E-ISSN:2460-7746; P-ISSN: 2528-282X

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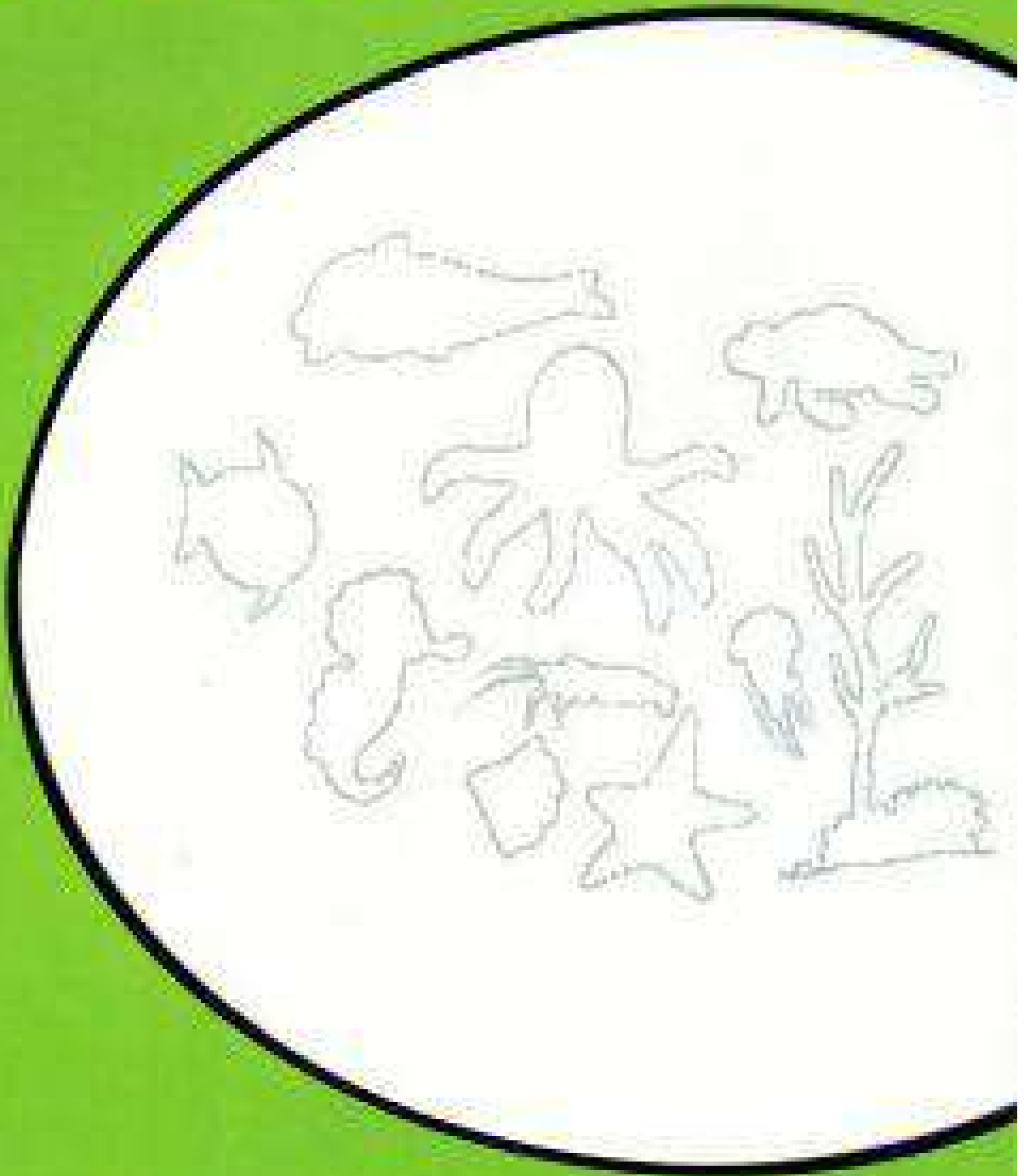

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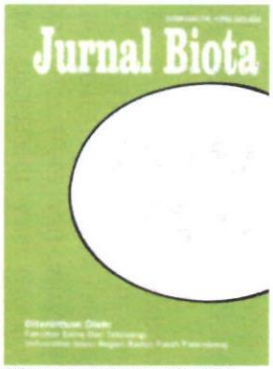

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Jurnal Biota Vol. 9 No. 2 (2023) https://doi.org/10.19109/Biota.v9i2.13746

Research Article

Anti-Proliferative Compound Candidate of White Turmeric (*Curcuma zedoaria*)

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Received: 09/09/2022	Revised: 26/10/2022	Accepted: 27/10/2022

ABSTRACT

Malignant disease or cancer progression burden the community after decades. This tumor formation or tumorigenesis involves cell proliferation. Protein Kinases (MAPK9s) is key proteins of regulating the growth and viability of cells physiologically and pathologically. Rhizomes of Curcuma zedoaria or zedoary or white turmeric is used as a health supplement. The aim of this study was to obtained candidate proteins for anti-proliferative using the docking method between the protein MAPK9 and the active compound obtained from the crude extract of white turmeric i.e. demethoxycurcumin, curcumenol and germacrone. The result s hows that complex MAPK-demethoxycurcumin have the lowest binding affinity -8.4 Kkal/mol, while MAPK-curcumenol was -8 Kkal/mol, and MAPK-Germacrone was -6.2 Kkal/mol, it determined the potential activity cell proliferation.

Key words: *Active compound; Curcuma zedoaria; Demethoxycurcumin; In-silico; MAPK.*

Introduction

Exposure to infectious agents as well as malignant disease or cancer, increases the health burden on the community. The immune response plays an important role in protecting the body from exposure to various antigenic substances. The use of vaccines is essential in controlling and preventing infectious diseases as well as treating several malignancies. In general, vaccines contain protein or materials that resemble microorganisms causing disease, or it was made from dead or attenuated microorganisms, from toxins that produced or proteins on the surface of these microorganisms (Dai et al., 2019). Adjuvant helps vaccines stimulate an immune response (Zhang et al., 2018). The role of

adjuvants in vaccines enhance the immune system by increasing the activity of immune system component that called immunostimulants (Labh & Shakya, 2014).

The development of tumor cells into cancer, first hypothesized by Virchow in 1863, it is a product of continuing inflammation. Cancers associated with inflammation shows several stages of tumorigenesis (Gonzalez et al., 2018). This tumor formation or tumorigenesis involves cell proliferation or also disrupted anti-apoptotic pathways. Mitogen-activated Protein Kinases (MAPK9s) is key proteins of regulating the growth and viability of cells physiologically and pathologically. Aberrant MAPK9 signaling plays a role in the development and progression of cancer (Low & Zhang, 2016). The MAPK9s family is a central messenger molecule consisting of 2 subfamilies: ERKs (ERK1 and ERK2), c-jun N-terminal kinases (JNKs) and p38MAPK9s as extracellular signaling transmitters to the nucleus that mediate regulation of genes related to cellular activity of apoptosis, proliferation, differentiation and inflammation (Susanti et al., 2019).

White turmeric is often used for its rhizomes and is widely used as a health supplement, especially in Asia (Chandrasekaran et al., 2013). The rhizome of white turmeric contains essential oils, polysaccharides and curcuminoids that have been isolated and contain 1,7bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadine-3,5-dione, demethoxycurcumin and bisdemethoxycurcumin (Yu et al., 2020).

Curcumin is one of the main curcumonoids, known of its activity to inhibit the immunostimulant function, inhibition of Mitogen-Activated Protein Kinase (MAPK9) activation and translocation of Nuclear Factor kappa B (NF-κB) (Catanzaro et al., 2018). Germacrone is active component of white turmeric, it has antioxidant antiviral, anti-inflammatory and anti-cancer activity. Germacrone affects autophagy through inhibitation of I3K III/ Beclin-1/ Bcl-2 pathway and activation of PI3K I/ Akt/mTOR pathway (He et al., 2019; Zhang et al., 2020). Curcumenol was identified induces apoptosis (Hamdi et al., 2014).

The important compounds of herbs have been explored, especially their activity in immunomodulators. In-silico approach is the first step in developing a potential drug or even vaccine. The result of this study was to obtained candidate proteins for anti-proliferative using the docking method between the protein involved in the immune system, MAPK9 and the active compound obtained from the crude extract of white turmeric namely demethoxycurcumin which can inhibit MAPK9 activity. The inhibition of MAPK9 activity is known as anti-inflammatory activity.

Materials and Methods

Preparation Methods

Software installation with PyMOL, Discovery Studio 2016 and PyRx (Ekawasti et al., 2021). The notebook specifications that will be used for minimal installation are dual core processor, 2 GHz, 2GB RAM, system type Windows 8 Enterprise – 64 bit operating system.

Preparation of tools for extraction such as knife, blender, Erlenmeyer tube, and rotary vacuum evaporator. For identification of active compounds were used Gas Chromatography-Mass Spectrophotometry (GC-MS), oven, and GC-MS solution software. PyMOL software for molecular visualization, Discovery Studio 2016 for 3D molecular structure creation, and PyRx for molecular docking. The materials prepared were ethanol for the extraction of white turmeric and the reagents used in GC-MS.

Extraction of Active Compounds

White turmeric has been obtained from UPT Materia Medica, Batu, East Java. Simplicia obtained in the form of white turmeric powder of 1000 grams. The extraction process uses 96% ethanol as solvent. From 500 grams of powder were isolated 58 grams of turmeric extract. White turmeric extract is stored in the refrigerator in a vacuum condition.

Extraction was used white turmeric rhizome and washed in running water, cut into smaller pieces, then dried in a cold

place. The dried pieces are mashed with a blender, filtered, so that a yellowish powder was obtained. The white turmeric powder was then extracted using ethanol for 72 hours. The extract was obtained by evaporation with a vacuum evaporator (Kaushik & Jalalpure, 2011).

Data Isolation

Sample profiling of white turmeric extract as much as 500 mg extract was dissolved in 2 mL n-Hexane pro analysis solvent. The solution was then mixed with a vortex for 3 minutes, followed by sonification for 5 minutes. After that, the solution was filtered with a 0.45µL membrane filter, and the filtrate was injected into the GC-MS instrument at 1 L. GC-MS instrument used is Agilent 6980N Network GC System with autosampler.

The molecular bio-computation method in silico will be carried out by identifying the chemical structure of the active compound which identified by GC-MS. Pubchem database was used to obtained active compounds according to profiling with GC-MS, while target protein involved in anti-proliferative were obtained with NCBI database or uniprot. The data is saved in .pdb format and displayed in PyMOL. The molecular docking test was carried out on the PyRx to determine the binding activity between the active compound and the target protein. The candidate compounds used in this study were demethoxycurcumin, curcumenol and germacrone. Target proteins in this study was MAPK9.

Data Analysis

A qualitative analysis was carried out by the active compounds from the GC-MS test. In GC-MS was *determined* the number of active compounds and the dominant active compounds in the white turmeric rhizome extract. The results of the in-silico test were analyzed by comparing the binding affinity, hydrogen bonding, hydrophobicity, the pose of the binding on the active site of the receptor, and the residu or amino acid in the binding site for the active compound to the unique ligand compound.

Results and Discussion

Profiling compound of white turmeric rhizome was done by GC-MS. The active compound found in the extract seen in table 1.

Tabel 1. Gas Chromatography- Mass Spectrophotometry Analysis of White Turmeric rhizome

No.	Compound	Real time	%	Group
1.	Methyl Pentane	1.53	3.30	Heksana
2.	Esani	1.62	56.86	Heksana
3.	Beta-elemen	15.39	0,06	sesquiterpene
4.	Beta Eudesmene	16.66	0.13	Sesquiterpene
5.	Aromadendren	17.26	0.02	Sesquiterpene
6.	3-ethyl-6 methoxycarbonyl-2- naphthol	18.07	0.17	Phenolic
7.	Dehydroaromadendrene	18.34	0.01	Sesquiterpene
8.	10-isopropenyl-3-7 cyclodecadien	19.15	0.11	sesquiterpene
9.	**Unknown (germacrone)** (Rita et al. 2019)	**19.58**	**0.22**	sesquiterpene
10.	7-isopropyl-1,4-dimethyl-2-azulenol	19.98	0.02	Ketone
11.	**Unknown (curcumin)** (Arivoli et al. 2019)	20.88	0.22	curcuminoid
12.	**Unknown (curcumenol)** (Arivoli et al. 2019)	22.06	0.09	Sesquiterpene
13.	Linderazulene	22.50	0.20	Sesquiterpene

Selection and Preparation of Active Compound

Selection of active or candidate compounds based on literature study. They were Demetoxycurcumin, curcumenol and

germacrone. The structure of the test compound as a candidate was taken through the database, namely for demethoxycurcumin https://pubchem.ncbi.nlm.nih.gov/compound/5469424 then for curcumenol https://pubchem.ncbi.nlm.nih.gov/compound/167812 the last is germacrone which is https://pubchem.ncbi.nlm.nih.gov/compound/6436348. These compounds saved from 3D dimensional structure in "sdf" format, using the PyMOL to convert in "pdf" format. Furthermore, the 3-dimensional structure of standard drugs doxorubicin and indomethacin can be downloaded from https://pubchem.ncbi.nlm.nih.gov/compound/31703 and https://pubchem.ncbi.nlm.nih.gov/compound/3715.

The results of GC-MS profiling of white turmeric extract contain hexane, terpenoid, phenolic, and ketone groups. In Table 1. It shows that the highest content is esani which belongs to the hexane group or aromatic chain. The sesquiterpene group was detected often from the extract. From the results of GC-MS, there are unknown compounds, when matched with the retention time or real time with the literature, may come from several compounds. The chemical structure of the candidate compounds can be seen in Figure 1.

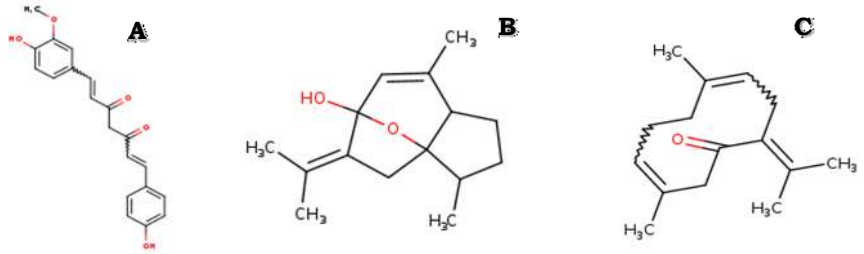

Figure 1. Chemical Structure of Candidate Compounds: Demethoxycurcumin (A); Curcumenol (B); Germacrone (C). (https://pubchem.ncbi.nlm.nih.gov).

Searching of Target Protein and Preparation

MAPK9 (PDB Code: 3NPC) was used for protein target, while the ligands are 3 candidate compounds, demetoxycurcumin (Pubchem ID: 9952605), curcumenol (Pubchem ID 167812), Germacrone (Pubchem ID: 6436348) and unique ligand B96, as well as standard drugs Doxorubicin (Pubchem ID 31703) and indomethacin (Pubchem ID 3715). The three-dimensional structure of each protein was taken from the RCSB Protein Data Bank database for MAPK9 https://www.rcsb.org/structure/3NPC. The criteria for each protein used an X-Ray structure with a resolution of < 3.00, in homo sapiens organisms. Furthermore, the data is stored in the form of "pdb". The 3-dimensional structure of each protein can be seen in Figure 2.

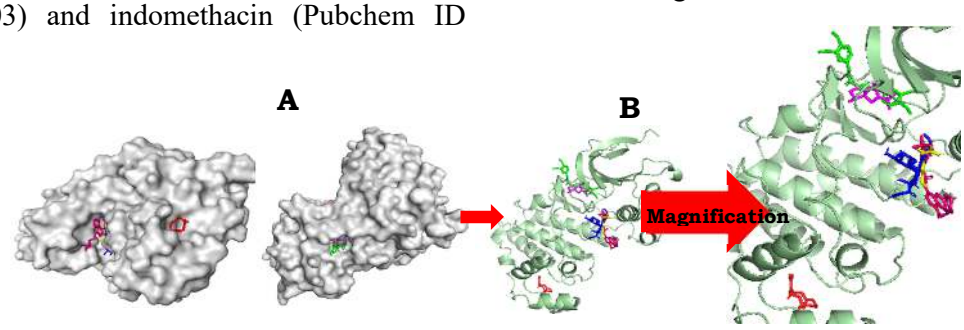

Figure 2. Complex of target protein with all ligands: MAPK9 (source: Pymol). Protein in surface structure 3D (A); B. Protein in licorice structure 3D.

Figure 2. Shows that MAPK9 protein has 3 binding sites for ligand B29 (pink), Doxorubicin (blue), and indomethacin (yellow), while demethoxycurcumin (green) and curcumenol (purple) on the other hand, the last is germacrone (red) bound on the other side (Figure 2. A right and left). This is appropriate if the amino acid similarity between the ligands of these compounds is observed in Table 3. The interaction between MAPK9, doxorubicin, indomethacin and curcumin proteins can be seen in Figure 3.

Unique ligands: chains with a spectrum of color are unique ligands for each protein Demethoxycurcumin: the green colored chain is demethoxycurcumin; Curcumenol: purple colored chain; Germacrone: red colored chain; B96: pink colored chain; Doxorubicin: blue colored chain. Indomethacin: yellow colored chain.

In Figure 3. The pathway between protein and ligands can be observed its interaction with the Stitch database. Doxorubicin can activate MAPK9 protein, and vice versa. While the interaction of MAPK9 protein with curcumin is not directly, but through JUN protein inhibition, as well as indomethacin is not directly related to MAPK9 protein activity.

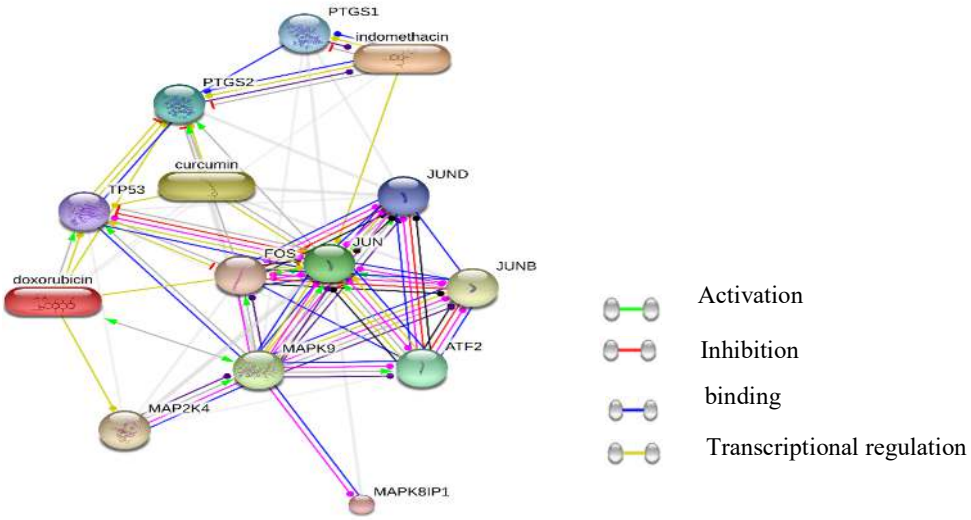

Figure 3. Interaction between MAPK9, doxorubicin, indomethacin and curcumin proteins using Stitch database http://stitch.embl.de/.

The MAPK protein group has an important role in the survival of cancer cells and is associated with oncogenesis, tumor progression and drug resistance. c-Jun N-terminal Kinase (JNK) is one of the proteins in the MAPK group, which is activated by a series of phosphorylation events. Activated JNK will further phosphorylate Jun protein. Continuous JNK activation indicates tumorigenesis through c-Jun activation (Pua et al. 2022).

C-Jun overexpression correlates with worsening of nasopharyngeal cancer. Downregulating c-Jun increases phosphorylation of the protein involved, so that silencing of c-Jun in CNE-2R cell cultures can activate the PI3K/AKT/mTOR signaling pathway, by inhibiting the expression of phosphorylated PI3K, AKT and mTOR (Sun et al., 2021). Curcumin appears to exhibit Jun-inhibiting activity which is associated with decreased migration and invasion of cells.

Based on docking of anti-proliferative candidate compounds, namely demethoxycurcumin, germacrone and curcumenol that interact with MAPK9 protein, the binding affinity energy values can be seen in Table 2.

Table 2. Binding Affinity Value of Protein-Ligand Interaction

Target Protein MAPK9	
Active compound/ ligand	***Binding Affinity* (Kkal/mol)**
Demethoxycurcumin	**-8.4**
Curcumenol	-8
Germacrone	-6.2
B96	-9.6
Doxorubicin	-8.7
Indomethacin	-8.1

Table 2. showed the value of binding affinity or bond energy between proteins and ligands. Binding affinity is the ability of a protein to bind to its ligand. MAPK9 binds with demethoxycurcumin ligand at the lowest binding affinity, which is -8.4 Kcal/mol, compared to other candidate compounds, i.e. curcumenol and germacrone, with binding affinities was -8.0 and -6.2 Kcal/mol, respectively. The lower of binding affinity, the more stable the bond and the more potential to interact each other naturally. Therefore, in all ligands and protein interactions, position 0 (RMSD= 0) was chosen which has the lowest binding affinity. The lower binding affinity value indicates the stronger the bond between the compound and its receptor due to the stability and strength of non-covalent interactions between the protein and its receptor (Prasetiawati et al., 2021).

Curcumin has a chemical structure similar to demetoxycurcumin related to the MAPK9 pathway, one of whose biological activities is related to JUN phosphorylation. Mitogen-activated protein kinases are involved in several processes such as cell proliferation, differentiation, migration, transformation and programmed cell death. Extracellular stimulation such as proinflammatory cytokines or other physical stress can stimulate the signaling protein kinase/c-Jun N-terminal kinase (SAP/JNK) pathway.

It is known that curcumin is one of the main curcumonoids that inhibits the immune-stimulating function of dendritic cells and interferes with the maturation of myeloid dendritic cells, interferes with the production of pro-inflammatory cytokines (IL-13) due to inhibition of the activation of Mitogen-Activated Protein Kinase (MAPK9) and the translocation of Nuclear Factor kappa B (NF-κB) (Catanzaro et al., 2018).

The phytopolyphenol pigment curcumin from *Curcuma zedoaria* can show immunomodulating effects by regulating B cells, dendritic cells, macrophages, Myeloid-derived suppressor cells (MDSCs), T-cells in some cancer. Curcumin inhibit immunosuppressive responses by decreasing the population of MDSCs and T-cell regulatory (Zhong et al., 2022).

Germacrone has anti-inflammatory, antioxidant, and antiviral and anticancer activities. the role of germacrone in the protective mechanism of autophagy induction. Classical upstream inhibitory 3-MA autophagy can inhibit type III PI3K activity. Germacrone can inhibit autophagy by inhibiting the I3K III/Beclin-1/Bcl-2 pathway and activating the PI3K I/Akt/mTOR pathway (Zhang et al., 2020). The bioactive component of curcumenol was identified as a cytotoxic component and induces apoptosis (Hamdi et al., 2014). The binding affinity between curcumenol (-8 Kcal/mol) and germacrone (-6.2 Kcal/mol), curcumenol has stronger binding activity to MAPK protein, compared to germacrone, determined the potential activity cell proliferation.

The docking between MAPK9 and its unique ligand, B96, has the lowest binding affinity (-9.6 Kcal/mol). Binding affinity with standard drug Doxorubicin is -8.7 Kcal/mol, and Indomethacin is -8.1 Kcal/mol. According to (Rivankar 2014), doxorubicin is a cancer therapeutic agent for a long time, which has a broad spectrum of antineoplastic activity. In this study, the results of docking MAPK9 with doxorubicin had a binding affinity that was

greater than that of B96. Meanwhile, indomethacin is known to have anti-neoplastic efficacy and is one of the strongest non-steroidal-anti-inflammatory drugs (NSAIDs) (Okda et al., 2020), but the binding energy between MAPK9 and demethoxycurcumin (-8.4 Kcal/mol) is higher than its unique ligand (-9.6 Kcal/mol), which means that demethoxycurcumin binds to the active site of MAPK9 weaker than B98, but still has the ability to bind.

The active site of MAPK9 protein has the strongest binding to demethoxycurcumin compared to other candidate compounds. This indicates that demethoxycurcumin has more potential ability to binding to MAPK9 compared to curcumenol and germacrone, so that it acts as an inhibitory competitor or can inhibit the activity of MAPK9. MAPK9 plays a role in the production of proinflammatory cytokines and decreases the signal to initiate inflammation (Manzoor et al., 2014).

Table 3. Amino Acid Residues in Demethoxycurcumin, Curcumenol and germacrone interact with MAPK9 Protein

Protein	Active compounds	Amino Acid Residues	Interaction
MAPK9	Demethoxy-curcumin	Ile32, Val40, Ala53, Met108, Leu168, Ala42, Leu110	Hydrophobic
		Met111, Ala113, **Glu73***, **Asp169***	Hydrogen bond
	Curcumenol	Val158, Leu168, Ala53, Met108, Leu110, Met111, Val40, Phe170	Hydrophobic
	Germacrone	Lys308, Leu241, Ile304, Val303	Hydrophobic
	B96	Leu76, Leu77, Val80, Leu142, Leu147	Hydrophobic
		Lys68, Arg72, Ile148, Glu344	Hydrogen bond
		Asp169*	Electrostatic
	Doxorubicin	Leu76, Leu77	Hydrophobic
		Asp151, Arg150, **Glu73***	Hydrogen bond
		Asp169*	Electrostatic
	Indomethacin	Leu77, Val80, Ile85, Leu142, Leu76	Hydrophobic
		Arg69, Gln37, Ile148	Hydrogen bond

The MAPKs group is a central messenger molecule consisting of 2 subfamilies: ERKs (ERK1 and ERK2), c-jun N-terminal kinases (JNKs) and p38MAPKs as extracellular signal transmitting to the nucleus that mediate regulation of genes related to cellular activity of apoptosis, proliferation, differentiation and inflammation (Susanti et al., 2019).

The MAPK9 protein complex with demethoxycurcumin has 7 amino acids with hydrophobic bond interactions, and 4 amino acid residues that form hydrogen bonds. There are 9 amino acids between MAPK9-curcumenol and 4 amino acids between MAPK9-germacrone which all interact to form hydrophobic bonds. While the MAPK9 complex with B96 showed that there was 1 amino acid that was the same as demethoxycurcumin, i.e. Asp169 (*), which was not shown from other protein candidates. There are 2 amino acid residues in common between the MAPK9 complex and the standard drug doxorubicin and demethoxycurcumin, they were Glu73 and Asp169.

Demethoxycurcumin has the ability to interact with MAPK9 by forming hydrogen bonds on the amino acid Asp169, the hydrogen bonds are the same as the hydrogen bonds formed during docking between the unique ligand B29 and MAPK9 protein. It means the active site where the B29 and demethoxycurcumin bind to the MAPk9 is the same, so that demethoxycurcumin have the same affinity as B29 in inhibiting the MAPK9 protein (Susanti et al., 2019). Demethoxycurcumin has the potential to inhibit MAPK9 protein in the same amino acid as its unique ligand inhibitory activity, B96.

White turmeric rhizome extract showed anticancer, anti-inflammatory, analgesic, antiallergic, antiparasitic effects, especially against Entamoeba histolytica, antibacterial and antifungal (Dosoky et al., 2018). It has also been reported that *C. zedoaria* has a moderate antimutagenic effect on benzopyrene agents. Several studies have also confirmed several benefits of *C. zedoaria* such as antifungal, antiulcer, antimutagenic, hepatoprotective and cytotoxic (Srivastava et al., 2011).

According to Sari et al., (2020), the hydrogen bonds formed between MAPK9 protein with demethoxycurcumin and B29 at the Asp169 residue stabilize the conformation between the ligand and its receptor. Hydrogen bonding was found only in demehoxycurcumin-MAPK9 complex that not found in curcumenol and germacrone complex with MAPK, which means that demethoxycurcumin was the most stable in binding to MAPK. The similarity of amino acid residues can be predicted that demethoxycurcumin have similarities with the B29 and doxorubicin as comparison compounds, so that it can predicted as drug candidates for anti-proliferative.

Conclusion

According to docking analysis between MAPK9 and all ligands, demethoxycurcumin showed best prediction for anti-proliferative compound, shows lowest binding affinity, there is hidrogen bond, and found same amino acid residu i.e Asp169 between demethoxycurcumin, B96 and doxorubicin.

Acknowledgment

Research fund support by LPPM Wijaya Kusuma Surabaya University with Agreement Letter Number: 052/LPPM/UWKS/IX/2020.

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