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Original Article

In silico approach for predicting the bioactive compound of *Cyperus rotundus* to inhibit NF-kB and iNOS signaling pathways

Abordagem in silico para prever o composto bioativo de *Cyperus rotundus* para inibir as vias de sinalização Nf-kB e iNOS

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Abstract

This study aims to evaluate the anti-cancer-related inflammation activity of *Cyperus rotundus* bioactive compounds. The component of *C. rotundus* was analyzed using LC-HRMS. The drug-likeness of all compounds were analyzed using swissADME webserver. In addition, the analysis of inhibition potential of compounds against NF-κB and iNOS were carried out using molecular docking in PyRx software. This study found 1-Nitro-2-phenoxybenzene, ethyl 4-(acetylamino)-3-phenyl-2-thioxo-2,3-dihydro-1,3-thiazole-5-carboxylate, and nootkatone passed all the parameters of drug-likeness including Lipinski, ghose, veber, egan, and muege. Based on molecular docking, verbascoside A and n-Pentyl isopentyl phthalate has the lowest binding affinity against iNOS (-10 and -8.9 kcal/mol, respectively). In addition, verbascoside A and maltopentaose have binding affinity of -7.6 and -6.6 kcal/mol, respectively, for NF-κB. The anti-cancer activity of verbascoside A, maltopentaose, and n-Pentyl isopentyl phthalate, according to PASS analysis were anti-inflammatory, antineoplastic, chemopreventive, and chemoprotectant. The cytotoxic effect prediction showed that these compounds were relatively selective to kill tumor cell but not non-tumor cell. Rat toxicity analysis showed maltopentaose was non-toxic, where n-Pentyl isopentyl phthalate was only toxic (class IV) for intravenous administration. perMM analysis showed verbascoside A and n-Pentyl isopentyl phthalate can translocate and across the cell membrane.

Keywords: anti-cancer, *Cyperus rotundus*, inflammation, in silico, molecular docking.

Resumo

Este estudo tem como objetivo avaliar a atividade inflamatória relacionada ao câncer de compostos bioativos de *Cyperus rotundus*. O componente de *C. rotundus* foi analisado utilizando LC-HRMS. A semelhança com o medicamento de todos os compostos foi analisada usando o servidor web swissADME. Além disso, a análise do potencial de inibição dos compostos contra NF-κB e iNOS foi realizada utilizando docking molecular no software PyRx. Este estudo descobriu que 1-Nitro-2-fenoxibenzeno, etil 4-(acetilamino)-3-fenil-2-tioxo-2,3-di-hidro-1,3-tiazol-5-carboxilato e nootkatone passaram em todos os parâmetros do medicamento-semelhança, incluindo Lipinski, ghose, veber, egan e muege. Com base no acoplamento molecular, o verbascosídeo A e o ftalato de n-pentil isopentil têm a menor afinidade de ligação contra iNOS (-10 e -8,9 kcal/mol, respectivamente). Além disso, o verbascosídeo A e a maltopentaose têm afinidade de ligação de -7,6 e -6,6 kcal/mol, respectivamente, para o NF-κB. A atividade anticancerígena do verbascosídeo A, maltopentaose e ftalato de n-pentil isopentil, de acordo com a análise PASS, foi antiinflamatória, antineoplásica, quimiopreventiva e quimioprotetora. A previsão do efeito citotóxico mostrou que esses compostos eram relativamente seletivos para matar células tumorais, mas não células não tumorais. A análise de toxicidade em ratos mostrou que a maltopentaose não era tóxica, enquanto o n-pentil isopentil ftalato era tóxico apenas (classe IV) para administração intravenosa. A análise perMM mostrou que o verbascosídeo A e o n-pentil isopentil ftalato podem translocar-se através da membrana celular.

Palavras-chave: anticancerígeno, *Cyperus rotundus*, inflamação, in silico, acoplamento molecular.

1. Introduction

Inflammation is a prevalent indication reported throughout all stages of cancer. The process of inflammation plays a pivotal role in the regulation of cancer formation, exerting both promotive and suppressive effects on

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tumorigenesis. Additionally, inflammation serves as a response mechanism to various therapeutic interventions (Zhao et al., 2021). The Hallmark of Cancer is a term to explain the formation and the progression of cancer, one of these Hallmark is tumor-promoting inflammation (Khusnurrokhman and Wati, 2022). In cancer, inflammation occurs continuously caused by the necrotic cells which secrete proinflammatory signal and recruit inflammatory cells (Kumar and Sethi, 2023). Necrotic cells can occur caused by cancer cell itself or as the consequence of chemotherapy drugs (Zhao et al., 2021; Khusnurrokhman and Wati, 2022). Therapy-induced inflammation is recently been concerned, including chemotherapy, radiotherapy, and immunotherapies (Greten and Grivennikov, 2019).

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a crucial transcription factor involved in the regulation of the inflammatory response. The activation of NF-κB has been shown to enhance cellular proliferation, disrupt apoptosis, initiate cellular migration and invasion, promote angiogenesis, and facilitate metastasis(Pavitra et al., 2023). The signaling pathway of NF-κB predisposes the expression of target genes, such as TNF-α, IL-6, Bcl-xl, Bcl-2, XIAP, and VEGF, which are responsible for cell survival and proliferation, and angiogenesis (Fakhri et al., 2021). The NF-κB pathway has been observed to have a role in enabling tumor cells escape programmed cell death through the upregulation of anti-apoptotic proteins (Verzella et al., 2020).

Another molecule responsible for inflammation in cancer is inducible nitric oxide synthase (iNOS). The expression of iNOS is also regulated by NF-κB and linked to metastatic ability of tumor cells. iNOS gene induction by NF-κB occurs due to the interaction of NF-κB and interferon regulatory factor 1 (IRF-1) (Kielbik et al., 2019). The high expression and activity of iNOS found in many tumors and positively correlated with the degree of malignancy (Nath and Kashfi, 2020). iNOS generates nitric oxide (NO) in tumor which is used for survival, growth, and migration of tumor cells (Girotti and Fahey, 2020). Therefore, NF-κB and iNOS inhibition is a promising target for inflammation-related cancer therapy.

Since the use of drug chemotherapy has several side effects, alternative therapies such as herbal medicine need to be explored. *Cyperus rotundus* also known as purple nut grass, one of the most common *Cyperus* plants, is a medicinal plant used in several countries in Asia including China, India, Japan, Indonesia, and other (Taheri et al., 2021). Recently, modern pharmacology revealed the anti-cancer activity of *C. rotundus* including inducing apoptosis and cell cycle arrest (Shao et al., 2023; Simorangkir et al., 2019), tumor cell chromatin condentation (Lin et al., 2019), and blocking immune checkpoint molecule (Nafisah et al., 2022). The pharmacological property of *C. rotundus* is contributed by the phytochemical composition. Phytochemical composition is influenced by several factors including extraction method, sample particle size, and agro-climatic condition (Prasedya et al., 2021). This study aims to evaluate the potency of ethanolic extract of *C. rotundus* tuber bioactive compounds on inflammation-related cancer therapy through *in silico* study.

2. Material and Methods

2.1. Plant material

The collection of *C. rotundus* plants was carried out at Tirtomarto Village, Ampelgading, Malang, Indonesia, located at coordinates 8°15'00.1"S; 112°53'00.6"E. The plants were then determined to obtained the plants certification by East Java Provincial Government Health Office, UPT Materia Medica Batu (nº. 074/118/102.7/2017).

2.2. Plant preparation and extraction

The tuber of *C. rotundus* were selected and used in this study. The tubers were cut into small pieces, washed thoroughly, and dried. Next, the tubers were grounded until become powder. The powder of *C. rotundus* was extracted using absolute ethanol (1:5, m:v) inside an aluminum-covered flask to keep it free from light for 24 h and shaken by rotary shaker at 120 rpm. The extract was then filtered using Whatman No. 1 filter paper. The filtrate was evaporated using rotary evaporator to remove the solvent and obtain a concentrated extract. Extract of *C. rotundus* was then stored at 4 °C until being used.

2.3. Bioactive compounds identification using LC-HRMS

The bioactive compounds content in *C. rotundus* tuber extract were analyzed using LC-HRMS (Liquid Chromatography-High Resolution Mass Spectrometry). The HPLC instrument was Thermo Scientific Dionex Ultimate 3000 RSLCnano with microflow meter. The solvent used in this analysis were 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The analytical column was Hypersil GOLD aQ 50 × 1 mm × 1.9 u particle size with flow rate 40 µL/min. In addition, the high-resolution mass spectrometer instrument was Thermo Scientific Q Exactive with 70,000 resolutions. The processing data software used was Compound Discoverer with mzCloud MS/MS Library.

2.4. Drug-likeness analysis using ADME

Drug-likeness of *C. rotundus* bioactive compounds was analyzed using SwissADME webserver (SIB, 2023). The SMILES of each bioactive compound which need to be inserted for analysis were obtained from PubChem (NLM, 2023). The parameters used for drug-likeness analysis were Lipinski, Ghose, Veber, Egan, and Muege represented by number of violations.

2.5. Molecular docking

The interaction of *C. rotundus* bioactive compounds against the target protein were analyzed using molecular docking simulation. The simulation was performed using Autodock Vina integrated in PyRx software. The target protein used in this study were human inducible nitric oxide synthase (PDBID: 4NOS) and NF-κB (PDBID: 1NFI). The 3D structures of target protein were retrieved from Protein Data Bank (RCSB PDB, 2023), where the 3D structure of *C. rotundus* compounds and control were retrieved from PubChem database. The control used in this study was the inhibitor of iNOS and NF-κB such as 2-[(1r)-3-Amino-1-Phenyl-Propoxy]-4-Chloro-Benzonitrile (CID: 51003749)

and andrographolide (CID: 5318517), respectively. The molecular docking was done in grid center X: -1.5792 Y: 96.11901 Z: 17.9123 for iNOS with active sites glu377, Trp372, gly371, Phe369, val352, and grid center X: -4.983 Y: 51.7502 Z: 2.2663 for NF-κB with blind docking. The three strongest interactions were then used for further analysis.

2.6. Molecular dynamic (MD) simulation

Molecular dynamic simulation was performed using WEBGRO molecular dynamics (UAMS, 2023) with GROMOS96 43a1 as a forcefield. The system was set up in a cubic form filled with water with a system pressure of 1 bar. The system was adapted to the physiological conditions of human cells, namely a temperature of 310K and a salt content of 0.15M. System optimization was carried out using the NVT/NPT method. The simulation was run for 5 nanoseconds with 1000-time AutoSaving. The MD parameters analysed were Root-Mean Deviation (RMSD), number of hydrogen bonds, and radius of gyration.

2.7. Potential activity, cytotoxicity and rat toxicity prediction using PASS online

Bioactive compound's biological potential activity was analyzed using PASS (Prediction of Activity Spectra for Substances) Online (Way2Drug, 2023). The analysis was based on the structure activity-relationship with an accuracy ~95% (Ayipo et al., 2023). Besides, this study also evaluated the toxicity of compounds on rat and tumor or non-tumor cells using Rat Toxicity and Cell Line Cytotoxicity-Prediction (CLC-Pred) Services in PASS Online. The activity and toxicity of each compound were shown by probable activity (Pa) value.

2.8. Cell membrane penetration identification

Cell membrane penetration of *C. rotundus* bioactive compounds analysis was carried out using PerMM online software (University of Michigan, 2023). During the analysis, environmental parameter such as pH 7.4 and temperature 310 K (physiological condition) was set. The penetration ability of compounds to the cell membrane was represented by the energy transfer value. The penetration process was visualized using Discovery Studio Software.

3. Results

3.1. C. rotundus bioactive compounds identification using LC-HRMS

Based on LC-HRMS analysis, there were at least 15 compounds found in *C. rotundus* extract such as Bis(2-ethylhexyl) phthalate, verbascoside A, maltopentaose, n-Pentyl isopentyl phthalate, L-Homocysteine, L(+)-Ornithine, L-Aspartic acid, (-)-Caryophyllene oxide, choline, guanine, 1-Nitro-2-phenoxybenzene, ethyl 4-(acetylamino)-3-phenyl-2-thioxo-2,3-dihydro-1,3-thiazole-5-carboxylate, nootkatone, phthalic acid, and maleamate (as shown in Table 1). Each compound was identified with a specific retention time (RT) by the HP-LC instrument. These compounds were included in the classification of carboxylic acid, carboxylic ester, oligosaccharide, sulfur compound, amino acid, sesquiterpenes, amines, purines, and phenols, based on the classification on PubChem. The molecular weight of compounds contained in *C. rotundus* was in the range of 100 to 828 g/mol, where maleamate was the lowest and maltopentaose was the highest.

Table 1. Bioactive compounds found in *C. rotundus* extract.

Compound	RT [min]	CID	Formula	Molecular Weight
Bis(2-ethylhexyl) phthalate	23.381	8343	C24H38O4	390.6 g/mol
Verbascoside A	1.031	15736674	C31H40O16	668.6 g/mol
Maltopentaose	1.065	124005	C30H52O26	828.7 g/mol
n-Pentyl isopentyl phthalate	13.919	71307505	C18H26O4	306.4 g/mol
L-Homocysteine	7.499	91552	C4H9NO2S	135.19 g/mol
L(+)-Ornithine	1.129	6262	C5H12N2O2	132.16 g/mol
L-Aspartic acid	0.974	5960	C4H7NO4	133.10 g/mol
(-)-Caryophyllene oxide	18.739	1742210	C15H24O	220.35 g/mol
Choline	1.181	305	C5H14NO+	104.17 g/mol
Guanine	1.475	135398634	C5H5N5O	151.13 g/mol
1-Nitro-2-phenoxybenzene	1.081	16661	C12H9NO3	215.20 g/mol
Ethyl 4-(acetylamino)-3-phenyl-2-thioxo-2,3-dihydro-1,3-thiazole-5-carboxylate	10.803	2813732	C14H14N2O3S2	322.4 g/mol
Nootkatone	17.91	1268142	C14H14N2O3S2	322.4 g/mol
Phthalic acid	23.386	1017	C8H6O4	166.13 g/mol
Maleamate	0.96	5280451	C4H5NO3	115.09 g/mol

3.2. Analysis of C. rotundus bioactive compounds drug-likeness

All bioactive compounds contained in *C. rotundus* tuber extract were evaluated the drug-likeness including Lipinski, Ghose, Veber, Egan, and Muege parameters. Based on the analysis, out of fifteen listed bioactive compounds (as shown in Table 1), twelve of them did not show any violation against Lipinski (see Figure 1). In addition, compounds 1-Nitro-2-phenoxybenzene (16661), ethyl 4-(acetylamino)-3-phenyl-2-thioxo-2,3-dihydro-1,3-thiazole-5-carboxylate (2813732), and nootkatone (1268142) did not violate all the parameters of drug-likeness (Lipinski, Ghose, Veber, Egan, and Muege) (see Figure 1). However, verbascoside A (15736674) violated all the parameters with the highest violation found in Ghose parameter.

3.3. Molecular docking simulation of C. rotundus bioactive compounds

A molecular docking simulation was performed to evaluate the potency of *C. rotundus* bioactive compounds to tackle chronic inflammation, here we use iNOS and NF-κB as the target protein. The interaction was evaluated according to the binding affinity, type, and site of interaction. Based on molecular docking analysis, verbascoside A (15736674) and n-Pentyl isopentyl phthalate (71307505) have strongest interaction showed by lowest binding affinity compared to control inhibitor (2-[(1r)-3-Amino-1-Phenyl-Propoxy]-4-Chloro-Benzonitrile) against iNOS (4nos) (-10, -8.9, and -8.6 kcal/mol, respectively). In addition, the lowest binding affinity against NF-κB (1nfi) was verbascoside A (15736674) (-7.6 kcal/mol), followed by control inhibitor, andrographolide (-7.1 kcal/mol) and maltopentaose (124005) (-6.6 kcal/mol) (as shown in Table 2). These three bioactive compounds were then used for further analysis.

The three lowest binding energy listed in Table 2 (bold) were visualized to know the interaction type and binding site of the ligand and each receptor (see Table 3, Figure 2). The residues involved in the interaction of control inhibitor and protein (iNOS and NF-κB) were considered as key residues in the inhibition mechanism of control against the target protein. Then, comparing to investigated bioactive compounds, verbascoside A (15736674) and maltopentaose (124005) have similar binding site which was expected to have similar inhibition mechanism with control inhibitor (shown by bold residues; Asn842, Thr854, Gly719, Gly796, Val726, Lys745, Ala743, Leu844, Leu718, Cys797, Phe723, Arg841, and Leu792) against NF-κB (Table 3). In addition, compared to verbascoside A (15736674), n-Pentyl isopentyl phthalate (71307505) showed more potency in inhibition iNOS due to the involvement of key residues Ser276, Asn190, Pro189, Arg278, Ala188 (as shown in Table 3).

3.4. Molecular dynamic (MD) simulation of C. rotundus bioactive compounds

Molecular dynamics simulations represent the stability of the molecular structure of interactions between proteins and ligands. RMSD represents the stability of the protein-ligand complex during the simulation. The simulation results showed that the iNOS-71307505 and iNOS-15736674 complexes had RMSD values below 3 Å, which indicated that the complex structure was stable during the simulation. The number of hydrogen bonds and radius of gyration of the two complexes were also not significantly different from the iNOS-inhibitor complex (Figure 3a). These results indicated that the interaction between compounds 71307505 and 15736674 with iNOS was stable. The same thing happens to the NF-kB-15736674 and NF-kB-124005 complexes. The RMSD values of these two complexes at the end of the simulation were lower than those of the NF-kB-inhibitor complex, which indicated the high stability of these complexes. The number of hydrogen bonds in both complexes was not significantly different from the NF-kB-inhibitor complex. Meanwhile, the radius of gyration showed no real difference between the NF-kB-compound complex and the NF-kB-inhibitor starting from 2 ns (Figure 3b). These results indicate that the structures of the NF-kB-15736674 and NF-kB-124005 complexes were stable during the simulation.

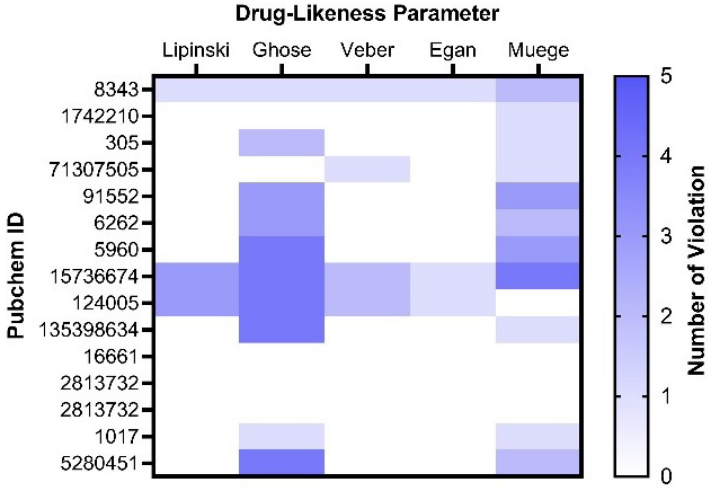

Figure 1. Drug-Likeness of *C. rotundus* bioactive compounds.

Table 2. Binding affinity of *C. rotundus* bioactive compounds against iNOS and NF-κB.

Compound	Pubchem ID	Binding Afinity (Kcal/mol)	
		4nos	1nfi
Verbascoside A	15736674	**-10**	**-7.6**
n-Pentyl isopentyl phthalate	71307505	**-8.9**	-5.7
1-Nitro-2-phenoxybenzene	16661	-8.1	-6
Bis(2-ethylhexyl) phthalate	8343	-7.9	-5.6
Maltopentaose	124005	-7.5	**-6.6**
Ethyl 4-(acetylamino)-3-phenyl-2-thioxo-2,3-dihydro-1,3-thiazole-5-carboxylate	2813732	-7.4	-5.6
Phthalic acid	1017	-7.1	-5.8
(-)-Caryophyllene oxide	1742210	-6.8	-5.7
Guanine	135398634	-6.1	-5.6
L-Aspartic acid	5960	-5.4	-4.8
Maleamate	5280451	-5.1	-5
L(+)-Ornithine	6262	-4.6	-4.6
L-Homocysteine	91552	-4.1	-4.2
Choline	305	-3.5	-3.4
Inhibitor	51003749/5318517	**-8.6**	**-7.1**

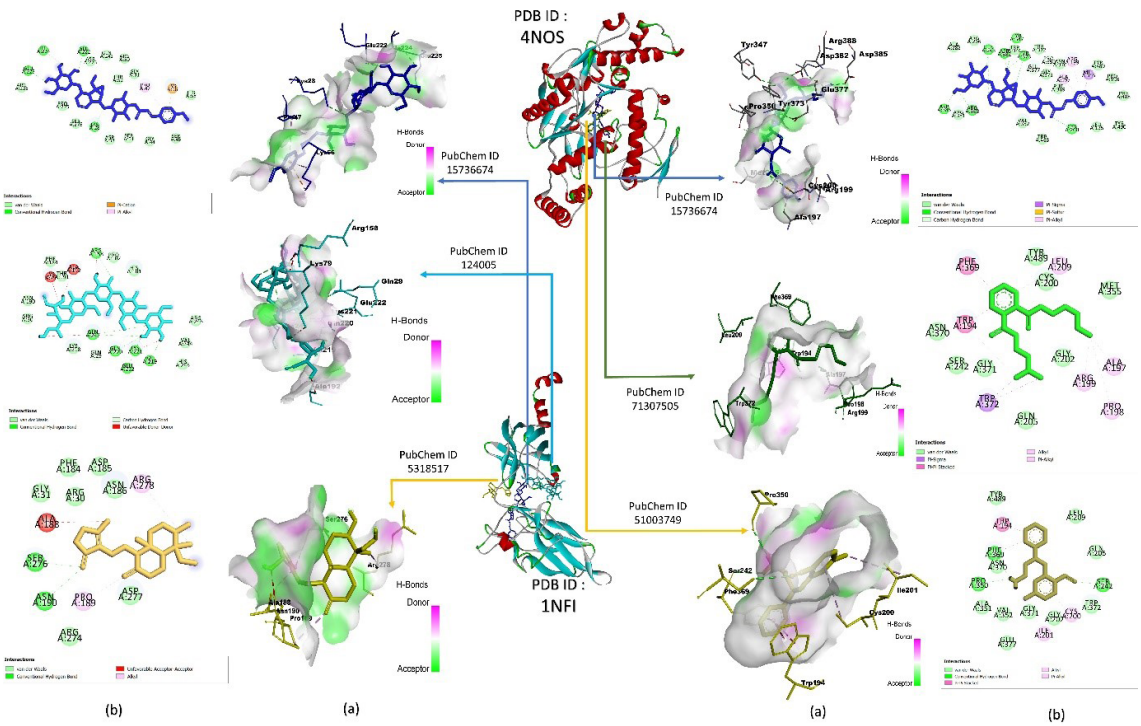

Figure 2. Visualization of ligand and receptor interaction, (a) 3D and (b) 2D interaction.

3.5. Analysis of potential activity, cell line and rat toxicity of C. rotundus bioactive compounds

The three bioactive compounds such as verbascoside A (15736674), maltopentaose (124005), and n-Pentyl isopentyl phthalate (71307505) were then further analysed for potential activity, cytotoxicity effect on tumor or non-tumor cell line, and rat toxicity using PASS online services. Based on the analysis, there was some potential activity shown by different Pa value, including hepatoprotectant, cardioprotectant, anti-inflammatory, antioxidant,

Table 3. Interaction and binding site of ligand and protein (NF-κB and iNOS).

Ligand	Interaction type	Residue (1nfi)	Interaction type	Residue (4nos)
15736674	Van der waals (7)	**Asn842**, Asp855, **Thr854**, Ala722, Gly721, **Gly719**, **Gly796**	Hydrogen bond (4)	Glu222, Ile224, Glu225, Lys28
	Hydrophobic (8)	**Val726**, **Lys745**, **Ala743**, **Leu844**, **Leu718**, **Cys797**, **Phe723**, **Arg841**	Hydrophobic (2)	Pro47, Lys56
124005	Van der waals (3)	**Leu792**, **Gly796**, Asp855	-	-
	Hydrogen bond (5)	Gln791, **Met793**, **Thr790**, **Thr854**, **Asn842**		
	Hydrophobic (6)	**Leu718**, **Ala743**, **Val726**, **Leu844**, **Phe723**, **Lys745**		
71307505	-	-	Hydrogen bond (2)	**Ser276**, **Asn190**
			Hydrophobic (2)	**Pro189**, **Arg278**
			Unfavourable bond (1)	**Ala188**
Inhibitor	Van der waals (8)	**Gly796, Leu792, Met793, Pro794, Thr790, Gly719, Cys797, Thr854**	Van der Waals (7)	**Arg274, Asp277, Gly31, Phe184, Asp185, Arg30, Asn186,**
	Hydrogen bond (1)	**Lys745**	Hydrogen bond (2)	**Ser276, Asn190**
	Hydrophobic (5)	**Leu718, Phe723, Val726, Ala743, Leu844**	Hydrophobic (2)	**Pro189, Arg278**
	Halogen (2)	**Arg841, Asn842**	Unfavourable bond (1)	**Ala188**

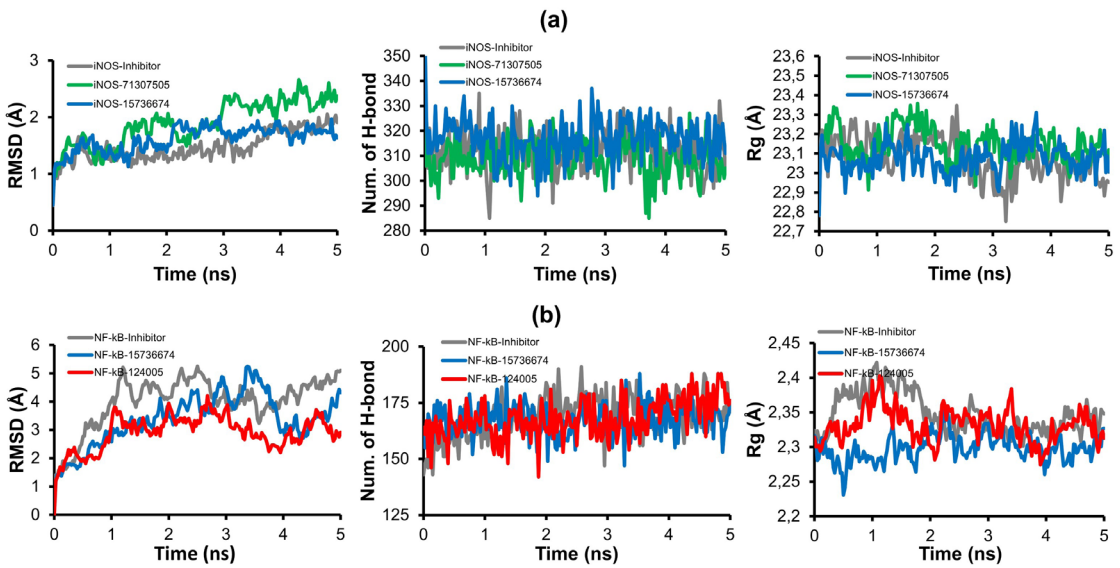

Figure 3. Molecular dynamic simulation reveal the structural stability based on RMSD, number of hydrogen bond, and radius of gyration of all complexes, (a) iNOS-ligand and (b) NF-kB-ligand.

anaesthetic, chemopreventive, chemoprotective, chemosensitizer, antihemorrhagic, haemostatic, free radical scavenger, antineoplastic, proliferative disease treatment, and membrane permeability inhibitor (see Figure 4a). In addition, the highest probable activity (Pa value) of verbascoside A (15736674) were hepatoprotectant (0.925), anti-inflammatory (0.866), and antineoplastic (0.801). The highest Pa value showed by maltopentaose (124005) were hepatoprotective (0.876), antineoplastic (0.806), and membrane permeability inhibitor (0.726). However, the potential activity of n-Pentyl isopentyl phthalate (71307505) with highest Pa value were anaesthetic (0.915) and membrane permeability inhibitor (0.775).

Then, this study also found anti-cancer potential of *C. rotundus* bioactive compounds according to the prediction of cytotoxic effect against tumor cell lines. The result showed verbascoside A (15736674) was toxic to breast carcinoma (T47D cell line, Pa = 0.785), cervival carcinoma (HeLa cell line, Pa = 0.646), and lung carcinoma (SW1573 cell line, Pa = 0.595) (see Figure 4d). Besides, maltopentaose

(124005) showed the highest Pa value of tumor cell line cytotoxicity against breast ductal carcinoma (BT-549 cell line, Pa = 0.688), lung carcinoma (A459 cell line, Pa = 0.676), and glioblastoma (SF-268 cell line, Pa = 0.625). However, n-Pentyl isopentyl phthalate (71307505) has a lower Pa value compared to other compounds, 0.445 for lung carcinoma (DMS-114 cell line), 0.39 for gastric carcinoma (MKN-7 cell line), and 0.387 for melanoma (LOX IMVI cell line). This study also evaluated the cytotoxic effect of each compound against non-tumor cells. This study found that *C. rotundus* bioactive compounds were relatively safe for non-tumor cells showed by very low Pa value (0-0.4) except maltopentaose (124005) and verbascoside A (15736674) which were potentially toxic to embryonic lung fibroblast with Pa value 0.721 and 0.65, respectively (see Figure 4c).

Since dose determination is crucial in evaluating the lethal dose of a drug, this study analyzed rat toxicity using *in silico* approach. The analysis demonstrated that maltopentaose (124005) was non-toxic for rat in all route of administration (IP; intraperitoneal, IV; intravenous, Oral, and SC; subcutaneous) (as shown in Table 4). In addition, verbascoside A (15736674) was categorized as toxic class 4 in AD for IP and IV, class 3 for SC, and class 5 for oral. Furthermore, n-Pentyl isopentyl phthalate (71307505) was only toxic (class 4) in IV route of administration.

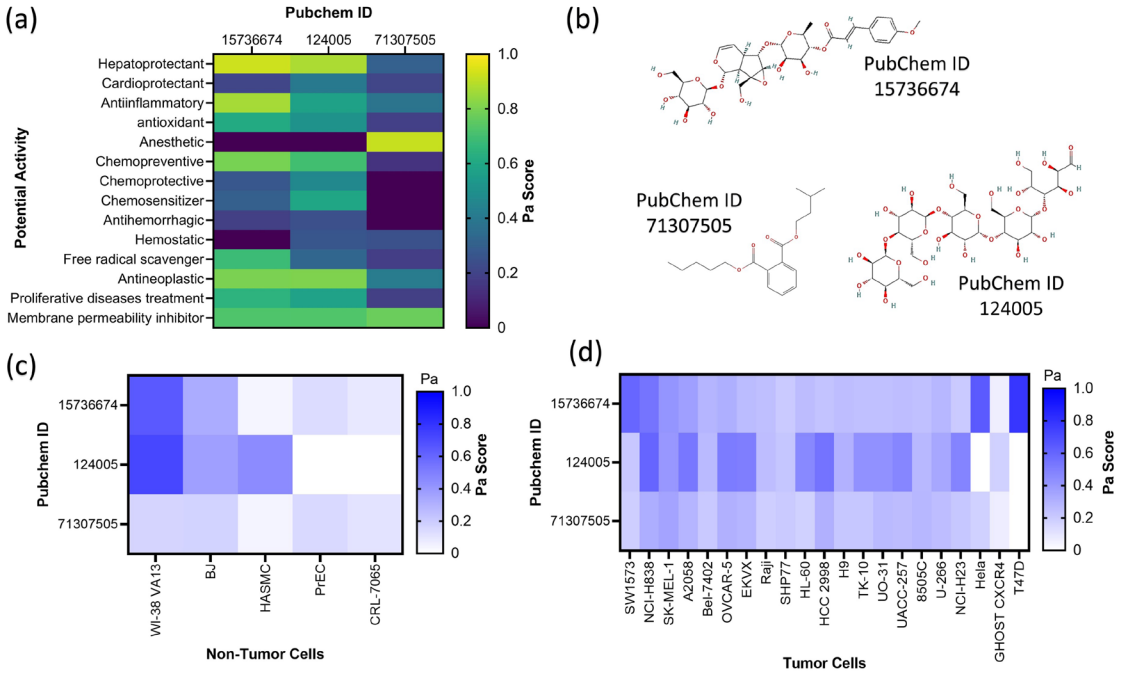

Figure 4. Potential biological and cytotoxic activity of *C. rotundus* bioactive compounds. (a) potential activity result by PASS online screening; (b) the three compounds that met the druglikeness and probable activity parameters; (c) potential activity from *C. rotundus* to non-tumor cells analysis by PASS online screening (d) potential activity from *C. rotundus* to tumor cells analysis by PASS online screening.

Table 4. Rat toxicity of *C. rotundus* bioactive compounds.

Rat Toxicity		Pubchem ID		
		15736674	**124005**	**71307505**
LD 50 (mg/Kg)	Intraperitoneal (IP) route of administration	218,600 in AD	4406,000 in AD	1874,000 in AD
	Intravenous (IV) route of administration	52,490 in AD	5604,000 in AD	114,600 in AD
	Oral route of administration	3093,000 out of AD	1,01E4 in AD	1,014E4 in AD
	Subcutaneous (SC) route of administration	135,800 in AD	9952,000 in AD	3843,000 in AD
Toxicity Classification	Intraperitoneal (IP) route of administration	Class 4 in AD	Non Toxic in AD	Non Toxic in AD
	Intravenous (IV) route of administration	Class 4 in AD	Non Toxic in AD	Class 4 in AD
	Oral route of administration	Class 5 out of AD	Non Toxic in AD	Non Toxic in AD
	Subcutaneous (SC) route of administration	Class 3 in AD	Non Toxic in AD	Non Toxic in AD

AD = Applicability Domain.

3.6. Cell membrane penetration ability of C. rotundus bioactive compounds

The penetration ability of drug against the cell membrane was crucial to be evaluated since the target protein was not on the surface of the cell. The penetration ability was presented by energy transfer value as shown by Figure 5b. The higher transfer energy indicates more difficulties of compound to across cell membranes. Based on perMM analysis, maltopentaose (124005) can't passively translocate through the lipid bilayer of membranes. Whereas, verbascoside A (15736674) and n-Pentyl isopentyl phthalate (71307505) can passively translocate with free energy of binding (DOPC) of -5.40 kcal/mol and -6.05 kcal/mol, respectively. The penetration of the process of each compound through the lipid bilayer were shown in Figure 5.

4. Discussion

C. rotundus is a medicinal plant found in tropical and sub-tropical countries including Indonesia, China, India, Korea, Japan, and other countries (Nafisah et al., 2023; Xue et al., 2023). *C. rotundus* tuber ethanolic extract, specifically obtained from East Java, Indonesia, contained carboxylic acid, carboxylic ester, oligosaccharide, sulfur compound, amino acid, sesquiterpenes, amines, purines, and phenols. Among all, 1-Nitro-2-phenoxybenzene (16661), ethyl 4-(acetylamino)-3-phenyl-2-thioxo-2,3-dihydro-1,3-thiazole-5-carboxylate (2813732), and nootkatone (1268142) passed all the parameters of drug-likeness, including lipinski, ghose, veber, egan, and muege. These drug-like compounds demonstrate good properties of being a drug pharmacodynamically and pharmacokinetically (Loureiro et al., 2019). All bioactive compounds of *C. rotundus* were then further analyzed using *in silico* study to evaluate the potency in tackling chronic inflammation and cancer. Chronic inflammation is one of common conditions found in cancer which is associated with the use of chemotherapeutic drugs, such as cisplatin. Cisplatin is one of the most common anti-cancer drugs with very effective effect in treating cancer but consequently induces inflammation through activating multiple mechanisms such as NF-κB, COX-2, and TNF-α (Zhao et al., 2021). Tumor-associated inflammation is reported to involve and affect cancer development, metastasis, and even drug resistance (Wen et al., 2022). NF-κB is one of the mediators of inflammation in cancer, with another transcription factor such as AP1 and STAT3, inducing chemokine expression associated with inflammatory response (Zhang et al., 2021). The activation of canonical NF-κB regulates proinflammatory gene expression which contributes to cancer progression and development, thus, NF-κB is a promising therapeutic target in cancer (Yu et al., 2020). Moreover, NF-κB activation induces iNOS transcription which is also highly induced in tumor cells. Indirectly, NF-κB can induce iNOS through the proinflammatory product molecules such as IL-6, IL-1, and TNF-α. iNOS associated with inflammation, tumor formation, and metastasis through the production of nitric oxide (Pasha et al., 2021). Thus, inhibiting iNOS can become one of anti-cancer strategies.

This study demonstrated the inhibition potential of *C. rotundus* bioactive compounds against NF-κB and iNOS through molecular docking and dynamic analysis. The inhibition mechanism of verbascoside A (15736674) and maltopentaose (124005) against NF-κB was similar to andrographolide, shown by involvement of key residues in the interaction of ligand and receptor. Through this interaction, andrographolide blocked NF-κB by enhancing the dephosphorylation of NF-κB subunit p65. Moreover, the binding energy of verbascoside A (15736674) and maltopentaose (124005) were lower compared to andrographolide, indicating a stronger interaction against NF-κB. The strong interaction of both complexes was due to the large number of van der Waals, hydrogen bond, and hydrophobic interactions. In addition, n-Pentyl isopentyl phthalate (71307505) showed inhibition potential against

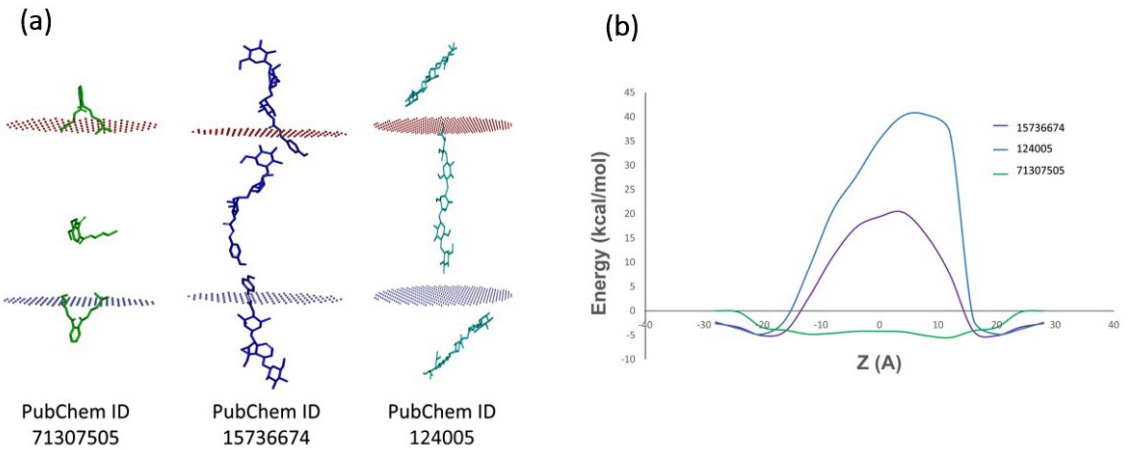

Figure 5. Cell membrane penetration ability of *C. rotundus* bioactive compounds, (a) simulation of the penetrating from *C. rotundus* bioactive compounds in the lipid bilayer; (b) value of the energy transfer from *C. rotundus* bioactive compounds penetrating the phospholipid membrane.

iNOS through Ser276, Asn190, Pro189, Arg278, and Ala188 amino acid residues. Even though verbascoside A (15736674) has stronger interaction compared to the control inhibitor, the interaction did not involve any key residues for the inhibition mechanism of iNOS by 2-[(1r)-3-Amino-1-Phenyl-Propoxy]-4-Chloro-Benzonitrile.

Potential activity analysis showed the three bioactive compounds of *C. rotundus,* verbascoside A (15736674), maltopentaose (124005), and n-Pentyl isopentyl phthalate (71307505) have anti-cancer activity such as anti-inflammatory, chemopreventive, chemoprotective, and antineoplastic. The anti-cancer potential was further analyzed by evaluating the cytotoxic effect on tumor cell *in silico*. This study found the three bioactive compounds were sensitive to different cancer cell lines, where n-Pentyl isopentyl phthalate (71307505) was very sensitive to gastric carcinoma MKN-7 cell line, maltopentaose (124005) was potentially very toxic to breast ductal carcinoma cell line (BT-549), and verbascoside A (15736674) was highly toxic to T47D breast carcinoma cell line. The three bioactive compounds were selectively toxic to cancer cells but not tumor cells, which is crucial in anti-cancer drug discovery. The compounds demonstrated low toxicity to non-tumor cell line showed by lower Pa value compared to tumor cell line. A Previous study reported that *C. rotundus* extract was selective in killing murine breast cancer cell line (4T1) but not normal human fibroblast cell line (TIG-1) (Nafisah et al., 2023). Another study also demonstrated cytotoxic effect of *C. rotundus* against MCF-7 breast cancer cell line and affects the growth of vero cells (normal cell) (Simorangkir et al., 2019). The toxic effect of this extract also evaluated in rat using *in silico* approach. The result showed maltopentaose (124005) was non-toxic to Rat through IP, IV, oral, and SC administration routes. n-Pentyl isopentyl phthalate (71307505) was also safe except for IV which was classified as toxic class 4. However, verbascoside A (15736674) was toxic in class V to III in all routes. According to globally harmonized system of classification of labeling chemicals (GSH), toxicity was classified as (a) class VI: non-toxic (LD>5000), class V: may be harmful if swallowed ($2000 < LD50 \leq 5000$), class IV: harmful if swallowed ($300 < LD50 \leq 2000$), class III: toxic if swallowed ($50 < LD50 \leq 300$), class II: fatal if swallowed ($5 < LD50 \leq 50$), and class I: fatal if swallowed ($LD50 \leq 5$) (Igbokwe et al., 2024).

The penetration ability of bioactive molecules is crucial if the target of the drug is inside the cells (Oliveira et al., 2022). This molecule interacts with cell membranes and translocate through active or passive mechanism across the lipid bilayer (Lomize and Pogozheva, 2019). The penetration ability was presented by energy transfer value, where the lower the energy the easier the molecule can translocate and penetrate (Wargasetia et al., 2023). This study found that verbascoside A (15736674) and n-Pentyl isopentyl phthalate (71307505) can translocate across lipid bilayer, but not maltopentaose (124005). During the translocation process, each compound changed its conformational structure to fit and across the membrane. There are some factors that influence the ability of molecule to penetrate the cell membrane, such as conformational flexibility, size, and polarity (Casalini, 2021). This study opens an entryway to drug discovery and the potentiality of *C. rotundus* extract as anti-cancer-related inflammation therapy.

The inability of maltopentaose (124005) to passively move through the lipid bilayer of membranes, as indicated by perMM analysis, suggests that maltopentaose cannot diffuse across the lipid bilayer without the assistance of a transporter or channel. This is in line with the characteristics of transporters, which are polytopic transmembrane proteins that catalyze the translocation of substrates from one side of the membrane to the other. Transporters have specific substrate-binding sites and undergo conformational changes upon substrate binding, leading to the translocation of molecules across the membrane (Scalise et al., 2020).

In contrast, substances like verbascoside A (15736674) and n-Pentyl isopentyl phthalate (71307505) can passively translocate through the lipid bilayer. The free energy of binding for these substances (-5.40 kcal/mol for verbascoside A and -6.05 kcal/mol for n-Pentyl isopentyl phthalate) indicates favorable binding interactions that allow them to move across the lipid bilayer without the need for a transporter or channel (Róg et al., 2021) .

The significance of free energy of binding in perMM analysis lies in its ability to predict and understand the movement of molecules across membranes (Lomize et al., 2019). By calculating the free energy of binding, one can determine whether a substance can passively translocate through the lipid bilayer or if it requires specific transport mechanisms to cross the membrane. This information is crucial for studying membrane permeability, drug design, and understanding the dynamics of molecular transport processes in biological systems (Róg et al., 2021).

In summary, this study demonstrated that *C. rotundus* tuber extract bioactive compounds, such as verbascoside A, maltopentaose, and n-Pentyl isopentyl phthalate, might be potentially tackle inflammation in cancer, through the inhibition of iNOS and NF-κB. These bioactive compounds also showed anti-cancer activity including anti-inflammatory, chemopreventive, chemoprotectant, antineoplastic, and cytotoxic to breast and lung carcinoma cell lines. Further in vitro research should be performed to confirm and validate the anti-cancer activity of C. *rotundus* through the inhibition of iNOS and NF-κB.

This research exhibits promising potential compounds as indicated by their favorable free energy of binding values and several analyses of potential were identified by an in silico approach. These potential compounds could serve as starting points for the development of novel therapeutics or drug delivery systems. Future pre-clinical trials in vivo and in vitro could investigate their efficacy and safety profiles for various medical applications, such as treatment of inflammatory conditions, neurological disorders, or cancer.

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