Indonesian Journal of Pharmacy

VOL 35 (1) 2024: 116–125 | RESEARCH ARTICLE

LC-HRMS Metabolite Profiling of *Lunasia amara* Stem Bark and *In Silico* Study in Breast Cancer Receptors

Agus Saputra[1,2*], Ietje Wientarsih[1,4], Mohamad Rafi[3,4,5], Lina Noviyanti Sutardi[1] and Silmi Mariya[6]

1. School of Veterinary Medicine and Biomedical Sciences, IPB University
2. Department of Anatomy, Physiology, Pharmacology, and Biochemistry, Faculty of Medicine and Veterinary Medicine, Universitas Nusa Cendana, Jalan Penfui, Kupang NTT, Indonesia
3. Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Jalan Tanjung, Kampus IPB Dramaga, Bogor 16880, Indonesia
4. Tropical Biopharmaca Research Center, IPB University, Kampus IPB Taman Kencana, Bogor 16128, Indonesia
5. Advance Research Laboratory, IPB University, Kampus IPB Dramaga, Bogor 16680, Indonesia
6. Primate Research Center, IPB University, Jalan Lodaya II/5 Bogor 16151, Indonesia

Article Info

Submitted: 11-02-2023
Revised: 23-08-2023
Accepted: 04-09-2023

*Corresponding author
Agus Saputra

Email:
saputra.a@staf.undana.ac.id

ABSTRACT

L. amara is a medicinal plant used as an aphrodisiac. Several studies show it contains a compound with biological activities such as inhibition of cell proliferation, an anticancer mechanism. This study aimed to profile the metabolites and predict their activities against two breast cancer receptors (ERα (3ERT) and HER2 (3PPO)) with an *in silico* approach. The metabolite profile of a water and 80% ethanol extract was analyzed by UHPLC-Q-Orbitrap-HRMS. We also investigated the radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Putative identification of metabolites in *L. amara* revealed 46 metabolites (4 unknown), which were predominantly quinoline alkaloids. Some of the compounds from glycosides and phenol groups were also identified. The antioxidant capacity test results showed that the 80% ethanol extract had a higher radical scavenging capacity than the aqueous extract. Based on molecular docking results, the highest affinity for the ERα receptor was found in the tested compound tetrahydropapaveroline and exceeded that of the native 4-OHT ligand. For the HER2 receptor, graveolinine had the highest affinity but was still below that of the native lapatinib ligand. Ligand interactions with Leu 387 and Glu 419 on the active site of the ERα receptor and Phe1004 on the HER2 receptor are thought to play an important role in increasing the energy affinity. Overall, all compounds showed higher affinity for HER2 receptors than ERα. According to our findings, molecular docking and UHPLC HRMS can validate the presence of lunamarine and graveoline metabolites in *L. amara* samples with anticancer action.

Keywords: Lunasia, bioinformatic, breast cancer, lunacridine, HER2

INTRODUCTION

L. amara (*Rutaceae* family) is a small tree found in tropical forests in the Philippines, Eastern Java, Borneo, Sulawesi, East Nusa Tenggara, Moluccas, Papua New Guinea, and Australia (Macabeo & Aguinaldo, 2008). It is a popular traditional medicine used in Indonesia to enhance sexual aggressiveness or as a pro-fertility agent, either as small dried wood or in a mixture with herbs known as jamu (Luthfi *et al.,* 2017). Pharmacological studies have shown that *L. amara* extract affects the central nervous system and has antibacterial, antioxidant, aphrodisiac, antituberculosis, DNA intercalation, caspase activation, and topoisomerase II decatenation effects. According to previous studies, alkaloid quinoline compounds, such as lunacridine, lunacrine, lunasine, lunamarine, and 5-hydroxygraveroline, are responsible for *L. amara* activity. Lunacrine and lunasine affect the strength of muscles in response to stimulation (Macabeo & Aguinaldo, 2008). The compound 2'-O-trifluoroacetyl lunacridine can inhibit topoisomerase II in non-carcinoma lung cell

fibroblasts, ATCC MRC-5, and two carcinoma cell lines (NCI H226 and HeLa). Furthermore, the concentration of 2'-O-trifluoroacetyl lunacridine considerably influences caspase activity. Activated caspases are essential for apoptosis (Prescott *et al*., 2007). Lunamarine and 5-hydroxygraveroline inhibit human liver microsomal dextromethorphan O-demethylation activity, which is a prototype marker of cytochrome P450 2D6 (CYP2D6) (Takahashi *et al*., 2012). Based on inhibition of cell proliferation, this mechanism leads to anti-cancer activity (Hanahan & Weinberg, 2011). Cancer is currently one of the leading causes of death, and without further advances or the development of novel drugs for treatment, it is predicted to remain the leading cause of death. Breast cancer is the fastest growing cancer in women, other than lung cancer (Dibha *et al*., 2022). Breast cancer is the uncontrolled growth and multiplication of cells that begin in the breast tissue and is quite heterogeneous and stratified into three major subtypes. The type currently being studied is characterized by the expression of steroid hormone receptors (estrogen receptor (ER) and progesterone receptor (PR)) and receptor tyrosine kinase of human epidermal growth factor receptor type 2 (HER2) (Holliday & Speirs, 2011). Mutations in the invasive breast carcinoma (BRCA1) genes tend to result in triple-negative breast cancer (TNBC). TNBC is characterized by a deficiency or absence of ER, PR, and HER2 amplification and is associated with a poor prognosis (Yim-im *et al*., 2014). This makes TNBC more fatal than other breast cancer types due to its ability to metastasize to the central nervous system and lungs and a lack of effective targeted therapeutics (Sun *et al*., 2011).

Therefore, the remaining major challenge in breast cancer treatment is identifying the defective signalling networks underlying this aggressive breast cancer subtype. Every year, many natural products are studied to determine whether there are new candidates for potential antitumor drugs. When a molecule is isolated and chemically characterized, in silico studies are the first step in basic research that leads to the next evaluation stage: in vitro and in vivo studies. The primary mechanism of action of a biological target and its pharmacokinetic profile can be determined using in silico tests. The molecular docking approach can be used to model the interactions between a small molecule and protein at the atomic level, allowing us to characterize the behaviour of small molecules at target protein-binding sites and elucidate fundamental biochemical processes (Dar & Mir, 2017). However, there are no reports of a comprehensive study of *L. amara* metabolite profiling and in silico activity studies of breast cancer biomarkers. Therefore, this study aims to investigate the metabolite profile of *L. amara* stem bark using UHPLC HRMS and molecular binding to breast cancer receptors ERα and HER2. These discoveries will be used to develop novel cancer precision medicines and therapeutic techniques based on lunasia, particularly for the treatment of breast cancer.

MATERIALS AND METHODS

L. amara stem bark was collected from a forest in the Chibal district of the Manggarai Regency, East Nusa Tenggara, Indonesia. The plant was identified by the National Research and Innovation Agency (BRIN) and voucher specimens (BMK0157092016) were stored at TropBRC, IPB University. Ethanol was obtained from Merck (Darmstadt, Germany). Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample Preparation and Extraction

The stem bark of *L. amara* was sliced, air-dried, and powdered. Approximately 25 g of dried powder was thoroughly extracted three times with 250 mL of 80% ethanol and water for three days at room temperature. The filtrate extracts were dried by rotary evaporation at 50°C.

Metabolite separation and putative identification using UHPLC-Q-Orbitrap HRMS

Metabolites from *L. amara* stem bark extracts were separated using a Vanquish Flex UHPLC-Q Exactive Plus Orbitrap-High Resolution Mass Spectrometer equipped with an Accucore™ phenyl hexyl (100 × 2.1 mm, 2.6 µm) column. The mobile phase used was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a gradient elution system: 0.0–3.0 min (5–20% B), 3.0–20.0 min (20–45% B), 20.0–25.0 min (45–95% B), 25.0–28.0 min (95% B), 28.0-28.1 min (95–5% B), 28.1–30.0 min (5% B). The flow rate was kept constant at 0.2 mL/min, with an injection volume of approximately 2 µL. Other parameters for the UHPLC-Q-Orbitrap HRMS analysis were as follows: the source of MS ionization was ESI (+) using a Q-Orbitrap mass analyzer with a m/z range of 133-2000 m/z. The collision energies used for fragmentation were 18, 35, and 53 eV. The spray voltage was approximately 3.8 kV, the capillary temperature was 320°C, and the flow rates of the

sheath gas and auxiliary gas were 15 and 3 mL/min, respectively. For the positive-ion mode, we used scan-type full MS/dd MS 2. Putative identification of the metabolites was performed using the obtained mass spectra and processed using Compound Discoverer version 3.2. We used an in-house database compiled from various scientific articles on the genus Lunasia to identify metabolites. We selected spectra, aligned retention times, detected unknown compounds, grouped unknown compounds, predicted compositions, searched mass lists, filled gaps, normalized areas, and marked background compounds.

DPPH radical scavenging activity

The antioxidant activities of the extracts were determined using the DPPH assay described by Salazar-Aranda *et al*. (2009). Each 40 µL extract was mixed with 120 µL of freshly prepared 125 µM DPPH solution in 96 well plates. The mixture was then incubated in the dark for 30 min at room temperature. A microplate reader (Epoch-BioTek, Winooski, MA, USA) was used to measure the decrease in the absorbance at 515 nm. The negative control was prepared by combining 40 µL of ethanol with 120 µL of freshly prepared DPPH solution at 125 µM. Trolox was used as a positive control and prepared at 9 concentrations (50, 75, 100, 150, 200, 250, 300, 350, and 400 µM). The measurement was repeated three times and the DPPH radical scavenging capacity was expressed as mol Trolox/g dry powder.

Molecular Docking Simulation

Molecular docking studies were performed using metabolites from the stem bark *L. amara* as ligands and triple-negative breast cancer receptors. The ligand and native ligand's (tamoxifen and lapatinib) three-dimensional structures were obtained from www.pubchem.ncbi.nlm.nih.gov and the receptor was downloaded from the protein data bank (www.pdb.org) with codes PDB 3ERT (ERα) and PDB 3PP0 (HER2). Ligand-receptor docking scores were obtained using the PyRx AutoDock Vina software (Trott & Olson, 2010). The Discovery Studio Visualizer was used to visualize the structure and binding docking positions between the ligand and receptor.

Data Analysis

The mass spectra from UHPLC-Q-Orbitrap HRMS were processed using Compounds Discoverer 3.2 (Thermo Fisher, Waltham, USA) with an in-house database compiled from various scientific articles about the Lunasia genus and Rutaceae family to identify the metabolites. We selected spectra, aligned retention times, detected unknown compounds, grouped unknown compounds, predicted compositions, searched mass lists, filled gaps, normalized areas, and marked background compounds for putative distinguishing proof of the Lunasia genus metabolites. The MS2 was confirmed to identify the metabolites.

RESULTS AND DISCUSSION

Extraction yield and antioxidant capacity

Maceration is one of the extraction methods used to extract metabolites. Maceration can be performed at low or high temperatures depending on the purpose of extraction. The type of solvent used affects the extraction yield and the type of metabolites extracted (Rafi *et al*., 2020). In this study, the extract of *L. amara* stem bark was obtained using two polar solvents: water and 80% ethanol. The extraction yields of 80% ethanol and water were 9.19% and 8.28%, respectively.

The DPPH free radical scavenging assay is a well-established method for determining the antioxidant potential of compounds, extracts, and other biological sources. In the DPPH assay, the extract reduces a violet-colored DPPH solution to a yellow-colored product (diphenylpicryl hydrazine), and the absorbance is measured after a set period of time (Rafi *et al*., 2021). This method is rapid, simple, inexpensive, and is widely used to measure the ability of compounds to act as free radical scavengers or hydrogen donors (Kedare & Singh, 2011).

Table I. The capacity of antioxidant activity with DPPH method

Extract	Antioxidant capacity(µmol Trolox/g dry sample)
Water	7.65±0.19
80% Ethanol	16.72±0.08

Mean± SD (n=3)

The antioxidant capacity depends on the type of compound and its concentration. Phenols, alkaloids, and flavonoids exhibit strong antioxidant activity. In (Table I), 80% ethanol has higher antioxidant activity (16.72 µmol Trolox/g dry sample) than water (7.65 µmol Trolox/g dry sample). Ethanol and water extracts were both dominated by quinoline alkaloid compounds but at different concentrations (Table II).

Table II. Putative identification of metabolites in *L. amara* stem bark extracts

Rt (minute)	Molecular Weight	Formula	Mass error	Identification	E 80%	H_2O
1.13	103.09982	$C_5H_{13}NO$	1.02	Choline	+	+
1.16	265.11563	$C_{10}H_{19}NO_7$	-1.97	D-1-[(3-Carboxypropyl)amino]-1-deoxyfructose	+	+
1.166	117.07894	$C_5H_{11}NO_2$	-0.3	Betaine	+	-
1.183	115.06333	$C_5H_9NO_2$	0.04	D-(+)-Proline	+	+
1.213	192.06276	$C_7H_{12}O_6$	-3.26	D-(-)-Quinic acid	+	+
1.229	129.07878	$C_6H_{11}NO_2$	-1.52	D-(+)-Pipecolinic acid	+	+
4.155	354.09454	$C_{16}H_{18}O_9$	-1.54	Chlorogenic acid	+	-
4.378	204.08922	$C_{11}H_{12}N_2O_2$	-3.22	DL-Tryptophan	+	-
5.064	354.09438	$C_{16}H_{18}O_9$	-1.99	Scopolin	+	+
5.082	162.03119	$C_9H_6O_3$	-3.09	7-Hydroxycoumarine	+	+
5.273	354.09501	$C_{16}H_{18}O_9$	-0.19	Neochlorogenic acid	+	-
5.714	594.15774	$C_{27}H_{30}O_{15}$	-1.22	Apigenin 6,8-di-C-glucoside	+	+
5.841	173.08364	$C_{11}H_{11}NO$	-2.47	Pyroquilon	+	+
5.842	232.15678	$C_{14}H_{20}N_2O$	-3.39	Unknown	+	+
5.99	273.13543	$C_{16}H_{19}NO_3$	-3.88	4-(tert-butyl)phenyl 3,5-dimethylisoxazole-4-carboxylate	+	+
6.193	564.147	$C_{26}H_{28}O_{14}$	-1.6	Schaftoside	+	+
6.244	368.11007	$C_{17}H_{20}O_9$	-1.8	3-Feruloylquinic acid	+	-
6.755	317.12501	$C_{17}H_{19}NO_5$	-4.14	Piperlongumine	+	+
6.842	263.09363	$C_{17}H_{13}NO_2$	-3.81	7-Methyl-2-phenylquinoline-4-carboxylic acid	+	+
7.196	303.14566	$C_{17}H_{21}NO_4$	-4.6	Hydromorphinol	+	+
7.279	192.04185	$C_{10}H_8O_4$	-2.14	Scopoletin	+	+
7.683	259.12001	$C_{15}H_{17}NO_3$	-3.21	Kokusaginine	+	+
8.773	317.12532	$C_{17}H_{19}NO_5$	-3.16	cis-Piplartine	+	+
9.107	293.10407	$C_{18}H_{15}NO_3$	-3.83	ethyl 4-hydroxy-2-phenylquinoline-6-carboxylate	+	+
10.113	259.11995	$C_{15}H_{17}NO_3$	-3.44	Ilepcimide	+	-
10.169	279.0886	$C_{17}H_{13}NO_3$	-3.38	Graveoline	+	+
11.345	273.13518	$C_{16}H_{19}NO_3$	-4.81	Lunacrine	+	-
11.717	287.1509	$C_{17}H_{21}NO_3$	-4.34	Lunine	-	+
11.929	309.15676	$C_{16}H_{23}NO_5$	-2.81	Lunamarine	+	-
12.86	279.08891	$C_{17}H_{13}NO_3$	-2.28	Graveolinine	+	+
13.626	289.13041	$C_{16}H_{19}NO_4$	-3.45	Hydroxylunacrine	+	+
13.851	259.08358	$C_{14}H_{13}NO_4$	-3.41	Skimmianine	+	+
14.952	287.11474	$C_{16}H_{17}NO_4$	-3.53	Tetrahydropapaveroline	+	+
15.548	301.16704	$C_{18}H_{23}NO_3$	-2.5	Pteleprenine	-	+
15.593	273.13531	$C_{16}H_{19}NO_3$	-4.34	Piperlonguminine	+	+
15.596	546.27186	$C_{32}H_{38}N_2O_6$	-2.07	Unknown	+	+
16	335.13578	$C_{17}H_{21}NO_6$	-3.29	Hydroxylunidine	+	+
16.442	199.06268	$C_{12}H_9NO_2$	-3.27	Dictamnine	+	+
16.871	321.15664	$C_{17}H_{23}NO_5$	-3.07	Unknown	+	+
17.506	285.13557	$C_{17}H_{19}NO_3$	-3.24	Norcodeine	+	+
20.391	271.11981	$C_{16}H_{17}NO_3$	-3.8	Normorphine	+	+
20.69	303.14577	$C_{17}H_{21}NO_4$	-4.25	Hydroxylunine	+	+
20.701	335.17229	$C_{18}H_{25}NO_5$	-2.94	Senecionine	+	+
20.93	319.14081	$C_{17}H_{21}NO_5$	-3.64	Unknown	+	+
21.657	305.16164	$C_{17}H_{23}NO_4$	-3.49	Lunacridine	+	+
21.659	287.15107	$C_{17}H_{21}NO_3$	-3.74	Dihydromorphine	+	+

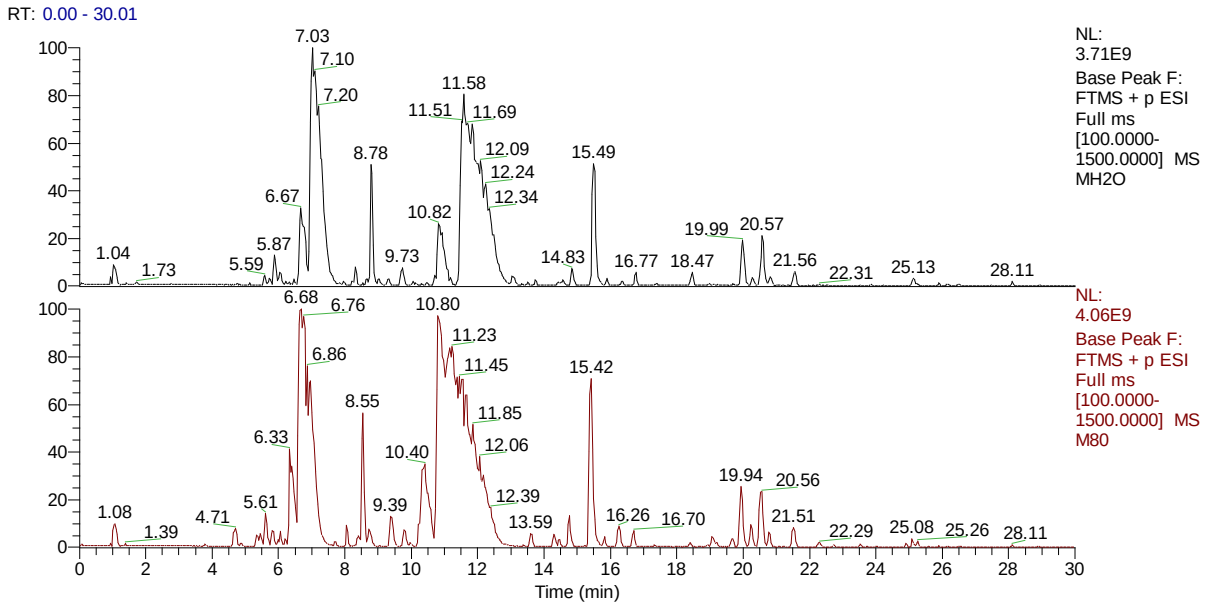

Figure 1. UHPLC chromatogram of *L. amara* stem bark water (1) and 80 % ethanol extracts (2)

The number of double bonds, nitrogen atoms, and hydroxyl groups (–OH) in the aromatic ring structure determines the antioxidant activity of alkaloid compounds (Saeed *et al*., 2023). This structure has been observed in various alkaloids, including lunamarine, lunine, lunacridine, graveolinine, and lunacrine. This group of compounds is considered to contribute the most and is correlated with radical scavenging activities because they can act as electron donors. Free radicals can be neutralized using readily available (pi) electrons. This reaction helps reduce free radical reactivity, which can lead to cellular damage.

The antioxidant properties of a compound are generally associated with its anticancer activity (Grigalius & Petrikaite, 2017). One of the causes of cancer is an increase in reactive oxygen species or free radicals (unstable molecules) in cells (Hanahan & Weinberg, 2011). These free radicals can cause cell damage, including gene mutations, which increases the risk of cancer. Antioxidants can capture free radicals, lower their energy, and convert them into stable molecules (Cockfield & Schafer, 2019).

Putative identification of the metabolites of *L. amara Blanco*

The untargeted screening and putative identification of metabolites from the *L. amara* stem bark extract were performed using UHPLC-Q-Orbitrap HRMS. LC-MS/MS generates large amounts of data in the form of spectra and chromatograms. The MZmine program was used to process the chromatograms, and the data were analyzed to identify metabolites. The components were identified by comparing the measured mass to the theoretical m/z of the metabolites in online public databases (ChemSpider and mzCloud) and search results from the literature were used as an internal database.

The base peak chromatograms from the UHPLC separation profiles of *L. amara* stem bark water and ethanol 80% extracts were relatively similar, but some metabolites differed in peak intensity (Figure 1). The putative identification of each extract has detected 38 metabolites in water extract and 44 metabolites in 80% ethanol extract (Table II). The discovered metabolites belong to a group of alkaloids, glycoside flavonoids, coumarins, and phenols.

Alkaloids are the predominant compounds found in many plants of the *Rutaceae* family, including quinoline alkaloids, which have wide molecular diversity. Quinoline alkaloids are associated with several biosynthetic pathways of aromatic amino acid precursors such as anthranilic acid, lysine, acridone, and ornithine (Macabeo & Aguinaldo, 2008). In the present study, 26 alkaloids were identified. Based on their biogenetic origin, four main groups of quinoline alkaloids have been identified.

3-Dimethylallyl-2-Quinolones.

3-dimethylallyl-2-quinolones are the most common and well-studied alkaloids. They are produced biogenetically through the pre-condensation of anthranilic acid and acetate to yield quinolone, a highly oxidized quinoline derivative. Lunacridine, hydroxylunidine, and pteleprenine were identified at retention times of 21.657, 16, and 15.548 min, respectively (Table II). Pteleprenine was not detected in *L. amara*. Other alkaloids such as lunolone, lunidine, and lunidonine, which are known to be present in the Lunasia genus, were not detected.

Furoquinolines.

This group of metabolites is thought to be chemically simple because they contain an aryl group and are usually differentiated by ring substitution in the benzenoid structure and the presence of a methoxy group at C-4. Two compounds from Lunasia have been reported to be of this type: kokusagine and skimmianine. However, putative identification from the data identified only skimmianine at a retention time of 13.851 min (Table II). Other metabolites, kokusaginine and dictamnine, which belong to the Rutaceae family, were also identified and detected at retention times of 7.683 and 16.442 min, respectively.

Furoquinolones.

Based on their basic ring structures, this group of basic compounds is classified as either furoquinoline or 2-isopropyl-2,3-dihydrofuroquinoline. The most important source of these two compounds is Lunasia. Four compounds from this group, lunacrine, lunine, hydroxylunacrine, and hydroxylunine were detected at retention times of 11.345, 11.717, 13.626, and 20.69 *min*, respectively (Table II). However, other water-soluble alkaloids such as lunacrinol and lunasine, which are known to be present in the genus Lunasia, were not detected.

2-arylquinolines and 4-quinolones

This group of alkaloids is formed through the biogenetically-derived condensation of anthranilic acid and other aromatic acids such as phenylalanine and tyrosine. Two 2-arylquinoline alkaloids, graveoline and graveolinine, were identified at retention times of 10.169 and 12.86 *min*, respectively, while of the 4-quinolone alkaloids, only lunamarine, was identified at a retention time of 11.929 *min*. Two alkaloids known in this group, 4-methoxy-2-phenylquinoline and eduleine, were not detected.

Several alkaloids that have never been reported in *L. amara* were identified based on the similarity of the MS2 fragmentation pattern with the database. Isoquinoline alkaloids, such as tetrahydropapaveroline, normorphine, senecionine, and dihydromorphine, were detected at retention times of 14.953, 17.506, 20.391, 20.701, and 21.659 *min*, respectively. Alkaloids from the amino acid synthetic pathways, lysine, piperlongumine, cis-piplartine, ilepcimide, and piperlonguminine, were also detected at retention times of 6.755, 8.773, 10.113, and 15.593 *min*, respectively.

Three glycosides, scopolin, apigenin 6,8-di-C-glucoside, and apigenin-6-glucoside-8-arabinoside (schaftoside), and one phenolic compound, 4-(tert-butyl) phenyl 3,5-dimethylisoxazole-4-carboxylate were also identified. Some amino acids such as choline, betaine, proline, and DL tryptophan were also detected at high concentrations. This may explain why Lunasia is rich in alkaloids.

Molecular docking

Molecular docking has gained importance as a drug discovery tool. The goal of molecular docking is to predict the structure of a ligand-receptor complex using computational methods (Meng *et al*., 2011). Docking studies were conducted against the best therapeutic targets for breast cancer therapy, such as estrogen receptor alpha (ERα) and human epidermal growth factor receptor-2 (HER2). Both receptors were downloaded from the Protein Data Bank with IDs 3ERT and 3PP0.

3ERT and 3PP0 are two examples of receptors that are widely used for in silico testing. 4-hydroxytamoxifen is the native ligand for 3ERT, whereas that of 3PP0 is lapatinib. Tamoxifen and lapatinib are two drugs used in patients with breast cancer. Tamoxifen is a drug used for hormone-based treatment of luminal A breast cancer, while lapatinib is a small molecule that inhibits several tyrosine kinase receptors involved in tumor cell growth and is used to treat advanced breast cancer and other solid tumors (May, 2014). Lapatinib therapy is associated with transient increases in serum aminotransferase levels as well as rare cases of clinically obvious acute liver injury. Lapatinib reversibly inhibits the phosphorylation of epidermal growth factor receptors (EGFR) (Gunjan Vasant *et al*., 2020).

Table III. Molecular docking of *L. amara* metabolites with ERα and HER2 receptors

Ligand	Receptor			
	3ERT		3PP0	
	Energy (kcal/mol)	Residue binding	Energy (kcal/mol)	Residue binding
4-hydroxytamoxifen	-7.6	Leu354, trp383, leu525, ala530, val533, leu536	-	-
Lapatinib	-	-	-11.1	leu726, val734, ala751, lys753, met774, ser783, arg784, leu785, leu796, thr798, gln799, met801, cys805, arg849, leu852, asp863, phe1004
Lunacridine	-5.9	Trp383, tyr526, lys529, leu536	-7.4	leu726, val734, ala751, lys753, cys805, arg849, asn850, leu852, asp863
Lunacrine	-7.5	Met343, leu346, ala350, leu384, leu387, met388, leu391, leu525,	-8.8	leu726, gly729, val734, lys753, cys805, leu852
Lunamarine	-7.5	Met343, thr347, ala350, trp383, met522, leu525, leu536	-9.2	leu726, val734, ala751, lys753, met801, cys805, leu852, thr862, asp863, phe1004,
Graveolinine	-8.4	Met343, leu346, ala350, met421, leu525	-9.7	leu726, val734, ala751, lys753, thr798, leu852
Tetra-hydropapaveroline	-8.8	Leu346, glu353, leu387, leu391, glu419, gly420, gly521, leu525,	-8.3	leu726, val734, ala751, lys753, asp863
Norcodeine	-8.3	Met343, leu346, ala350, leu525,	-7.2	Lys762, ala763, glu766, ile767, glu770, arg868

According to the docking simulation results (Table III), the affinity energy of lunacridine, lunacrine, and lunamarine with the ERα receptor is lower than that of the tamoxifen native ligand (-7,6 kcal/mol), whereas it is higher for graveoline, tetrahydropapaveroline, and norcodeine. Lunacridine interacts with amino acids outside the active site, such as Leu536, Tyr526, and Lys529 (Figure 2A). The bond structure and strength of lunacrine and lunamarine were similar to those of tamoxifen, with the same hydrophobic interactions with the active-site amino acids Leu525, Ala350, and Met343 (Figure 2B, 2C). Interactions with these amino acids were also observed in tetrahydropapaveroline, graveoline, and norcodeine. The highest energy affinity was observed for tetrahydropapaveroline (-8.8 kcal/mol). The presence of hydrogen bonds with amino acids Leu387 and Glu419 (Figure 2E) is thought to play a role in receptor-ligand complex stabilization. The overall interactions of the ligand with the amino acid residues generates free Gibbs bond energy. Hydrogen bonding with Glu353 plays a role in tumor development by suppressing angiogenesis (Mutiah *et al*., 2020).

3PP0 is the crystal structure of the human HER2 kinase domain. This receptor, as well as ERα, is an important marker for breast cancer. Based on the data (Table III), all ligands have lower affinity energy than the native lapatinib ligand (-11.2 kcal/mol). The stability of the lapatinib receptor complex is enhanced by hydrogen bonding with Thr798, Gln799, Met801, Arg 849, and Asp863 and hydrophobic interactions with Leu726, Val734, Met744, Ala751, Lys753, Ser783, Arg784, Leu785, Leu796, Cys805, and Leu852. Ser783, Arg784, Leu785, Leu769, Gly770, Ala771, Met774, and Phe864 are found in the phosphate-binding site of the 3PP0 receptor. This is a deep, semi-enclosed site in the ATP-binding pocket (Yim-im *et al*., 2014)

Graveoline and lunamarine have the highest affinity energies, -9.7 and -9.2 kcal/mol, respectively. Graveoline is bound to polypeptide chain A, whereas lunamarine is bound to polypeptide chain B. Carbon-hydrogen bonds with the amino acid Thr798 and hydrophobic interactions with Val734, Ala751, Leu726, Lys753, and Leu 852 (Figure 3D) contribute to the stability of the graveoline bond.

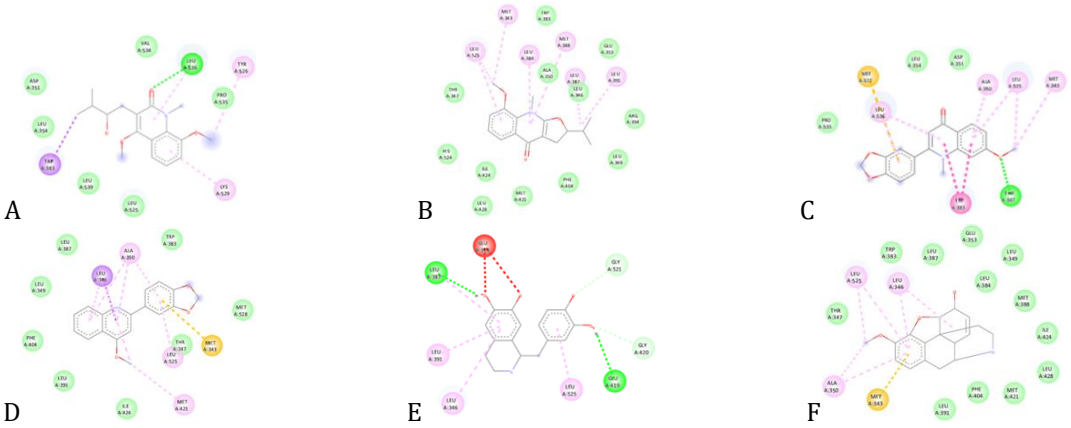

Figure 2. Docking interactions between amino acids from ERα receptor with (A) lunacridine, (B) lunacrine, (C) lunamarine, (D) graveolinine, (E) tetrahydropapaveroline, and (F) norcodeine

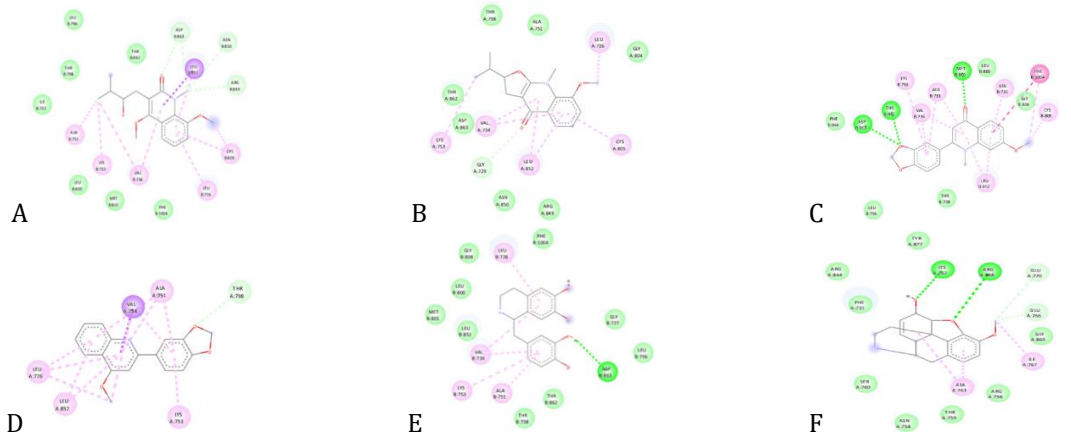

Figure 3. Docking interactions between amino acids from HER2 receptor with (A) lunacridine, (B) lunacrine, (C) lunamarine, (D) graveolinine, (E) tetrahydropapaveroline, and (F) norcodeine

The presence of hydrogen bonds with Thr862, Met801, and Asp863, as well as hydrophobic interactions with Phe1004 (Fig. 3C) increased the structural stability of the lunamarine complex with the receptor.

CONCLUSION

In this study, 80% ethanol produced extracts of *L. amara* stem bark with higher antioxidant capacities than water. Putative identification yielded 46 metabolites that comprise alkaloids, glycosides, amino acids, and phenols. According to molecular docking studies on breast cancer receptors, alkaloid compounds have lower affinity energies for the 3ERT receptor than for the 3PP0 receptor. Graveoline and lunamarine are the ligands with the highest anti-HER2 breast cancer biomarker activity. Based on these findings, further research is needed on various breast cancer cell lines, such as MCF7, T47D, and MDA MB 231, which are positive for the overproduction of receptor tyrosine kinase (HER2).

ACKNOWLEDGMENTS

The authors gratefully acknowledged the Advance Research Laboratory and Tropical Biopharmaca Research Center, IPB University for data support in this research.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Cockfield, J. A., & Schafer, Z. T. (2019). Antioxidant Defenses: A Context-Specific Vulnerability of Cancer Cells. *Cancers*, 11(8), 1208. https://doi.org/10.3390/cancers11081208

Dar, A. M., & Mir, S. (2017). Molecular Docking: Approaches, Types, Applications and Basic Challenges. *Journal of Analytical & Bioanalytical Techniques*, 08, 356. https://doi.org/10.4172/2155-9872.1000356

Dibha, A., Wahyuningsih, S., Ansori, A., Kharisma, V., Widyananda, M., Parikesit, A., Sibero, M., Probojati, R., Murtadlo, A., Trinugroho, J., Sucipto, T., Turista, D., Rosadi, I., Ullah, M., Jakhmola, V., & Zainul, R. (2022). Utilization of Secondary Metabolites in Algae Kappaphycus alvarezii as a Breast Cancer Drug with a Computational Method. *Pharmacognosy Journal*, 14(3), 536–543. https://doi.org/10.5530/pj.2022.14.68

Grigalius, I., & Petrikaite, V. (2017). Relationship between Antioxidant and Anticancer Activity of Trihydroxyflavones. *Molecules*, 22(12), 2169. https://doi.org/10.3390/molecules22122169

Gunjan Vasant, B., Gufran, A., Sarita Kumari, Y., Pooja, M., & Brahmeshwar, M. (2020). Lapatinib-loaded nanocolloidal polymeric micelles for the efficient treatment of breast cancer. *Journal of Applied Pharmaceutical Science*, 10(9), 023-029. https://doi.org/10.7324/JAPS.2020.10903

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, 144(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Holliday, D. L., & Speirs, V. (2011). Choosing the right cell line for breast cancer research. *Breast Cancer Research,* 13(4), 215. https://doi.org/10.1186/bcr2889

Kedare, S. B., & Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology,* 48(4), 412–422. https://doi.org/10.1007/s13197-011-0251-1

Luthfi, M. J., Kamalrudin, A., & Noor, M. M. (2017). Effects of Lunasia amara Blanco (Sanrego) on Male Fertility: A Preliminary Study on Sperm Proteomic Analysis. *Journal of Applied Pharmaceutical Science,* 7(8), 085-091. https://doi.org/10.7324/JAPS.2017.70812

Macabeo, A. P. G., & Aguinaldo, A. M. (2008). PHCOG REV.: Plant Review Chemical and Phytomedicinal Investigations in Lunasia amara. *Pharmacognosy Reviews*, 2(4), 10.

May, F. (2014). Novel drugs that target the estrogen-related receptor alpha: Their therapeutic potential in breast cancer. *Cancer Management and Research,* 2014(6), 225-252. https://doi.org/10.2147/CMAR.S35024

Meng, X.-Y., Zhang, H.-X., Mezei, M., & Cui, M. (2011). Molecular Docking: A Powerful Approach for Structure-Based Drug Discovery. *Current Computer Aided-Drug Design,* 7(2), 146–157. https://doi.org/10.2174/157340911795677602

Mutiah, R., Indrawijaya, Y. Y., & Puspita, D. (2020). Study in Silico Compounds In 96% Ethanol Extract of Chrysanthemum cinerariifolium (Trev.) Leaves Towards Alfa Estrogen Receptors. *Indonesian Journal of Cancer Chemoprevention,* 11(3), 144. https://doi.org/10.14499/indonesianjcancchemoprev11iss3pp144-153

Prescott, T. A. K., Sadler, I. H., Kiapranis, R., & Maciver, S. K. (2007). Lunacridine from Lunasia amara is a DNA intercalating topoisomerase II inhibitor. *Journal of Ethnopharmacology,* 109(2), 289–294. https://doi.org/10.1016/j.jep.2006.07.036

Rafi, M., Meitary, N., Anggraini Septaningsih, D., & Bintang, M. (2020). Phytochemical Profile And Antioxidant Activity Of Guazuma ulmifolia Leaves Extracts Using Different Solvent Extraction. *Indonesian Journal of Pharmacy,* 31(3) 171-180. https://doi.org/10.22146/ijp.598

Rafi, M., Rismayani, W., Sugiarti, R. M., Syafitri, U. D., Tri, W., & Rohaeti, E. (2021). FTIR-based Fingerprinting Combined with Chemometrics for Discrimination of Sonchus arvensis leaf Extracts and Correlation with Their Antioxidant Activity. *Indonesian Journal of Pharmacy,* 32(2), 132-140. https://doi.org/10.22146/ijp.755

Saeed, Y. S., Ali, J. F., & Mohammed, M. A. (2023). Chemical composition, antioxidant, and antibacterial activity of Ruta graveolens (Rutaceae). Biodiversitas *Journal of Biological Diversity,* 24(6), 3162-3168. https://doi.org/10.13057/biodiv/d240609

Salazar-Aranda, R., Pérez-López, L. A., López-Arroyo, J., Alanís-Garza, B. A., & Waksman de Torres, N. (2009). Antimicrobial and Antioxidant Activities of Plants from Northeast of Mexico. *Evidence-Based Complementary and Alternative Medicine*, 2009, 1–6. https://doi.org/10.1093/ecam/nep127

Sun, T., Aceto, N., Meerbrey, K. L., Kessler, J. D., Zhou, C., Migliaccio, I., Nguyen, D. X., Pavlova, N. N., Botero, M., Huang, J., Bernardi, R. J., Schmitt, E., Hu, G., Li, M. Z., Dephoure, N., Gygi, S. P., Rao, M., Creighton, C. J., Hilsenbeck, S. G., & Westbrook, T. F. (2011). Activation of Multiple Proto-oncogenic Tyrosine Kinases in Breast Cancer via Loss of the PTPN12 Phosphatase. *Cell*, 144(5), 703–718. https://doi.org/10.1016/j.cell.2011.02.003

Takahashi, N., Subehan, Kadota, S., & Tezuka, Y. (2012). Mechanism-based CYP2D6 inactivation by acridone alkaloids of Indonesian medicinal plant Lunasia amara. *Fitoterapia*, 83(4), 774–779. https://doi.org/10.1016/j.fitote.2012.03.011

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, 31, 455–461. https://doi.org/10.1002/jcc.21334

Yim-im, W., Sawatdichaikul, O., Semsri, S., Horata, N., Mokmak, W., Tongsima, S., Suksamrarn, A., & Choowongkomon, K. (2014). Computational analyses of curcuminoid analogs against kinase domain of HER2. *BMC Bioinformatics,* 15, 261. https://doi.org/10.1186/1471-2105-15-261