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Original Article

Antioxidant and Anticancer Activities of *Spatholobus littoralis* Stem Extract: An *in Vitro* and *in Silico* Computational Investigation

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Abstract: The stem of *Spatholobus littoralis* has been used to treat cancer and is empirically utilized by the Dayak people in Kalimantan, Indonesia. This study aims to explore the antioxidant activity of Bajakah stems extracted using nine different solvent variations and to test their anticancer activity and toxicity *in silico*. The in vitro methods used to assess antioxidant activity are the DPPH and ABTS assays. Applications used for ADMET and computational analysis methods include SwissADME, Deep-PK, ProTox 3.0, STITCH, SwissTargetPrediction, STRING, and PyRx 0.8. The test results showed different antioxidant activity profiles. Meanwhile, the toxicity test of the contained bioactive compounds indicates a good safety level with a minimum IC50 value of 1034 mg/kg. The bioactive compounds exhibit good affinity, with the highest values for the bioactive daidzein at receptors CDK-2 and ROCK1, with values of -9.5 and -8.8, respectively. It can be concluded from this study that the stem of *S. littoralis* has potential as an anticancer agent, although its modest antioxidant potential.

Keywords: anticancer, deep learning, CDK-2, ROCK 1, toxicity, molecular docking

1. INTRODUCTION

The prevalence of cancer in 2022 reached 20 million new cases, making it the leading cause of death worldwide, with a total of 9.7 million fatalities. By 2040, new cases will increase to 29.9 million, with 15.3 million deaths [1]. This issue needs to be addressed by discovering and developing anticancer agents that have been proven effective. The Dayak people have used the plant *Spatholobus littoralis*, known as Bajakah Tampala, to treat cancer and other ailments based on ethnomedicine studies [2]. Bajakah Tampala is utilized by the Tamambaloh Dayak tribe, the Dayak people in the Arut Selatan and Kumai sub-districts, the Linoh Dayak tribe, the Kutai tribe, the Garong village community in Central Kalimantan, and the Liwu Metingki village community in Southeast Sulawesi [3], [4], [5], [6], [7], [8], [9].

The potential success of developing *S. littoralis* as an anticancer drug and a source of antioxidant compounds is based on research conducted at the genus level. The genus *Spatholobus* contains 141 compounds that exhibit ideal characteristics of drug-like molecules, with 175 phytochemicals isolated (with flavonoids as the primary component), demonstrated anticancer activity, and no toxic effects [10]. This study aims to explore the antioxidant potential of *S. littoralis* stems using nine different solvents and to evaluate their anticancer activity and toxicity *in silico*.

2. MATERIALS AND METHODS

2.1. Material and chemical

The *S. littoralis* stems used in this study were sourced from Balangan Regency, South Kalimantan, Indonesia. The specimen was verified by the Herbal Materia Medica Laboratory Unit (UPT Laboratorium Herbal Materia Medica) in Batu, Malang, under verification number 000.9.3/2425/102.20/2024, confirming the suitability of the sample for the research. Materials and reagents used in the study include 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) (Merck), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Merck), distilled water, 96% ethanol (Merck), methanol (Merck), ethyl acetate (Merck), chloroform (Merck), n-hexane (Merck), ascorbic acid, trolox, and ether (Merck).

2.2 Preparation of plant extract

The stems of S. littoralis (Hassk.) were cleaned of dirt using water. Subsequently, they were air-dried at room temperature until completely dry. The stem parts of *S. littoralis* (Hassk.) that have been cleaned and dried undergo size reduction into powder with a particle size of 0.420 – 1.68 mm. The powder is dried in an oven at 50°C until the moisture content is less than 5%. The maceration process uses a solvent-to-powder ratio of 1:5. The solvents used include 100% aquadest, 70% ethanol, 96% ethanol, 50% ethanol, methanol, ethyl acetate, chloroform, n-hexane, and ether. The maceration process was carried out for 3 days (72 hours) and repeated up to a maximum of 3 times. Evaporation was performed using a rotary evaporator at a maximum temperature of 50°C until the macerate was obtained. The drying of the macerate was continued using a water bath until the extract was obtained [11].

2.3. Antioxidant activity

The DPPH and ABTS assays are used to test antioxidant activity. The equipment includes a reservoir, micropipette, vortex mixer, centrifuge, 100 µL multichannel pipettor, and microplate reader.

2.3.1. DPPH assay

Prepare the stock solution for the samples using DMSO as the solvent, then homogenize it by vortexing. Aquadest will create the standard ascorbic acid solution. Pipette 80 µL of the sample and standard into the 96-well plate, starting from the lowest concentration to the highest. Add 80 µL of 0.1 mM DPPH solution to each well. Incubate in the dark for 30 minutes while maintaining a temperature of 25°C. After incubation, measure the absorbance at a wavelength of 492 nm with

medium shaking for 30 minutes [12]. Calculate the DPPH scavenging activity or percent inhibition using the following equation:

$$\text{DPPH Scavenging Activity} = \frac{(A0 - A1)}{\text{A0}}\ x\ 100\%$$

Where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

2.3.2. ABTS assay

The samples were dissolved in DMSO as the solvent and homogenized by vortexing. Dissolved in distilled water, Trolox was used as the antioxidant activity assay standard. Add the ABTS solution, mixed with ethanol, into a 96-well plate (in triplicate) at a volume of 300 μL. Measure the absorbance at a wavelength of 630 nm, targeting an absorbance of 0.7. Pipette 10 μL of the sample, control, and standard into the 96-well plate, starting with the lowest concentration to the highest. Add 290 μL of the ABTS solution under dark conditions to the wells containing the samples, controls, and standards. Incubate for 6 minutes at room temperature in the dark. Measure the absorbance at a wavelength of 630 nm using a microplate reader [12]. The calculation of ABTS scavenging activity or percent inhibition is done using the following equation:

$$\text{ABTS Scavenging Activity} = \frac{(A0 - A1)}{\text{A0}}\ x\ 100\%$$

Where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

2.4. Absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis

The compounds contained in the stem of *S. littoralis* were further analyzed regarding drugability and pharmacokinetics based on previous research [13]. The analysis to identify the absorption, distribution, metabolism, elimination, and toxicity (ADMET) profile utilized the SwissADME database (http://www.swissadme.ch/) and Deep-PK (https://biosig.lab.uq.edu.au/deeppk/), accessed on September 28, 2024 [14], [15]. The results of the ADMET analysis on the SwissADME and pkCSM servers were further processed on the ProTox 3.0 server (https://tox.charite.de/protox3) to analyze bioactive compounds predicted to penetrate the blood-brain barrier (BBB) and not be expelled from the central nervous system by P-glycoprotein [16].

2.5. Computational analysis

The bioactive analysis using the STITCH database (http://stitch.embl.de/) was conducted to predict the relationship between bioactive compounds and cancer-related genes. This web tool is in the initial screening stage [17]. The next step is screening the bioactive activity against a broader range of gene targets using the SwissTargetPrediction web tool (http://www.swisstargetprediction.ch/) [18], [19]. The gene targets are further analyzed to illustrate their activity through Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses using the STRING web tool (https://string-db.org/) [20], [21]. The relationship between the bioactive compounds and gene targets is visualized using Cytoscape 3.10.3 [22], [23].

Bioactive compounds connected to cancer-related genes underwent docking analysis on cyclin-dependent kinase 2 (CDK-2, PDB: 1di8) and rho-associated protein kinase 1 (ROCK 1, PDB: 3twj). These receptors were chosen due to their association with antioxidant and anticancer activities [24]. PyRx 0.8 was used for molecular docking analysis, including Open Babel and AutoDock Vina, utilized during the docking process. PyMOL 3.0.4 and BIOVIA Discovery Studio Visualizer 2021 were the visualization tools used [25].

3. RESULTS AND DISCUSSION

3.1. Antioxidant activity

Antioxidant activity assessment was carried out using two different approaches, namely the DPPH and ABTS methods. The results of antioxidant testing of 9 extracts extracted using various solvents can be seen in Figure 1. The percent scavenging between the sample and the standard showed a significant difference, indicating that the concentration required by the S. littoralis stem extract is much higher than the standard. Certain extracts showed inadequate regression results for IC50 calculation, such as ether, methanol, 50% ethanol, and 70% ethanol extracts in the DPPH method. Additionally, the ABTS method revealed no IC50 values for n-hexane, chloroform, and 70% ethanol extracts. This is likely due to the low concentration of active compounds in the extracts or the inability of the method to capture the specific activity of these compounds. Further testing using alternative antioxidant methods is necessary to confirm the activity of extracts that did not yield IC50 results. IC50 data for antioxidant activity can be found in Table 1.

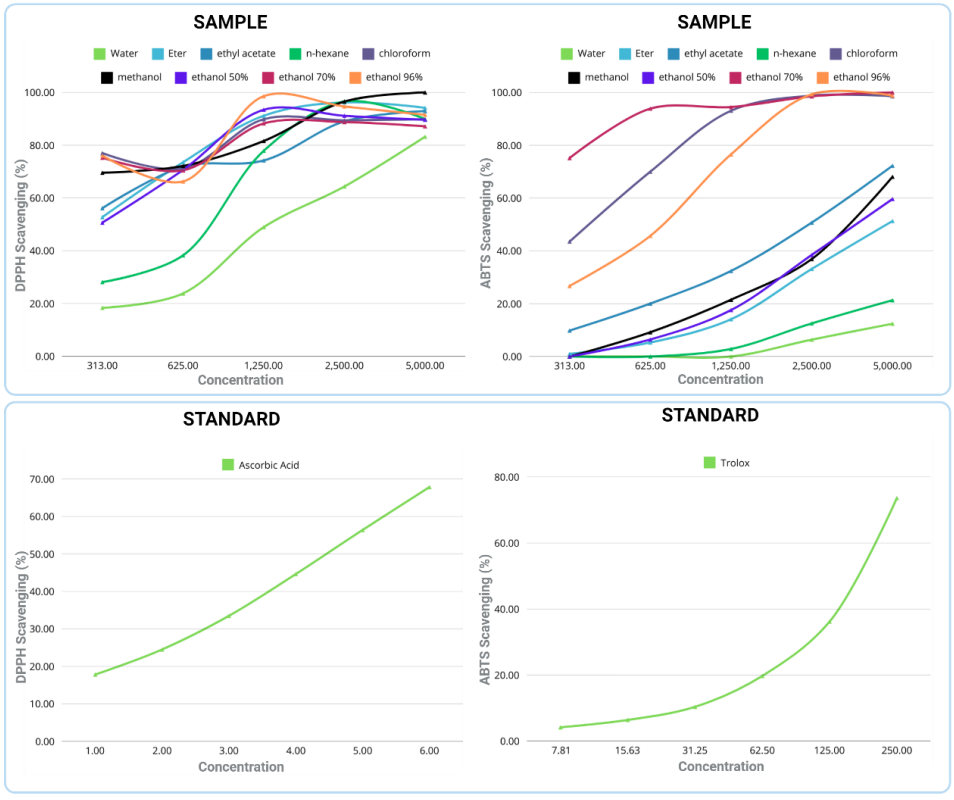

Figure 1. Antioxidant activity of *S. littoralis* stem extract

Table 1. Antioxidant activity of extract S. littoralis stem

Sample	IC50 (ppm)	
	DPPH	ABTS
100% aquadest	1.716.97	28.748.75
ether	-	4.248.19
ethyl acetate	256.60	2.514.09
n-hexane	792.76	-
chloroform	543.32	-
methanol	-	4.178.88
50% ethanol	-	2.981.18
70% ethanol	-	-
96% ethanol	416.15	1.090.24
Ascorbic acid	4.46	-
Trolox	-	169.20

3.2. ADMET analysis

The bioactives identified in the 70% ethanol and water extracts fractionated using 1-butanol solvent have similarities to 17 compounds, as shown in Figure 2. The bioactive compounds analyzed were based on a previous study conducted by Sianipar et al., [2024].

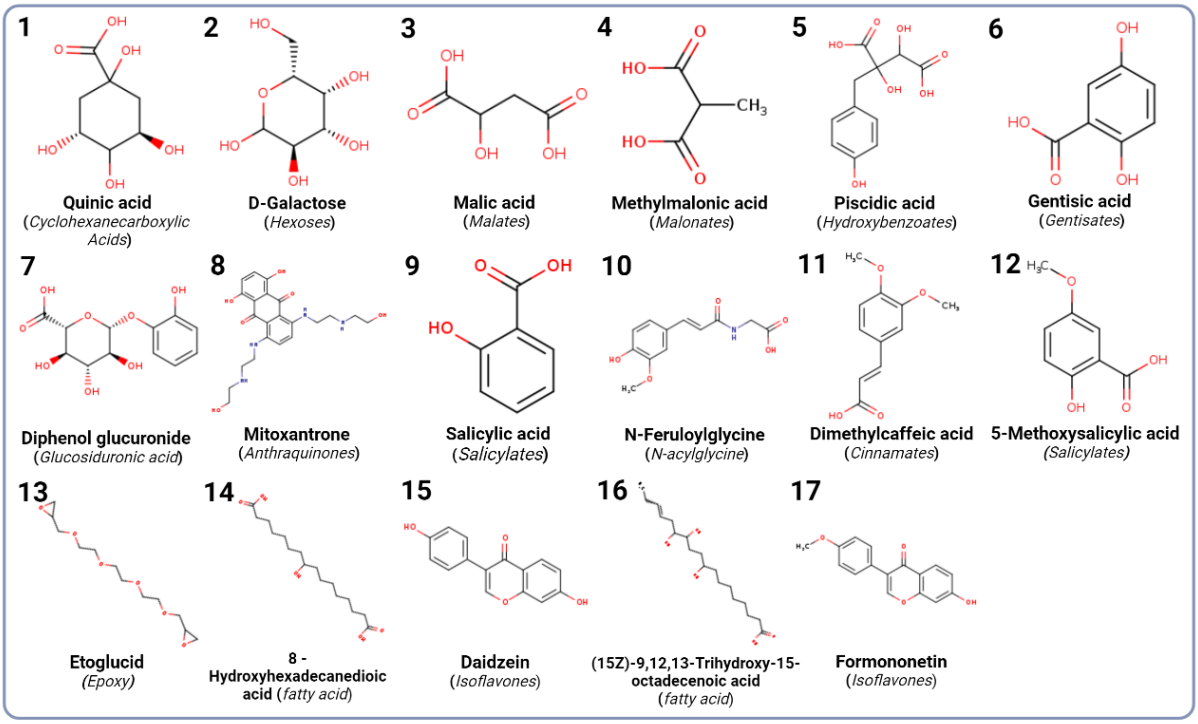

Figure 2. Bioactive from 70% ethanol extract and water [13]

The bioactive compounds contained in the 70% ethanol and water extracts were tested and classified, as shown in Figure 3. Figure 3(A) is a bioavailability radar that illustrates the similarity of the tested compounds to their role as drugs. The pink area indicates that compounds located in this area have drug-like properties according to several criteria, including lipophilicity (XLOGP3 between −0.7 and +5.0), size (MW between 150 and 500 g/mol), polarity (TPSA between 20 and 130 Å²),

solubility (log S not higher than 6), saturation (fraction of carbons in sp^3 hybridization not less than 0.25), and flexibility (no more than nine rotatable bonds) [26].

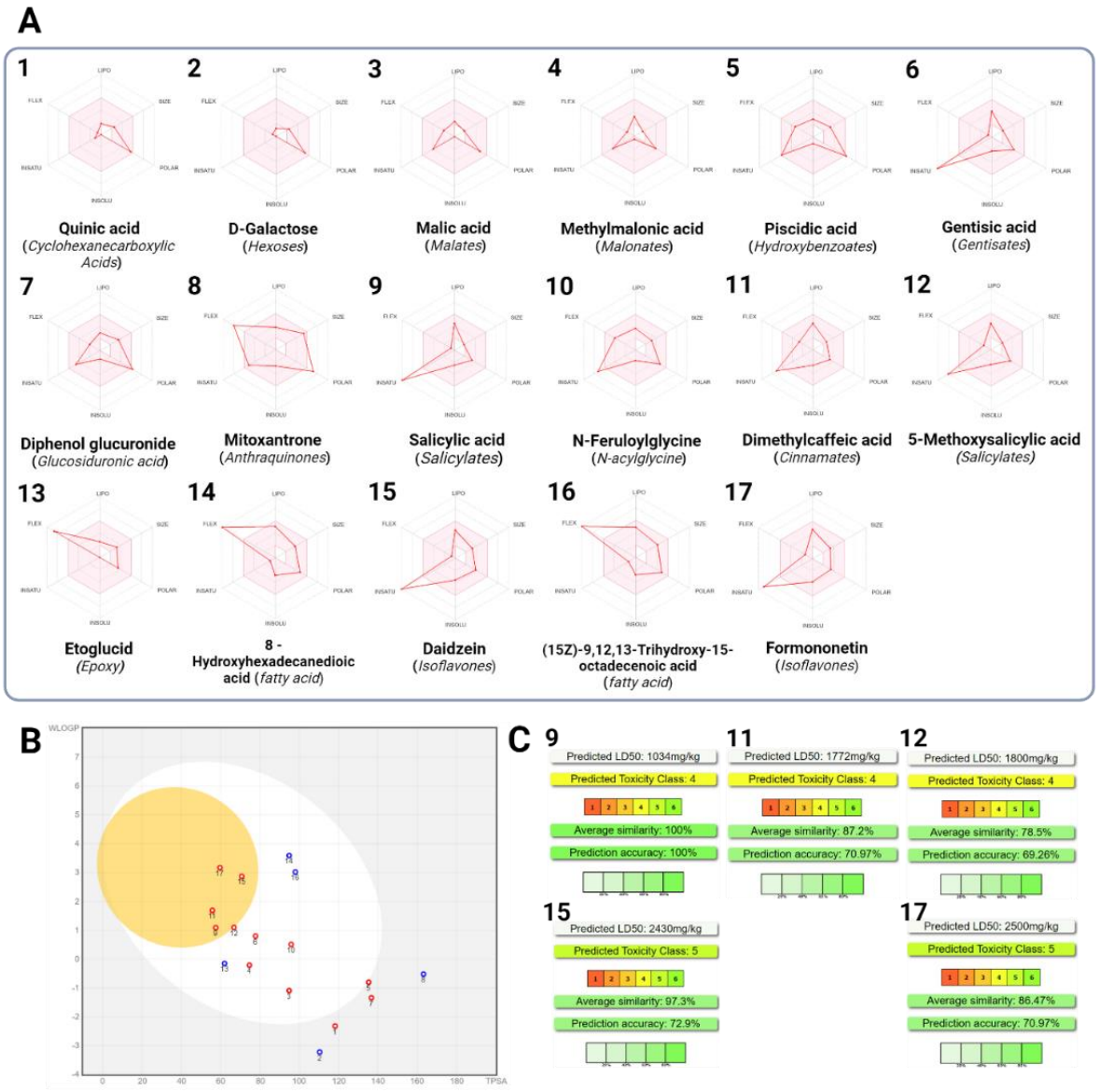

Figure 3. (A) bioavailability profile, (B) boiled-egg model, (C) LD50 prediction

The classification in Figure 3(B), at the point located in the boiled-egg yolk, indicates that the bioactive compounds are predicted to be able to penetrate the blood-brain barrier (BBB). In contrast, bioactives in the white area indicate that they are predicted to be absorbed through the gastrointestinal tract. Meanwhile, the red dots indicate that the bioactive compounds will not be expelled from the central nervous system (CNS) by P-glycoprotein (P-gp).

Compounds that potentially cross the blood-brain barrier (BBB) and are difficult to expel by P-glycoprotein through the efflux pump mechanism underwent LD50 prediction testing. The results of the LD50 prediction for bioactive compounds suspected to be problematic include bioactives numbered 9, 11, 12, 15, and 17, as shown in Figure 3(A). The LD50 prediction results indicate that the

suspected problematic bioactive compounds still fall within the medium range, with a minimum LD50 value of 1034 mg/kg.

The results of the analysis using the deep learning method to describe the pharmacokinetic and toxicity profiles can be seen in Table 1. The deep learning model used in this stage is based on graph neural networks and graph-based signatures. The application of this model can generate 73 endpoints, including 64 ADMET and nine general properties.

3.3. Computational analysis

The relationship between bioactive compounds and cancer-related genes was analyzed as an initial screening before the molecular docking test. The analysis results using the STITCH database, which explores the relationship between genes and bioactive compounds. Bioactives associated with cancer-affiliated genes include mitoxantrone and daidzein. Mitoxantrone interacts with several cancer-related genes, such as ABCB1, ABCG2, TOP2A, and TOP2B [27], [28], [29], [30]. Meanwhile, daidzein is linked to the cancer-related gene NOS3, which has been identified as a novel target for gastric cancer treatment [31].

The relationship between bioactives and genes is illustrated in Figure 4(A), with 192 genes linked to the bioactives. Among these, 12 genes are simultaneously associated with the bioactives mitoxantrone and daidzein, including EGFR, HTR2C, ADORA1, SLC6A2, ACHE, PPARA, F10, PARP1, GSK3B, CSNK2A1, PIM1, and AURKB. The gene ontology analysis in Figure 4(B) reveals that these genes are localized in cellular components related to cancer, such as membrane rafts [32] and protein kinase complex [33]. The analysis of biological processes shows that the bioactive compounds are associated with pathways such as the one-carbon metabolic process [34], peptidyl-serine phosphorylation [35], and response to organonitrogen compound [36]. For molecular functions related to cancer, the compounds are linked to insulin-like growth factors (IGFs) such as IGF-I and IGF-II [37], protein kinase activity [33], [38], G-protein-coupled receptors (GPCRs) [39], and Protein tyrosine kinase activity [40]. The GO analysis highlights that the 192 genes associated with the bioactive compounds are significantly localized within membrane rafts and the protein kinase complex, underlining the crucial role of these bioactive compounds in regulating cellular processes, particularly in the context of cancer.

KEGG analysis related to cancer includes pathways such as protein metabolism [41], prostate cancer [42], microRNAs in cancer [43], EGFR tyrosine kinase inhibitor resistance [44], PI3K-akt signaling pathway [45], pathway in cancer [46], progesterone-mediated oocyte maturation [47], and acute myeloid leukemia [48]. The KEGG analysis indicates that the bioactive compounds are associated with several significant signaling pathways and biological processes involved in cancer.

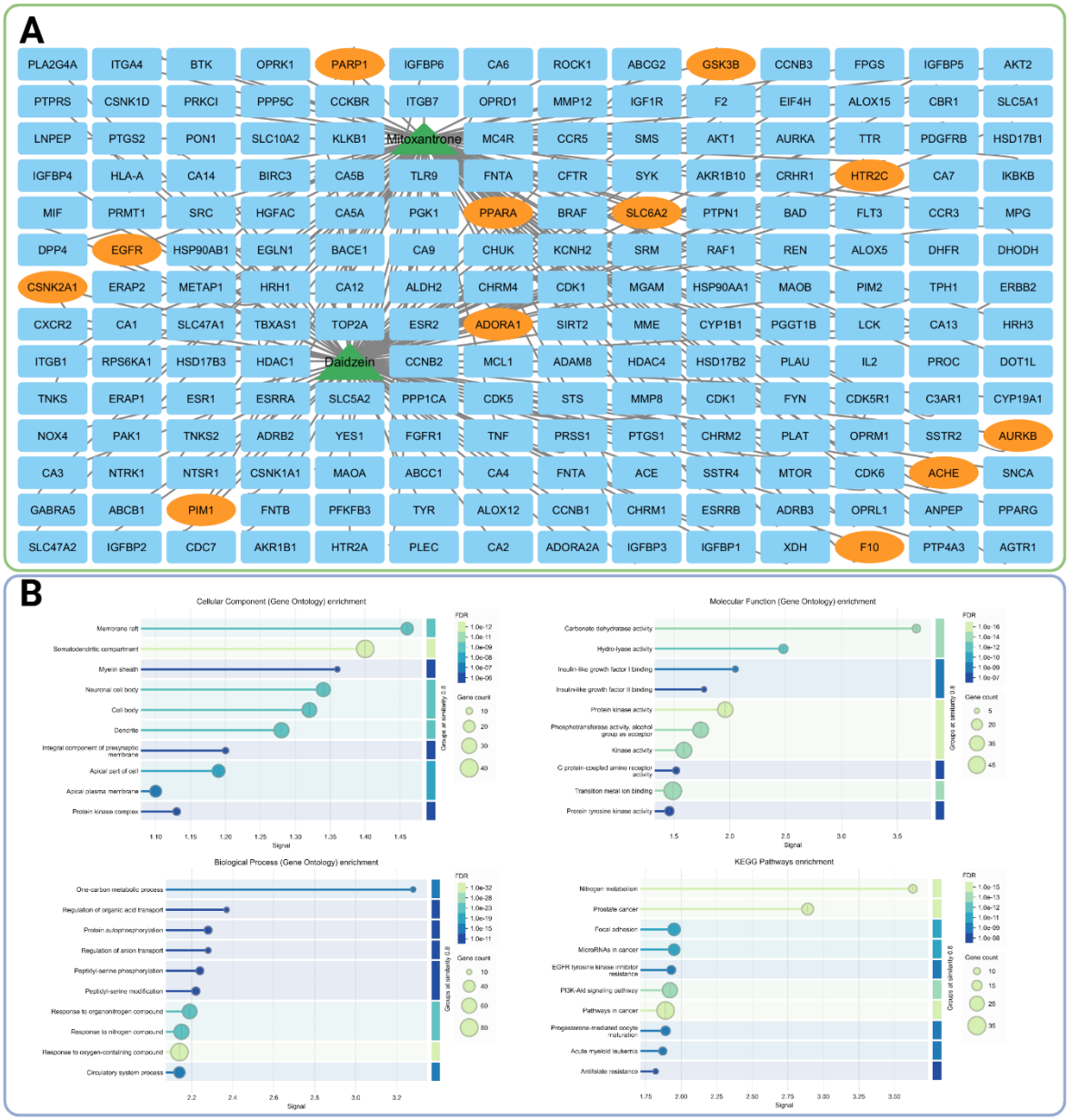

Figure 4. The relationship between genes and bioactive contained in *S. littoralis* stem. (A) Bioactive Gene Target Relationships and (B) Protein-Protein Interaction (PPI) Network, Gene Ontology (GO), and KEGG Pathway Analysis

The molecular docking analysis of the compounds found in *S. littoralis* showed good affinity with negative values. The highest binding affinity was observed between the protein CDK-2 and daidzein, with an affinity value of -9.5, and between the protein ROCK1 and daidzein, with an affinity value of -8.8. The interaction between the receptor and bioactive compounds can be seen in Figure 5, and the docking scores of *S. littoralis* can be found in Table 2. CDK-2 plays a crucial role in deregulating several cancers, where CDK2 inhibition leads to antitumor activity [49]. Additionally, daidzein exhibits a stronger affinity than mitoxantrone by inhibiting ROCK1, which disrupts the regulation of cytoskeletal dynamics, thereby preventing cell migration, proliferation, and survival in

various cancer types [50]. Studies indicate that ROCK1 plays a significant role in cancer progression, as evidenced by its overexpression [51].

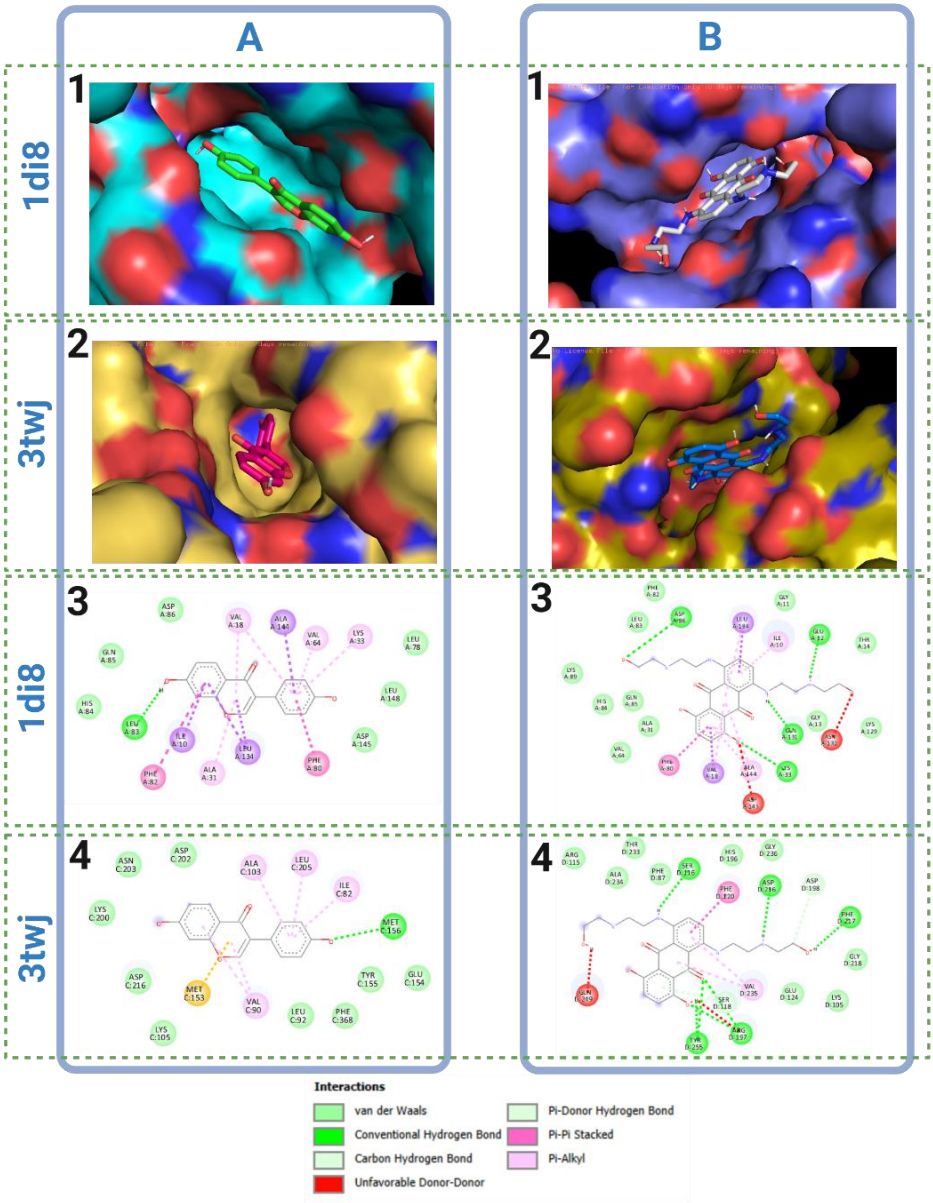

Figure 5. Illustration of 3D (1-2) and 2D diagrams (3-4). Bioactive daidzein (A) and mitoxantrone (B).

Table 2. Docking Scores and Hydrogen Bond Interactions of *S. littoralis*

Code	Components	Protein	Interaction energy (kcal/mol)	Hydrogen Bond Interaction
A	daidzein	CDK-2 (PDB ID: 1DI8)	-9.5	LEU A:83
B	mitoxantrone	CDK-2 (PDB ID: 1DI8)	-8.3	ASP A:86; GLU A:12; GLN A:131; LYS A:33
A	daidzein	ROCK1 (PDB ID: 3TWJ)	-8.8	MET C:156
B	mitoxantrone	ROCK1 (PDB ID: 3TWJ)	-7.8	SER D:116; ASP D:216; PHE D:217; TYR D:255; ARG D:197

4. CONCLUSION

The empirical use of *S. littoralis*, based on ethnomedicinal studies in the Dayak community, has driven the need for validation through in-vitro and in-silico research. Using *S. littoralis* extract with various solvents has resulted in different antioxidant profiles. The antioxidant activity test using the DPPH and ABTS methods on extracts prepared with nine solvents resulted in varying profiles. Molecular testing on the compounds in *S. littoralis's* stem indicates the potential for antioxidant and anticancer activities. The bioactive compound contributing to these activities is daidzein. Toxicity testing predicts that the bioactive compounds found in the stem of *S. littoralis* do not exhibit harmful toxicity.

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Conflicts of interest: The authors declare no conflict of interest.

Table 3. Pharmacokinetic and toxicity profile of bioactive compounds from *S. littoralis*

Bioactive	Absorption/ Human Oral Bioavailability 20%	Distribution/ Plasma Protein Binding	Distribution/ Steady State Volume of Distribution	Metabolism/ CYP 1A2 substrate	Metabolism/ CYP 2C19 substrate	Metabolism/ CYP 2D6 Substrate	Metabolism/CYP 3A4 Substrate	Excretion/ Clearance	Toxicity/ Liver Injury	General Properties/ Log(P)	General Properties/ pKa Acid	General Properties/ pKa Basic
Quinic acid	Bioavailable	36.16	0.08	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	-2.81	3.7	6.73
D-Galactose	Bioavailable	32.01	0.15	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	-2.91	8.66	3.45
Malic acid	Bioavailable	-4.99	0.42	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	-1.26	2.67	8.21
Methylmalonic acid	Bioavailable	4.64	0.44	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Toxic	-0.51	0.91	8.98
Piscidic acid	Non-Bioavailable	15.59	0.24	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	-0.97	2.86	8.19
Gentisic acid	Bioavailable	19.56	0.1	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Toxic	1.73	5.2	1.26
Diphenol glucuronide	Non-Bioavailable	62.24	0.3	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	-0.41	3.18	6
Mitoxantrone	Non-Bioavailable	48.04	1.51	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	1.18	6.1	7.3
Salicylic acid	Bioavailable	21.65	0.08	Non-Substrate	Non-Substrate	Non-Substrate	Substrate	None	Toxic	2.49	3.23	-0.93
N-Feruloylglycine	Bioavailable	35.79	0.11	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	0.5	4.72	5.34
Dimethylcaffeic acid	Bioavailable	28.9	0.26	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Toxic	1.98	4.34	2.95
5-Methoxysalicylic acid	Bioavailable	27.93	0.27	Non-Substrate	Substrate	Non-Substrate	Non-Substrate	None	Safe	1.93	3.31	4.52
Etoglucid	Bioavailable	-22.32	1.21	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	-0.95	6.43	0.12
8-Hydroxyhexadecanedioic acid	Non-Bioavailable	17.35	0.58	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	3.19	4.53	8.18
Daidzein	Bioavailable	74.64	0.48	Non-Substrate	Non-Substrate	Substrate	Non-Substrate	None	Toxic	3	7.97	6.63
(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	Non-Bioavailable	10.65	0.55	Non-Substrate	Non-Substrate	Non-Substrate	Non-Substrate	None	Safe	2.74	5.41	6.21
Formononetin	Bioavailable	77.81	0.37	Substrate	Non-Substrate	Substrate	Non-Substrate	None	Toxic	3.53	8.45	3.37

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