Evaluation of polyphenol and antioxidant properties of *Blumea balsamifera* extract as potential therapeutic for breast cancer

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Abstract. *Blumea balsamifera* (Bb) is a plant used as herbal medicine in Southeast Asia, and it has been used due to its antibacterial, anti-inflammatory, anticancer, etc. However, there is currently limited evidence that Bb leaf extract from Batu, Indonesia, contains beneficial compounds against breast cancer. Hence, this study evaluates the active compounds in extract and their potential as therapeutic agents for breast cancer. The total phenolic and flavonoid content was determined based on quantified colourimetry analysis followed by DPPH assay to evaluate antioxidant activity and phytochemicals screening in the extract, which was characterised by LC-HRMS analysis. Furthermore, computational methods are used to predict the pharmacological properties of compounds in the extract, particularly against breast cancer. The results showed a total phenolic content of 103.85+1.5 mgGAE/g and a total flavonoid content of 225.99+17.68 mgQE/g, with an antioxidant activity of 255.17+13.11µg/mL. 11 compounds were identified, but only four (Aurantio-obtusin, Isorhamnetin, Quercetin, and Hemiphloin) were computationally analysed. Molecular docking and dynamics simulation indicate that these phytochemicals bind to their target, possibly limiting their activity. Therefore, Bb has potential as a natural product remedy for breast cancer and contributes significantly to our knowledge of the plant by providing essential data for its future development.

1 Introduction

Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS) are byproducts of normal cell metabolism. Natural antioxidant pathways can mitigate the negative effects of ROS. These free radicals play a variety of roles in human health and disease. As a result, it is possible to slow disease progression and lower the risk of chronic diseases by boosting the body's natural antioxidant defence system or supplementing with dietary antioxidants. Antioxidants play a crucial role in preventing disease by neutralising harmful free radicals. While synthetic antioxidants have potential drawbacks, natural antioxidants derived from

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plants offer a promising alternative. Researchers are actively seeking new natural antioxidants with potent radical scavenging properties and minimal side effects [1].

Blumea balsamifera (Bb), also known as 'Sembung legi' in Indonesian, was a plant used as herbal medicine in several Asian countries, including Indonesia, China, Malaysia, Thailand, Philippines, and Vietnam. This plant is used in traditional medicine due to its pharmacological properties, such as antibacterial, anti-inflammatory, anticancer etc. [2]. It belongs to the *Blumea* genera and can grow in tropical and subtropical environments. It grew as an herbaceous plant, reaching a height of 3-4 meters and emitting a pungent camphor aroma. Its leaves were long with pointed tips, serrated leaf margins, fine hairs on the leaf surface, and scattered single leaflets [3, 4].

Bb contains many phytochemical compounds, including phenolic and flavonoid groups [5]. Previous studies have shown dihydroflavonol contained in Bb leaf extract work synergistically with Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) protein to induce apoptosis of adult T-cell leukaemia/lymphoma (ATLL) cancer cells [6]. Another study showed that several compounds from the flavonoid group are toxic to the oral cavity (KB) and lung cancer cell lines (NCI-H187). The compounds include luteolin-7-methyl ether, quercetin, dihydroquercetin-7,4'-dimethyl ether, 5,7,3'5'-tetrahydroxyflavone, and blumeatin [7].

The usage of natural products in medicine has led to the search for new potential drugs derived from this plant. The medicinal value of plants depends on their phytochemicals, which have various physiological effects on the human body. As a result, phytochemical screening can identify compounds present in plants that could be used as the basis for current medication development [8]. Phytochemical evaluation involves identifying and quantifying bioactive compounds contained in herbal extracts using a tool, for example, liquid chromatography combined with mass spectrometry [9]. LC-MS involves the process of separating compounds through a chromatographic column that is simultaneously characterised by m/z values. Both methods provide comprehensive data information on the compounds in the analysed sample [10].

In recent years, *in silico* methods have emerged as powerful tools for predicting the biological activities of compounds. These methods involve using computational techniques to analyse the molecular structure and properties of compounds and predict their potential interactions with biological targets. Molecular docking analysis predicts compound-protein interactions using computer simulations. This method is important in the design of candidate new drugs by simulating compound binding and its effect on protein structure. The combination with molecular dynamics provides a deeper understanding of the dynamic compound-protein interactions, helping in designing more effective and safe candidate drugs [11].

While limited evidence exists for the efficacy of Bb leaves from Batu, Indonesia, against breast cancer, the increasing incidence of this disease necessitates exploring novel treatment options. Therefore, this study aimed to identify the phytochemicals and antioxidant activity in *B. balsamifera* extract and explore the potential therapeutic of phenolic compounds in *B. balsamifera* to inhibit breast cancer progression.

2 Materials and methods

2.1 Extraction

B. balsamifera leaves dried powder purchased from UPT Balai Materia Medica, Batu, East Java. The extraction process is based on a previous study [12]. Dried leaves powder (5 g) was inserted into the MAE vessel. Ethanol 96% (50 mL) was added into the MAE vessel

containing the extract in a ratio of 1:10. The instrument is run by warming up to 50 °C for 5 minutes, holding set to 50 °C for 10 minutes, and cooling down for 5 minutes. The obtained extract was filtered was put into a rotary vacuum evaporator at 50 rpm, 40 °C. The crude extract was then stored at 4 °C before further use.

2.2 Total phenolic content

The total phenolic test is based on previous research protocols [12, 13]. The extract was 100 µg/mL, while gallic acid concentrations (standard) were 0.625, 1.25, 2.5, 5, 10, 20, 40 and 80 µg/mL. Extract and standard (10 µL each) were added to a 96-well plate, followed by Folin-Ciocalteu (100 µL). Na_2CO_3 (100 µL) was added to the sample and incubated for 90 minutes at room temperature in a dark room. The absorbance of the sample was measured at 735 nm with a microplate reader. The total phenolic content of extracts was calculated by converting the gallic acid standard curve (mg GAE/g).

2.3 Total flavonoid content

The total flavonoid test follows previous research protocols [14, 15]. The extract was used at 100 µg/mL, while the quercetin was used as a standard at 0.6125, 1.25, 5, 10, 20, 40, and 80 µg/mL. Extracts and quercetin (50 µL) were added to a 96-well plate, followed by ethanol 96% (150 µL) and $AlCl_3$ (10 µL). CH3COONa 1 M (10 µL) was added to the sample and incubated for 40 minutes at room temperature in a dark room. The absorbance of the sample was measured at 405 nm using a microplate reader. The total flavonoid content of extracts was calculated by converting the quercetin standard curve (mg QE/g).

2.4 Antioxidant activity

The antioxidant activity protocol was based on the previous study [12]. The extract concentrations were 25, 50, 100, 200, and 400 µg/mL, while ascorbic acid as a standard at 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 µg/mL. Extracts and gallic acid (100 µL) were added to a 96-well plate, followed by DPPH 0.4 mM solution (100 µL). The sample was incubated for 30 minutes at room temperature in a dark room. The absorbance of the sample was measured at 490 nm using a microplate reader. The antioxidant activity of extracts was determined using the percentage IC_{50} value ratio of extract to ascorbic acid (%).

2.5 LC-HRMS analysis

The preparation protocol and compound profile analysis of the extract follows the Metabolomics Laboratory protocol at Bogor Agricultural University (IPB, Bogor). Extract (5 mg) was dissolved in methanol 1 mL, then filtered through a nylon membrane (0.2 µm). LC-MS performed by Vanquish Tandem Q Exactive Plus Orbitrap HRMS UHPLC device (Thermo Scientific, USA). The column used was an Accucore C18, with 100 x 2.1 mm, 1.5 µm (Thermo Scientific, USA). The Eluents were H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). The eluent gradient ranged from 0-1 minute (A 5%), 1 to 25 minutes (A 5-95%), 25 to 28 minutes (B 95%), and 28 to 33 minutes (B 5%). UHPLC was programmed with a flow rate of 0.2 mL/min and a column temperature of 30 °C. Sample 2 µL was injected into a column on a UHPLC. HRMS was configured with a mass range of 100-1500 m/z and a negative ionisation mode. HRMS data was analysed using Compound Discoverer 3.2 and mzCloud.

2.6 Druglikeness, bioactivity, and target protein prediction

Eleven bioactive compounds were retrieved from LC-HRMS analysis. Subsequently, their biological activities were analysed using the PASS server(http://way2drug.com/passonline/). The assessment of biological activity was based on Pa values, with a cut-off set at Pa > 0.3. The results of the biological activity analysis were displayed as a Heatmap using GraphPad Prism. The pharmacological properties of the bioactive compounds were assessed using the SwissADME web (http://www.swissadme.ch). The analysis used each compound's canonical smiles from the PubChem database and submitted to SwissADME. SWISS Target Prediction (http://www.swisstargetprediction.ch/) was used to identify protein targets associated with breast cancer in each compound. Target proteins were chosen based on their association with breast cancer and frequent alteration.

2.7 Molecular docking and structural visualisation

Ligands were retrieved from PubChem and saved in three-dimensional SDF format. Ligand energy was minimised by Open Babel in PyRx software. Native ligands as control were extracted from each of the proteins using PyMol software. The three-dimensional protein structure was sourced from the PDB RCSB database (https://www.rcsb.org.). Water molecules and native ligands were removed from proteins using Biovia Discovery Studio 2019 Client software. The targeted docking process was performed between these compounds and the active site of each protein using AutoDock Vina, which was integrated into PyRx. The docking data were then visualised using the Biovia Discovery Studio 2019 Client software.

2.8 Molecular dynamics

Molecular dynamics simulation was analysed using Yet Another Scientific Artificial Reality Application (YASARA), a software commonly used for such simulations, with certain modifications as described in [12]. The system parameters were tuned to match the cellular physiological conditions, with settings at 310 °K, pH 7.4, 1 atm pressure, and a NaCl concentration of 0.9%, maintained throughout a 50 ns period. The macros program included "md_run" for executing simulations, "md_analyze" for assessing RMSD, and "md_bindingenergy" for evaluating the binding energy of protein-ligand complexes during molecular dynamics.

3 Results and discussions

3.1 Total phenolic content, total flavonoid content and antioxidant activity

Table 1 shows that *B. balsamifera* extract (BBE) has phenolic content in correlation with flavonoid content. The concentrations of phenolic compounds and flavonoids in extracts vary depending on the solvent used for extraction. This occurs because plant-derived compounds are classified as polar or non-polar [16]. As a result, solvent selection is critical to the efficiency of the polyphenol extraction process from plants. Furthermore, the precise concentrations of phenols and flavonoids in the BBE may differ from those reported in previous studies [17, 18]. Other factors, such as differences in growing locations, influence component levels, and different growing environments can affect the plant's metabolic system, resulting in varying quantities of compounds produced [17].

However, the phenolic and flavonoid content has a low correlation to the antioxidant properties of BBE. The molecular structure of flavonoids has a significant influence on the antioxidant activity of plant extracts. Key structural features contributing to this activity include the ortho-dihydroxy structure on the B-ring, the 2,3 double bond with 4-oxo functional group on the C-ring, and the 3- and 5- hydroxyl groups with 4-oxo functionalities on the A and C rings. The ortho-dihydroxy structure on the B-ring enables efficient electron donation and radical stabilisation, while the 2,3 double bond with the 4-oxo functional group on the C-ring facilitates electron delocalisation. The 3- and 5-hydroxyl groups with 4-oxo functionalities on the A and C rings are required for maximising radical scavenging potential [19]. In addition, co-existing pigments in extracts that absorb in the same wavelength range as DPPH radicals (around 517 nm) may interfere with the absorbance readings [20]. Consequently, high flavonoid content alone does not guarantee strong antioxidant activity, as the specific molecular structure of flavonoids and the presence of interfering pigments are significant factors.

Table 1. Total phenolic, flavonoid and antioxidant activity of BEE.

Assay	Value
TPC (mgGAE/g)	103.85 ± 1.5
TFC (mgQE/g)	225.99 ± 17.68
DPPH (µg/mL)	255.17 ± 13.11

3.2 LC-HRMS analysis

The bioactive compounds detected in the *B. balsamifera* extract by LC-HRMS are listed in Table 2. The compound 4,5-dicaffeoylquinic acid is known to have several bioactivities, namely antioxidant, anti-inflammatory, prostate anticancer, anti-diabetic and cognitive function improvement [21–23]. Aurantio-obtusin is known to be effective in inhibiting the growth of liver cancer cells by decreasing fat production and inducing ferroptosis, which is cell death due to the accumulation of Fe in the cells [24]. Lecanoric acid is effective in inhibiting the growth of colon cancer cells by stopping the cell cycle in the M phase so that the cancer cell division process is inhibited. In addition, the induction of death by this compound is only specific to cancer cells [25]. Corchorifatty acid F showed efficacy as an antifungal against the pathogenic fungus *Pyricularia oryzae* in resistant rice varieties [26].

Isorhamnetin has anti-tumour activity against several cancer cells, including cervical, lung, colon, breast, pancreatic, nasopharyngeal, liver, and gastric cancer cells. The anti-tumour activity of this compound by inhibiting cancer cell proliferation induces apoptosis and regulates tumour suppressor genes, proto-oncogene, and signalling pathways [27]. Tuberonic acid glucoside is a regulator of tuberonic acid levels in response to environmental conditions [28]. Quercetin can induce the inhibition of important molecular pathways in cells, such as MAPK/ERK1/2, p53, JAK/STAT, and others. These signalling pathways regulate cell growth, induction of cell death and cell cycle arrest [29].Quercetin-3β-D-glucoside has anti-arteriosclerotic, anti-inflammatory, antiviral, and antioxidant properties both in vivo and in vitro. It also improves the anti-inflammatory properties of M2a macrophages and controls the immune response to pro-inflammatory stimuli [30].

Salicylic acid can inhibit cancer cell growth by inducing stress on the endoplasmic reticulum that induces death signals [31]. Chlorogenic acid, based on the previous study, has the potential to be an anticancer by halting the cell cycle, inducing apoptosis, and suppressing cancer cell proliferation [32]. Hemiphloin has pharmacological effects for humans, including antioxidant, anti-inflammatory, anticancer, cardioprotective, and neuroprotective [33]. Overall, the results suggest that Bb extract contains bioactive compounds with potential

health benefits. In addition, phenolic compounds found in the extract have promising activities to inhibit cancer cells.

3.3 Druglikeness and bioactivity prediction

The general method to assess compound drug-likeness involves property-based filters and substructure analysis, which set specific boundaries for certain molecular properties of compound or drug candidates. According to the drug-likeness screening, all of the compounds have passed the Lipinski rules [34] that are indicated in Table 3, indicating good bioavailability and cell membrane penetration. While most compounds exhibited favourable GIA, all of the compounds were unable to cross the BBB. Solubility was determined using ESOL [34, 35], with most compounds classified as soluble, facilitating drug development and formulation. ESOL (estimated solubility) is a computational chemistry model used to predict a compound's solubility in water. The model belongs to the Quantitative Structure-Property Relationship (QSPR) category, which is a model that links the chemical structure of a compound with its solubility properties. The solubility scale is divided into several categories, including Log S value < -10 (insoluble), -10 < Log S value < -6 (less soluble), -4 < Log S value < -2 (moderate soluble), -2 < Log S value < 0 (soluble), Log S value > 0 (very soluble) [35]. P-gp interaction was evaluated, revealing that all of the compounds were not substrates of P-gp. P-gp (permeability glycoprotein) influences the ADMET properties of xenobiotics (drugs or chemicals) as it regulates the uptake and metabolism of compounds within the cell, which acts similar to an efflux pump to remove the substrate from the cell [34]. These findings suggest that *B. balsamifera* compounds have promising drug-like properties.

The bioactivity screening results for four compounds through PASS Online, identified four compounds activities were associated with the cancer pathway (Fig. 1). The compounds were further screened to assess their potential bioactivity, with the aim of predicting their capability to inhibit the progression of breast cancer. The bioactivities related to the progression of breast cancer predicted from compounds were MMP9 inhibitor, JAK2 inhibitor, apoptosis agonist, caspase 3 stimulant, caspase 8 stimulant, TP53 enhancer, antineoplastic (breast cancer), Bcl2 antagonist, Bcl-xL inhibitor, breast cancer-resistant protein inhibitor, CDC25A inhibitor, CDC25B inhibitor, CDC25C inhibitor, CDC25 phosphatase inhibitor, CDK4/cyclin D1 inhibitor, CDK6 inhibitor, Topoisomerase I inhibitor, Topoisomerase II inhibitor, Estrogen antagonist, Protein kinase C inhibitor, antimutagenic, antineoplastic (breast cancer), anticarcinogenic, antimetastatic, and chemosensitiser. Besides, bioactivities related to antioxidant activities were HIF-1α inhibitor, free radical scavenger, and antioxidant.

3.4 Molecular docking

Molecular docking was used to analyse the interactions between compounds in *B. balsamifera* with target proteins selected based on SwissTarget (EGFR, ERα, IGF1R). EGFR is a highly activated transmembrane protein receptor that can trigger a series of reactions in cells that can promote uncontrolled cell growth and the formation of cancer cells [36]. ERα plays a role in cell regulation related to proliferation and survival and induces cancer cell growth and metastasis [37]. IGF1R plays a role in cell regulation related to survival, growth, cell cycle, and differentiation of cancer cells [38]. Table 4 shows the binding affinity values for all complexes. The complexes with the lowest binding affinity values for each compound included EGFR-quercetin, ERα-hemiphloin, and IGF1R-isorhamnetin.

Fig. 2 depicts detailed interactions, with all three potential compounds binding to the target protein's active site. Quercetin binds to EGFR at the same residue as the inhibitor,

including Leu718, Val726, Ala743, Ile744, Glu762, Met766, Leu788, Ile789, Thr790, Leu792, Met793, Gly796, Leu844, and Thr854. Hemiphloin binds to ERα at the same residue as the inhibitor, including Met343, Leu346, Thr347, Ala350, Glu353, Trp383, Leu384, Leu387, Met388, Leu391, Leu525, and Met528. IGF1R binds to IGF1R at the same residue as the inhibitor, including Leu975, Gln977, Gly978, Val983, Ala1001, Lys1003, Val1033, Met1049, Glu1050, Leu1051, Met1052, Gly1055, Asp1056, Met1112, Met1126, Thr1127, and Ile1130. The active compound has the same interaction position as an inhibitor. It is considered to have similar activity to interfere with the protein's function.

3.5 Molecular dynamics

Molecular dynamic simulation was used to determine the stability of the protein-ligand complexes between three potential compounds and their targets [39]. The simulations evaluated root mean square deviation (RMSD) of protein-ligand complexes, ligand movement RMSD and molecular dynamics binding energy. The RMSD values of the three selected complexes during simulations displayed low fluctuations, indicating that the ligand-protein complexes' structure and the ligand's movement in the binding pocket of the target protein during the simulation remained stable (Fig. 3). The binding energy values during the molecular dynamic simulation displayed stable interaction between the ligand-protein with the more positive value means more stable the ligand-protein interaction [39]. The binding energy values show no fluctuations, suggesting the complex interaction was stable. Overall, the molecular dynamics reveal that the interaction of active compounds to target protein remains stable.

Table 2. Identified compounds in *B. balsamifera* based LC-HRMS analysis.

Name	Formula	m/z	Exper. mass	Cal. mass	Mass error	Fragment product	Ion Mode	Class	Ref.
4,5-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516	516.1261	516.1257	0.775	354. 09143; 191.05513; 179.03386	$[M-H]^-$	phenolic	[40]
Aurantio-obtusin	$C_{17}H_{14}O_7$	330.0675	330.0739	330.0740	-0.3029	314.04266; 299.01913; 271.02429; 243. 02872	$[M-H]^-$	Anthraquinone	[41,42]
Lecanoricacid	$C_{16}H_{14}O_7$	318.0662	318.0737	318.0740	-0.9431	167.03383; 149.02328; 123.04377	$[M-H]^-$	phenolic	[43–45]
Corchorifatty acid F	$C_{18}H_{32}O_5$	328.2237	328.2251	328.2250	0.3046	229.14493; 211.13298; 183.13773; 97.05895; 85.02833; 57.033111	$[M-H]^-$	fatty acid	[46]
Isorhamnetin	$C_{16}H_{12}O_7$	316.0583	316.0583	316.0583	0.00	330.02695; 271.02402	$[M-H]^-$	flavonoid	[47,48]
Tuberonic acid glucoside	$C_{18}H_{28}O_9$	388.1733	388.1733	388.1733	0.00	207.10168; 163.11220	$[M-H]^-$	terpenoid	[49]
Quercetin	$C_{15}H_{10}O_7$	302.18	302.0427	302.0427	0.00	273.04022; 229.04938; 151.00256; 121.02830; 107.00581	$[M-H]^-$	flavonoid	[50]
Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.0936	464.0955	464.0955	0.00	301.03433; 300.02676; 271.02368	$[M-H]^-$	flavonoid	[46,47]
Salicylic acid	$C_7H_6O_3$	138.0311	138.0311	138.0317	-4.3468	108.89841; 93.03335	$[M-H]^-$	phenolic	[46]

Name	Formula	m/z	Exper. mass	Cal. mass	Mass error	Fragment product	Ion Mode	Class	Ref.
Chlorogenic acid	$C_{16}H_{18}O_9$	354.0878	354.0952	354.0951	0.2824	191.05511; 179.03352; 161.02330; 135.04399	$[M-H]^-$	phenolic	[47]
Hemiphloin	$C_{21}H_{22}O_{10}$	434.1154	434.1213	434.1213	0.00	343.08188; 313.07135; 271.06116; 151.00255; 119.04906	$[M-H]^-$	flavonoid	[47]

Table 3. Druglikeness prediction of BEE selected compounds.

Compounds	**Physicochemical Properties (Lipinski rule)**					**Water Solubility**		**Pharmacokinetics**		
	TPSA	MLOGP	HBA	HBD	MW	Log S (ESOL)	Class	GI	BBB	P-gp
Aurantio-obtusin	113.29 Å²	-0.22	7	3	330.07387	-3.8	Soluble	High	No	No
Isorhamnetin	120.36 Å²	-0.31	7	4	316.0583	-3.36	Soluble	High	No	No
Quercetin	131.36 Å²	-0.56	7	5	302.04265	-3.16	Soluble	High	No	No
Hemiphloin	177.14 Å²	-1.83	10	7	434.12133	-2.58	Soluble	Low	No	No

TPSA: Topological polar surface area; MW: molecular weight; GI: gastrointestinal tract; BBB: blood brain barrier; P-gp: P-glycoprotein

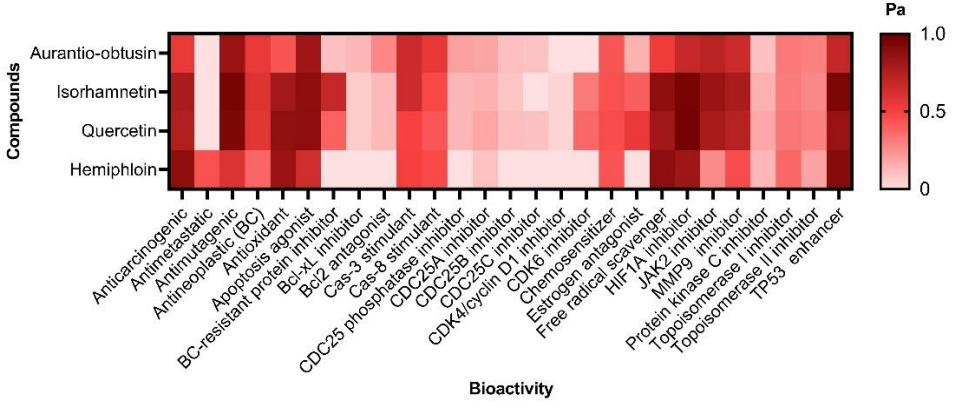

Fig 1. Bioactivity related to breast cancer prediction of selected compounds. BC = breast cancer.

Table 4. Molecular docking results between bioactive compounds and their target proteins

Compound	Protein target	PDB ID	Inhibitor	Ref.	Binding affinity (kcal/mol)	
					Protein-compound	Protein-inhibitor
Aurantio-obtusin	EGFR	4I23	Dacomitinib	[51]	-7.88	-8.158
	ERα	3ERT	Tamoxifen	[52]	-5.194	-9.428
Isorhamnetin	EGFR	4I23	Dacomitinib	[51]	-8.367	-8.158
	IGF1R	2OJ9	Benzimidazole	[53]	-7.978	-9.478
Quercetin	EGFR	4I23	Dacomitinib	[51]	-8.861	-8.158
	IGF1R	2OJ9	Benzimidazole	[53]	-7.475	-9.478
Hemiphloin	ERα	3ERT	Tamoxifen	[52]	-6.776	-9.428

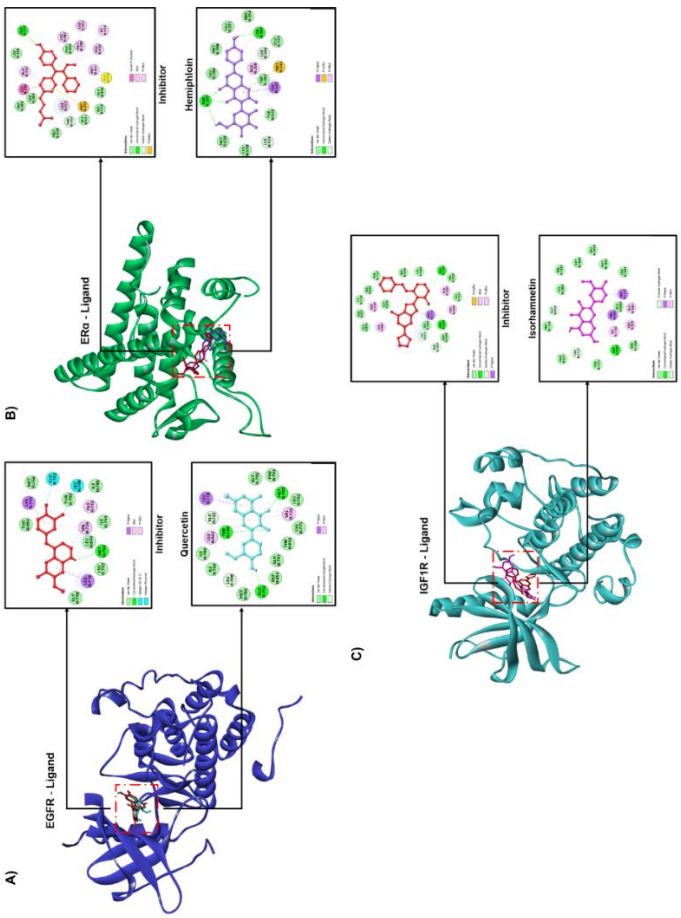

Fig 2. Molecular interaction of the ligand with target. A) EGFR, B) ERα, C) IGF1R.

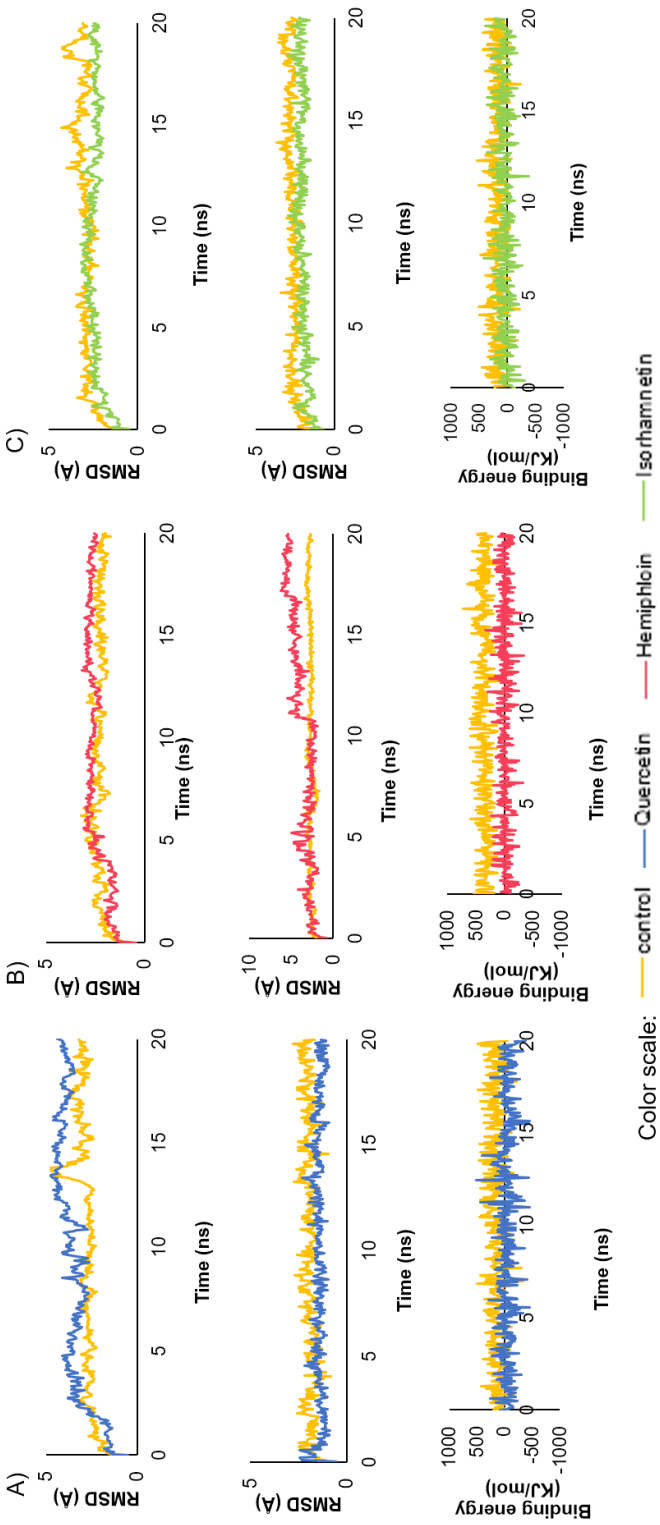

Fig 3. Molecular dynamics of the ligand with target. A) EGFR, B) ERα, C) IGF1R
From above to below – RMSD backbone, RMSD ligand movement, and Binding energy

4 Conclusion

The findings of this study demonstrate that B. balsamifera has a diverse range of identified compounds with health-promoting properties. Furthermore, the phenolics in BBE show promising anticancer activity. The molecular docking and molecular dynamics simulations provide further evidence for the potential therapeutic applications of BBE for breast cancer. Further research is needed to explore the potential health benefits of BBE fully and investigate its mechanisms of action. In vitro and in vivo studies are also necessary to evaluate the efficacy and safety of BBE extract before it is used in further therapeutic applications.

This research was funded by Professor Grants from LPPM (Grants No. 02172.4/UN10.F0901/B/KS2024), Universitas Brawijaya, Malang. We also thank the AI Center of Universitas Brawijaya for providing access to high-performance computing facilities to perform molecular dynamics simulations.

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