silico herbal agents but had a low probability experimentally (Tjitda et al., 2023).

Screening drug-likeness and ADMET

Parameters seen from physicochemical properties based on the Lipinski rule of five include molecular weights of no more than 500 g mol^{-1}, lipophilicity values of no more than 5, hydrogen bond donors of no more than 5, acceptor bonds of no more than 10, and molar refractivity (MR) values in the range of 40-130 (Lipinski et al., 2001). Compounds of N-acetylnornuciferine, N-formylanonaine, N-trans-coumaroyltyramine, and Tyramine based on ADME analysis obtained that the molecular weight of the four *T.crispa* alkaloid-derived compounds has met Lipinski's rule of less than 500 g mol^{-1}. The lipophilicity value (CLogP) obtained meets Lipinski's rule with a lipophilicity value of no more than 5 (Table 2). The lipophilicity values indicate that the four compounds are easily absorbed in the body (Suryana et al., 2022).

The number of hydrogen bond donors and acceptor bonds in the four compounds is also in accordance with Lipinski's rule, which is each obtained molecular weight below 500 g mol^{-1}, CLogP less than 5, hydrogen bond donor is not more than 5, and hydrogen bond acceptor is not more than 10 (Lipinski et al., 2001). This indicates that the compound has conformational stability with the target protein. The four ligands' molar refractivity values are 40-130, meaning that the MR values have met Lipinski's rules. This indicates that the four ligands have good steric properties and compounds that easily interact with receptors (Syahputra, 2014).

N-acetylnornuciferine, N-formylanonaine, N-trans-coumaroyltyramine, and tyramine compounds above meet all the criteria in the Drug-likeness rule with a bioavailability value of 0.55. This shows that the four compounds can be well absorbed in the body. A compound can be said to be well absorbed in the body if it has a bioavailability value of ≥0.55 (Daina et al., 2017). The best result of the four ligands is the N-Acetylnornuciferine compound because there are no reports of dangerous side effects or other deviations, so it meets the criteria in all existing rules. Therefore, the N-Acetylnornuciferine compound can be formulated orally (Benet et al., 2016).

Boiled-egg analysis aims to look at the ability of target compounds to penetrate the blood-brain barrier (BBB) and passive absorption in the gastrointestinal region (HIA) (Daina & Zoete, 2016). White areas indicate high gastrointestinal passive absorption, while yellow zones indicate high brain penetration. Blue dots indicate substrate P-glycoprotein, while red dots indicate non-substrate P-glycoprotein. Boiled-egg analysis results showed that the compounds N-acetylnornuciferine (Molecule 1) and N-formylanonaine (Molecule 2) were in the Yolk area with blue dots (Figure 1). This indicates that the two alkaloid-derived compounds can penetrate the blood-brain border passively through P-glycoprotein. Meanwhile, N-trans-coumaroyltyramine (Molecule 3) and Tyramine (Molecule 4) are in the Yolk area with red dots. This indicates that the two alkaloid-derived compounds can penetrate the blood-brain border passively without going through the P-glycoprotein substrate (Geldenhuys et al., 2015).

The Pre-ADMET analysis results show that the four test compounds in the AMES test are mutagenic. Generally, compounds with mutagenic results are carcinogenic, such as the results on the compound N-formylanonaine. Still, in the other three test compounds the results of carcinogens in both rats and mice are negative, meaning they are non-carcinogenic (Table 3).

Table 1. Colorectal anticancer activity prediction using PASS online

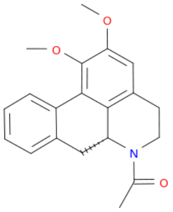
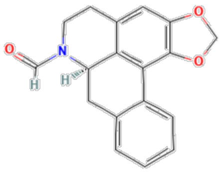
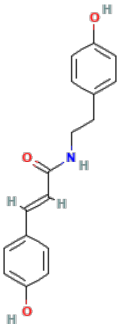
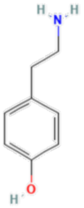
Compound	Chemical structure	PubChem ID	Pa Value
N-acetylnornuciferine		101630664	0.317
N-formylanonaine		158516	0.312
N-trans-coumaroyltyramine		5372945	0.318
Tyramine		5610	0.308

Table 2. Screening drug-likeness result

Ligand	PubChem ID	MW (g mol^{-1})	CLogP	HBD	HBA	MR
N-acetylnornuciferine	101630664	323.39	2.99	0	3	96.96
N-formylanonaine	158516	293.32	2.76	0	3	85.62
N-trans-coumaroyltyramine	5372945	283.32	2.46	3	3	82.49
Tyramine	5610	137.18	1.17	2	2	40.95

Note: MW= Molecular weight, CLogP= =lipophilicity, HBD= Hydrogen bond donor, HBA= Hydrogen bond acceptor, MR= Molar refractivity

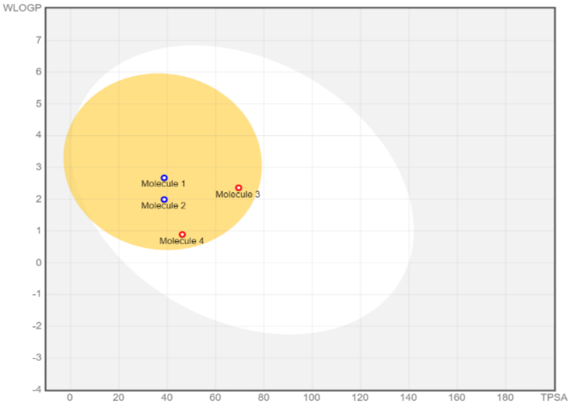


Figure 1. Boiled egg analysis result of alkaloid derivatives in *T.crispa*

Table 3. Toxicity analysis results

Ligand	hERG inhibitor	AMES Test	Carcino
N-acetylnornuciferine	Medium risk	Mutagen	Negative
N-formylanonaine	Medium risk	Mutagen	Positive
N-trans-coumaroyltyramine	Medium risk	Mutagen	Negative
Tyramine	Low risk	Mutagen	Negative

N-acetylnornuciferine, N-formylanonaine, and N-trans-coumaroyltyramine compounds have a medium risk in inhibiting hERG, in contrast to Tyramine compounds which have a low risk. So, if analyzed from the ADME test results that meet the Lipinski Rule of Five and from the Pre-ADMET results which are not carcinogenic, compounds that have the potential as colorectal anticancer are N-acetylnornuciferine, N-trans-coumaroyltyramine, and Tyramine with the condition that N-acetylnornuciferine and N-trans-coumaroyltyramine compounds should not be consumed by people with heart disease and the doses used are small. While the Tyramine compound in the use of its dose needs to be considered, namely with a low dose to avoid the mutagens occurrence. For development with better results due to consideration of the results of ADME analysis on all eligible compounds, it is necessary to conduct further research in terms of minimizing the content in compounds that cause mutagenic, carcinogenic, and reduce the risk of hERG inhibition (Kurniasih et al., 2022).

Molecular docking

The initial stage of protein preparation is to remove water molecules (H_2O) to leave amino acids in the target protein so that during the docking process, only test compounds interact with amino acids (Huey et al., 2012). Water molecules must be removed to maximize the interaction between the test compound and the target protein (Kitchen et al., 2004). Interactions between ligands and receptors are generally in the form of hydrogen bonds, so adding hydrogen is necessary to optimize the interactions.

The molecular docking method was performed on the mTOR (Mammalian Target of Rapamycin) receptor with PDB code (4DRJ) and RAP as a native ligand (Table 4). The results are obtained in the form of RMSD value. The RMSD value explains the deviation value of the error that occurs when docking, so the smaller the RMSD value indicates that the deviation of the error in docking is small (Frimayanti et al., 2021).

Table 4. RMSD (*Root Mean Square Deviation*) of each ligand

Ligand	Binding affinity (kcal mol^{-1})	rmsd/ub (Å)	rmsd/lb (Å)
N-Acetylnornuciferine	-8.5	2.635	1.761
N-formylanonaine	-8.9	5.207	2.990
N-trans-coumaroyltyramine	-8.3	2.412	1.935
Tyramine	-5.0	3.140	2.688
RAP (receptor-associated protein)	-7.1	2.126	1.590

The binding free energy is useful to describe how the test ligand binds to the receptor. If the lower the free energy value of the bond, the more stable the ligand bonds with the receptor (Meiyanto, 2012). Based on the analysis, the N-formylanoine compound has the most stable structural conformation (Table 4). Compounds with the lowest total energy value can have a good final conformation and are considered to have a stable structure (Suryana et al., 2022). The validation results between the four test ligands with mTOR found that N-acetylnornuciferine is the best ligand candidate with a stronger binding affinity than RAP as a native ligand ($-8.5 < -7.1$ kcal mol^{-1}), without a significant difference in RMSD with the native ligand (≤ 2Å) (Table 4). The results indicate that the validated ligand and protein met the valid criteria. The validation of the molecular docking method is qualified while the RMSD (Root Mean Square Deviation) value ≤ 2Å (Kalita et al., 2019). The smaller RMSD value indicates that the predicted ligand position is improving because it is closer to the original conformation (Dany et al., 2013).

The mTOR receptor binds strongly to RAP as the native ligand. In addition, interaction analysis between the mTOR receptor, the active compound N-acetylnornuciferine, and the native ligand RAP was conducted to determine the interaction between the three (Figure 2). The interactions formed usually consist of hydrogen bonds, Van der Waals bonds, and hydrophobic bonds. Hydrogen bonding is a strong bond compared to Van der Waals bonding. This is due to the formation of hydrogen bonds, even though the distance between the ligand and the receptor is quite far. Hydrophobic interactions also play a role in determining the stability of the ligand towards the receptor (Frimayanti et al., 2021).

The interactions found between typical compounds and alkaloid-derived active compounds with mTOR receptors are 3 non-bond interactions in the form of hydrophobic bonds. This shows that the N-acetylnornuciferine compound forms a hydrophobic bond. This bond is important in ligand and receptor interactions because it can generate entropy (Dwirosalia et al., 2021). The position of the hydrophobic bond on the non-polar side aims to minimize the interaction of the nonpolar residue with water (Frimayanti et al., 2021). The results of the interaction of mTOR receptors and test ligands show that the three can interact with each other, where active compounds bind to native ligands as receptor connectors so that they have potential as colorectal anticancer candidates.

The results of the interaction of the mTOR receptor with each active compound are seen in 3D visualization that the four active compounds can interact with the mTOR receptor. Still, the location of the active compounds in binding to the receptor appears different, where the N-acetylnornuciferine, N-formylanonaine, and N-trans-coumaroyltyramine compounds bind in the middle of the receptor only at different points. Still, the tyramine compound binds to the other side of the mTOR receptor.

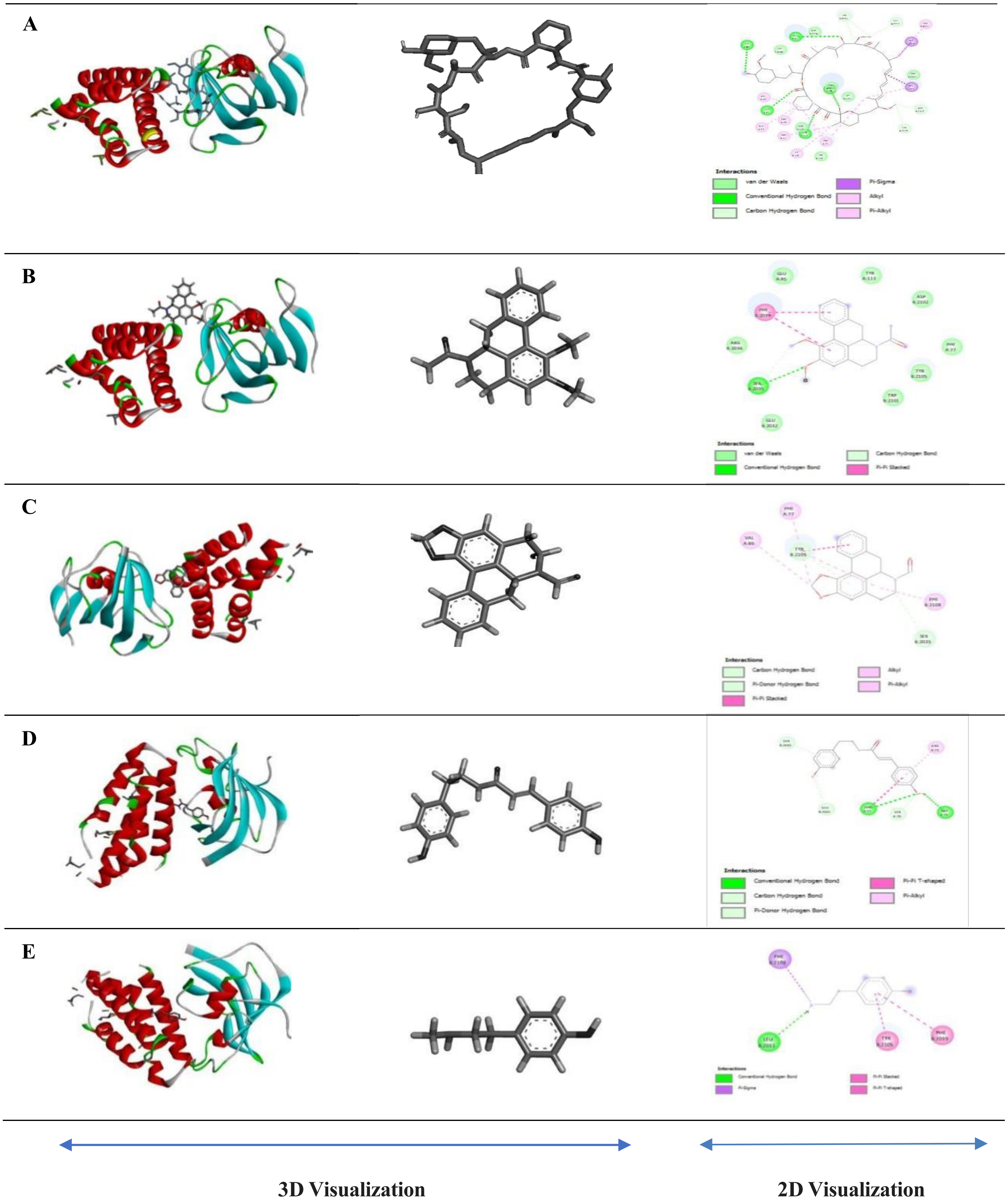


Figure 2. Interaction between mTOR receptor and (A) RAP (Native Ligand); (B) N-acetylnornuciferine; (C) N-formylanonaine; (D) N-trans-coumaroyltyramine; (E) tyramine

Conclusion

In silico tests using molecular docking showed mTOR inhibition by typical compounds and active compounds of *T.crispa* alkaloid derivatives. N-acetylnornuciferine, N-trans-coumaroyltyramine, and tyramine compounds have good potential as colorectal anticancer agents, but with the condition that N-acetylnornuciferine and N-trans-coumaroyltyramine compounds are consumed in low doses and are not recommended for people with heart disease, as well as tyramine compounds that need to be considered in the use of doses to avoid mutagenesis. The best protein-ligand complex needs to be further analyzed by molecular dynamics to determine the stability.

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