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Phytoconstituents and Molecular Docking of Ethanol Extract of *Sargassum aquifolium* J. Agardh with Different Apoptosis Proteins Involved in Hepatocellular Carcinoma

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Hepatocellular carcinoma **(HCC) is the most common type of primary liver cancer worldwide. Several signaling pathways are involved in the development and apoptosis of cancer cells. Brown macroalgae, Sargassum aquifolium, is a marine functional food source known to have anticancer activity by interfering with carcinogenesis and acting as a tumor suppressor. This study aims to screen phytoconstituents from ethanol extract of S. aquifolium and the anticancer effects of its compounds on five protein receptors involved in the development and apoptosis of HCC in silico approach. Phytochemical screening was performed by GC-MS and molecular docking was performed on selected compounds from S. aquifolium with growth receptors such as VEGFR2, EGFR; apoptotic proteins such as Bcl-2, Caspase-3, and Caspase-9. GC-MS analysis, by comparing spectra to the NIST database, identified nineteen bioactive compounds. Among these, only four exhibited potential as candidates for HCC therapy. This study showed that better docking scores were obtained by linoleic acid when compared to palmitic acid, dodecanoic acid, formic acid, and phytol. Linoleic acid demonstrates promising binding affinity for receptors associated with HCC, suggesting its utility as a therapeutic inhibitor. To fully realize this potential, however, future studies should focus on the isolation of this phytoconstituent and the evaluation of its bioactivity through in vivo models. Such research would yield more comprehensive insights and further illuminate its pharmacological prospects.**

Keywords: ADME-Tox; GC-MS; Molecular docking;, Pharmacology; Phytochemical; Sargassum aquifolium.

Hepatocellular carcinoma (HCC) is a primary liver cancer that develops from abnormal hepatocytes, and accounts for approximately 80% of liver cancer cases worldwide.[1] HCC is the fifth most common cancer worldwide and the most common malignancy in adult males.[2] Reports suggested that approximately 7.5 Lakh new cases of HCC are diagnosed globally each year.[3] The

development of HCC has been linked to hepatitis infection and exposure to aflatoxin produced by *Aspergillus flavus*.[4] A case of liver cancer patients in Guangxi, China, showed high levels of aflatoxin B_1 in serum and tumor tissue.[5] HCC is most commonly found in patients with chronic hepatitis B and C.[6] Treatments carried out to treat HCC are through surgery, transplantation, heating of cancer cells, and chemotherapy.[7]

Surgery is now the main treatment for HCC as it produces the best outcome of all treatments with a 5-year survival rate of 60-80%. Liver excision and transplantation are the initial options for individuals with early malignancies with curative intent.[8] Besides the surgical excision, patients with HCC are treated with chemotherapy drugs such as fluorouracil,[9] adriamycin,[10] and cisplatin.[11] Although targeted drugs can be given to treat HCC, some patients develop drug resistance towards those and have a poor prognosis after long-term therapy. Cellularly, several protein receptors involved in HCC development have been studied such as VEGFR-2 and EGFR, and their apoptotic proteins such as B-cell lymphoma-2 (Bcl-2), caspase-3, and caspase-9. The transmembrane receptor tyrosine kinase VEGFR-2/KDR can be overexpressed through vascular endothelial growth in advanced HCC. EGFR is one of the first cancer-causing genes discovered and a major target in cancer therapy including HCC.[12,13] Likewise, apoptosis-related proteins such as Bcl-2 are involved in apoptosis, necrosis and autophagy that regulate cell death[14] and caspases are proteins that regulate cell development, differentiation and apoptosis with caspase-3 acting as the final apoptosis executor.[15]

Certain compounds used in traditional medicine have the potential to to treat HCC with relatively lower cytotoxicity than the cytotoxicity due to clinical drugs treatment.[16] For example, the astaxanthin compound is a member of xanthophylls, because it contains not only carbon and hydrogen atoms, but also oxygen atoms. As an anticancer, the astaxanthin compound has a mechanism to inhibit HCC growth factors such as VEGFR-2 and EGFR, as well as apoptosis proteins such as Bcl-2, caspase-3, and caspase-9.[17] Sources of traditional medicines, such as the astaxanthin compound, can be found from marine sources, especially seaweed which is rich in bioactive compounds with potential anticancer abilities.[18,19] Brown seaweed containing fucoidan polymers has been reported as a source of natural compounds [20] that could treat oral cancer.[21] Fucoidan from brown macroalgae and carrageenan from red macroalgae was reported to exhibit anti-metastatic, apoptotic, and antiviral properties that can inhibit cancer growth.[22,23] Previous studies reported sodium alginate produced from brown seaweed as a potential biopolymer in drug delivery,[19] a source of antioxidants,[24] antibacterial activities,[25] and its combination with *Blumea balsamifera* leaves extract as an anti-hypercholesterolemia.[26]

However, until now the mechanism of action played by *S. aquifolium* compounds in inhibiting the development of HCC has not been reported. This study aims to analyze the phytoconstituents of the ethanol extract of *S. aquifolium* and its potential targets that can underlie the mechanism of action of its active compounds in the development of HCC. To obtain optimal drug efficacy, the accuracy of active compounds in interacting with receptors is very necessary through analysis of signaling pathways related to HCC by *in silico* molecular docking.[17]

MATERIALS AND METHODS

Seaweed collection and extraction

Brown algae, *S. aquifolium*, was collected from Sindhu Beach, Denpasar City in the intertidal zone at the lowest ebb and flow conditions and transported to the research laboratory, Universitas Dhyana Pura. Authentication of the brown algae samples was submitted to the botanist of the National Research and Innovation Agency (BRIN) and the specimens were preserved in the laboratory for future supporting references according to the method described in previous studies with specimen number: 25498.[19,24] 1.5 kg of wet weight of *S. aquifolium* was dried using an oven at a temperature of 50! for three days, coarsely ground using a grinder, filtered with a No. 40 mesh sieve. A total of 100 grams of *S. aquifolium* simplicia was extracted in 1,000 mL of ethanol solution (Sigma Aldrich Co. USA) by the maceration method. The extraction was carried out for three days and stirred every day to increase the absorption of the compound. After three days, the sample was filtered and the filtrate obtained was concentrated and dried

using a rotary vacuum evaporator. The final extract yield was determined based on the dry weight of powdered *S. aquifolium.*[27]

GC-MS/MS of seaweed extract

The sample was filtered using a 0.45 ìm micro membrane and the sample was injected into the GC-MS instrument as much as 1 ìL. The optimization conditions of the GC-MS instrument specifications are the instrument used is the Agilent 6980N Network GC system with an autosampler with an Agilent 5973 inert MSD detector, J&W Scientific Column, HP-5MS, 0.25mm × 30m × 0.25 µm, Split inlet 1/100, 250, Over programmed 50 (5 minutes) 10 280 (15 minutes), column flow 1 mL/min (constant), aux of 250, MS Quad = 150 MS Source = 230 Scan mode = 20-600 amu Solvent delay = 0 minutes Library = Wiley version 7.0 Injection volume = 1 µL.

ADME-Tox study

Compounds identified in GC-MS were evaluated for their absorption, distribution, metabolism, and excretion using the SwissADME module, accessible on the SIB (Swiss Institute of Bioinformatics) website (https://www.sib.swiss) , as well as partition coefficients, solubility, and various other characteristics. Furthermore, Osiris DataWarrior (Version 5.2.1) was used to estimate the toxicity and drug-like properties of the compounds.[28]

Molecular docking analysis

Biological dataset

These computational experiments were conducted using a series of tools including Ligprep, Sitemap, Grid Generation, Grid XP Dock, MMGB-SA analysis, and other computational techniques.

Ligand optimization

A set of phytoconstituents was obtained from the GC-MS table of *S. aquifolium* extract. For docking purposes, compounds were tested for their drug-like properties based on Lipinski's Rule of Five screening. The selected compounds with drug-like properties were visualized using ChemSketch (Freeware) in .sdf and mol formats.[29] The 3D structures of selected compounds were retrieved in SDF format from the PubChem chemical database of NCBI. Next, using Pymol, these SDF format structures were converted to PDB format and prepared for docking by removing water molecules, forming hydrogen bonds, and adding required charges AutodockTools-1.5.7. These structures were saved in PDBQT format for docking.

Protein optimization

RCSB Protein Data Bank provides the structures of VEGFR-2 kinase (PDB ID. 3VHK), EGFR kinase (PDB ID. 5UGB), BCL-2 (PDB ID. 6O0K), Caspase-3 (PDB ID. 2XYG), and human caspase-9 (PDB ID. 3V3K). Caspase is a hydrolase receptor and Bcl-2 is an apoptosis regulatory receptor. Meanwhile, VEGFR-2 and EGFR act as HCC transferase receptors. All water molecules were removed from the protein structures using AutodockTools-1.5.7, and polar hydrogen atoms and charges were added. Bond angles, bond sequences, and topologies were fixed to optimize the structures. In detail, the list of receptor proteins used in this test is shown in Table 1. For docking, these structures were saved in PDBQT format.[30]

Receptor grid generation

A three-dimensional grid box was created to allow the ligand to dock with the receptor. To create the grid box, a protein and a ligand in pdbqt format are selected. In addition to adjusting the dimensions of the grid box to match X, Y, and Z, a spatial adjustment of 0.5 A—almost a quarter of the length of a single-bonded carbon atom—was made. In the center of the grid box, the ligand was present and correctly positioned within the active site of the receptor protein.[28]

Molecular docking

Vina 1.2.5 docking software was used to convert each receptor and its native ligand into a .pdb file for redocking technique. The mean square deviation (RMSD) was calculated by redocking with the native ligand. To validate the grid box coordinates, we set a limit of RMSD <2 Å. As recommended in previous studies, AutoDock Vina 1.2.5 was used to perform three-dimensional interaction simulations between ligands and receptors selected in this study.[31]

Molecular dynamics simulations

The docking results were verified using molecular dynamics simulations using CABS-flex 2.0 (https://biocomp.chem.uw.edu.pl/CABSflex2/index) to ensure the stability of the molecular complex. The RMSD value, which serves as an indicator of stability, was used to select the ligand-protein combinations evaluated in the simulations. Protein rigidity, constraints, number of cycles, C-alpha weight and side chain constraints,

temperature range, trajectory, and random number generator seed were among the characteristics of the molecular dynamics simulations.[32]

RESULTS

Phytoconstituents of ethanol extract of *S. aquifolium*

GCMS screening showed the presence of abundant secondary metabolites in *S. aquifolium* that may provide some therapeutic effects. Gas chromatogram was performed for about 32 min and the fractions from GC were examined by mass spectrometry. The detected bioactive compounds served as ligands for docking analysis. *S. aquifolium* extract contained 19 bioactive compounds with some of the same compounds and were therefore excluded from the data (Table 2). GC-MS study of the ethanol extract of *S. aquifolium* revealed a total of 19 different compounds with diverse phytochemical activities. Table 2 lists the chemical components, including retention time (RT) and peak area (%).

Selection of drug-likeness properties

The results of phytochemical screening of *S. aquifolium* extract were then selected for their drug-like properties based on screening with the Lipinski Rule of Five. A total of three compounds were screened for their drug-like properties with a bioavailability value of 0.55, namely palmitic acid, dodecanoic acid, and linoleic acid are shown in Table 3. Lipinski's Rule of Five was utilized in distinguishing between drug-like and non-drug-like molecules. This rule predicts a high probability of success or failure due to drug-likeness for molecules that obey 2 or more rules i.e., Molecular mass <500 Daltons, High Lipophilicity (expressed as LogP <5), <5 hydrogen bond donors,<10 hydrogen bond acceptors, and Molar refractivity should be between 40-130.

In silico analysis and validation

The results of molecular docking using PyRx software showed that VEGFR-2 (PDB ID: 3VHK) had the best binding affinity to compounds in *S. aquifolium*. Linoleic acid had the best docking results against all receptor proteins with binding values of VEGFR2 (-6.3 kcal/mol), EGFR (-5.5 kcal/mol), BCL-2 (6.0 kcal/mol), caspase-3 (-4.9 kcal/mol), and caspase-9 (-5.3 kcal/mol) (shown in Table 4).

Linoleic acid docking on VEGFR-2 showed hydrogen interactions with six amino acids involved, namely Ile_{888}(A), His_{1026}(A), Leu_{1019}(A), Val_{899}(A), Val_{898}(A), Val_{916}(A) (Figure 1). Linoleic acid docking with EGFR showed hydrogen bonds with five amino acids involved, namely Leu_{718}(A), Leu_{844}(A), Val_{726}(A), Ala743(A), Leu792(A). The bond of linoleic acid with Bcl-2 showed hydrogen interactions with five amino acids, namely Arg146(A), Phe_{104}(A), Tyr_{108}(A), Leu_{137}(A), Val_{148}(A). Linoleic acid with caspase-3 shows hydrogen interactions involving three amino acids, namely Lys_{82}(A), Leu_{81}(A) His_{277}(B) and linoleic acid with caspase-9 shows hydrogen

Table 1. List of HCC receptor proteins used in molecular docking trials

Number	Receptors	PDB ID	Classification of Receptor	Mutation
1.	Crystal structure of the VEGFR2 kinase domain in complex with a back pocket binder	3VHK	HCC Transferase	No
2.	Crystal structure of the EGFR kinase domain in complex with 4-(4-{[2-{[(3S)-1-acetylpyrrolidin-3 -yl]amino}-9-(propan-2-yl)-9H-purin-6-yl]amino}phenyl)-1-methylpiperazin-1-ium	5UGB	HCC Transferase	No
3.	Crystal structure of BCL-2 with venetoclax	6O0K	Apoptosis	No
4.	Caspase-3:CAS329306	2XYG	Hydrolase	No
5.	Human caspase 9 in complex with bacterial effector protein	3V3K	Hydrolase	No

bonds involving one amino acid, namely Arg_{87}(B) (Table 5).

Molecular dynamic simulations of the linoleic acid compound against the five protein receptors are shown in Table 6 and Figure 2. The highest root mean square deviation (RMSD) value was shown by the bond of linoleic acid – caspase-9 (2.175), followed by the linoleic acid – caspase-3 bond (2.157), linoleic acid – EGFR (1.971), linoleic acid – VEGFR-2 (1.940), and linoleic acid – Bcl-2 (1.709).

DISCUSSION

Hepatocellular carcinoma (HCC) is a common cancer with a high mortality rate. Growing evidence suggests that gut dysbiosis and metabolic abnormalities contribute to the development of HCC. Drugs such as sorafenib, pembrolizumab, and nivolumab are currently used to treat HCC, but their long-term use may be limited due to side effects such as dermatotoxic responses, gastrointestinal reactions, systemic reactions, and

Table 2. Results of GC-MS chromatogram analysis of the ethanol extract fraction of brown macroalgae *S. aquifolium*

No.	Retention Time	Name of Compound	Peak Area %
1	2.335	Formic Acid	12.11
2	3.130	Hydrochloric acid	12.85
3	3.478	Acetic acid	0.03
4	3.843	Acetyl carbinol	0.01
5	4.2.17	Vinylformic acid	0.02
6	11.383	Glycinamide	0.00
7	5.417	Formamide	0.02
8	6.429	2-Furancarboxaldehyde	0.01
9	8.975	Disiloxane	0.01
10	9.673	Phosphonic acid	0.00
11	9.716	Ethanedioic acid	0.01
12	9.828	Hexanoic acid	0.01
13	9.969	Ethanediimidic acid	0.00
14	23.816	Palmitic Acid	0.95
15	19.034	Dodecanoic Acid	0.03
16	24.044	Linoleic Acid	0.07
17	24.170	Oleic Acid	0.03
18	24.889	Stearic acid	0.02
19	25.266	Phytol	0.09

Table 3. Drug-likeness parameter measurement results based on Lipinski's Rule of Five Screening

Compound	Drug-likeness Parameters				
	Mass	Hydrogen Bond Donor	Hydrogen Bond Acceptors	LogP (High Lipophilicity)	Molar Refractivity
Palmitic acid	256	1	2	5.55	77.94
Dodecanoic acid	200	1	2	3.99	59.47
Linoleic acid	280	1	2	5.88	86.99
Formic acid	312	5	6	-0.05	77.14
Phytol	296	1	1	6.36	95.56

vascular dysfunction.[2,33] Several crystal structures of domains such as VEGFR-2, EGFR, BCL-2, caspase-3, and caspase-9 have been studied elsewhere previously.

Previous studies have explained that among 19 compounds of *Ocimum basilicum* extract, 8 compounds including palmitic acid were selected because they met Lipinski's Rule of Five.[34] Palmitic acid is a saturated fat containing 16 carbon atoms which is generally abundant in animal and vegetable fats.[35,36] Dodecanoic acid or lauric acid is a medium-chain fatty acid molecule with twelve carbon atoms found in virgin coconut oil in concentrations ranging from 46 - 52%.[37] Lauric acid is also found in foods such as human milk, fruits, palm oil, and including seaweed from the types *Sargassum* sp. and *Ulva* sp..[38–40]

Lauric acid has good antioxidant properties in cells, so it can protect cells and inhibit neuroinflammation, kill human colon cancer cells up to 93% by producing oxidative stress and apoptosis in cells, and can protect the liver from

Table 4. Molecular docking results

Number	Compound types	Docking results with several tested receptors (kcal/mol)				
		VEGFR2 (PDB ID: 3VHK)	EGFR (PDB ID: 5UGB)	BCL-2 (PDB ID: 6O0K)	Caspase-3 (PDB ID: 2XYG)	Caspase-9 (PDB ID: 3V3K)
1.	Palmitic acid	-5.5	-4.9	-5.0	-4.0	-4.3
2.	Dodecanoic acid	-6.3	-4.8	-4.5	-4.2	-3.8
3.	Linoleic acid	-6.3	-5.5	-6.0	-4.9	-5.3
4.	Formic acid	-3.0	-2.9	-2.9	-2.8	-2.8
5.	Phytol	-5.2	-5.3	-6.1	-4.3	-5.0

Table 5. Diagram of protein-ligand complex interactions (linoleic acid with receptor proteins related to HCC)

Interaction Complez	Type of Interaction	Amino Acids Involved in Interaction
(a) Linoleic acid-VEGFR2	vdw	Ile892(A), Ile1044(A), Asp1046(A), Glu885(A), Phe1047(A), Leu1035(A), Val848(A)
	HI	Ile888(A), His1026(A), Leu1019(A), Val899(A), Val898(A), Val916(A)
	PHI	Cys1045(A), Lys868(A)
(b) Linoleic acid-EGFR	vdw	Thr854(A), Thr790(A), Asp855(A), Met766(A), Phe856(A), Leu788(A), Met793(A), Gly796(A), Cys797(A), Gly719(A)
	HI	Leu718(A), Leu844(A), Val726(A), Ala743(A), Leu792(A)
	PHI	Glu762(A), Lys745(A)
(c) Linoleic acid-BCL-2	vdw	Gly145(A), Trp144(A), Phe198(A), Ala100(A), Glu152(A), Phe153(A)
	HI	Arg146(A), Phe104(A), Tyr108(A), Leu137(A), Val148(A)
	PHI	Ala149(A)
(d) Linoleic acid-caspase-3	vdw	Pro42(A), Glu43(A), Met39(A), Ser112(A), Thr77(A), Asp40(A), Leu223(B), Ala227(B), Lys224(B), Asp228(B), Tyr276(B), Phe275(B)
	HI	Lys82(A), Leu81(A) His277(B)
	PHI	Met44(A)
(e) Linoleic acid-caspase-9	vdw	Leu179(B), Glu89(B), Phe84(B), Val86(B), Arg99(B), Glu85(B), Val86(H), Arg99(H), Asn81(H), Glu149(H), Lys183(H), Glu85(H)
	HI	Arg87(B)
	PHI	Asn81(B), Arg80(B), Lys183(B)

Note: vdw= Van der Walls interaction; HI=hydrophobic interaction; PHI=polar hydrogen interaction

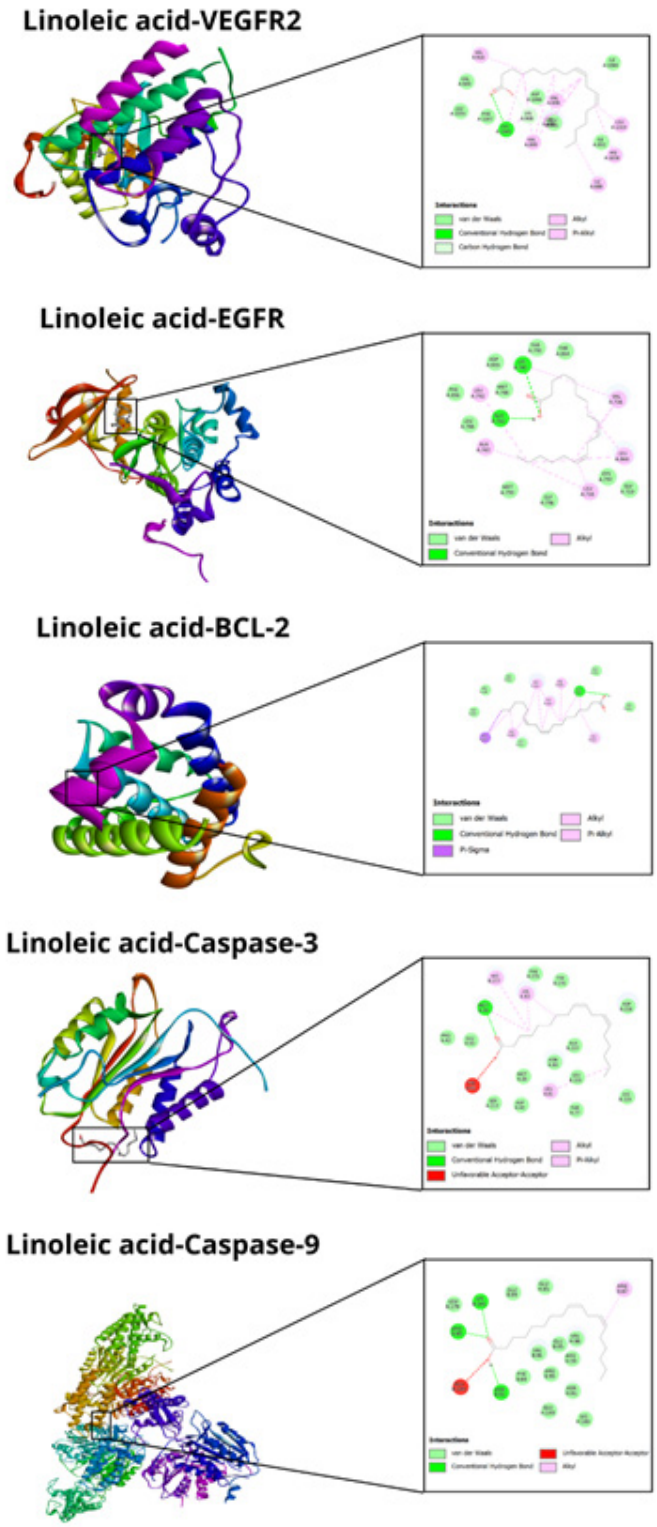

Fig. 1. Chemical interaction of ligand-receptor complexes

hepatotoxicity caused by ethanol induction by increasing HNF4á regulation and apoptosis.[41–43] Linoleic acid can be converted through chain elongation and desaturation into long-chain fatty acids including arachidonic acid.[44,45] Research showed that chitosan nanoparticles containing *Melastomastrum capitatum* leaf extract can deliver linoleic acid to OV7 cancer cells compared to MCF-7 cell lines. Linoleic acid loaded in chitosan also showed the highest selective index and increased caspase-3 activity.[46]

Molecular docking is a major theoretical tool used in molecular modeling to predict molecular binding, interaction intensity, and signal types in signal transduction and drug production processes. Molecular docking plays an important role in structural molecular biology and computer-aided drug design by predicting important binding models between ligands and known protein structures.[47] Our *in silico* analysis showed that the binding affinity value to VEGFR-2 which is similar to dodecanoic acid is also shown by linoleic acid (-6.3 kcal/mol). In theory, angiogenesis involving vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 is very important in the development of cancer and cardiovascular disease. VEGFR-2 is able to encourage vascular and regulate proliferation, migration, and survival of endothelial cells in vasculogenesis and angiogenesis.[48] Targeting VEGFR-2 played by linoleic acid allows for a new therapeutic strategy. This tethering is intended to be able to suppress VEGFR-2 which is a tyrosine kinase receptor required in tumor cells.[49] A previous study tested the effectiveness of astaxanthin extract *in silico* showed a high binding affinity value to the VEGFR-2 receptor (-6.46 kcal/mol when compared to positive drugs (Sorafenib) in general (-4.92 kcal/mol).[17]

Table 6. Molecular dynamics simulation results

Complex Name	Root Mean Square Deviation (RMSF)(Å)
Linoleic acid – VEGFR-2	1.940
Linoleic acid– EGFR	1.971
Linoleic acid –Bcl-2	1.709
Linoleic acid–caspase-3	2.157
Linoleic acid–caspase-9	2.175

Bcl-2 is a protein expressed by the *bcl*-2 gene that regulates cell death. Overexpression of *bcl*-2, especially when paired with certain genes such as *myc*, can trigger aggressive B-cell malignancies by suppressing apoptosis.[50] Interestingly, we found

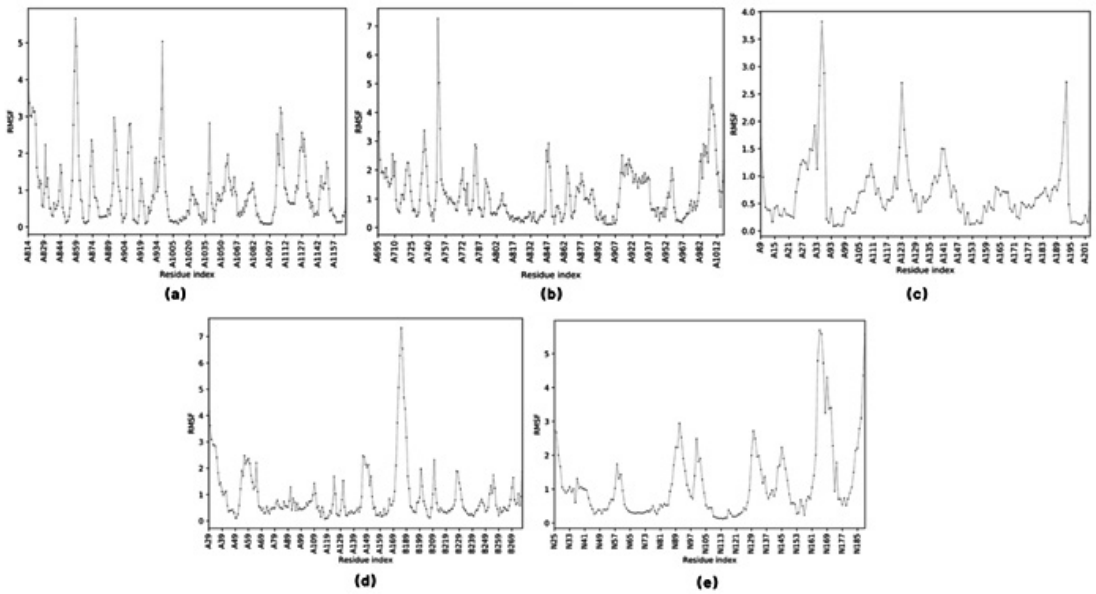

Fig. 2. RMSF Value. (a, Linoleic acid – VEGFR-2; b, Linoleic acid – EGFR; c, Linoleic acid – VEGFR-2-Bcl-2; d, Linoleic acid – caspase-3; e, Linoleic acid – caspase-9.

that linoleic acid has a high binding affinity value to the Bcl-2 receptor. Caspase-3 and caspase-9, are required for cell apoptosis, but have the potential to increase cancer cell proliferation and invasion in certain cases.[51] EGFR is a cell signaling molecule whose mutation or overexpression is associated with cancer, making it an important target for cancer treatment.[52]

In summary, this study demonstrates that the phytoconstituents from the ethanol extract of *S. aquifolium* are closely associated with the inhibition of protein receptors related to HCC. *In silico* analysis suggests that linoleic acid may act as a key regulator in the energy metabolism of HCC. These findings not only provide a novel perspective for understanding the mechanism of action of seaweed-derived bioactive compounds in regulating the metabolism of HCC development, but also offer a theoretical foundation for the development of novel functional foods aimed at specifically modulating HCC metabolic pathways and their regulatory networks. Subsequent *in vitro* and *in vivo* experiments are warranted to further explore the specific functions of these compounds.

Our results showed that the ethanol extract of *S. aquifolium* is a promising candidate for use in several anticancer therapeutic techniques, especially hepatocellular carcinoma (HCC). However, detailed *in vitro* and *in vivo* studies are still needed as an addition to increase the validity of its efficacy and safety profiles before considering the use of *S. aquifolium* in clinical applications. From the above study, five compounds were found to be potential candidates for HCC inhibitors with the highest value shown by linoleic acid as a potent VEGFR-2 inhibitor.

CONCLUSION

Molecular docking study between growth factors and apoptosis proteins from HCC with *S. aquifolium* compounds clearly demonstrated the binding and interaction of amino acids at the active site between ligands and receptors. Since linoleic acid was found to bind growth and apoptosis proteins with the least free energy compared to the other four compounds, linoleic acid might activate apoptosis proteins, which in turn could inhibit the growth of HCC through inhibition of VEGFR-2, EGFR, and Bcl-2 in HCC, thus acting as a potent anticancer agent. To confirm the anti-HCC potential of the drug candidates discovered through the limited computational analysis, further benchworks and clinical studies are needed. The further investigations will provide a more comprehensive understanding of the safety and efficacy of the compounds discovered in treating HCC. On the other hand, exploring the efficacy of these key molecules in HCC animal models may pave new avenues for future clinical trials and the development of novel treatments for degenerative diseases. These phytoconstituents are useful from *S. aquifolium* source for the development of new HCC drugs targeting five HCC-related ligands, as they can be further modified and improved for better HCC treatment.

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Conflict of Interest

The author(s) do not have any conflict of interest

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

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Not Applicable.

Author Contributions

Anak Agung Ayu Putri Permatasari and Putu Angga Wiradana: Conceptualization, Methodology, Writing – Original Draft, Project Administration, Funding Acquisition; I Gede Widhiantara: Supervision, Writing – Review & Editing, Project Administration; Ni Kadek Yunita Sari and I Made Gde Sudyadnyana Sandhika: Data Collection and Analysis; Mochammad Aqilah Herdiansyah, Win Darmanto, and Novaria Sari Dewi Panjaitan: Data Collection, Analysis, Visualization, and Supervision.

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