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EDITORIAL WORDS

apt. Mohammad Rizki Fadhil Pratama, S.Farm., M.Si.

Editor in Chief
Borneo J Pharm

Assalamu'alaikum Wr. Wb.

Alhamdulillahirabbil 'alamin. The next edition of **Borneo Journal of Pharmacy** (*Borneo J Pharm*), has been published at August 2024. This edition contains ten articles: Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Analytical Pharmacy-Medicinal Chemistry, Microbiology Pharmacy, Natural Product Development, Clinical-Community Pharmacy, and Pharmaceutical Education. This edition includes writings from four countries: India, Indonesia, Italy, and Malaysia. The authors come from several institutions, including Universitas Muhammadiyah Malang, Universitas Ahmad Dahlan, Universitas Muhammadiyah Banjarmasin, Universitas Jember, Università degli Studi del Piemonte Orientale "Amedeo Avogadro", University of Kalyani, Azienda Ospedaliero Universitaria Maggiore della Carita, Universitas Airlangga, Institut Ilmu Kesehatan Bhakti Wiyata Kediri, Universitas Islam Bandung, Universitas Mulawarman, Universitas Negeri Yogyakarta, Universitas Gadjah Mada, Universitas Kristen Duta Wacana, Universitas Global Jakarta, and Management and Science University.

Editorial boards are fully aware that there are still room for improvement in this edition, hence with all humility willing to accept constructive suggestions and feedback for improvements to the publication for the next editions. The editorial board would like to thank all editors and reviewers, and contributors of the scientific articles who have provided the repetoire in this issue. We hope that all parties, especially the contributors, could re-participate for the publication in the next edition on November 2024.

Wassalamu'alaikum Wr. Wb.

Palangka Raya, August 2024

Editor-in-Chief

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1. Writing is the result of research or review of critical analysis study in the field but not limited to Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Analytical Pharmacy-Medicinal Chemistry, Natural Product Development, Clinical-Community Pharmacy, Management Pharmacy, and other Pharmacy aspects which have never been published in other scientific publication media.
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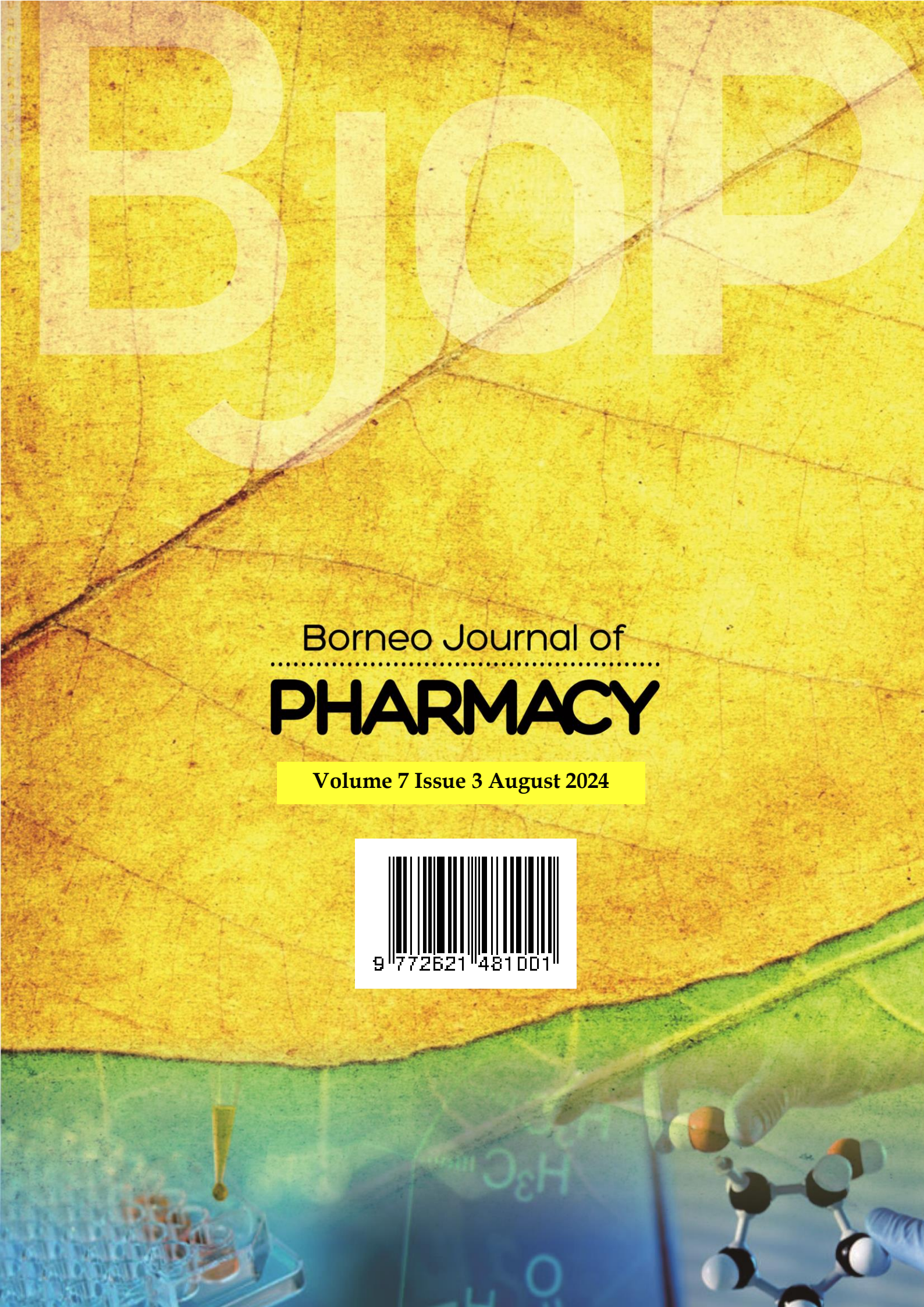
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Research Article

Integrative Network Pharmacology Unveils *Limonia acidissima* as a Potential Natural Product for Targeting Cancer

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Keywords:
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Limonia acidissima
MAPK
Network pharmacology
PI3K-Akt

Abstract

Cancer remains a formidable health challenge worldwide, with complex molecular mechanisms driving its initiation, progression, and therapeutic resistance. In this study, we employed bioinformatics analyses to elucidate the molecular underpinnings of cancer biology, focusing on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Our GO analysis revealed the enrichment of key biological processes such as protein phosphorylation, regulation of programmed cell death, and transmembrane receptor signaling pathways, underscoring the critical roles of signaling cascades and regulatory mechanisms in tumorigenesis. Similarly, molecular functions such as protein kinase activity and ATP binding were identified as significantly enriched, highlighting the importance of protein kinases and molecular interactions in cancer development and progression. The KEGG pathway analysis further delineated dysregulated signaling pathways associated with cancer, including the MAPK and PI3K-Akt signaling pathways, implicating these pathways as central regulators of cancer progression. These findings deepen our understanding of cancer biology and offer potential targets for therapeutic intervention. Integrating multi-omics data and systems biology approaches may provide deeper insights into the intricate networks underlying cancer pathogenesis, paving the way for developing more effective treatments for cancer patients.

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INTRODUCTION

Cancer represents a formidable global health challenge characterized by uncontrolled cell growth and proliferation[1,2]. Despite advancements in conventional treatments such as surgery, chemotherapy, and radiotherapy, cancer remains a complex disease with formidable resistance mechanisms[3]. In recent years, there has been a surge in innovative therapeutic strategies, including stem cell therapy, targeted therapy, ablation therapy, nanoparticles, natural antioxidants, radionics, chemodynamic therapy, and ferroptosis-based therapy[4]. Integrating traditional medicinal knowledge with modern scientific methodologies offers a promising avenue for developing novel and effective cancer treatments[5].

Limonia acidissima, a deciduous tree belonging to the Rutaceae family, is indigenous to the Indian subcontinent and Southeast Asia[6]. Recognized for its rich phytochemical profile, including alkaloids, flavonoids, tannins, and terpenoids, *L. acidissima* has been traditionally used for its medicinal properties[7]. Emerging evidence supports the anticancer potential of *L. acidissima* extracts, as demonstrated by their efficacy against various cancer cell lines[6].

Cancer remains a significant global health challenge despite advancements in therapeutic strategies. The heterogeneous nature of cancer cells, their ability to evade immune responses, and the emergence of drug resistance underscore the urgent

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need for innovative treatment approaches[8,9]. Network pharmacology, a systems biology-based approach, offers a comprehensive framework for understanding the intricate interplay between drugs and biological systems[10]. Its application in elucidating the mechanisms of action of natural products has gained momentum in recent years[11]. By employing computational tools, network pharmacology can unveil the complex interactions of multiple bioactive compounds within biological networks[12,13].

Limonia acidissima has demonstrated promising anticancer activity in preclinical studies[6,14]. However, the precise molecular mechanisms underlying its therapeutic efficacy remain elusive. This study aims to employ a network pharmacology approach to elucidate the potential molecular targets and signaling pathways modulated by *L. acidissima* constituents. By bridging the gap between experimental data and mechanistic understanding, this research seeks to contribute to the development of novel therapeutic strategies for cancer treatment.

MATERIALS AND METHODS

Materials

This study utilized several online resources for data retrieval and analysis. These included PubChem (https://pubchem.ncbi.nlm.nih.gov/), SwissTargetPrediction (http://swisstargetprediction.ch/), GeneCards (https://www.genecards.org/), Venny (https://bioinfogp.cnb.csic.es/tools/venny/), and STRING (https://string-db.org/). Cytoscape 3.10.1 (https://cytoscape.org/) was employed for network visualization and analysis. Additionally, the CytoHubba plugin 0.1 was utilized within Cytoscape for network centrality analysis[15]. A comprehensive literature review was conducted to identify secondary metabolite compounds commonly found in *L. acidissima*. The results of this review[14] are summarized in **Table I**.

Table I. Secondary metabolite compounds of *L. acidissima*[14].

No	Compounds	PubChem ID	Parts
1	2,6-dimethoxy benzoquinone	68262	Fruit
2	3-formylindole	10256	Stem
3	4-hydroxybenzoic acid	135	Fruit
4	4-methoxy-1-methyl-2-quinolone	182073	Stem
5	4-methoxy-2-quinolone	600167	Stem
6	5-(3-acetoxypropenyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3- dihydroxybenzofuran-3-ylmethyl acetate	73207012	Stem
7	5-hydroxy-2-(-hydroxyphenyl)-7-methoxy-6-(3-methylbut-2-enyl)chroman-4- one	146026481	Root
8	Acidissimin	6442730	Fruit, root
9	Acidissiminol	14506785	Fruit
10	Acidissiminol epoxide	5363185	Fruit
11	Aurapten	1550607	Root
12	Bergapten	2355	Fruit, stem, root, leaf
13	Columbianetin	92201	Stem
14	Demethylsuberosin	5316525	Fruit, stem
15	Dihydrosuberenol	14077805	Root
16	Dihydroxyacidissiminol	101676196	Fruit
17	Edulitine	826073	Stem
18	Gallic acid	370	Fruit
19	Gallocatechin	65084	Fruit
20	Hederatriol	44144287	Stem
21	Isopimpinellin	68079	Fruit, stem, root
22	Limodissimin A	163183899	Stem
23	Limonin	179651	Stem
24	Lupeol	259846	Stem
25	Marmesin	334704	Stem, root
26	N, N-dimethyltryptamine	6089	Stem
27	N-benzoyltyramine	577614	Fruit
28	Obacunone	119041	Stem
29	Orientin	5281675	Leaf
30	Osthenol	5320318	Fruit, stem, root
31	Osthol	10228	Root
32	Physcion	10639	Stem
33	Psoralen	6199	Fruit, stem, root
34	Rutaevin	441805	Stem

35	Saponarin	441381	Fruit, leaf
36	Seselin	68229	Stem
37	Stigmasterol	5280794	Stem, root, leaf
38	Suberenol	5375166	Stem
39	Syringaldehyde	8655	Stem
40	Syringaresinol	100067	Stem
41	Tanakamine	101413702	Stem
42	Tanakine	57357311	Stem
43	Tembamide	177583	Stem
44	Vitexin	5280441	Fruit, leaf
45	Xanthotoxin	4114	Fruit, stem, root
46	Yangambin	443028	Stem

Methods

The SMILES (Simplified Molecular Input Line Entry System) code for each identified secondary metabolite compound was retrieved from the PubChem database[16]. Subsequently, these SMILES codes were input into SwissTargetPrediction to predict potential protein targets[17]. Cancer-related proteins were identified using GeneCards[18]. The intersection of protein targets predicted by SwissTargetPrediction and those associated with cancer (from GeneCards) was determined using Venny[19]. Finally, the STRING database[20] was utilized to construct a pharmacological network illustrating potential protein-protein interactions among the identified proteins, providing insights into the potential mechanisms of action of secondary metabolites from *L. acidissima* in relation to cancer.

Data analysis

Protein-protein interaction data, encompassing predicted interactions between *L. acidissima* secondary metabolites and cancer-related proteins, were retrieved from the STRING database. To elucidate the functional implications of these interactions, Gene Ontology (GO) enrichment analysis for biological processes, molecular functions, and cellular components, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted. The interaction network was visualized and analyzed using Cytoscape[15]. Key hub proteins within this network were identified using the CytoHubba plugin[21], specifically employing the Maximal Clique Centrality (MCC) algorithm to identify the most influential nodes.

RESULTS AND DISCUSSION

Network pharmacology of secondary metabolite of L. acidissima

A comprehensive phytochemical analysis of *L. acidissima* identified 46 secondary metabolites distributed across its various plant parts (fruit, stems, roots, and leaves) (**Table I**)[14]. To predict potential protein targets for these metabolites, SwissTargetPrediction was employed, with a probability value threshold of >0 for further analysis.[22] GeneCards was utilized to identify cancer-related proteins, revealing 629 potential targets for *L. acidissima* metabolites among a broader set of 9,894 cancer-associated proteins. A Venn diagram analysis (**Figure 1**) identified a subset of 449 proteins implicated in cancer that also exhibited potential interactions with *L. acidissima* metabolites.

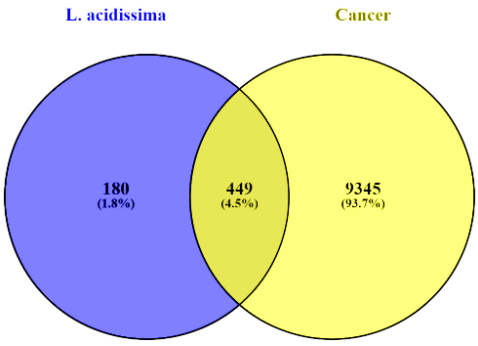

Figure 1. The Venn diagram intersection between proteins predicted to be able to interact with secondary metabolites of *L. acidissima* and proteins associated with cancer.

A pharmacological network analysis was conducted utilizing STRING (**Figure 2**), a comprehensive database containing over nine million proteins from diverse sources[22]. This analysis enabled the prediction of protein-protein interactions within the context of the 449 identified proteins[23]. The resulting network elucidates the interconnectivity between the selected target proteins and associated biological pathways.

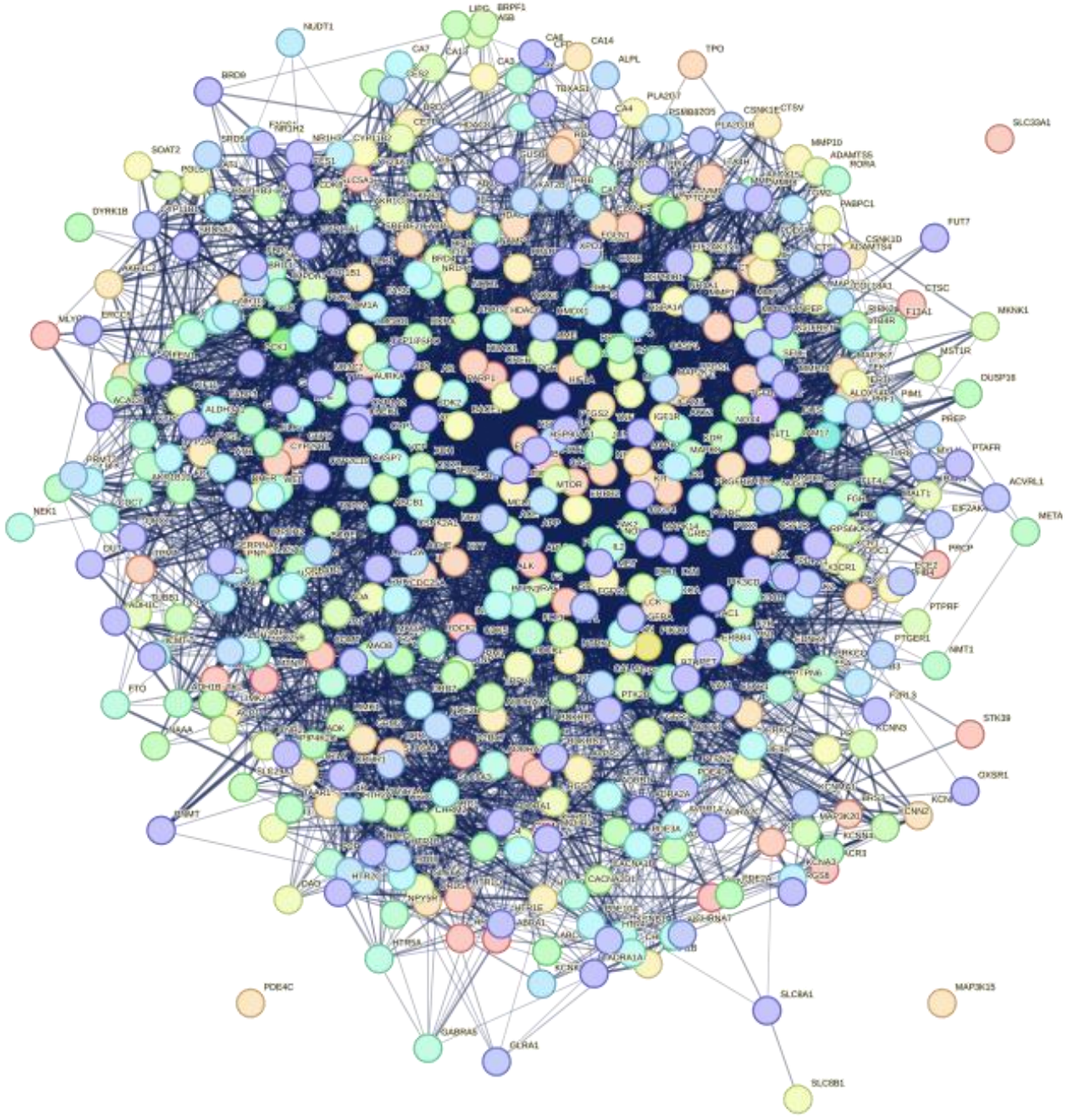

Figure 2. The network pharmacology using STRING.

Gene ontology and KEGG enrichment analysis

To elucidate the biological mechanisms underlying the identified gene sets, GO and KEGG enrichment analyses were conducted. GO categorizes genes into three ontologies: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC)[24]. The KEGG provides manually curated pathways representing molecular interactions and reactions[25]. The significance of enrichment results was assessed using the False Discovery Rate (FDR) value, which represents the

expected proportion of false positives among identified gene sets. A lower FDR value indicates a higher degree of confidence in the results[26]. In this study, FDR values are expressed as -log(p-values). A higher -log(p-value) corresponds to a lower probability of error (**Figure 3**).

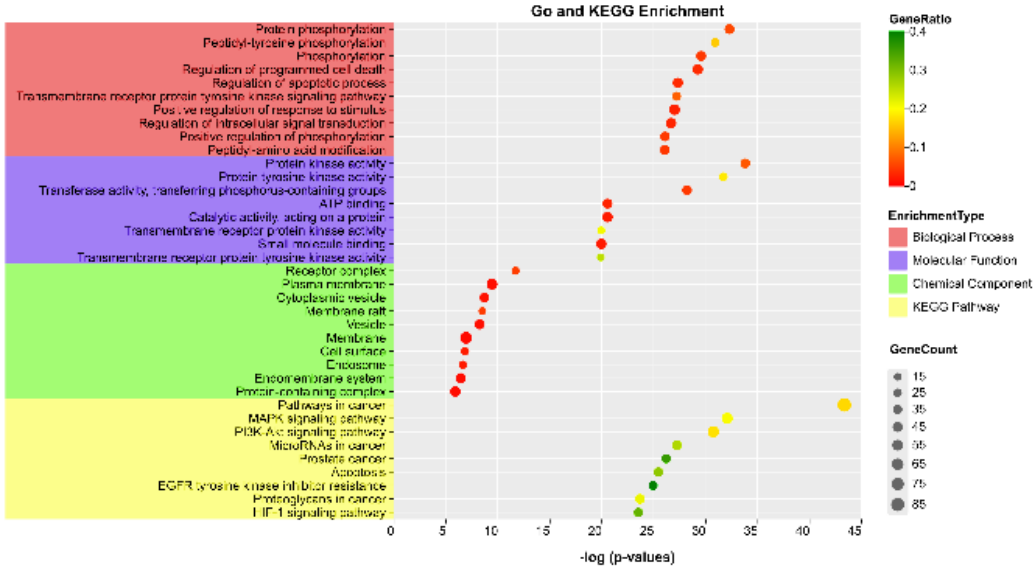

Figure 3. Gene Ontology and KEGG enrichment analysis.

Our analysis of the gene expression data revealed several biological processes associated with cancer. Notably, protein phosphorylation emerged as a central mechanism implicated in these processes[27]. Phosphorylation, a key regulatory mechanism in cells, involves the addition of phosphate groups to proteins, influencing a wide range of cellular functions, including signaling, proliferation, differentiation, and apoptosis[28]. Among the enriched biological processes, peptidyl-tyrosine phosphorylation and the transmembrane receptor protein tyrosine kinase signaling pathway stood out[29]. Tyrosine phosphorylation of proteins, especially within the context of RTKs, plays a critical role in regulating cell growth, survival, and migration[30]. Aberrant protein phosphorylation is a common hallmark of cancer[31,32]. Understanding the molecular mechanisms underlying peptidyl-tyrosine phosphorylation and RTK signaling pathways could potentially lead to the identification of novel therapeutic targets for precision cancer therapy[33]. By targeting these pathways, researchers may be able to develop more effective and targeted treatments for cancer patients.

Apoptosis, a programmed cell death mechanism, is a critical regulator of cell survival in cancer. Dysregulation of apoptosis pathways allows cancer cells to evade cell death and proliferate uncontrollably[34,35]. Intracellular signaling cascades play a pivotal role in transmitting information from the extracellular environment to the cell, influencing various cellular processes including proliferation, differentiation, and survival[36]. Aberrant signaling pathways can contribute to cancer development by promoting uncontrolled cell growth and survival[37]. Cancer cells often exhibit heightened sensitivity to external stimuli, enabling them to adapt to the complex tumor microenvironment and facilitate tumor progression and metastasis[38]. This enhanced responsiveness reflects the dynamic nature of cellular responses to diverse environmental cues and stress signals[39].

Molecular function analysis revealed that the identified proteins are enriched in functions associated with cancer, particularly those related to protein kinases. Protein kinases play pivotal roles in cellular signaling by catalyzing the phosphorylation of target proteins, making them attractive targets for cancer therapy[40]. Several kinase inhibitors, especially those targeting tyrosine kinases, have been approved for clinical use or are under investigation[41]. Transferase enzymes, responsible for transferring functional groups between molecules, including phosphate-containing groups, further highlight the significance of phosphorylation in cellular signaling and regulation[42]. The enrichment of transferase activity, particularly those involving phosphorus-containing groups, emphasizes the crucial role of phosphorylation events in cancer-related processes[43].

ATP binding is a pivotal molecular process underpinning diverse cellular functions, including energy metabolism, signal transduction, and protein synthesis[44]. Kinases, a class of enzymes involved in phosphorylation reactions, utilize ATP as a phosphate donor[45]. Targeting ATP-binding sites in key regulatory proteins, particularly receptor tyrosine kinases, offers a promising therapeutic strategy for cancer. These proteins play a critical role in transmitting extracellular signals into intracellular responses[47,48]. Aberrant activation of receptor tyrosine kinases, such as EGFR and VEGFR, drives tumor growth, angiogenesis, and metastasis[49]. Targeting these receptors and their downstream signaling pathways has yielded effective cancer treatments, with several receptor tyrosine kinase inhibitors approved for clinical use[50].

Small molecule binding encompasses a range of molecular interactions between ligands and their binding partners[51]. Small molecules, including drugs and endogenous metabolites, can modulate protein function and activity by binding to specific sites[52]. Leveraging small molecule-protein interactions is a powerful approach to drug discovery and therapeutic intervention in cancer. Targeted therapies that selectively inhibit oncogenic signaling pathways while minimizing off-target effects can be developed through this strategy[53].

Our CC analysis identified several proteins associated with cancer, emphasizing the significance of cell-surface receptors in cellular communication and signaling[54]. Receptor complexes function as molecular hubs, facilitating the binding of extracellular ligands and initiating intracellular signaling cascades that govern crucial cellular processes, including growth, differentiation, and survival[55]. Dysregulation of these receptor signaling pathways is a hallmark of cancer, with aberrant activation of growth factor receptors contributing to tumor progression and metastasis[56]. Targeting these receptor complexes and their downstream signaling pathways represents a promising therapeutic approach for disrupting oncogenic signaling networks and inhibiting tumor growth[57].

The plasma membrane and membrane rafts serve as critical platforms for cell-cell interactions, signal transduction, and molecular trafficking[58]. These membrane microdomains, enriched in cholesterol and sphingolipids, facilitate the clustering and organization of signaling molecules, including receptors and kinases, thereby modulating their activity and downstream signaling pathways[59]. Alterations in the composition and organization of the plasma membrane have been implicated in cancer development and metastasis, underscoring the importance of membrane-associated processes in tumor biology[60]. Dysregulation of the endomembrane system has been implicated in various pathological conditions, including cancer[61,62]. The endomembrane system encompasses a network of membrane-bound organelles, including the endoplasmic reticulum, Golgi apparatus, and vesicles, responsible for crucial cellular functions like protein trafficking, folding, and sorting[63-66]. Disruptions in this system can lead to abnormal receptor turnover, signaling, and drug sensitivity in cancer cells, suggesting its potential as a therapeutic target[67].

Pathway analysis, such as the "Pathways in Cancer" database, provides valuable insights into the molecular mechanisms underlying tumorigenesis[68]. Key signaling pathways implicated in cancer include the MAPK and PI3K-Akt pathways, which regulate cell proliferation, survival, and metastasis[69,70]. Additionally, dysregulated apoptotic pathways contribute to tumor cell survival and therapy resistance, emphasizing the need for therapeutic interventions targeting these pathways[71]. MicroRNAs also play a pivotal role in cancer biology, influencing gene expression and tumor progression. Targeting dysregulated miRNAs or their downstream targets represents a promising therapeutic strategy[72].

Resistance to targeted therapies, as exemplified by EGFR tyrosine kinase inhibitor resistance, remains a major obstacle in cancer treatment. Developing novel therapeutic strategies to circumvent these resistance mechanisms is imperative[73]. The tumor microenvironment significantly influences cancer progression, with proteoglycans playing a pivotal role in regulating tumor cell behavior, angiogenesis, and immune evasion[74]. Targeting proteoglycan signaling pathways or their downstream effectors within the tumor microenvironment presents promising therapeutic avenues[75]. Furthermore, hypoxia-induced signaling through the HIF-1 pathway promotes tumor growth and metastasis, highlighting another potential target for therapeutic intervention[76]. By elucidating dysregulated pathways through comprehensive pathway analysis, we can identify promising therapeutic targets and develop effective strategies to improve cancer treatment outcomes[77].

MCC analysis

To identify the most influential proteins within the pharmacological network, a hub network analysis was conducted using the MCC method. This approach identifies groups of proteins forming tightly interconnected sub-networks, known as

maximal cliques. The centrality of proteins within these cliques, which reflects their importance in maintaining or influencing network connections, was then assessed[21].

MCC enrichment analysis using CytoHubba revealed several key hub genes that may play pivotal roles in cancer pathogenesis and progression. These top-ranking hub genes included BCL2, SRC, ESR1, HIF1A, MDM2, MTOR, BCL2L1, AKT1, CD274, and CASP3 (**Figure 4**). The BCL2 family of proteins, including BCL2 and BCL2L1, plays a crucial role in regulating apoptosis[78]. SRC and AKT1 are protein kinases involved in signaling pathways associated with cell proliferation, survival, and metastasis[79]. Estrogen receptor alpha (ESR1) is a well-established biomarker and therapeutic target in hormone receptor-positive breast cancer[80]. Hypoxia-inducible factor 1 alpha (HIF1A) is a master regulator of cellular responses to hypoxia, a common feature of the tumor microenvironment[81]. Activation of HIF1A promotes angiogenesis and metastasis, facilitating tumor adaptation to low-oxygen conditions[82]. MDM2 and MTOR are key regulators of cell growth and proliferation, with critical roles in cancer development and progression[83]. CD274 (programmed death-ligand 1) and CASP3 (caspase 3) are intriguing hub genes implicated in immune evasion and apoptotic pathways, respectively[84].

The identification of BCL2, SRC, ESR1, HIF1A, MDM2, MTOR, BCL2L1, AKT1, CD274, and CASP3 as hub genes through MCC enrichment analysis highlights their potential significance in cancer pathogenesis and offers promising avenues for therapeutic intervention. Further experimental validation and functional studies are warranted to elucidate the specific mechanisms by which these hub genes contribute to tumorigenesis and to explore their potential as druggable targets in cancer therapy.

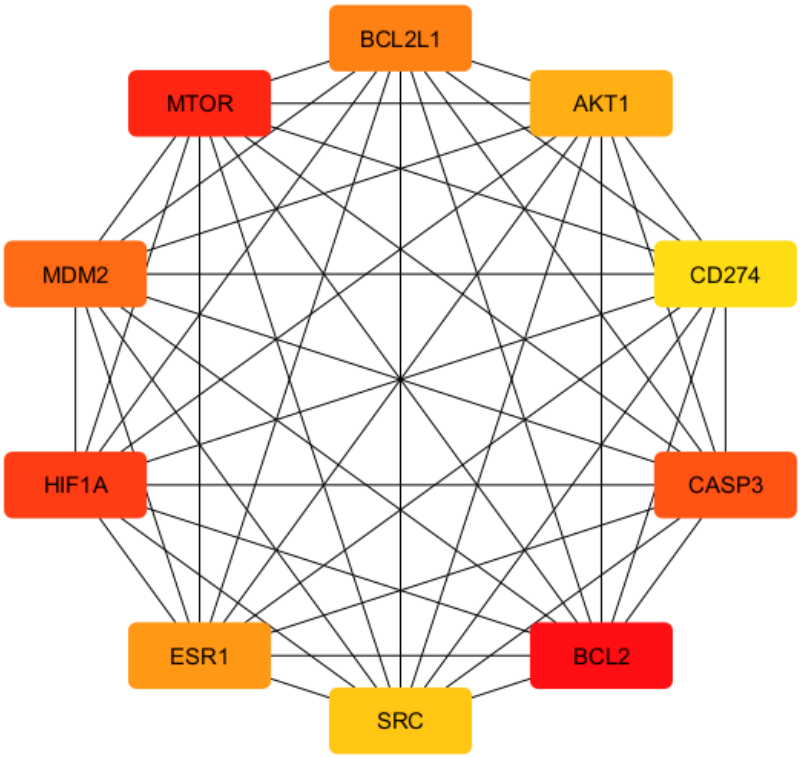

Figure 4. The top 10 proteins predicted to interact with secondary metabolites of *L. acidissima* and be associated with hypertension using the MCC method.

CONCLUSION

This study employed Gene Ontology and KEGG pathway analysis to elucidate the molecular mechanisms underpinning cancer. Our findings reveal significant enrichment in biological processes and molecular functions associated with cancer, emphasizing the pivotal role of signaling cascades and regulatory mechanisms in tumorigenesis. Notably, the MAPK and PI3K-Akt signaling pathways emerged as key regulators of cancer progression. These insights advance our understanding of cancer biology and offer potential therapeutic targets for the development of more effective treatments.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Research Article

Antioxidant Activity, Total Phenolics, and Total Flavonoids Content of Bajakah Tampala (*Spatholobus littoralis*): The Indigenous Herbal Medicine from Kalimantan

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Abstract

Bajakah tampala (*Spatholobus littoralis*), a medicinal plant traditionally used in Indonesia, particularly on Kalimantan Island, has garnered interest for its potential health benefits. However, scientific evidence remains scarce. This study investigated the antioxidant activity of *S. littoralis* extract and its total phenolic and flavonoid content. Ethanol extraction and evaporation were used to prepare the extract. The DPPH method assessed antioxidant activity, while Folin–Ciocalteu and $AlCl_3$ complexation methods quantified total phenolics and flavonoids, respectively. The *S. littoralis* extract exhibited strong antioxidant activity with an IC_{50} value of 54.19 ± 8.15 µg/mL. Additionally, the extract contained substantial levels of phenolics (0.649 ± 0.026% GAE) and flavonoids (1.084 ± 0.043% QE). These findings suggest a link between the high phenolic and flavonoid content of *S. littoralis* extract and its observed strong antioxidant activity.

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INTRODUCTION

Borneo or Kalimantan Island, renowned for its vast tropical rainforests, is a hotspot of plant biodiversity in Southeast Asia, rivaling even the Amazon. This diverse ecosystem provides a natural habitat for a wide array of medicinal plants, including *Spatholobus littoralis* Hassk, which is commonly found in the island's interior and forested areas[1,2]. *Spatholobus littoralis*, commonly known as *bajakah tampala*, is a native plant found in Kalimantan, Indonesia. This plant has been traditionally used by the Dayak people for generations as a remedy for various ailments[3,4]. The Dayak culture encompasses a rich tradition of utilizing medicinal plants, and *S. littoralis* is one such example[5,6].

Spatholobus littoralis, a promising medicinal plant, has demonstrated antioxidant, anti-inflammatory, anticancer, and antibacterial properties in previous studies[7-11]. These findings suggest its potential for development as a widely applicable traditional medicinal preparation. Previous research[8] has identified several secondary metabolites in *S. littoralis*, including 3,7-trihydroxyflavone, eriodictyol, plantymenin, dihydroquercetin, butin, neoisoliquiritigenin, dihydrokaempferol, liquiritigenin, and 6-methoxyeriodictyol. These compounds may contribute to the plant's therapeutic effects.

Antioxidants play a crucial role in preventing the progression of degenerative diseases, including diabetes, hypertension, dyslipidemia, and cardiovascular disease[12,13]. Free radicals, reactive oxygen species that can damage cellular components like lipids, proteins, and DNA, contribute to various health issues, including degenerative diseases, premature aging, inflammation, and even tumor formation[14]. While the human body naturally produces antioxidants to combat free radicals,

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excessive oxidative stress can necessitate external antioxidant supplementation. Phenolic and flavonoid compounds have been identified as potent antioxidants but remain understudied[15,16]. This study aims to investigate the antioxidant activity of *S. littoralis* extract and determine its correlation with total phenolic and flavonoid content.

MATERIALS AND METHODS

Materials

Spatholobus littoralis plant samples were collected from Banjarmasin, South Kalimantan during 2023. The plant specimens were authenticated by a botanist at the Laboratory of Biological Education, Universitas Lambung Mangkurat, and assigned the voucher specimen number 074/UN8.1.2.3.2/PG/Lab.PMIPA/Bio/2023.

Methods

Extract preparation

Spatholobus littoralis powders were extracted using ethanol in a 1:10 sample-to-solvent ratio. The maceration process was repeated with fresh ethanol to maximize extract yield. The obtained extract was concentrated using a rotary evaporator at 50°C.

Antioxidant activity assay

The antioxidant activity of the *S. littoralis* extract was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay[17]. A series of *S. littoralis* extract concentrations were added to a DPPH solution. The antioxidant compounds within the extract donated hydrogen atoms to the DPPH free radicals, resulting in a reduction of the DPPH radical. This reduction was quantified by measuring the decrease in absorbance at 517 nm. The antioxidant activity was expressed as the IC_{50} value, which represents the concentration of the extract required to inhibit the DPPH radical by 50%[18].

Total phenolic content assay

The total phenolic content (TPC) of the *S. littoralis* extract was quantified using a spectrophotometric method with gallic acid as a standard. The extract was oxidized with Folin-Ciocalteu reagent, resulting in a blue-purple color change. The absorbance of the resulting solution was measured at 765 nm using a spectrophotometer[19].

Total flavonoid content assay

The total flavonoid content (TFC) of the plant extracts was determined using the aluminum chloride ($AlCl_3$) colorimetric method. This method involves the reaction between flavonoids and $AlCl_3$, resulting in the formation of a colored complex[20]. The intensity of the yellow color produced was measured spectrophotometrically at 510 nm. Quercetin was used as a standard.

Data analysis

The IC_{50} values were determined by correlating the sample concentration with the percentage inhibition. The TPC was expressed as a percentage of gallic acid equivalent (GAE). The TFC was expressed in terms of quercetin equivalents (QE).

RESULTS AND DISCUSSION

As depicted in **Figure 1**, *S. littoralis* exhibits a cylindrical, unbranched stem with a greenish-brown color and a diameter of 3.2 cm. The leaves are simple, alternate, pinnately compound, with an inverted triangular shape, smooth surface, flat edges, and a pointed tip, measuring 6 cm in length and 2.4 cm in width. The flowers are monoecious, arranged in fistulous inflorescences with 5 cm flower stalks, possessing four calyxes and a color spectrum ranging from white to red.

Antioxidant activity assay

The antioxidant activity of *S. littoralis* ethanol extract was evaluated using the DPPH free radical scavenging assay. As illustrated in **Figure 2**, the extract demonstrated potent antioxidant activity, with an IC_{50} value of 54.19 ± 8.15 µg/mL. This value falls within the range of 50-100 µg/mL, characteristic of strong antioxidants[21]. The antioxidant properties of *S. littoralis*

may contribute to the prevention of oxidative stress, which is associated with various degenerative diseases[22]. By reducing oxidative stress, this plant extract could potentially mitigate the progression of these diseases.

The DPPH assay is a widely employed method for assessing the antioxidant potential of plant-based extracts. It relies on the ability of compounds to scavenge free radicals, reducing the DPPH radical. In this study, compounds capable of scavenging and reducing DPPH radicals were considered antioxidants. A higher extract concentration generally correlates with increased antioxidant activity[23].

a b

Figure 1. Morphology of (**a**) stem and (**b**) leaf of *S. littoralis*.

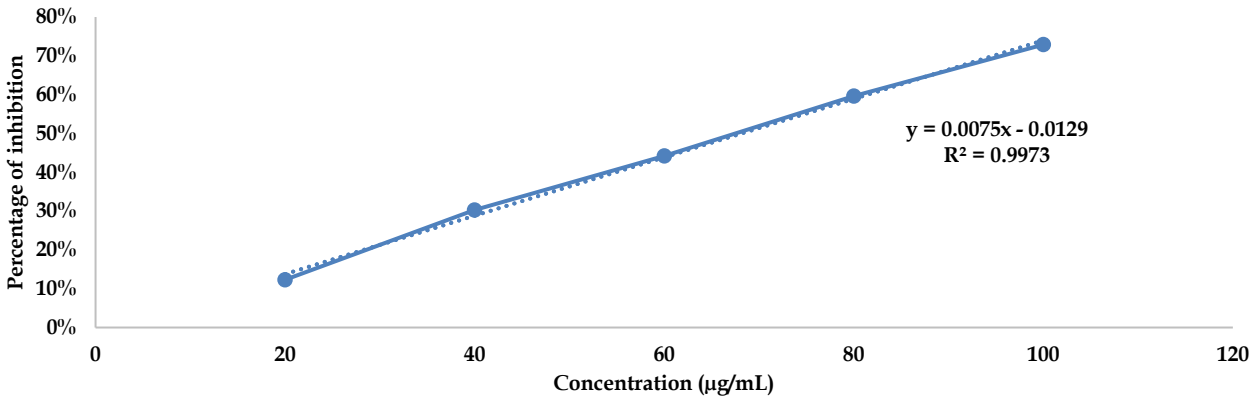

Figure 2. Antioxidant activity of *S. littoralis* extract.

Total phenolic content

The total phenolic content of the *S. littoralis* extract was determined to be 0.649 ± 0.026 %GAE as shown in **Figure 3**. This finding is consistent with previous reports from Central Kalimantan, which identified a phenolic content of 0.7 %GAE in *S. littoralis*[24]. Interestingly, the geographical origin of *S. littoralis* did not significantly influence its total phenolic content.

The presence of phenolic compounds, including flavonoids, phenols, terpenoids, and cardiac glycosides, is likely responsible for the strong antioxidant activity exhibited by the *S. littoralis* extract[25]. The high phenolic content in this extract is directly correlated with its potent antioxidant properties, as demonstrated in previous studies[24]. These phenolic compounds have also been shown to contribute to the cytotoxic and anti-inflammatory effects of *S. littoralis* extract[9].

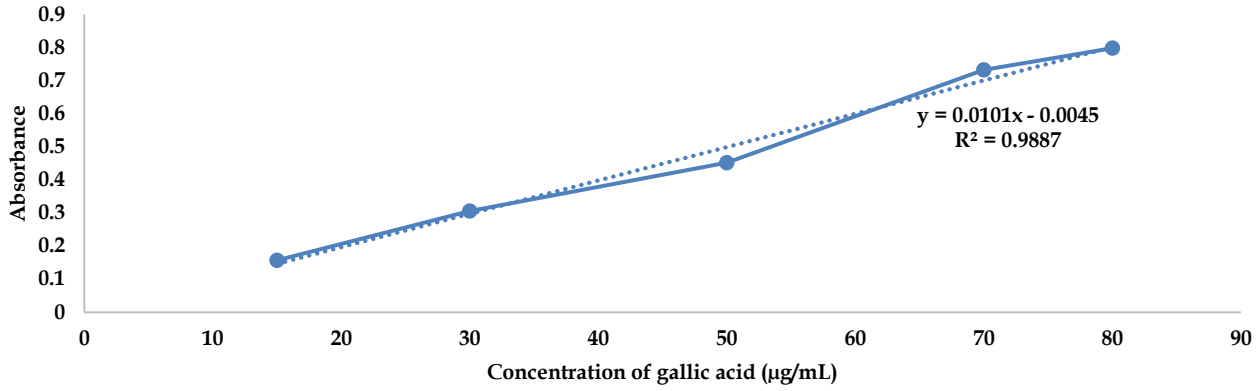

Figure 3. Gallic acid standard curve for determination of total phenolic content of *S. littoralis* extract.

Total flavonoid content

The antioxidant activity, total phenolic content, and total flavonoid content of *S. littoralis* extract were determined and presented in **Table I**. Flavonoids, known for their antioxidant properties, are prevalent in herbal medicines[26]. The conjugated double bonds and hydroxyl groups within flavonoid compounds contribute to their electron-donating capacity, enabling them to act as free radical scavengers.

The total flavonoid content in *S. littoralis* extract was quantified using quercetin as a standard, expressed as %QE. The standard curve for quercetin is depicted in **Figure 2**. The results indicate a total flavonoid content of 1.084 ± 0.043 %QE in *S. littoralis* extract. Previous research has reported that the leaves of *Uncaria acida*, another member of the Rubiaceae family, exhibit the highest flavonoid content compared to other plant parts[27]. This finding underscores the importance of preserving the leaves of *S. littoralis* for potential medicinal applications. While the Dayak people traditionally utilize the roots of *S. littoralis*, our findings suggest that the leaves may be a more valuable source of flavonoids. This information could inform future research and potential commercialization efforts.

Table I. Antioxidant activity, total phenolic, and total flavonoid contents of *S. littoralis* extract.

Parameter	Value
Antioxidant activity ($IC_{50;}$ µg/mL)	54.19 ± 8.15
Total phenolic content (%GAE)	0.649 ± 0.026
Total flavonoid content (%QE)	1.084 ± 0.043

The results of this study demonstrate the significant antioxidant capacity of *S. littoralis* extract as evaluated by the DPPH method. Flavonoids and phenolic compounds, known for their hydroxyl group, are key contributors to this antioxidant activity[8]. While the DPPH method provides valuable insights, further confirmation of *S. littoralis* extract's antioxidant activity through additional *in vitro* assays is warranted. The ABTS and FRAP methods could be employed to assess antioxidant capacity using different mechanisms[28]. Moreover, *in vivo* studies investigating the impact of *S. littoralis* extract on endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are essential to fully elucidate its antioxidant potential[29].

CONCLUSION

This study demonstrates the potent antioxidant activity of *S. littoralis* extract against DPPH free radicals. This activity can be attributed to the extract's high concentrations of phenolic and flavonoid compounds, which were quantified at 0.649 ± 0.026 %GAE and 1.084 ± 0.043 %QE, respectively. These findings highlight the potential of *S. littoralis* as a valuable source of natural antioxidants for various applications.

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AUTHORS' CONTRIBUTION

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Software: -
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Writing - original draft: Habib Basyanur Murdi, Dwi Utami, Mustofa Ahda, Andika
Writing - review & editing: Nurkhasanah Mahfudh, Siti Nashihah

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Research Article

Structure Modification of Cinnamic Acid to (*E*)-1-(3,4-dihydroisoquinoline-2(1H)-yl)-3-phenylprop-2-en-1-one and Antioxidant Activity Test by DPPH Method

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Acylation reaction
Antioxidant
Cinnamic acid
Structure modification

Abstract

Antioxidants can protect cells from free radical damage by stabilizing them. One of the compounds that has antioxidant activity is cinnamic acid. Cinnamic acid and its derivatives have several activities: antibacterial, anticancer, and antioxidant. However, the ability of cinnamic acid to capture free radicals is still relatively low. One of the efforts that can be made to increase the antioxidant activity of cinnamic acid is to modify its structure. Structure modification is an effort to improve the pharmacological activity of a compound through chemical synthesis reactions. The cinnamic acid structure can be modified by changing the carboxylic -OH group into an amine group through an N-atom acylation reaction. This study was conducted by reacting cinnamoyl chloride (**1a**), which is a cinnamic acid derivative with 1,2,3,4-tetrahydroisoquinoline (**2b**) which is a compound of isoquinoline group to produce (*E*)-1-(3,4-dihydroisoquinoline-2(1H)-yl)-3-phenylprop-2-en-1-one (**3b**) and then tested for antioxidant activity using DPPH method. The resulting product compound was yellow crystals with a yield of 81.56%. The antioxidant activity produced by the product is more significant than that of cinnamic acid compounds at the same concentration.

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INTRODUCTION

Free radicals, highly reactive species with unpaired electrons, can cause oxidative stress when produced in excess. The human body possesses defense mechanisms, including enzymes like glutathione peroxidase, catalase, and superoxide dismutase, to counteract free radical damage[1,2]. However, oxidative stress, resulting from an imbalance between free radical production and elimination, is implicated in various serious health conditions, including cancer, atherosclerosis, aging, immunosuppression, inflammation, ischemic heart disease, diabetes, and neurological disorders[3].

Antioxidants, substances capable of neutralizing free radicals by donating electrons, play a crucial role in protecting cells from oxidative damage[3]. Cinnamic acid, a compound commonly used as a food flavoring agent, exhibits antioxidant properties[4-6]. Its unsaturated double bonds facilitate hydrogen atom donation, neutralizing free radicals. However, cinnamic acid's antioxidant activity is relatively limited. Structure modification represents a promising strategy to enhance its antioxidant capacity[7,8].

Structure modification, a key drug development strategy, involves chemically altering existing compounds to enhance their pharmacological activity[9,10]. The cinnamic acid scaffold, characterized by its carboxylic OH group, offers opportunities for modification through N-atom acylation to produce novel derivatives. Tetrahydroisoquinoline compounds hold significant

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promise in bioorganic chemistry and medicine. This scaffold has been widely explored in pharmaceutical research, demonstrating diverse biological activities including anti-inflammatory, neurotropic, antibiotic, antipsychotic, antituberculosis, estrogenic receptor blocker, and antioxidant properties[11,12]. The detrimental effects of reactive oxygen species (ROS) on cellular macromolecules have fueled research efforts to develop novel antioxidant compounds[13,14]. To counteract oxidative damage, extensive investigations have been conducted to identify and characterize potential antioxidants.

In this study, we concentrated on the design, synthesis, characterization, and evaluation of the antioxidant activity of the newly cinnamic acid-tetrahydroisoquinoline hybrid compound. The structures of the synthesized compounds were verified based on FTIR and ^{1}H-NMR spectral data. The structural combination of cinnamic acid and tetrahydroisoquinoline is expected to increase the antioxidant activity of cinnamic acid as the lead compound.

MATERIALS AND METHODS

Materials

The synthesis of target compound involved the use of cinnamoyl chloride (Sigma-Aldrich), 1,2,3,4-tetrahydroisoquinoline (Sigma-Aldrich), triethylamine (Merck), tetrahydrofuran (Merck), and ethyl acetate (Merck). Purification was achieved using *n*-hexane and distilled water. Analytical techniques included melting point determination (using a melting point tester), Fourier-Transform Infrared spectroscopy (FTIR; Shimadzu), thin-layer chromatography-densitometry (TLC-densitometry; Camag), and nuclear magnetic resonance spectroscopy (NMR; JEOL Resonance).

Methods

Synthesis of target compound

(*E*)-1-(3,4-dihydroisoquinoline-2(1H)-yl)-3-phenylprop-2-en-1-one (**3b**) was synthesized via a condensation reaction between cinnamoyl chloride (**1a**) and 1,2,3,4-tetrahydroisoquinoline (**2b**) using triethylamine as a catalyst (**Figure 1**). Cinnamoyl chloride (0.004 mol, 0.67 g) and 1,2,3,4-tetrahydroisoquinoline (0.008 mol, 1.07 g) were combined in an ice bath under stirring conditions (220 rpm). Triethylamine (0.008 mol, 0.81 g) was added as a catalyst. The reaction was monitored by TLC and terminated when the cinnamoyl chloride spot disappeared. The reaction mixture was quenched with a saturated sodium bicarbonate solution followed by 50 mL of distilled water. The precipitated product was filtered using a Buchner funnel. The crude product was recrystallized from hot methanol. Recrystallized crystals were filtered using a Buchner funnel and washed with 10 mL of methanol (twice). The final product was dried in an oven at a constant temperature of 50°C. The yield of the synthesized compound was determined by weighing the dried product.

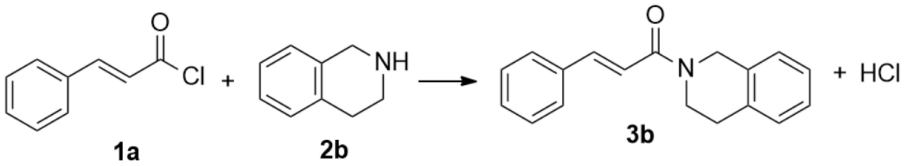


Figure 1. The synthetic route to a target compound (**3b**).

Optimization of reaction time and eluent

Reaction progress was monitored using TLC. Five aliquots were taken from the reaction mixture at specific time points (0, 1, 2, 3, and 4 hours). To optimize the TLC separation, four different eluent systems were evaluated. The comparisons are presented in **Table I**.

Table I. Comparison of eluents for optimization.

Eluent code	Eluent	Comparison
A	*n*-hexane : ethyl acetate	10 : 1
B	*n*-hexane : ethyl acetate	1 : 1
C	*n*-hexane : ethyl acetate : acetone	10 : 1 : 5
D	*n*-hexane : ethyl acetate : acetone	7 : 1 : 5

Data analysis

Organoleptic test

Visual assessment of the product's shape and color was conducted as part of the organoleptic evaluation.

Product compound purity test: melting range test

The melting point of the sample was determined using a capillary tube method. A small amount of the sample was packed into a capillary tube, which was then inserted into the melting point apparatus. The portion of the tube containing the sample was positioned in the center of the apparatus. The temperature was gradually increased, and the melting point was recorded as the temperature at which the sample transitioned from a solid to a liquid state.

Product compound purity test: TLC-densitometry

Thin-layer chromatography was employed to assess the purity of the sample solution. A small amount of the sample was applied to a TLC plate, and the developed chromatogram was visually inspected. The presence of a single spot indicates a pure compound. To further confirm purity, densitometric analysis was performed[15].

Target compound identification: FTIR

The spectra were recorded using the KBr pellet technique in a Shimadzu spectrometer. The pellets were prepared by mixing 1.5-2 mg of 3b with 350 mg of KBr. The 13 mm diameter pellets were prepared in a standard device[16].

Target compound identification: ^{1}H-NMR

Samples were dissolved in $CDCl_3$ within NMR tubes. 1H-NMR spectroscopy was conducted using a JEOL RESONANCE 400 MHz spectrometer. The NMR parameters adhered to the protocols established by Kornberger *et al*.[17] in their previous research.

Antioxidant test using DPPH method

Stock solutions of the target compound (**3b**) and cinnamic acid were prepared at a concentration of 1000 ppm in methanol. Working solutions were then prepared by diluting the stock solutions to concentrations of 100 ppm and 500 ppm using methanol. A 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was prepared in methanol. The DPPH solution was stored in a dark bottle to prevent photodegradation.

The antioxidant activity of **3b** and cinnamic acid solutions was determined using the DPPH free radical scavenging assay. Aliquots (0.2 mL) of each test solution at varying concentrations were mixed with 0.8 mL of DPPH solution. The mixture was then incubated for 30 minutes at room temperature in darkness. Absorbance measurements were recorded at 516 nm using a UV-Vis spectrophotometer[18].

RESULTS AND DISCUSSION

Synthesis of target compound

Compound **3b** was synthesized via a nucleophilic acyl substitution reaction between **1a** and **2b**. The reaction mechanism proceeds through two stages: nucleophilic addition to the carbonyl group and subsequent chloride ion elimination (**Figure 2**)[19]. Triethylamine served as a base to deprotonate intermediate species, facilitate HCl removal, and catalyze the reaction[20]. Tetrahydrofuran was selected as the solvent due to its aprotic nature, which enhances the reaction by minimizing hydrogen bond formation. THF's water solubility facilitates solvent removal through washing[21]. The overall yield of compound **3b** was 81.56%.

Optimization of reaction time and eluent

Reaction optimization revealed that compound **3b** was formed immediately upon mixing of starting materials (**Figure 3**), indicating an instantaneous reaction. Therefore, a reaction time of zero hours was determined to be optimal. The selected eluent system, consisting of *n*-hexane and ethyl acetate in a 1 : 1 ratio (eluent B), demonstrated excellent chromatographic separation, as evidenced by the absence of overlapping spots on the TLC plate (**Figure 4**)[22]. This eluent system effectively

separated compounds with Rf values within the desired range of 0.2-0.8[23,24]. The Rf value of the target compound, **3b**, was determined to be 0.71 using eluent B, confirming its suitability for the separation.

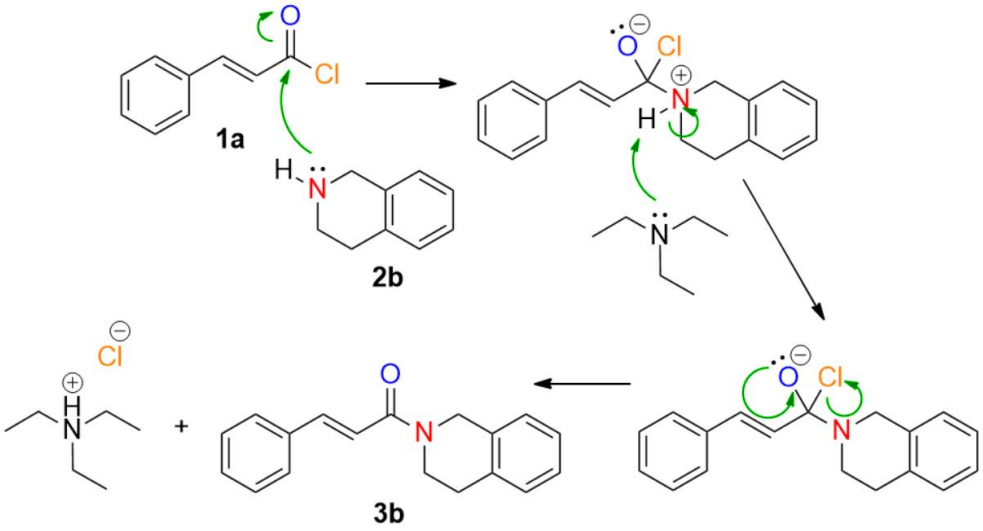


Figure 2. Mechanism for the synthesis of compound **3b**.

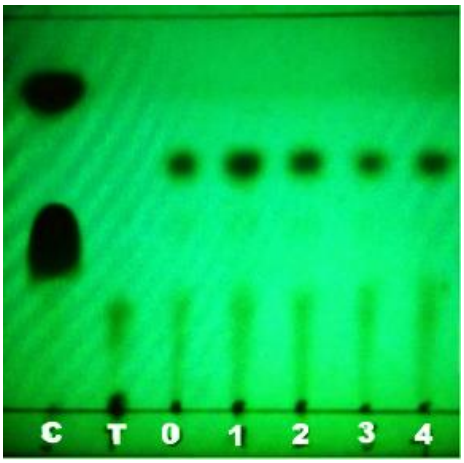
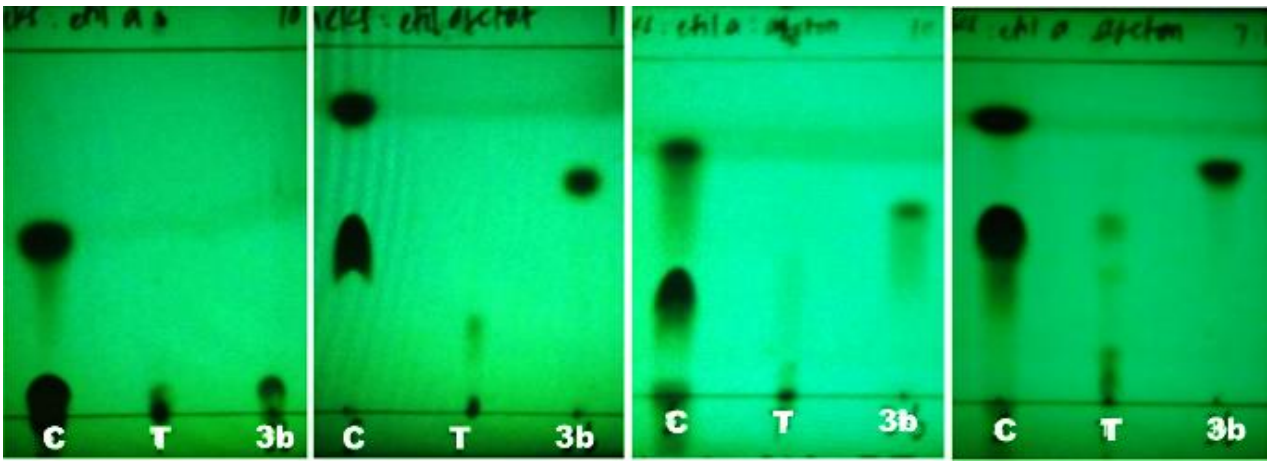


Figure 3. Reaction time optimization results using TLC under UV light 254 nm (**C**: cinnamoyl chloride; **T**: 1,2,3,4-tetrahydroisoquinoline; **0, 1, 2, 3,** and **4**: sampling hours 0, 1, 2, 3, 4).



A **B** **C** **D**

Figure 4. TLC spot from eluent optimization results (eluent **A, B, C**, and **D**); **C**: cinnamoyl chloride; **T**: 1,2,3,4-tetrahydroisoquinoline; **3b**: target compound.

Organoleptic test

The synthesized product exhibited a crystalline form with a distinctive yellow color, as depicted in **Figure 5**.

Figure 5. The crystal of target compound **3b**.

Product compound purity test: melting range test

The purity of a compound can be assessed by its melting range, with a narrow range (typically 1-2°C) indicating high purity[25]. The target compound in this study exhibited a melting range of 72-74°C (**Table II**), confirming its purity.

Table II. Melting range test results of target compounds.

Replication	Melting range (°C)
1	72-74
2	72-73
3	72-74
Average	**72-74**

Product compound purity test: TLC-densitometry

TLC-densitometry analysis revealed three distinct peaks corresponding to the cinnamoyl chloride (**1a**), the tetrahydroisoquinoline (**2b**), and the target compound **3b** (**Figure 6**). The Rf values for these compounds were 0.57, 0.45, and 0.93, respectively. Notably, the chromatogram of the target compound lacked any peaks at the Rf values of **1a** and **2b**, confirming the successful formation of the desired product.

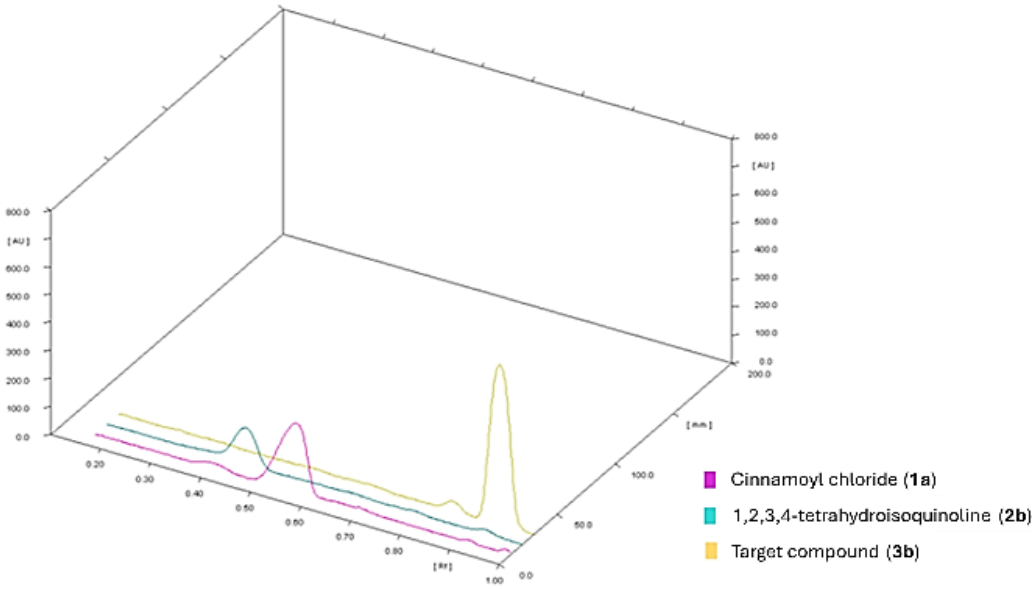


Figure 6. Spectra of compounds **1a**, **2b**, and target compound **3b** at a wavelength of 289 nm.

Target compound identification: FTIR

The synthesized target compound exhibited a distinct functional group compared to the starting compound, an amide group. Amide groups are characterized by their C=O stretching vibration observed at 1680-1630 cm^{-1} in FTIR spectra[26]. The FTIR spectrum of compound **3b** displayed an absorption peak at 1640.35 cm^{-1}, confirming the successful formation of the target product (**Figure 7** and **Table III**).

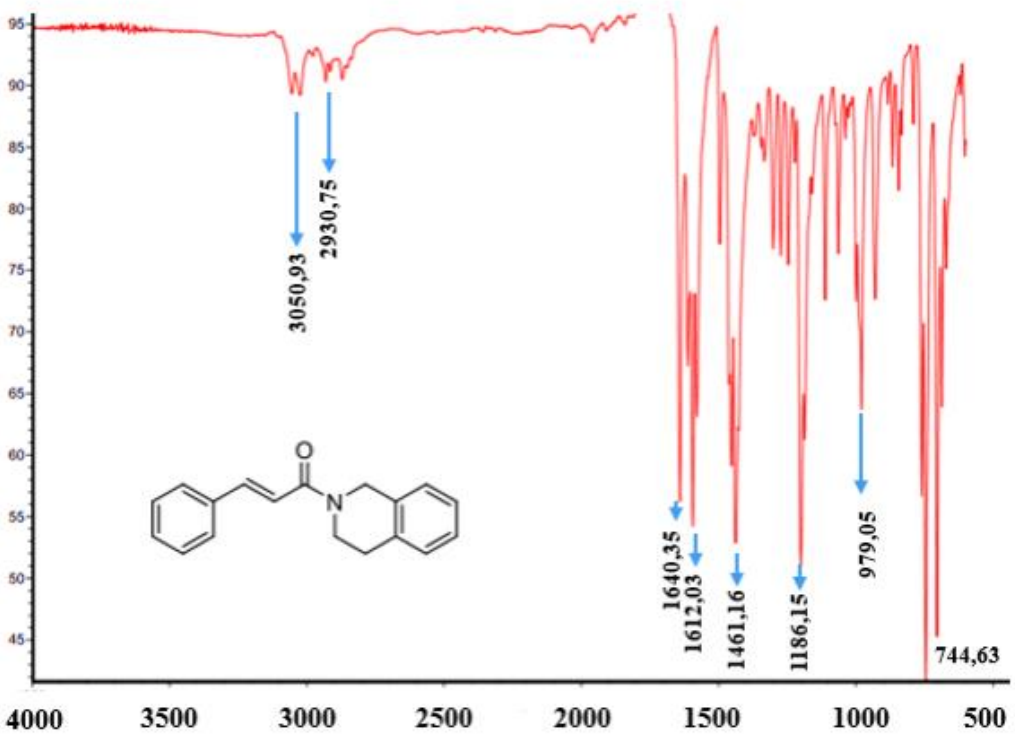


Figure 7. IR spectra of the target compound.

Table III. Interpretation of IR spectra of the target compound.

Functional group	Compound 3b Wavenumber (cm^{-1})	Theoretical Wavenumber (cm^{-1}) (9)
C-H aromatic	744.63	900-690
C-H alkene	979.05	1000-650
C-N	1186.15	1350-1000
C=C aromatic	1461.16	1600 and 1475
C=C	1612.03	1680-1600
C=O amide	1640.35	1680-1630
C-H	2930.75	3000-2850
C-H aromatic	3053.93	3150-3050

Target compound identification: ^{1}H-NMR

^{1}H-NMR spectroscopy provides valuable information regarding the hydrogen atoms present in a molecule[26]. The spectrum of compound **3b**, depicted in **Figure 8**, exhibits eight distinct peaks. Peaks a, b, and c, located at chemical shifts of 2.939 ppm, 3.892 ppm, and 4.832 ppm, respectively, correspond to the -CH_2- group within the piperidine ring. Peaks d and e, appearing at chemical shifts of 6.933 ppm and 7.690 ppm, are attributed to the =CH- group. Peaks f, g, and h, observed within the range of 7.197-7.537 ppm, represent hydrogen atoms from aromatic groups.

The integration ratio in ^{1}H-NMR analysis provides insight into the relative abundance of hydrogen atoms in a compound[27]. For compound **3b**, the integration ratio was determined to be 2 : 2 : 2 : 1 : 1 : 4 : 3 : 2 (**Table IV**). This ratio indicates a total of 17 hydrogen atoms, which aligns with the expected number of hydrogen atoms in compound **3b**. Therefore, the ^{1}H-NMR analysis confirms the identity of the isolated compound as compound **3b**.

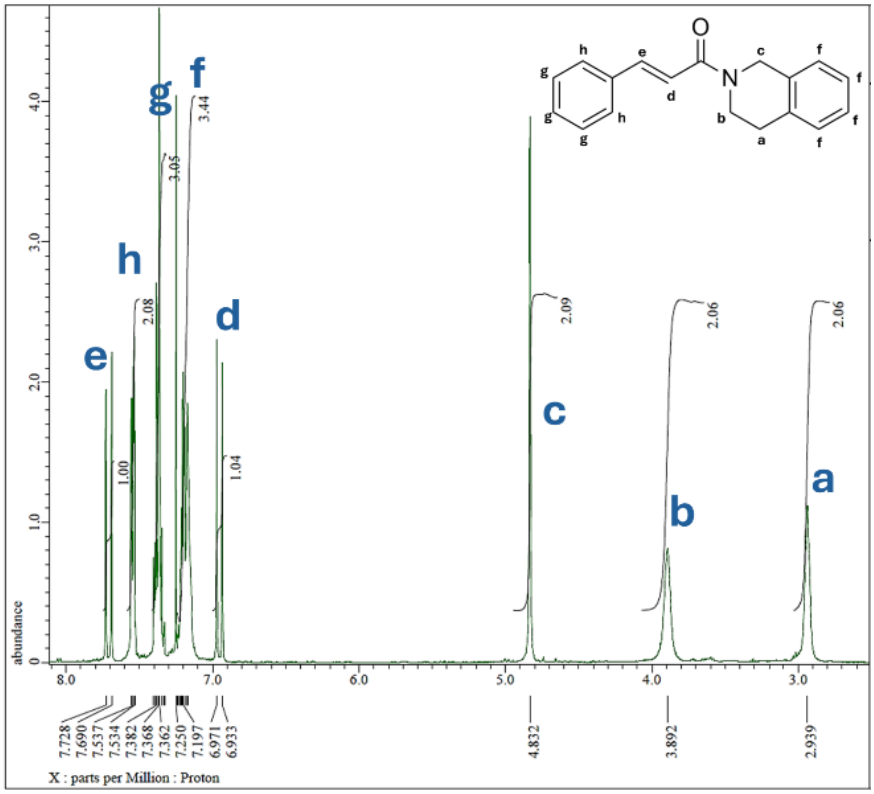


Figure 8. ^{1}H-NMR result of compound **3b**.

Table IV. Interpretation of ^{1}H-NMR spectra of the target compound.

Proton	Type of proton	Chemical shift (ppm)	Integration	Multiplicity
A	-CH_2-	2.94	2.06	Singlet
B	-CH_2-	3.89	2.06	Singlet
C	-CH_2-	4.83	2.09	Singlet
D	=CH-	6.93-6.97	1.04	Doublet
E	=CH-	7.69-7.73	1.00	Doublet
F	H aromatic	7.19-7.25	3.44	Multiplet
G	H aromatic	7.36-7.38	3.05	Multiplet
H	H aromatic	7.53-7.54	2.08	Multiplet

Antioxidant test using DPPH method

The DPPH free radical scavenging assay was employed to evaluate the antioxidant activity of the compounds. This method is based on the principle that antioxidants can donate electrons to neutralize free radicals, leading to a decrease in absorbance at 516 nm[28]. The % inhibition of DPPH serves as an indicator of antioxidant activity. Three concentrations of cinnamic acid (100, 500, and 1000 ppm) and compound **3b** were tested. The results, presented in **Table V**, demonstrate that compound **3b** exhibited significantly higher antioxidant activity compared to cinnamic acid[29], with % inhibition values of 10.61%, 24.24%, and 26.68% at 100, 500, and 1000 ppm, respectively, compared to cinnamic acid's 3.305%, 5.998%, and 6.936%. Statistical analysis using the T-test confirmed a significant difference in antioxidant activities between cinnamic acid and compound **3b**. This suggests that the structural modification involving the addition of the isoquinoline group enhances the antioxidant properties of the compound.

Table V. Compound concentration and antioxidant activity (% inhibition).

Compounds	Concentration (ppm)	% Inhibition ± SD (n=3)
Cinnamic acid	100	3.305 ± 0.245
Cinnamic acid	500	5.998 ± 0.648
Cinnamic acid	1000	6.936 ± 0.187
3b	100	10.61 ± 0.308
3b	500	24.24 ± 1.205
3b	1000	26.68 ± 0.648

CONCLUSION

This study successfully synthesized compound **3b** via a nucleophilic substitution reaction between compounds **1a** and **2b**, yielding an 81.56% yield. Subsequent evaluation of compound **3b's** antioxidant activity at concentrations of 100, 500, and 1000 ppm revealed significant antioxidant potential, with % inhibition values of 11% ± 0.308, 24% ± 1.205, and 27% ± 0.648, respectively. Notably, compound **3b** exhibited superior antioxidant activity compared to cinnamic acid at all tested concentrations. These findings highlight the potential of compound **3b** as a promising antioxidant agent.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Research Article

Affinity of Nintedanib Towards New Candidate Target for Idiopathic Pulmonary Fibrosis

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ADMET-SAR properties
COACH server-binding site predictor
Idiopathic pulmonary fibrosis
Nintedanib

Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive disease due to aggregation of fibroblasts on lung parenchyma. Nintedanib, an indolinone-derived tyrosine kinase inhibitor (TKi) has been approved for the treatment of IPF and it is a well-known inhibitor of platelet-derived growth factor (PDGF) receptor-α and -β, fibroblast growth factor (FGF) receptor-1–3 and vascular endothelial growth factor (VEGF) receptor-1–3. This study aims to evaluate the binding interaction between new therapeutic protein candidates for IPF such as autotaxin, galectin-3, interleukin-13, chitotriosidase-1, JNK, RhoE-ROCK-1, ROCK-2 against nintedanib. In this investigation we predicted, computed, and analyzed the binding interactions of the drug nintedanib using an *in silico* approach called molecular docking. Our docking studies demonstrated that RhoE-ROCK1 and autotaxin showed strong binding affinities towards nintedanib compared to known targets such as VEGFR2 and FGFR1. We can therefore hypothesize a further contribution of nintedanib to the improvement of pathology due to its affinity towards new targets in the pathogenesis of IPF. The next step will be to evaluate the effects of this affinity *in vitro* on specific cellular models.

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease characterized by the accumulation of fibrotic tissue, leading to impaired gas exchange and ultimately respiratory failure[1]. Despite ongoing research, the precise molecular mechanisms underlying IPF development remain elusive[2]. A variety of factors have been implicated in IPF pathogenesis, including cigarette smoking, viral infections, chronic aspiration, exposure to environmental pollutants, genetic predisposition, and certain drugs[3]. The histopathological hallmark of IPF is the presence of fibroblast foci, where myofibroblasts and excessive collagen deposition contribute to the distortion of lung architecture[1]. Alveolar macrophages play a pivotal role in initiating

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the fibrotic process by secreting proinflammatory and profibrotic cytokines that stimulate mesenchymal cell proliferation and collagen deposition[4,5].

Currently, only two drugs, pirfenidone[6] and nintedanib[7], are approved for IPF treatment. While these medications can slow disease progression as measured by forced vital capacity (FVC), they do not reverse the fibrotic tissue changes observed on high-resolution computed tomography (CT) scans. Consequently, they often fail to improve disease-related symptoms and quality of life for IPF patients[3]. Ongoing research efforts are focused on developing novel therapeutic approaches targeting various pathways involved in IPF pathogenesis. These include new drug delivery systems and molecules that act on distinct mechanisms[8,9].

Nintedanib, a tyrosine kinase inhibitor, targets platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) receptors, inhibiting their intracellular signaling by competitively binding to the ATP-binding site[10]. While nintedanib has demonstrated efficacy in treating idiopathic pulmonary fibrosis (IPF), this study aimed to explore its potential interactions with additional proteins implicated in IPF pathogenesis. Specifically, we investigated the binding affinity of nintedanib to RhoE-ROCK1, c-Jun N-terminal kinase (JNK), interleukin (IL)-13, human galectin-3 (Gal-3), autotaxin, human chitotrosidase-1 (CHIT-1) and the Rho-associated protein kinase-2 (ROCK2). These proteins have been implicated in the development or progression of IPF[8], suggesting that their modulation might offer therapeutic benefits.

Autotaxin, an enzyme responsible for lysophosphatidic acid (LPA) production, plays a crucial role in IPF by promoting fibroblast migration and inducing apoptosis of lung epithelial cells. Elevated levels of autotaxin and LPA have been observed in the bronchoalveolar lavage fluid and exhaled breath of IPF patients[11-14]. Targeting autotaxin could potentially mitigate the fibrotic process in IPF.

Galectin-3, a lectin involved in fibrosis, regulates the expression of transforming growth factor beta (TGF-β) receptors and has been found to be elevated in IPF patients[15]. The Rho-ROCK pathway, implicated in IPF fibrosis, is activated by oxidative stress and plays a role in regulating the cytoskeleton and cell motility[16]. Targeting the Rho-ROCK pathway has shown promise in reducing fibrosis in preclinical models[16-18].

Interleukin-13, a pro-fibrotic cytokine, is involved in asthma and has been associated with IPF pathogenesis[19-22]. While IL-13 inhibitors have shown efficacy in asthma, their effectiveness in IPF is limited. However, the combination of pirfenidone and lebrikizumab has demonstrated positive effects in reducing exacerbations in IPF patients[23].

Chitotrosidase-1, an enzyme involved in chitin degradation, has been implicated in fibrosis by modulating TGF-β and IL-13 signaling[24,25]. Targeting CHIT-1 has shown promise in reducing fibrosis in preclinical models[26]. Finally, the JNK pathway, involved in inflammation and fibrosis, is activated in IPF and has been shown to contribute to the fibrotic process[27,28]. JNK inhibitors have demonstrated efficacy in reducing fibrosis in preclinical models[29,30].

Existing literature lacks studies investigating the molecular interactions between nintedanib and ancillary proteins implicated in IPF. To address this knowledge gap, we employed molecular docking to predict and analyze the binding interactions of nintedanib with these proteins. By identifying potential alternative targets, this study aims to elucidate the mechanisms underlying nintedanib's efficacy and explore avenues for developing novel therapeutic strategies.

MATERIALS AND METHODS

Materials

The three-dimensional (3D) structures of target proteins, including RhoE-ROCK1 (PDB ID: 2V55), JNK (PDB ID: 3V6R), IL-13 (PDB ID: 4I77), human Gal-3 (PDB ID: 5H9P), autotaxin (PDB ID: 6W35), human CHIT-1 (PDB ID: 6Z8E), and ROCK2 (PDB ID: 7JNT), were retrieved from the Protein Data Bank (PDB). Additionally, the structures of VEGFR2 (PDB ID: 3C7Q) and chain A and B of FGFR1 (PDB ID: 1FGK) were obtained for use as positive controls, as nintedanib is known to bind these proteins. The 3D structure of the ligand nintedanib (PubChem CID: 135423438) was retrieved from the PubChem database. A summary of the software, web servers, and databases used in this study is provided in **Table I**.

Table I. The software, web servers, and databases used.

Software	link
Discovery Studio 2.5	Standalone version; Dassault Systèmes, Vélizy-Villacoublay, France
AutoDock 4.0	Standalone version; The Scripps Research Institute, La Jolla, CA, US
COACH Server	https://zhanggroup.org/COACH/
LigPlot+	https://www.ebi.ac.uk/thornton-srv/software/LigPlus/
Lipinski Filter	http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp
AdmetSAR server	http://lmmd.ecust.edu.cn/admetsar2
PubChem	https://pubchem.ncbi.nlm.nih.gov/
PDB	https://www.rcsb.org/

Methods

Preparation of receptor and ligand structures

Co-crystallized molecules and crystallographic water molecules were removed from the three-dimensional coordinate files using Discovery Studio Visualizer 2.5. This step was necessary to ensure that the protein structures were free of extraneous molecules for accurate docking calculations[31]. Subsequently, the protein structures were geometrically optimized using Discovery Studio Visualizer 2.5, employing a standard energy minimization protocol to alleviate steric clashes and bond distortions. The ligand molecule was also optimized using the same software and protocol.

Prediction of active sites

Accurate identification of a protein's active site is crucial in *de novo* drug design and molecular docking approaches. This knowledge facilitates the generation of near-native conformations for the receptor-ligand complex, ultimately contributing to the success of the docking study[32]. Here, the COACH server was employed to predict the active site amino acid residues for all target proteins[33]. Additionally, the active site residues of VEGFR2 and FGFR1 were retrieved from the literature for comparison.

Molecular docking analysis

A molecular docking study was performed using the AutoDock 4 software suite. AutoDock employs a Lamarckian genetic algorithm to predict the binding orientation and affinity of small molecules (ligands) to macromolecules (receptors)[34]. Ligand structures were prepared using Discovery Studio Visualizer 2.5. Polar hydrogen atoms were added, Kollman united atom type charges were assigned, fragmental volumes were calculated, and solvation parameters were set using AutoDockTools. These parameters were saved for subsequent docking simulations.

Binding site information for each ligand-receptor complex was identified using the COACH server and incorporated into AutoDock[33]. AutoGrid was utilized to generate a grid map encompassing the binding site residues of the receptor protein. This grid-based approach optimizes computational efficiency and improves docking accuracy. Lamarckian genetic algorithm searches were performed for each ligand-receptor complex using AutoDock. Each docking simulation yielded ten unique ligand conformations within the defined binding site.

Prediction of the physiochemical properties of the ligand

To assess the drug-like properties of nintedanib, we employed the admetSAR server and the Lipinski rule of five[28]. The Lipinski rule of five is a set of guidelines used to evaluate a ligand's potential for oral bioavailability. The admetSAR server provides a user-friendly interface to visualize various physiochemical and pharmacological properties of ligands relevant to drug discovery, including LogP (octanol-water partition coefficient), hydrogen bond donor and acceptor count, molecular weight, and rotatable bond count. These properties are crucial factors influencing a ligand's potential as a drug candidate. The admetSAR server was used to assess the combined effects of these properties, along with pharmacokinetics and pharmacodynamics, and generate a drug-likeness score.

Data analysis

The Discovery Studio Visualizer 2.5 was employed to visualize the interactions between the receptor and ligand within the protein-ligand complexes. LigPlot+, a web-based tool, was used to analyze the binding interaction profile in detail. This tool calculates the various non-covalent interactions (e.g., hydrogen bonds, hydrophobic interactions) established between the ligand and the receptor.

RESULTS AND DISCUSSION

Analysis of the active sites of the receptor

Amino acid residues within the active sites of proteins play crucial roles in ligand binding interactions. Understanding these interactions is vital for drug discovery and protein-ligand docking studies[35]. We employed the COACH server to predict potential ligand binding sites using the amino acid sequences and structural information of the receptor proteins. The results of these analyses are presented in **Tables II** to **VII**. Given the diverse methodologies employed by the various tools within the COACH server, we adopted a consensus approach to analyze the results. By considering the outputs from all tools, we aimed to identify consistent predictions regarding potential ligand binding sites. The consensus amino acid residues present in the binding site of the proteins were used for docking purposes. We used the method of directed docking by specifying the amino acid residues of the ligand proteins, by looking for amino acid residues that always appear in the results of each test parameter (COACH server, TM SITE, S-SITE, and COFACTOR). The results show that there are 8, 9, 7, 10, 9, and 8 consensus amino acids in CHIT-1, ROCK2, Gal-3, RhoE-ROCK1, JNK, and autotaxin, respectively.

Table II. COACH server results of human CHIT-1 (PDB ID: 6Z8E).

Parameter	Amino acid residues
COACH server	TYR27, PHE58, GLY98, TRP99, ASP138, GLU140, ALA183, MET210, TYR212, ASP213, TYR267, TRP358
TM SITE	TYR27, PHE58, GLY98, TRP99, ASP138, GLU140, ALA183, MET210, TYR212, ASP213, TYR267, ARG269, TRP358
S-SITE	TYR27, TRP31, PHE58, GLY98, TRP99, ASN100, ASP138, GLU140, TYR141, ALA183, MET210, TYR212, ASP213, TRP218, TYR267, ARG269, GLU297, MET300, MET356, TRP358, ALA359, LEU362
COFACTOR	TYR27, TRP31, PHE58, GLY98, TRP99, ASN100, PHE101, ASP138, GLU140, TYR141, ALA183, MET210, TYR212, ASP213, TYR267, ARG269, GLU297, MET300, TRP358, LEU362
Consensus binding site	TYR27, TRP99, ASP138, GLU140, MET210, TYR212, ASP213, TRP358

Table III. COACH server results of ROCK2 (PDB ID: 7JNT).

Parameter	Amino acid residues
COACH server	ILE98, GLY99, ARG100, GLY101, ALA102, VAL106, ALA119, LYS121, VAL153, MET169, GLU170, TYR171, MET172, PRO173, GLY175, ASP218, ASN219, LEU221, ALA231, ASP232
TM SITE	ILE98, GLY99, ARG100, GLY101, VAL106, ALA119, LYS121, GLU140, VAL153, MET169, GLU170, TYR171, MET172, PRO173, GLY175, ASP176, ASP218, LEU221, ALA231, ASP232
S-SITE	ILE98, GLY99, ARG100, GLY101, ALA102, PHE103, GLY104, VAL106, ALA119, LYS121, GLU140, VAL153, MET169, GLU170, TYR171, MET172, PRO173, GLY174, GLY175, ASP176, ASN179, ASP218, ASN219, LEU221, ALA231, ASP232
COFACTOR	ILE98, VAL106, ALA119, LYS121, VAL153, MET169, GLU170, TYR171, MET172, ASP218, ASN219, LEU221, ASP232, VAL372
Consensus binding site	ILE98, VAL106, ALA119, LYS121, VAL153, MET169, MET172, LEU221, ASP232

Table IV. COACH server results of Gal-3 (PDB ID: 5H9P).

Parameter	Amino acid residues
COACH server	HIS158, ASN160, ARG162, ASN174, TRP181, GLU184, ARG186
TM SITE	HIS158, ASN160, ARG162, VAL172, ASN174, TRP181, GLU184, ARG186
S-SITE	ARG144, ALA146, HIS158, ASN160, ARG162, GLU165, VAL172, ASN174, TRP181, GLU184, ARG186
COFACTOR	ARG144, HIS158, ASN160, ARG162, GLU165, ASN174, TRP181, GLU184, ARG186
Consensus binding site	HIS158, ASN160, ARG162, ASN174, TRP181, GLU184, ARG186

Table V. COACH server results of RhoE-ROCK1 (PDB ID: 2V55).

Parameter	Amino acid residues
COACH server	ILE82, GLY83, ARG84, GLY85, VAL90, ALA103, LYS105, VAL137, MET153, GLU154, TYR155, MET156, GLY159, ASP160, ASP202, ASN203, LEU205, ALA215, ASP216
TM SITE	LYS80, ILE82, GLY83, ARG84, GLY85, VAL90, ALA103, LYS105, MET153, GLU154, TYR155, MET156, PRO157, GLY159, ASP160, ASP202, ASN203, LEU205, ALA215, ASP216
S-SITE	ILE82, GLY83, ARG84, GLY85, ALA86, PHE87, GLY88, VAL90, ALA103, LYS105, VAL137, MET153, GLU154, TYR155, MET156, PRO157, GLY158, GLY159, ASP160, ASN163, ASP202, ASN203, LEU205, ALA215, ASP216
COFACTOR	ILE82, GLY83, ARG84, GLY85, GLY88, GLU89, VAL90, ALA103, LYS105, VAL137, MET153, GLU154, MET156, ASP202, LEU205, ALA215, ASP216
Consensus binding site	ILE82, GLY83, GLY85, VAL90,ALA103, LYS105, MET153, MET156, LEU205, ALA215, ASP216

Table VI. COACH server results of JNK (PDB ID: 3V6R).

Parameter	Amino acid residues
COACH server	ILE70, GLY71, SER72, VAL78, ALA91, LYS93, ILE124, MET146, GLU147, LEU148, MET149, ASP150, ALA151, ASN152, SER193, ASN194, VAL196, LEU206, ASP207
TM SITE	ILE70, GLY71, VAL78, ALA91, LYS93, ILE124, MET146, GLU147, LEU148, MET149, ASP150, ALA151, ASN152, SER193, ASN194, VAL196, LEU206, ASP207
S-SITE	ILE70, GLY71, SER72, GLY73, GLN75, GLY76, VAL78, ALA91, LYS93, GLJU111, MET115, ILE124, LEU144, MET146, GLU147, LEU148, MET149, ASP150, ALA151, ASN152, GLN155, SER193, ASN194, VAL196, LEU206, ASP207, PHE208
COFACTOR	VAL78, ALA91, LYS93, ARG107, GLU111, ILE124, LEU144, MET146, MET149, ASP150, ALA151, GLN155, LEU206, ASP207
Consensus binding site	VAL78, ALA91, LYS93, ILE124, MET149, ASP150, ALA151, LEU206, ASP207

Table VII. COACH server results of autotaxin (PDB ID: 6W35).

Parameter	Amino acid residues
COACH server	ILE168, ASP172, LYS209, THR210, PHE211, LEU217, ALA218, ASN231, PHE274, PHE275, TYR307, ASP312, HIS316, HIS475, MET513
TM SITE	ILE168, ASP172, LYS209, THR210, PHE211, LEU217, ALA218, ASN231, PHE274, PHE275, TYR307, ASP312, HIS316, HIS475, MET513
S-SITE	ILE168, SER170, ASP172, LYS209, THR210, PHE211, LEU214, LEU217, ALA218, ASN231, LEU244, TRP255, TRP261, PHE274, PHE275, ALA305, TYR307, ASP312, HIS316, ASP359, HIS360, HIS475, MET513
COFACTOR	ILE168, SER170, THR210, PHE211, LEU214, ALA218, ILE228, ASN231, LEU244, TRP255, TRP261, PHE274, ALA305, TYR307, ASP312, HIS316, HIS475
Consensus binding site	THR210, PHE211, ALA218, ASN231, TYR307, ASP312, HS316, HIS475

Molecular docking simulations (Site-specific and blind docking)

Molecular docking simulations were conducted using AutoDock 4.0 to predict the binding interactions between nintedanib and the target proteins. AutoDock 4 employs a genetic algorithm for docking simulations, generating a set of potential protein-ligand complex conformations. These conformations were ranked based on their binding free energy values, with those exhibiting the most favorable binding energies selected for further analysis (**Table VIII**). Given the known amino acid residues involved in nintedanib binding, site-directed docking was employed for these proteins. For other proteins where binding site information was unavailable, both blind and site-specific docking simulations were performed.

For each receptor protein, two sets of docking experiments were performed: site-specific (directed) and blind docking. Directed docking utilized previously identified binding site information to constrain the ligand's search space. In contrast, blind docking allowed the ligand to explore the entire receptor protein surface to identify the most favorable binding site. This dual approach provided a more comprehensive assessment of potential binding interactions[36].

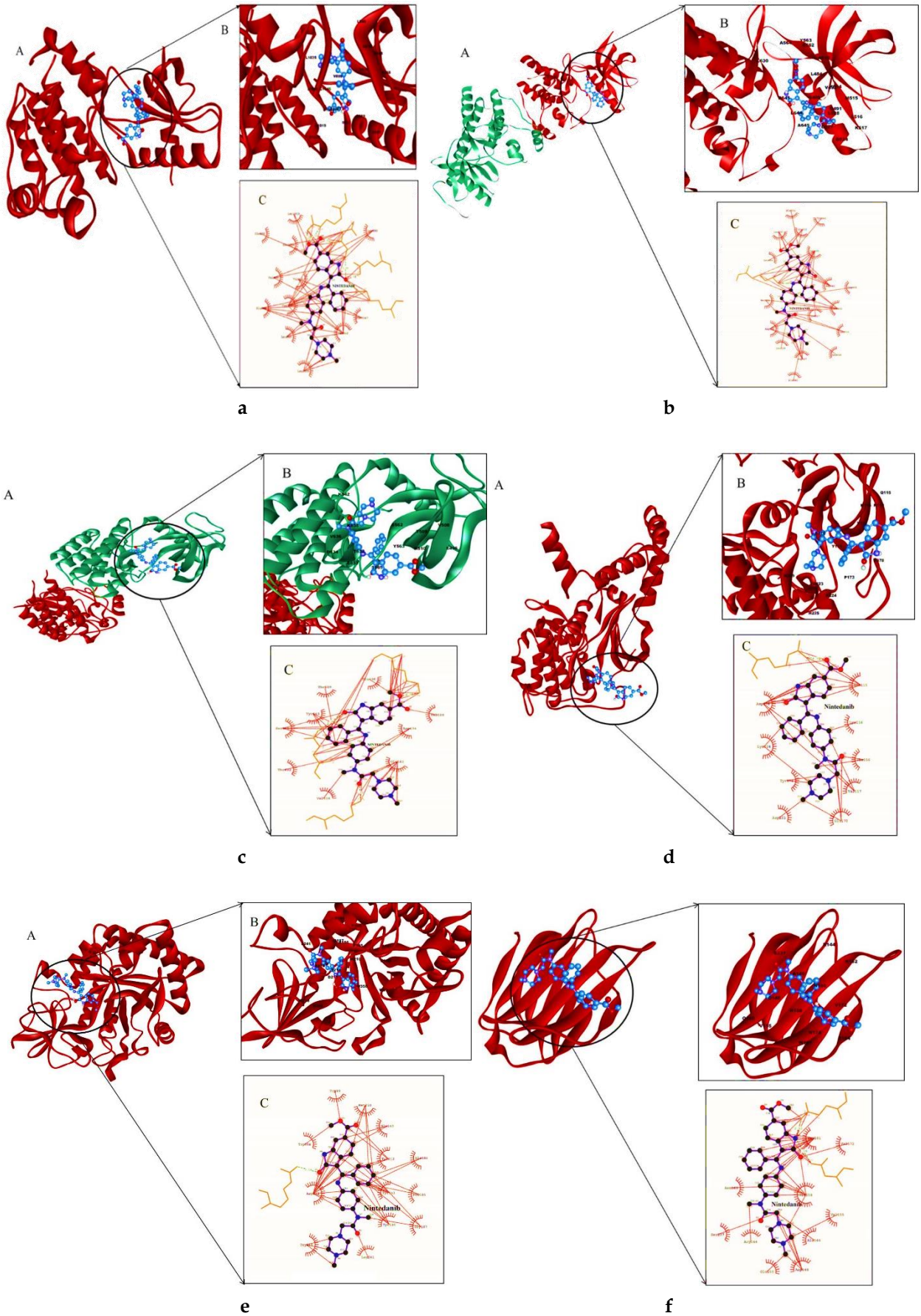
The binding free energy values obtained from both docking methods were analyzed to identify the most promising binding conformations. LigPlot+ was used to visualize the binding interactions between the ligand and the receptor proteins. While the COACH server could not identify specific binding sites for IL-13, we conducted blind docking simulations for this molecule. As nintedanib's binding sites on VEGFR2 and FGFR1 are well-established, control experiments did not involve blind docking for these receptors. Our prime focus in this study is to check the interaction pattern of the Nintedanib with the following proteins RhoE-ROCK1, JNK, IL-13, Gal-3, autotaxin, CHIT-1, and ROCK2, therefore the co-crystal/reference ligands were removed from their structures. While nintedanib displayed favorable binding affinities for all target proteins, RhoE-ROCK1, CHIT-1, and JNK emerged as the most promising targets based on their binding free energy values. As detailed in **Table IX**, we employed the following docking protocols for each protein. The binding interactions between nintedanib and these receptor proteins are depicted in **Figures 1** and **2**.

Table VIII. Binding free energy from docking results.

Receptors	Binding free energy (kcal/mol)	
	Site-specific docking	Blind docking
VEGFR2	-9.27	-
FGFR1 (ChainA)	-8.74	-
FGFR1 (ChainB)	-6.17	-
Autotaxin (6W35)	-11.45	-5.00
RhoE-ROCK1 (2V55)	-11.57	-8.26
JNK (3V6R)	-8.60	-6.87
CHIT-1 (6Z8E)	-8.30	-8.98
ROCK2 (7JNT)	-5.45	-8.90
Gal-3 (5H9P)	-4.69	-6.49
IL-13 (4I77)	-	-5.46

Table IX. Binding free energy from docking results.

Protein ID	Parameters	Site-specific docking	Blind docking
2V55	Grid Points(user specific)	X-points: 58	X-points: 126
		Y-points: 52	Y-points: 126
		Z-points: 82	Z-points: 126
	Central Grid point of maps	(-15.787, 46.861, 34.694)	(-31.351, 48.495, 19.430)
	Macromolecule file in Grid maps	2V55NEW.pdbqt	2V55.pdbqt
	Grid parameter file	2V55.gpf	2V55.gpf
	Minimum Coordinates in grids	(-27.155, 36.669, 18.622)	(-82.948, -3.102, -32.167)
	Maximum Coordinates in grids	(-4.419, 57.053, 50.766)	(20.246,100.092, 71.027)
3V6R	Grid Points (user specific)	X-points: 74	X-points: 126
		Y-points: 46	Y-points: 126
		Z-points: 98	Z-points: 126
	Central Grid point of maps	(16.554, -15.429, -23.232)	(21.633, -22.936, -18.874)
	Macromolecule file in Grid maps	3V6Rnew.pdbqt	3V6Rnew.pdbqt
	Grid parameter file	3V6R.gpf	3V6R.gpf
	Minimum Coordinates in grids	(2.679, -24.054, -41.607)	(-14.970, -59.539, -55.477)
6Z8E	Grid Points (user specific)	X-points: 60	X-points: 126
		Y-points: 30	Y-points: 126
		Z-points: 26	Z-points: 126
	Central Grid point of maps	(8.954, -2.708, -10.629)	(14.600, -4.887, -10.506)
	Macromolecule file in Grid maps	6Z8ENEW.pdbqt	6Z8E.pdbqt
	Grid parameter file	6Z8E.gpf	6Z8E.gpf
	Minimum Coordinates in grids	(-14.626, -14.498, -20.847)	(-16.018,-35.505, -41.124)
7JNT	Grid Points (user specific)	X-points: 28	X-points: 126
		Y-points: 28	Y-points: 126
		Z-points: 22	Z-points: 126
	Central Grid point of maps	(48.025, 60.570, 42.725)	(46.477, 73.347, 44.105)
	Macromolecule file in Grid maps	7JNTNEW.pdbqt	7JNT.pdbqt
	Grid parameter file	7JNT.gpf	7JNT.gpf
	Minimum Coordinates in grids	(34.025, 46.570, 31.725)	(6.409, 36.459, 4.037)
5H9P	Grid Points (user specific)	X-points: 48	X-points: 110
		Y-points: 36	Y-points: 120
		Z-points: 54	Z-points: 116
	Central Grid point of maps	(-19.226, 4.508, 1.724)	(-13.132, -0.460, 6.424)
	Macromolecule file in Grid maps	5H9Pnew.pdbqt	5H9P.pdbqt
	Grid parameter file	5H9P.gpf	5H9P.gpf
	Minimum Coordinates in grids	(-29.162, -2.944, -9.454)	(-33.757, -22.960, -15.326)
6W35	Grid Points (user specific)	X-points: 64	X-points: 126
		Y-points: 56	Y-points: 126
		Z-points: 80	Z-points: 126
	Central Grid point of maps	(16.142, 3.060, 20.584)	(-4.699, 3.083, 17.187)
	Macromolecule file in Grid maps	6W35new.pdbqt	6w351.pdbqt
	Grid parameter file	6W35.gpf	6w351.gpf
	Minimum Coordinates in grids	(4.142, -7.440, 5.584)	(-66.124, -58.342, -44.238)
4I77	Grid Points (user specific)	Not performed as no data for the binding sites were found from the COACH server	X-points: 126
			Y-points: 90
			Z-points: 100
	Central Grid point of maps		(0.775, 31.603, -24.165)
	Macromolecule file in Grid maps		4I771.pdbqt
	Grid parameter file		4I771.gpf
	Minimum Coordinates in grids		(-62.225, -13.397, -74.165)
1FGK (Chain-A)	Grid Points (user specific)	X-points: 74	Not performed
		Y-points: 82	
		Z-points: 126	
	Central Grid point of maps	(3.900, 6.729, 17.631)	
	Macromolecule file in Grid maps	FGK1.pdbqt	
	Grid parameter file	FGK1.gpf	
	Minimum Coordinates in grids	(-6.941, -5.284, -0.828)	
1FGK (Chain-B)	Grid Points (user specific)	X-points: 60	Not performed
		Y-points: 114	
		Z-points: 102	
	Central Grid point of maps	(70.806, 4.003, 15.899)	
	Macromolecule file in Grid maps	FGK1.pdbqt	
	Grid parameter file	FGK1.gpf	
	Minimum Coordinates in grids	(62.016, -12.698, 0.956)	
3C7Q	Grid Points (user specific)	X-points: 94	Not performed
		Y-points: 126	
		Z-points: 82	
	Central Grid point of maps	(20.635, 59.454, 37.551)	
	Macromolecule file in Grid maps	VEGFR2.pdbqt	
	Grid parameter file	VEFGFR2.gpf	
	Minimum Coordinates in grids	(8.509, 43.200, 26.973)	


A
B
C
a
b
c
d
e
f
Nintedanib

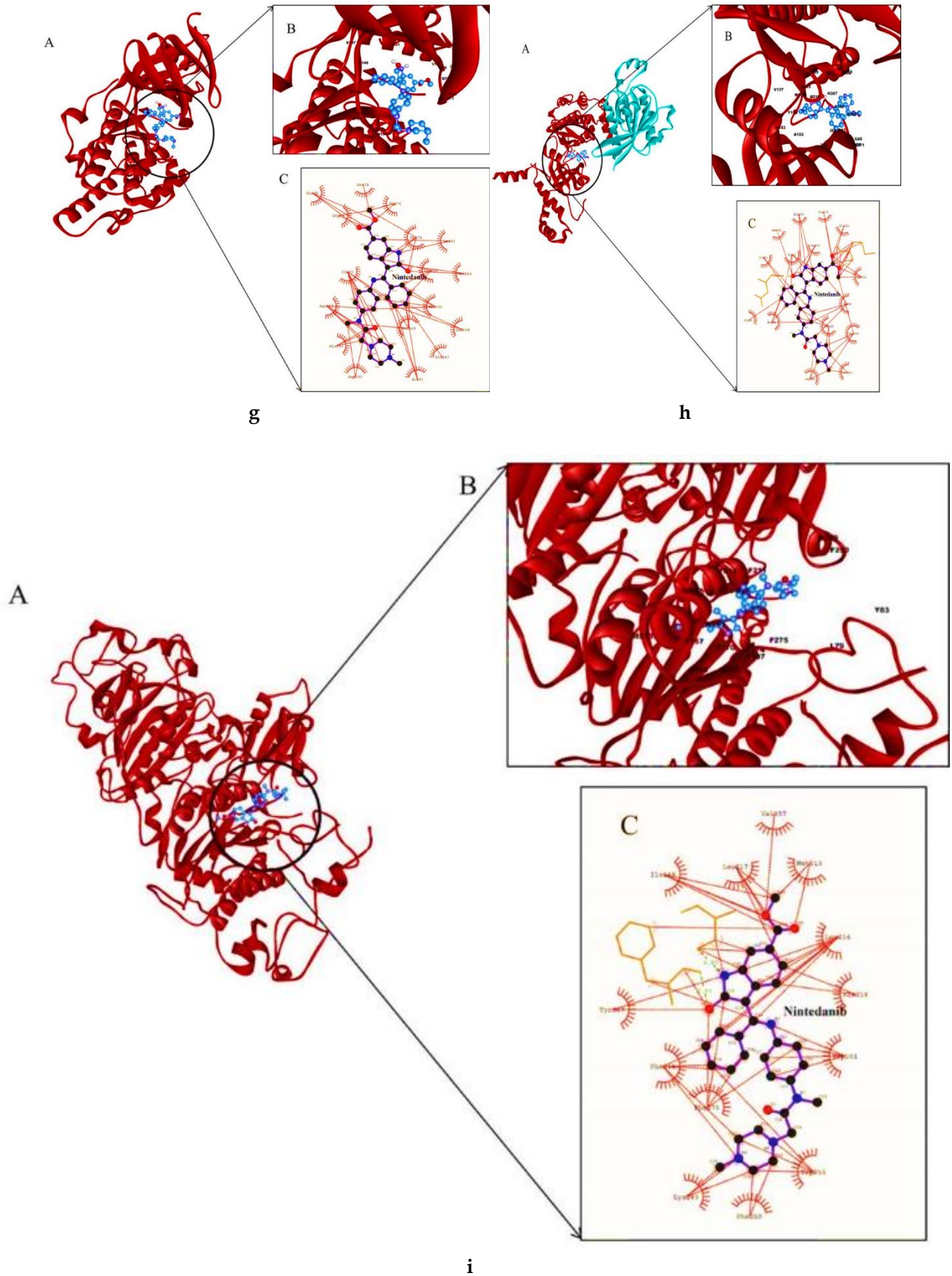
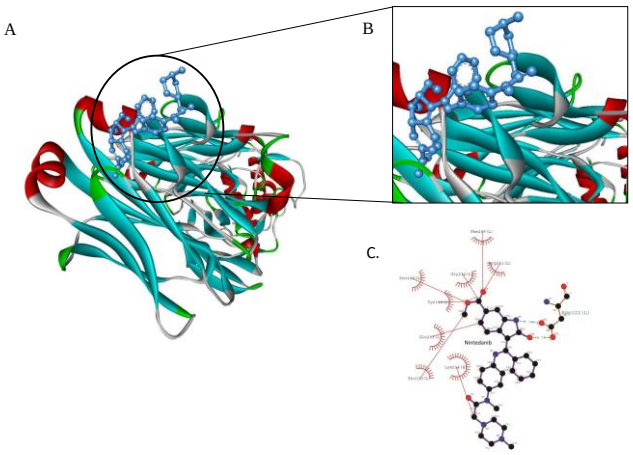
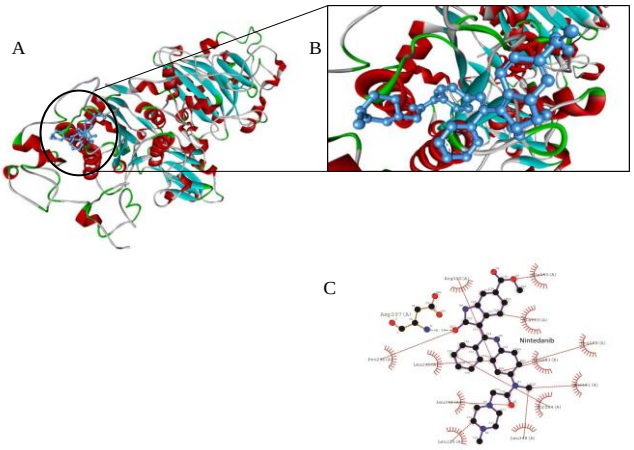


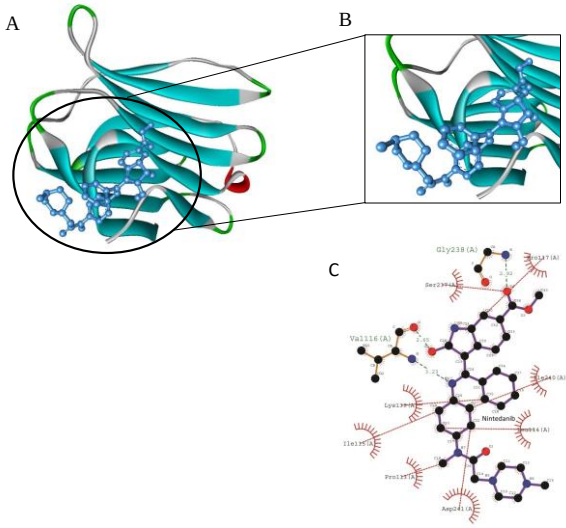
Figure 1. The binding interactions between the ligand and the different protein receptors (site-specific) of **a**: VEGFR2; **b**: FGFR1 Chain-A; **c**: FGFR1 Chain-B; **d**: ROCK2; **e**: CHIT-1; **f**: Gal-3; **g**: JNK; **h**: RhoE-ROCK1; and **i**: autotaxin. In each panel, **A-** represents the binding of the ligand with the receptor protein (the receptor protein is represented in the cartoon. The ligand is presented in ball stick model). **B-** represents the amino acid residues of the receptor protein binding with the ligand. **C-** represents the various types of non-covalent interactions during the binding of the ligand with the receptor.


A
B
C.
Nintedanib

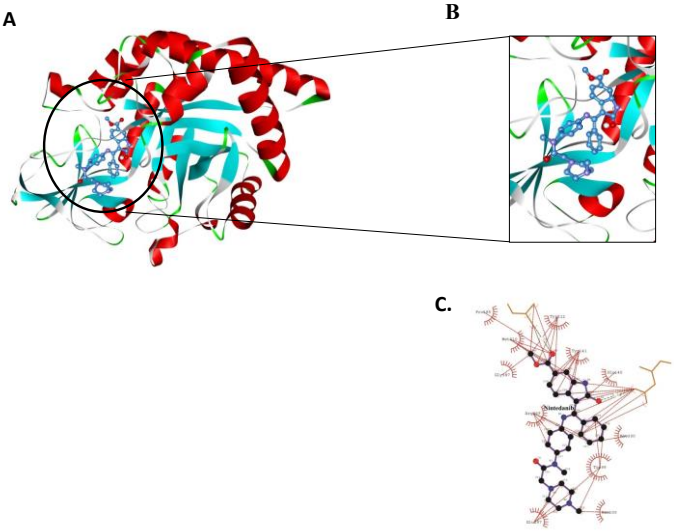
a


A
B
C
Nintedanib

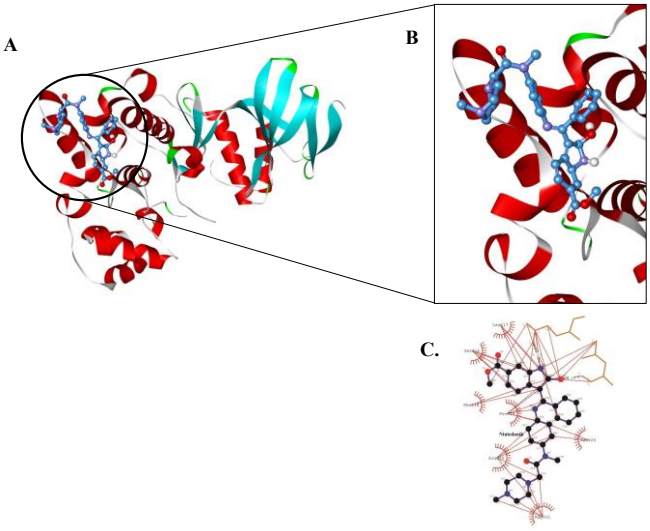
b


A
B
C
Nintedanib

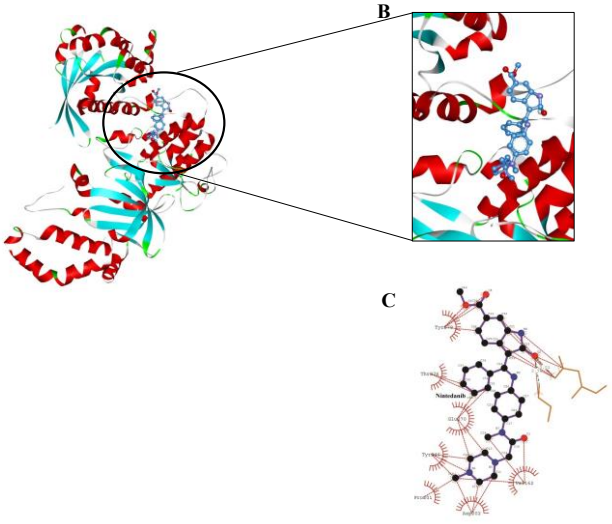
c


A
B
C.
Nintedanib

d


A
B
C.
Nintedanib

e


B
C
Nintedanib

f

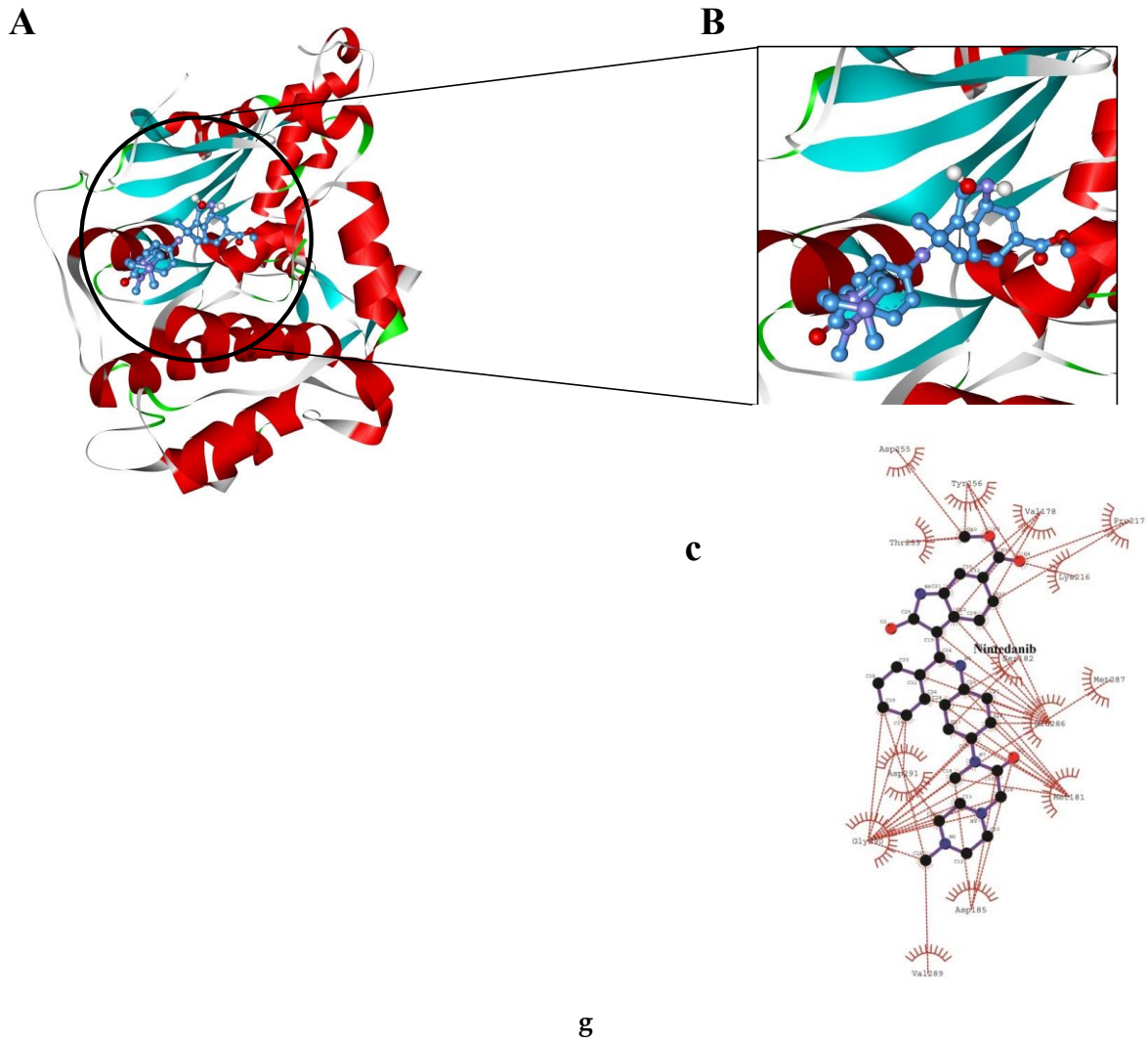


Figure 2. The binding interactions between the ligand and the different protein receptors (blind docking) of **a**: IL-13; **b**: autotaxin; **c**: Gal-3; **d**: CHIT-1; **e**: JNK; **f**: RhoE-ROCK1; and **g**: ROCK2. In each panel, **A-** represents the binding of the ligand with the receptor protein (the receptor protein is represented in the cartoon. The ligand is presented in ball stick model). **B-** represents the amino acid residues of the receptor protein binding with the ligand. **C-** represents the various types of non-covalent interactions during the binding of the ligand with the receptor.

Physiochemical and pharmacodynamics properties of the ligand

We conducted a comprehensive ADMET (absorption, distribution, metabolism, excretion, and toxicity) profile analysis for the ligand molecule (**Table X**). The ADMET study was unable to be conducted using other web servers due to limitations. SwissADME could not analyze nintedanib due to the length of its SMILES character string (exceeding 200 characters). Additionally, pkCSM was undergoing maintenance during the time of this study. Additionally, the Lipinski rule of five was applied to assess the ligand's drug-likeness properties (**Table XI**). These results indicate that nintedanib is most likely absorbed from the human intestine, has low oral bioavailability, may inhibit and be a substrate for P-glycoprotein, and is moderately toxic when administered orally in a single dose. The results indicate that the ligand possesses favorable characteristics for drug development, suggesting its potential as a promising therapeutic candidate. These results are relevant as reported by Wind *et al*.[7] previously.

Table X. List of drug-likeliness properties of nintedanib.

Parameters	Values
Molecular mass (Da)	541
Hydrogen bond donor	2
Hydrogen bond acceptors	9
LogP	2.2736
Molar refractivity (m^3/mol)	155.623642

Table XI. ADMET profile of nintedanib.

Parameters	Values
Acute oral toxicity (class)	III
Blood brain barrier	-
Human intestinal absorption	+
Human oral bioavailability	-
P-glycoprotein inhibitor	+
P-glycoprotein substrate	+

CONCLUSION

This study investigated the binding interactions of nintedanib with several ancillary proteins implicated in the pathogenesis of IPF, including RhoE-ROCK1, JNK, IL-13, Gal-3, autotaxin, and CHIT-1. Nintedanib is a well-established inhibitor of VEGFR2, PDGF, and FGFR1, which are key drivers of IPF. Our molecular docking simulations revealed that nintedanib exhibits strong binding affinities to both autotaxin and RhoE-ROCK1, comparable to its interactions with VEGFR2 and FGFR1. These findings suggest that nintedanib might exert additional beneficial effects in IPF treatment by targeting these alternative pathways. However, further in vitro studies are warranted to validate the functional implications of these binding interactions and elucidate the precise mechanisms through which nintedanib could influence IPF progression.

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Validation: Angshuman Bagchi
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Writing - original draft: Angshuman Bagchi, Luigia Grazia Fresu, Maria Talmon
Writing - review & editing: Hari Baskar Balasubramanian, Angshuman Bagchi, Maria Talmon

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Research Article

Thiourea Derivatives as Estrogen Receptor Alpha Inhibitors for Breast Cancer Therapy: An *In Silico* Evaluation with ADMET Prediction and Molecular Docking

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Abstract

Breast cancer remains a significant public health concern, necessitating the discovery of novel therapeutic agents. This study investigates the potential of thiourea derivatives, specifically HU, HTMX, and BMPTU compounds, as estrogen receptor alpha (ERα) inhibitors using computational approaches. Drug-likeness assessments using Lipinski's Ro5 confirmed the oral bioavailability of all compounds. Additionally, ADMET analysis indicated favorable pharmacokinetic properties, with minimal metabolic interactions and acceptable safety profiles, except for BMPTU2, which showed potential hepatotoxicity. Molecular docking simulations revealed strong binding affinities between BMPTU derivatives, particularly BMPTU2, BMPTU3, and BMPTU4, and key ERα residues. These interactions suggest their potential as ERα modulators, warranting further *in silico* and experimental validation. In conclusion, the findings highlight the potential of BMPTU derivatives, especially BMPTU2, BMPTU3, and BMPTU4, as promising lead compounds for developing novel ERα-targeted breast cancer therapies. Further optimization and validation are crucial to fully elucidate their therapeutic potential.

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INTRODUCTION

Breast cancer remains a significant global health burden, with millions of cases diagnosed annually and substantial mortality rates[1]. Despite advancements in conventional treatments like surgery, chemotherapy, and radiation therapy, drug resistance and adverse effects continue to hinder optimal patient outcomes[2]. The urgent need for novel therapeutic agents with enhanced efficacy and safety profiles has spurred research into compounds targeting specific molecular pathways involved in breast cancer progression[3]. Such targeted therapies hold the promise of improving treatment outcomes and potentially extending survival rates[4].

Thiourea derivatives have emerged as a promising class of compounds with diverse pharmacological activities, including anticancer potential[5-8]. These compounds have been shown to inhibit key enzymes, induce apoptosis, and modulate cellular signaling pathways, suggesting their potential as anticancer agents[9]. Their ability to interact with biological macromolecules further underscores their versatility in drug development, prompting further investigation into their therapeutic applications, particularly in the context of breast cancer[10].

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Estrogen receptor alpha (ERα) plays a pivotal role in the pathogenesis of hormone-sensitive breast cancers, making it a prime therapeutic target[11]. The ERα activation by estrogen triggers downstream signaling cascades that promote tumor growth, survival, and metastasis[12]. Consequently, inhibiting ERα signaling has emerged as a promising approach for treating ER-positive breast cancers, a significant subset of breast cancer cases[13].

Thiourea derivatives have demonstrated potential as ERα inhibitors, disrupting estrogen-mediated signaling pathways and attenuating tumor growth in preclinical studies[14]. This evidence underscores their potential as targeted agents for combating hormone-driven breast cancers. Thiourea derivatives have emerged as promising scaffolds for drug discovery, with N-benzoyl-N'-methoxyphenilthiourea (BMPTU) serving as a lead compound. This study explores the structural modification of BMPTU's aromatic ring, guided by the Topliss approach, to optimize its physicochemical properties as defined by the Hansch model[15].

Computational methods have revolutionized drug discovery by enabling efficient identification and optimization of potential drug candidates[16]. *In silico* techniques, such as molecular docking and absorption, distribution, metabolism, excretion, and toxicity (ADMET) prediction, offer valuable insights into drug-target interactions, pharmacokinetics, and toxicology[7]. By leveraging these computational tools, researchers can streamline drug development, reduce costs, and accelerate innovation[17,18]. Furthermore, *in silico* methods facilitate the exploration of chemical space, guiding the rational design of novel compounds with improved potency, selectivity, and bioavailability[19,20].

This study aims to harness the potential of *in silico* methods to identify novel thiourea derivatives with optimized pharmacological profiles for the treatment of breast cancer. Through integrating computational and experimental techniques, this research seeks to elucidate the therapeutic potential of thiourea derivatives as anti-breast cancer agents. By employing *in silico* docking studies, we aim to elucidate the molecular interactions between thiourea derivatives and ERα, providing mechanistic insights into their inhibitory effects. Additionally, ADMET predictions will help evaluate the pharmacokinetic properties and safety profiles of the identified compounds, guiding subsequent experimental validation. Ultimately, this interdisciplinary approach aims to accelerate the discovery and development of novel therapeutics for breast cancer, offering new hope for patients and advancing the paradigm of personalized medicine.

MATERIALS AND METHODS

Materials

All calculations and visualizations were performed on a laptop equipped with an AMD A6-7310 quad-core processor (2.00 GHz), 6 GB RAM, and a Windows 10 Pro (64-bit) operating system. The following software packages were utilized: Chem Bio Draw Ultra version 12 (PerkinElmer, Inc.), Chem Bio 3D Ultra version 12 (PerkinElmer, Inc.), Molegro Virtual Docker 5.5 (CLC bio), and pkCSM (https://biosig.unimelb.edu.au/pkCSM/prediction). Protein structures were retrieved from the Protein Data Bank (https://www.rcsb.org). As shown in **Table I**, hydroxyurea (HU), 4-hydroxytamoxifen (HTMX), and a BMPTU derivative were employed as chemical structures in this study. Hydroxyurea served as a positive control, while HTMX acted as both a positive control and an internal ligand within the PDB file[21].

Methods

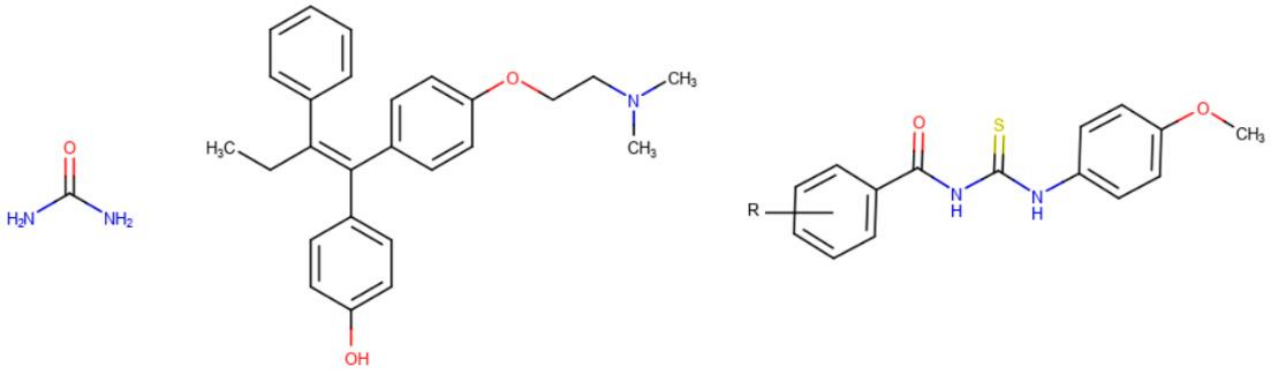
The ADMET properties of BMPTU derivatives were predicted using the pkCSM. The 3D structure of the ERα protein was obtained from the Protein Data Bank (PDB ID: 3ERT)[21,22]. This protein was selected due to its high resolution (1.9 Å), and the ligand HTMX was used to identify the receptor's binding site. The BMPTU derivative was constructed using Chem Bio Draw Ultra version 12 and energy-minimized using Chem Bio 3D Ultra Version 12 with the Merck Molecular Force Field 94 (MMFF94) method. Molecular docking was performed using Molegro Virtual Docker 5.5, with BMPTU docked in the same position as HTMX (x = 34.21; y = -2.42; z = 20.71; Radius 13) using the MolDock Score (GRID) scoring function and MolDock SE search algorithm.

Data analysis

Molecular docking simulations were performed to evaluate the binding affinity between the identified ligands and the target proteins. The docking results were ranked based on the calculated rerank score (RS), a measure of the binding energy

between the ligand and receptor. A lower RS value indicates a stronger predicted binding affinity, suggesting a greater potential for the compound to exhibit anticancer activity[23].

Table I. Chemical structures of HU, HTMX, and BMPTU derivative compounds.



HU **HTMX** **BMPTU**

No	R	Compound name	Compound code
1	-	Hydroxyurea	HU
2	-	4-hydroxytamoxifen	HTMX
3	H	N-benzoyl-N′-4-methoxyphenylthiourea	BMPTU1
4	4-NO_2	N-4-nitronbenzoyl-N′-4-methoxyphenylthiourea	BMPTU2
5	4-CN	N-4-cyanobenzoyl-N′-4-methoxyphenylthiourea	BMPTU3
6	3-NO_2	N-3-nitrobenzoyl-N′-4-methoxyphenylthiourea	BMPTU4
7	4-OCH_3	N-4-methoxybenzoyl-N′-4-methoxyphenylthiourea	BMPTU5
8	3,4-diCl	N-3,4-dichlorobenzoyl-N′-4-methoxyphenylthiourea	BMPTU6
9	2,4-diCl	N-2,4-dichlorobenzoyl-N′-4-methoxyphenylthiourea	BMPTU7
10	3-CF_3	N-3-trifluoromethylbenzoyl-N′-4-methoxyphenylthiourea	BMPTU8
11	4-CF_3	N-4-trifluoromethylbenzoyl-N′-4-methoxyphenylthiourea	BMPTU9
12	4-Br	N-4-bromobenzoyl-N′-4-methoxyphenylthiourea	BMPTU10
13	4-Cl	N-4-chlorobenzoyl-N′-4-methoxyphenylthiourea	BMPTU11
14	2-CH_3	N-2-methoxybenzoyl-N′-4-methoxyphenylthiourea	BMPTU12
15	2-Cl	N-2-chlorobenzoyl-N′-4-methoxyphenylthiourea	BMPTU13
16	2-OCH_3	N-2-methoxybenzoyl-N′-4-methoxyphenylthiourea	BMPTU14
17	4-$C(CH_3)_3$	N-4-tertiarybutylbenzoyl-N′-4-methoxyphenylthiourea	BMPTU15
18	4-NH_2	N-4-aminobenzoyl-N′-4-methoxyphenylthiourea	BMPTU16
19	3-Cl	N-3-chlorobenzoyl-N′-4-methoxyphenylthiourea	BMPTU17
20	4-CH_3	N-4-methylbenzoyl-N′-4-methoxyphenylthiourea	BMPTU18
21	4-OH	N-4-hidroxybenzoyl-N′-4-methoxyphenylthiourea	BMPTU19
22	4-F	N-4-fluorobenzoyl-N′-4-methoxyphenylthiourea	BMPTU20

RESULTS AND DISCUSSION

Drug-likeness

All compounds adhered to Lipinski's Rule of Five (Ro5), suggesting favorable oral bioavailability (**Table II**)[24]. Hydroxyurea, with its low molecular weight and favorable hydrogen bond donor/acceptor balance, is a promising candidate for oral administration. Its negative log P value indicates hydrophilicity. 4-hydroxytamoxifen, a known estrogen receptor modulator, also meets Ro5 but exhibits moderate lipophilicity (log P slightly exceeding 5), potentially influencing its distribution[25]. All BMPTU2, BMPTU3, and BMPTU4 demonstrated balanced lipophilicity with log P values around 2.7-2.9, despite slightly higher hydrogen bond acceptor counts. BMPTU5, BMPTU6, and BMPTU7 exhibited moderate lipophilicity (log P around 3.0-4.1), suggesting a good balance of hydrophilic and lipophilic properties. All BMPTU10, BMPTU11, and BMPTU13 showed similar moderate lipophilicity (log P around 3.5-3.8). In contrast, BMPTU15, BMPTU16, and BMPTU17 displayed higher hydrophilicity (log P around 0.1-0.4), which might affect membrane permeability but could favor solubility.

Drug development is a lengthy and costly process, with a high failure rate often attributed to the inability to therapeutically modulate drug targets[26]. Lipinski's Rule of Five, introduced in 1997, provides a set of guidelines for assessing the drug-likeness of compounds based on their physicochemical properties[27]. The Ro5 criteria stipulate that orally active drugs

typically possess a molecular weight below 500 Da, no more than five hydrogen bond donors and ten hydrogen bond acceptors, and a log P value less than or equal to 5[28]. By applying the Ro5, researchers can identify and eliminate compounds with low likelihood of success in clinical trials, thereby improving drug development efficiency and reducing costs[29].

Table II. The drug-likeness analysis using Lipinski's Ro5.

Compound code	Molecular weight	Hydrogen bond acceptors	Hydrogen bond donors	Log P	Lipinski's Ro5
	≤500 DA	≤10	≤5	≤5	
HU	76.055	2	3	-0.956	Yes
HTMX	371.524	2	0	5.996	Yes
BMPTU1	286.386	3	2	2.822	Yes
BMPTU2	331.351	5	2	2.730	Yes
BMPTU3	311.266	4	2	2.694	Yes
BMPTU4	331.353	5	2	2.730	Yes
BMPTU5	316.238	4	2	2.831	Yes
BMPTU6	355.246	3	2	4.129	Yes
BMPTU7	355.246	3	2	4.129	Yes
BMPTU8	354.353	3	2	3.841	Yes
BMPTU9	354.353	3	2	3.841	Yes
BMPTU10	365.252	3	2	3.585	Yes
BMPTU11	320.801	3	2	3.475	Yes
BMPTU12	303.383	3	2	3.130	Yes
BMPTU13	320.801	5	2	3.475	Yes
BMPTU14	316.382	4	2	2.381	Yes
BMPTU15	342.464	3	2	4.119	Yes
BMPTU16	301.371	4	2	2.404	Yes
BMPTU17	320.801	3	2	3.475	Yes
BMPTU18	300.383	3	2	3.130	Yes
BMPTU19	302.335	3	2	2.528	Yes
BMPTU20	304.346	3	2	2.961	Yes

All BMPTU derivatives adhere to Lipinski's Ro5, indicating their potential for oral bioavailability. Variations in the R group do not significantly affect their fundamental drug-like characteristics. The observed range of log P values suggests a balance between hydrophilicity and lipophilicity, which is crucial for optimal drug absorption and distribution. Compounds with higher log P values (e.g., BMPTU6 and BMPTU7) might exhibit enhanced membrane permeability but require careful consideration of potential solubility issues.

Hydroxyurea exhibited a pronounced hydrophilic character compared to the more lipophilic HTMX, highlighting the diverse physicochemical profiles within this drug class. The BMPTU derivatives demonstrated intermediate properties, suggesting a potential balance between solubility and permeability, which could translate into improved therapeutic profiles. Compounds like BMPTU6 and BMPTU7, with their moderate log P values and adherence to Lipinski's Ro5, emerged as promising candidates for further development due to their favorable physicochemical properties. Compounds with extreme log P values may require structural modifications to optimize their bioavailability while maintaining efficacy. Despite the favorable predictions based on Lipinski's Ro5, further analysis and toxicity studies are warranted to validate these initial assessments and ensure safety and efficacy.

All BMPTU derivatives evaluated in this study demonstrated favorable drug-like properties, indicating their potential suitability for oral administration. The diverse log P values observed among these compounds suggest a range of hydrophilicity and lipophilicity, providing a promising foundation for further pharmacokinetic and pharmacodynamic investigations. Similar to previous research on N-benzoyl-N'-phenylthiourea derivatives[30], all BMPTU compounds adhered to Lipinski's Ro5, exhibiting acceptable log P values, molecular weight, hydrogen bond donors (HBDs), and hydrogen bond acceptors (HBAs). To further explore the therapeutic potential of BMPTU derivatives as ERα inhibitors, subsequent analyses focusing on ADMET properties and molecular docking simulations are warranted. These investigations will provide valuable insights into the compounds' drug-like characteristics and their potential interactions with the ERα.

ADMET prediction

Hydroxyurea demonstrated favorable intestinal solubility and a safe profile, with limited brain-blood barrier penetration and minimal interactions with CYP2D6 or OCT2 (**Table III**). These characteristics suggest a low likelihood of central nervous system effects and a relatively high therapeutic dose. In contrast, HTMX exhibited high intestinal solubility but

significant brain-blood barrier penetration, indicating potential central nervous system effects. Its maximum recommended dose is lower than HU, suggesting a narrower therapeutic window. Both HU and HTMX exhibited minimal risk of drug-drug interactions due to their lack of interaction with CYP2D6 and OCT2.

All BMPTU derivatives demonstrated generally good intestinal solubility, ranging from 83.235 to 93.148, suggesting adequate absorption. However, their brain-blood barrier penetration varied, with most compounds showing low to moderate permeability. None of the derivatives were substrates for CYP2D6 or renal OCT2, minimizing the risk of drug-drug interactions in these pathways. Maximum recommended doses varied across derivatives (log mg/kg/day: 0.015 to 0.411), reflecting varying safety profiles. Notably, BMPTU2 displayed potential hepatotoxicity.

Table III. ADMET prediction.

No	Compound code	Absorption	Distribution	Metabolism	Excretion	Toxicity	
		Intestinal solubility	Blood-brain barrier penetration	CYP2D6 substrate	Renal OTC2 substrate	Max dose (log mg/kg/day)	Hepatotoxic
1	HU	73.601	-0.664	No	No	1.934	No
2	HTMX	96.885	1,329	No	No	0.313	No
3	BMPTU1	90.737	0.243	No	No	0.348	No
4	BMPTU2	83.836	-0.438	No	No	0.09	Yes
5	BMPTU3	93.148	-0.135	No	No	0.304	No
6	BMPTU4	83.235	-0.333	No	No	0.015	No
7	BMPTU5	92.321	-0.058	No	No	0.411	No
8	BMPTU6	89.531	0.2	No	No	0.36	No
9	BMPTU7	88.792	0.127	No	No	0.34	No
10	BMPTU8	89.665	0.059	No	No	0.269	No
11	BMPTU9	91.163	0.097	No	No	0.303	No
12	BMPTU10	89.927	0.22	No	No	0.355	No
13	BMPTU11	89.994	0.221	No	No	0.365	No
14	BMPTU12	91.765	0.149	No	No	0.31	No
15	BMPTU13	90.307	0.136	No	No	0.322	No
16	BMPTU14	91.861	0.167	No	No	0.332	No
17	BMPTU15	89.685	0.221	No	No	0.101	No
18	BMPTU16	91.174	-0.768	No	No	0.349	No
19	BMPTU17	91,178	0.079	No	No	0.369	No
20	BMPTU18	92.48	0.107	No	No	0.354	No
21	BMPTU19	90.896	-0.893	No	No	0.216	No
22	BMPTU20	91.44	0.246	No	No	0.323	No

BMPTU2 demonstrated moderate intestinal solubility but exhibited low BBB penetration and potential hepatotoxicity at a maximum dose of 0.09 log mg/kg/day. This suggests caution in its use due to the risk of liver toxicity and a narrow therapeutic window. BMPTU4, similar to BMPTU2, exhibited low hepatotoxicity but possessed a very narrow therapeutic window, with a maximum dose of only 0.015 log mg/kg/day. BMPTU7 and BMPTU8 displayed higher intestinal solubility and moderate BBB penetration, suggesting their potential suitability for conditions requiring moderate central nervous system (CNS) exposure. BMPTU11 and BMPTU13 demonstrated favorable pharmacokinetic properties, including good intestinal solubility and moderate BBB penetration, indicating potential CNS effects with an acceptable safety profile. BMPTU16 and BMPTU17 exhibited high intestinal solubility and promising safety profiles, making them potential candidates for further development due to their balanced ADMET properties.

All compounds demonstrated favorable oral absorption characteristics due to their good intestinal solubility, a crucial factor for developing orally administered drugs. While most compounds exhibited low to moderate blood-brain barrier (BBB) penetration, which is advantageous for avoiding CNS side effects, compounds with higher BBB penetration, such as TMX, could be explored for CNS-targeted therapies. The absence of CYP2D6 substrate interactions across all BMPTU derivatives suggests a reduced risk of metabolic drug-drug interactions, making them potentially safer for patients taking multiple medications. Additionally, the lack of interactions with renal OCT2 transporters indicates minimal renal excretion issues, which is beneficial for compounds requiring prolonged systemic exposure. Most compounds in this study showed no hepatotoxicity, indicating a favorable safety profile. However, BMPTU2 and BMPTU4 warrant caution due to their narrow therapeutic windows and potential for liver toxicity.

N-benzoyl-N'-methoxyphenilthiourea derivatives, compared to the established benchmarks of HU and HTMX, demonstrate improved intestinal solubility and more consistent BBB penetration. Moreover, their broader safety profiles,

characterized by a lack of CYP2D6 and OCT2 interactions, suggest potential advantages over existing therapies. Compounds such as BMPTU7 and BMPTU16, with their balanced ADMET profiles, emerge as promising candidates for further development. However, compounds exhibiting potential hepatotoxicity or narrow therapeutic windows warrant either structural modifications or rigorous monitoring in subsequent studies.

An ADMET analysis was conducted to assess the pharmacokinetic and safety profiles of the compounds[31]. The pkCSM database was utilized to predict intestinal solubility, BBB penetration, CYP2D6 substrate status, renal OCT2 substrate status, maximum recommended therapeutic dose, and hepatotoxicity. Intestinal solubility is a crucial factor in oral absorption[32]. BBB penetration is essential for compounds targeting CNS[33]. CYP2D6 substrate status determines the compound's susceptibility to metabolism by the CYP2D6 enzyme, which can impact drug-drug interactions[34]. Renal OCT2 substrate status indicates the compound's potential for renal excretion[35]. The maximum recommended therapeutic dose and hepatotoxicity parameters provide insights into safety and potential liver toxicity[36]. While the compounds exhibited varying ADMET profiles, most did not show significant hepatic toxicity.

The ADMET analysis revealed favorable pharmacokinetic properties for the BMPTU derivatives, including good absorption, minimal metabolic interactions, and acceptable safety profiles. These characteristics suggest promising potential for further development, especially for compounds like BMPTU7 and BMPTU16. Previous research on thiourea derivatives has identified hepatotoxic potential in some compounds, such as N-(benzoyl)-N'-phenyltiourea, N-(4-methylbenzoyl)-N'-phenyltiourea, N-(4-tertiarybutylbenzoyl)-N'-phenyltiourea, N-(4-propoxybenzoyl)-N'-phenyltiourea, N-(3,4-ditrifluoromethylbenzoyl)-N'-phenyltiourea, N-(3,5-ditrifluoromethylbenzoyl)-N'-phenyltiourea, N-(4-dimethylaminobenzoyl)-N'-phenyltiourea, and N-(3-nitrobenzoyl)-N'-phenyltiourea[30]. However, it's important to note that these derivatives also demonstrate good human intestinal absorption (>80%)[15]. Future research should focus on optimizing BMPTU derivative compounds to enhance their efficacy and safety. *In vivo* studies are crucial to validate their potential for therapeutic applications.

Molecular docking

Molecular docking simulations were employed to predict the binding affinity of BMPTU derivatives to ERα. Hydroxyurea, a known ERα modulator, served as a positive control. The docking scores obtained revealed that all BMPTU derivatives exhibited significantly stronger binding affinities to ERα compared to HU with RS range from -75.1959 to -96.820, suggesting their potential as ERα modulators[37,38]. Among the BMPTU derivatives, BMPTU2 demonstrated the highest binding affinity, indicating a promising lead compound for further development. BMPTU3 and BMPTU4 also exhibited strong binding affinities, warranting further investigation. BMPTU8 and BMPTU6 displayed moderate binding affinities, while BMPTU22, despite being the weakest among BMPTU derivatives, still exhibited a significantly higher affinity than HU (**Table IV**).

BMPTU derivatives demonstrated strong binding affinities for ERα, as evidenced by their negative RS scores. Compounds containing nitro (e.g., BMPTU2 and BMPTU4), cyano (e.g., BMPTU3), and methoxy groups (e.g., BMPTU5) exhibited particularly high binding affinities, suggesting their contribution to enhanced receptor interactions. Additionally, halogenated derivatives (e.g., BMPTU6 and BMPTU11) displayed strong binding affinities, highlighting the importance of these functional groups for receptor interaction. Hydroxyurea, a reference compound, exhibited low binding affinity, underscoring the superior binding potential of BMPTU derivatives.

Among the BMPTU derivatives, BMPTU2, BMPTU3, and BMPTU4 emerged as promising candidates with the highest binding affinities. BMPTU8 and BMPTU6 also demonstrated significant potential and warrant further investigation. Future research should focus on optimizing these lead compounds to enhance their binding affinities while maintaining favorable ADMET properties. Structural modifications could be explored to fine-tune interactions with the ERα receptor, potentially improving efficacy and reducing off-target effects.

To gain insights into the ligand-receptor interaction, the specific amino acid residues on the receptor that interact with the ligand were identified. These interactions can be categorized as hydrogen bonds, electrostatic interactions, or steric interactions. These interactions play a pivotal role in determining the binding affinity and stability of the ligand-receptor complex[39].

Table IV. Molecular docking results.

No	Compound code	Rerank score	Amino residue interaction		
			Hydrogen bond	Electrostatic interaction	Steric interaction
1	HTMX	-124.289	Arg394	Asp351	Ala350, Asp351, Glu353
2	BMPTU2	-96.82	-	-	Met343, Leu346, Leu387, Met388, Leu391
3	BMPTU3	-92.5796	His524	-	Leu346, Leu391, Leu525
4	BMPTU4	-89.2612	Arg394	-	Leu346, Thr347, Ala350, Leu387, Met388
5	BMPTU5	-89.058	Thr347, His524	-	Leu346, Thr347, His524, Leu525
6	BMPTU6	-88.9712	-	-	Leu346, Leu384, His524
7	BMPTU7	-86.554	Thr347	-	Met343, Leu346, Thr347, Met388, Phe404, Leu525
8	BMPTU8	-85.9682	Leu346, Ala350, His524	-	Met343, Leu346, Ala350, Leu387, Met388, Leu391, Gly420
9	BMPTU9	-85.4756	Thr347	-	Met343, Leu346, Thr347, Ala350, Gly420, Gly521, His524, Leu525
10	BMPTU10	-85.3747	Leu346, Thr347, Arg394	-	Ala350, Asp351, Leu387, Arg394
11	BMPTU12	-84.7357	Thr347	-	Leu349, Ala350, Met388, Leu387, Arg394, Leu391
12	BMPTU11	-84.5448	Thr347	-	Met343, Leu346, Ala350, Leu428, His524, Leu525
13	BMPTU13	-84.5182	His524	-	Leu346, Leu349, Leu387, Met388, Phe404, Ile424, His524, Leu525
14	BMPTU14	-83.2699	Arg394	-	Leu346, Met388, Leu391, Arg394
15	BMPTU15	-82.443	-	-	Met321, Leu387, Met388, Leu391, Ile424
16	BMPTU1	-82.046	Leu346, Arg394	-	Leu346, Ala350, Glu353, Leu384, Met388
17	BMPTU16	-80.7878	Glu353	-	Ala350, Asp351, Leu387
18	BMPTU17	-80.3469	Thr347, Arg394	-	Arg350, Glu353, Trp383, Leu384, Leu387, Leu391, Arg394
19	BMPTU18	-79.0584	His524	-	Leu346, Gly521, Leu525
20	BMPTU19	-76.6632	Leu346, His524	-	Leu346, Leu349, Leu387, Met388, Phe404, Ile424, His524, Leu525
21	BMPTU20	-75.1959	-	-	Leu346, Ala350, Glu353, Leu387, Arg394
22	HU	-34.895	Glu353, Leu387, Arg394	-	-

Molecular docking simulations revealed that HU forms hydrogen bonds with key residues such as Glu353, Leu387, and Arg394, contributing to its binding to ERα. However, its lower binding affinity may be attributed to a lack of significant electrostatic and steric interactions. 4-hydroxytamoxifen, in contrast, forms hydrogen bonds with Arg394 and engages in electrostatic interactions with Asp351. Additionally, steric interactions with Ala350, Asp351, and Glu353 contribute to its enhanced binding stability, explaining its potent modulatory effects on ERα.

The majority of derivatives studied formed multiple hydrogen bonds, primarily with Thr347, Arg394, and His524. Electrostatic interactions were less frequent but played a significant role when present. Steric interactions, involving residues like Leu346, Ala350, and Met388, also contributed to overall binding stability. BMPTU2 exhibited strong steric interactions with multiple leucine residues, while methionine contributed to its highest binding affinity among the derivatives. BMPTU3 demonstrated a hydrogen bond with His524 and significant steric interactions with leucine residues, resulting in strong ERα binding. BMPTU4, with its dual hydrogen bonds and extensive steric interactions, exhibited the most potent binding affinity to ERα.

Hydrogen bonding plays a crucial role in stabilizing ligand-receptor interactions. Residues like Arg394, Thr347, and His524 are commonly involved in these interactions. Compounds forming multiple hydrogen bonds often exhibit stronger binding affinities. While less frequent, electrostatic interactions, as observed with Asp351 in HTMX, can significantly enhance binding stability. Additionally, steric interactions, mediated by residues such as Leu346, Leu387, and Met388, contribute to the stability of the ligand-receptor complex. Compounds with extensive steric interactions, like BMPTU2 and BMPTU4, tend to have higher binding affinities.

Hydroxyurea exhibited fewer molecular interactions with the target protein compared to the BMPTU derivatives, aligning with its lower binding affinity. The BMPTU derivatives demonstrated significantly stronger binding, facilitated by additional steric and hydrogen bond interactions. 4-hydroxytamoxifen, with its extensive hydrogen bonding, electrostatic interactions, and steric interactions, established a high benchmark for binding affinity. Several BMPTU derivatives approached or surpassed this level of interaction complexity, suggesting their potential efficacy. Specific functional groups, including nitro, cyano, and methoxy, contributed to enhanced hydrogen bonding and steric interactions, leading to higher

binding affinities. Halogenated derivatives, such as those containing chloro groups, also exhibited significant steric interactions, crucial for binding stability.

ERα, a nuclear receptor, regulates gene expression in response to estrogen. Ligand binding induces conformational changes in ERα, enabling it to distinguish between agonists and antagonists[40]. Agonist binding to ERα leads to H12 adopting a conformation that recruits coactivators, facilitating gene transcription. Residues Glu353, Arg394, and His524 stabilize H12 in this conformation through hydrogen bonding. Conversely, antagonists disrupt H12 positioning, preventing coactivator recruitment and favoring corepressor binding, which inhibits transcription. Asp351 forms a salt bridge with antagonists, further stabilizing the antagonistic conformation[21].

The ERα ligand binding domain (LBD) undergoes distinct conformational changes depending on the ligand bound. Hydrogen bonding with His524 is crucial for differentiating agonists and antagonists. Mutations in His524, even if hydrogen bonding with Glu353 or Arg394 is maintained, can result in ERα antagonism[22,41]. Understanding these molecular interactions is essential for designing selective ERα modulators with therapeutic potential.

To elucidate the mode of action of BMPTU derivatives, we investigated their interactions with ERα. Our analysis revealed that HU forms hydrogen bonds with key residues similar to known ERα agonists, suggesting potential agonistic activity. However, its low binding affinity indicates weak potency. In contrast, HTMX, a known ERα antagonist, forms a hydrogen bond with Asp351 but lacks the critical hydrogen bond with His524, leading to displacement of the H12 helix[42]. Several BMPTU derivatives (BMPTU3, BMPTU5, BMPTU8, BMPTU13, BMPTU18, and BMPTU19) demonstrated hydrogen bonding with His524, suggesting potential agonistic activity. However, further *in silico* molecular dynamics simulations are necessary to confirm whether their steric interactions can effectively displace the H12 position. *In vitro* and *in vivo* studies are warranted to definitively validate these predictions and characterize the biological activity of BMPTU derivatives as ERα modulators.

CONCLUSION

This *in silico* study evaluated BMPTU derivatives as potential ERα inhibitors for breast cancer therapy. All compounds adhered to Lipinski's Ro5, suggesting favorable oral bioavailability. ADMET analysis revealed promising pharmacokinetic profiles, with minimal metabolic interactions and acceptable safety margins, except for BMPTU2, which warrants further assessment due to potential hepatotoxicity. Molecular docking simulations identified strong binding affinities between BMPTU derivatives, particularly BMPTU2, BMPTU3, and BMPTU4, and key ERα residues. These findings suggest that certain BMPTU derivatives could act as ERα agonists or antagonists, warranting further investigation through molecular dynamics simulations and experimental studies. Collectively, these results highlight the potential of BMPTU derivatives, especially BMPTU2, BMPTU3, and BMPTU4, as promising lead compounds for developing novel breast cancer therapies targeting ERα. Further optimization and validation are necessary to fully realize their therapeutic potential.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Research Article

In Vitro Evaluation of Prebiotic Potential of Red Ginger (*Zingiber officinale* var. *rubrum*) Rhizome Ethanol Extract on *Lactobacillus acidophilus* and *Escherichia coli*

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Abstract

Prebiotics, including carbohydrates and phenols, promote beneficial gut bacteria (probiotics). Red ginger (*Zingiber officinale* var. *rubrum*) rhizomes, rich in these compounds, have been traditionally used in medicine but their prebiotic potential remains unexplored. This study investigated the *in vitro* prebiotic effects of *Z. officinale* var. *rubrum* rhizomes on *Lactobacillus acidophilus* (beneficial) and *Escherichia coli* (opportunistic) bacteria. Prebiotic activity was assessed using a turbidimetric method, measuring bacterial growth via UV-Vis spectrophotometry at 600 nm. The prebiotic index and percentage inhibition were calculated to evaluate the impact on bacterial growth. Additionally, total phenol content was determined using the Folin-Ciocalteu method. Results indicate that *Z. officinale* var. *rubrum* rhizomes exhibit prebiotic properties, stimulating *L. acidophilus* growth (prebiotic index of 156.035 and percentage inhibition value of -153.128%) while inhibiting *E. coli* growth (54.343% inhibition). The rhizomes contained 31.15 mg GAE/g extract of total phenols and 23.55% carbohydrates. These findings suggest that *Z. officinale* var. *rubrum* rhizomes possess prebiotic potential, warranting further investigation for potential applications in gut health management.

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INTRODUCTION

Prebiotics are non-digestible compounds that selectively support the growth of beneficial bacteria (probiotics) in the large intestine[1]. This enhanced probiotic population contributes to various health benefits, including suppression of pathogenic bacteria, improved intestinal motility, enhanced calcium absorption, strengthened immune function, and reduced risk of cancer[2,3]. Common prebiotic compounds include carbohydrates or dietary fiber, such as inulin, fructooligosaccharides, isomaltooligosaccharides, lactosucrose, lactulose, pyrodextrins, transgalactooligosaccharides, and xylooligosaccharides. Notably, certain non-carbohydrate compounds, like phenolic compounds, have also been identified as prebiotics[4].

Red ginger (*Zingiber officinale* var. *rubrum*) is a widely used traditional medicinal plant, with its rhizome being the primary part of interest. This rhizome is rich in biologically active secondary metabolites, including flavonoids, phenols, terpenoids, and essential oils[5]. Among these compounds, phenolic compounds like oleoresin, gingerol, and shogaol are particularly noteworthy. *Zingiber officinale* var. *rubrum* exhibits the highest oleoresin content among ginger varieties, reaching up to 3% of its dry weight[6]. Additionally, the rhizome contains approximately 2-14% gingerol and 1-2% shogaol[7].

Due to the high phenolic content in *Z. officinale* var. *rubrum* rhizomes, they might have the potential to be prebiotics[8]. However, the prebiotic effects of *Z. officinale* var. *rubrum* rhizomes have not been thoroughly investigated. This study aims

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to evaluate the prebiotic potential of *Z. officinale* var. *rubrum* rhizome extract on beneficial bacteria (*Lactobacillus acidophilus*) and opportunistic pathogens (*Escherichia coli*). Additionally, this study aimed to quantify the total phenolic and carbohydrate content of *Z. officinale* var. *rubrum* rhizome ethanol extract.

MATERIALS AND METHODS

Materials

This study utilized a range of equipment and materials for the extraction, analysis, and microbiological evaluation of *Z. officinale* var. *rubrum* rhizomes. Laboratory equipment included Pyrex glassware, a Waring blender, a Mettler Toledo analytical balance, a Memmert oven, an IKA HB 10 basic rotary evaporator, a Shimadzu UV-1800 UV-Vis spectrophotometer, an Envilife vmx-s vortex, and a Memmert water bath. Reagents and materials employed were distilled water, gallic acid (a reference standard), sulfuric acid (analytical grade, Merck), ethanol (analytical grade, Merck), phenol (analytical grade, Merck), *Z. officinale* var. *rubrum* rhizomes sourced from the Medicinal Plant and Spice Garden in Manoko, Lembang, methanol (analytical grade, Merck), Man Rogosa Sharpe Agar (MRSA), Man Rogosa Sharpe Broth (MRSB), sodium carbonate (analytical grade, Merck), Nutrient Agar (NA), Nutrient Broth (NB), Folin-Ciocalteu reagent, and glucose (reference standard, Merck). The study utilized *L. acidophilus* ATCC 4356 and *E. coli* ATCC 25922, obtained from the Microbiology Laboratory of the Faculty of Pharmacy, Universitas Islam Bandung, as bacterial strains for microbiological analysis.

Methods

Plant extraction

Zingiber officinale var. *rubrum* rhizomes were obtained from local sources in Bandung. The plant material was authenticated at the Herbarium of the School of Life Sciences and Technology, Institut Teknologi Bandung (voucher specimen number: 1009/IT1.C11.2/TA.00/2023). Fresh rhizomes were sliced, dried, and subjected to maceration extraction using 96% ethanol as the solvent. The extraction process involved soaking the sliced rhizomes in ethanol for a specified duration, followed by filtration to obtain the ethanolic extract.

Determination of total phenols and carbohydrates

To quantify the phenol content in *Z. officinale* var. *rubrum* rhizomes, a spectrophotometric assay using Folin-Ciocalteu reagent and gallic acid as a standard was employed[9]. Similarly, the total carbohydrate content was determined using the spectrophotometric phenol-sulfuric acid method[10].

Prebiotic activity test

Prebiotic activity was assessed using UV-Vis spectrophotometer to measure optical density at 600 nm (OD600)[11,12]. OD600 values indicate bacterial growth in the culture medium. Additionally, prebiotic index (PI) and % inhibition were calculated using **Equations 1** and **2**. The following variables were used: $OD600_{control}$ (bacterial culture without *Z. officinale* var. *rubrum* extract), $OD600_{sample}$ (bacterial culture with *Z. officinale* var. *rubrum* extract), P_p^0, P_p^{24}, P_G^0, P_G^{24} P_{GF}^0, P_{GF}^{24}, (OD600 values for *L. acidophilus* at time points 0 and 24 hours in different media; p: prebiotics/*Z. officinale* var. *rubrum* extract; G: glucose-containing media; GF: glucose-free media), E_p^0, E_p^{24}, E_G^0, E_G^{24} E_{GF}^0, E_{GF}^{24} (OD600 values for *E. coli* at time points 0 and 24 hours in different media; p: prebiotics/*Z. officinale* var. *rubrum* extract; G: glucose-containing media; GF: glucose-free media).

$$\%inhibition = \frac{(OD600_{control} - OD600_{sample})}{OD600_{control}} \text{x} 100\% \quad [1]$$

$$Prebiotic\ index = \left[\frac{(P_p^{24} - P_p^0) - (P_{GF}^{24} - P_{GF}^0)}{(P_G^{24} - P_G^0) - (P_{GF}^{24} - P_{GF}^0)}\right] - \left[\frac{(E_p^{24} - E_p^0) - (E_{GF}^{24} - E_{GF}^0)}{(E_G^{24} - E_G^0) - (E_{GF}^{24} - E_{GF}^0)}\right] \quad [2]$$

Data analysis

Data collected from three experimental replicates were averaged to obtain mean values. The Kruskall-Wallis non-parametric test was employed to evaluate differences in OD600 values among the various prebiotic samples. The Mann-Whitney test was used to identify significant differences between specific pairs of samples. Statistical significance was determined at a p-value threshold of ≤0.05 using SPSS 26 software.

RESULTS AND DISCUSSION

A total of 132.43 g of *Z. officinale* var. *rubrum* ethanol extract was obtained from 730 g of dried rhizome, yielding an 18.14% extraction efficiency. This value falls within the acceptable range of 17.0% as per Indonesian Herbal Pharmacopoeia guidelines[13]. However, it's noteworthy that previous studies reported a lower extraction yield of 7.13% for *Z. officinale* var. *rubrum*[7]. This variation can be attributed to differences in the extraction process[14].

The maceration method, employed in this study, is a simple and cost-effective technique for extracting bioactive compounds from plant materials. The efficiency of maceration can be influenced by various factors, including the sample-solvent ratio[15]. Ethanol, a commonly used solvent in phytochemical extraction due to its Generally Recognized as Safe (GRAS) status and low vapor pressure, was chosen for this study[16]. The choice of solvent and the sample-solvent ratio can significantly impact the extraction yield, with higher solvent volumes generally leading to increased extraction of bioactive compounds. Additionally, the duration of the extraction process plays a crucial role. Prolonged extraction times allow for greater penetration of the solvent into the plant material, facilitating the diffusion of compounds[14]. Phytochemical screening of the *Z. officinale* var. *rubrum* rhizome revealed the presence of various secondary metabolites, as detailed in **Table I**.

Table I. Phytochemisty screening of *Z. officinale* var. *rubrum* rhizome.

Secondary metabolites	Result
Alkaloids	+
Polyphenols	+
Flavonoids	+
Saponins	-
Tannins	+
Monoterpenes and sesquiterpenes	+

(+): detected; (-): not detected

Phytochemical screening of *Z. officinale* var. *rubrum* confirmed the presence of alkaloids, polyphenols, flavonoids, tannins, monoterpenes, and sesquiterpenes, aligning with previous research[17]. Polyphenols, a class of compounds found in *Z. officinale*, exhibit prebiotic properties, promoting the growth of beneficial bacteria and conferring health benefits to humans[18]. The total phenolic content in *Z. officinale* plants varies widely, ranging from 0.2 to 155.3 mg gallic acid equivalent (GAE)/g extract[19]. Notably, *Z. officinale* var. *rubrum* has been reported to possess the highest phenolic content among *Z. officinale* varieties[20]. The phenolic compounds in *Z. officinale* var. *rubrum* primarily consist of vanilloids, including gingerol, shogaol, paradol, zingerone, gingerdione, and gingerdiol[5,21].

The ethanol extract of *Z. officinale* var. *rubrum* in this study exhibited a total phenolic content of 31.15 mg GAE/g, surpassing previous reports of 21.90 mg GAE/g[22] and 12.2533 ± 0.13 mg GAE/g[23] obtained using the infusion extraction method. Variations in total phenolic content among *Z. officinale* var. *rubrum* extracts can be attributed to factors such as the extraction solvent and the intrinsic chemical properties of the plant material. The polar nature of phenolic compounds in *Z. officinale* var. *rubrum* facilitates their effective binding by ethanol[24]. Additionally, geographic variations in *Z. officinale* var. *rubrum* growth conditions, including cultivar type, soil composition, cultivation practices, and maturity, can influence the total phenolic content[25].

Besides polyphenols, carbohydrates are also prebiotic compounds[26]. Carbohydrates are the primary component of *Z. officinale* var. *rubrum* rhizomes, constituting approximately 10.1% in fresh rhizomes and a significantly higher 85% in dried rhizomes[27-29]. Starch comprises 59.29% of the carbohydrates in *Z. officinale* var. *rubrum* rhizomes[28]. In this study, the ethanol extract of *Z. officinale* var. *rubrum* contained 23.55% total carbohydrates. To assess the impact of *Z. officinale* var. *rubrum* ethanol extract on bacterial growth, we measured OD600 values of *L. acidophilus* and *E. coli* cultures. OD600 values are directly correlated with bacterial population density. As shown in **Figure 1**, the extract significantly inhibited the growth of *E. coli* while promoting the growth of *L. acidophilus*.

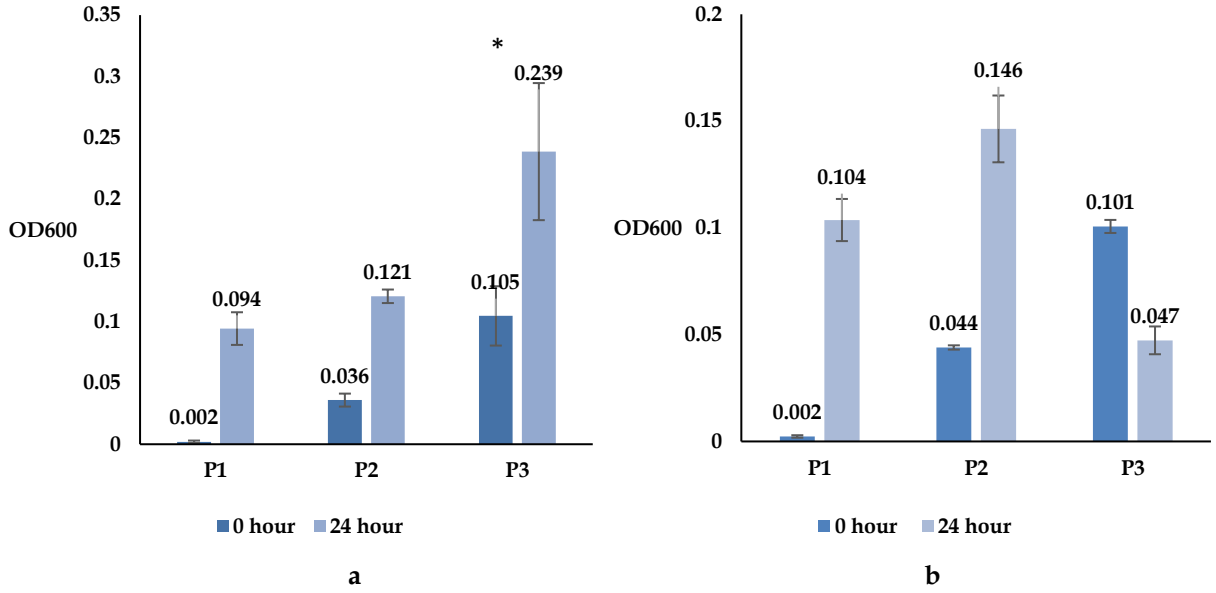

Figure 1. The OD600 values of *L. acidophilus* (**a**) and *E. coli* (**b**) in medium with no carbon source (P1), glucose (P2) and *Z. officinale* var. *rubrum* ethanol extract (P3). The bars are presenting the mean of OD600 values with standard deviation bar (n=3). * is significantly different compared to P1 (P=0.016) dan P2 (P=0.05).

Three experimental groups were established for this study: Group P1, cultured in a carbon-free medium; Group P2, supplemented with glucose; and Group P3, treated with *Z. officinale* var. *rubrum* ethanol extract. Glucose, a common bacterial growth medium component, served as a control[30,31]. After 24 hours of incubation, *L. acidophilus* populations exhibited significant growth in Group P3 (*Z. officinale* var. *rubrum* ethanol extract) compared to Groups P1 (no carbon source) and P2 (glucose). Conversely, *E. coli* populations decreased in Group P3, while increasing in Groups P1 and P2 (**Figure 1**).

The bacteria cultivated with glucose exhibited the highest increase in growth compared to the bacteria cultivated with *Z. officinale* var. *rubrum* ethanol extract and the control group. While glucose enhanced the growth of both *L. acidophilus* and *E. coli*, *Z. officinale* var. *rubrum* ethanol extract demonstrated a selective effect, promoting the growth of *L. acidophilus* while suppressing the growth of *E. coli*. This selective prebiotic effect is evident from the inhibition percentage values of -153.128% for *L. acidophilus* and 54.343% for *E. coli*, respectively, when cultured with *Z. officinale* var. *rubrum* ethanol extract. The phytochemical compounds present in *Z. officinale* var. *rubrum* ethanol extract may be responsible for this selective prebiotic effect by interacting with specific bacterial pathways and promoting the growth of beneficial bacteria like *L. acidophilus*[32].

The prebiotic index of *Z. officinale* var. *rubrum* ethanol extract was calculated to be 156.035. This indicates its ability to selectively stimulate the growth of beneficial bacteria compared to unfavorable bacteria, even in the presence of non-prebiotic substrates like glucose[33]. These findings suggest that *Z. officinale* var. *rubrum* ethanol extract is metabolized similarly to control prebiotics by probiotic strains. Notably, its PI was significantly higher than that of aqueous extracts of white ginger or *Zingiber officinale* Rosc. var. *officinale* (0.373-0.837)[34], emphasizing the superior prebiotic potential of *Z. officinale* var. *rubrum*.

CONCLUSION

This study demonstrates the prebiotic potential of *Z. officinale* var. *rubrum* ethanol extract, as evidenced by its PI value of 156.035 and its selective growth promotion of *L. acidophilus* over *E. coli*. Additionally, the extract contains a significant amount of total phenols (31.15 mg GAE/g extract) and total carbohydrates (23.55%).

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AUTHORS' CONTRIBUTION

Conceptualization: Bertha Rusdi
Data curation: Fadil Rido Gumelar, Farendina Suarantika, Bertha Rusdi
Formal analysis: Fadil Rido Gumelar, Farendina Suarantika, Bertha Rusdi
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Project administration: -
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Validation: Farendina Suarantika, Bertha Rusdi
Visualization: -
Writing - original draft: Fadil Rido Gumelar
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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Formulation of Nutraceutical Jelly Candy from a Combination of *Cucurbita moschata* Puree and *Averrhoa carambola* Juice as Antioxidant

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Jelly candy
Sweet starfruit
Yellow pumpkin

Abstract

Nutraceutical candy jellies are gaining popularity as a potential approach to deliver antioxidants in a palatable form. This study investigated the antioxidant activity of *Cucurbita moschata* puree combined with *Averrhoa carambola* juice, formulated into jelly candies. Design-Expert software V.13 was used to optimize the jelly candy base formula. The combined *C. moschata* puree and *A. carambola* juice exhibited strong antioxidant activity (IC_{50} = 29.580 ppm) at variation V1. The optimal base formula B3 consisted of 12% gelatin and 4% carrageenan. The formulated jelly candy possessed very strong antioxidant activity (IC_{50} = 44.771 ppm). These findings suggest the potential of *C. moschata* puree and *A. carambola* juice as ingredients in functional jelly candies.

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INTRODUCTION

Indonesia's rich biodiversity includes numerous medicinal plants with potential therapeutic applications. Many of these plants contain secondary metabolites that could serve as valuable components in pharmaceutical preparations[1]. Pharmaceutical preparations encompass a wide range of products, including chemical drugs, traditional medicines, cosmetics, and health supplements. Among the essential compounds required by the human body are antioxidants, which play a crucial role in neutralizing harmful free radicals. Free radicals are highly reactive molecules capable of causing complex cellular damage, leading to oxidative stress and contributing to the development of degenerative diseases[2].

Yellow pumpkin or *Cucurbita moschata*, a widely cultivated horticultural crop in Indonesia, holds potential as a source of bioactive compounds. Characterized by its taproot, hollow stems, five-lobed leaves, and yellow unisexual flowers, *C. moschata* has been a significant agricultural commodity in Indonesia, with production reaching 369,846 tons in 2010[3]. Despite its abundance, the full potential of *C. moschata* remains largely untapped.

Cucurbita moschata is rich in beta-carotene, a provitamin-A with potent antioxidant properties. Its antioxidant activity, measured at 30.75 ppm, is significantly higher than that of chayote, another *Cucurbita* species with an IC_{50} of 440.26 ppm[4]. Beyond beta-carotene, *C. moschata* contains a diverse array of secondary metabolites, including polyphenols, tannins, phenolic acids, and flavonoids such as quinic acid, p- coumaric acid, trans-cinnamic acid, cirsiliol, and luteolin, which further contribute to its antioxidant potential[5]. Additionally, *C. moschata* is a source of essential nutrients like carbohydrates, proteins, lipids, vitamins (including vitamin C and niacin), and minerals (Ca, K, Cu, Na, P, Fe, Zn)[6,7].

Sweet starfruit or *Averrhoa carambola*, a tropical fruit widely cultivated in regions like Indonesia, also holds potential for pharmaceutical applications. Characterized by its distinctive star shape, the fruit exhibits a yellow to light yellow hue,

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translucent texture, and a juicy flavor reminiscent of oxalic acid[8]. The *A. carambola* tree reaches heights of 6-9 meters, featuring numerous branches and compound leaves with 3-6 pairs of leaflets[9].

Phytochemical analysis of *A. carambola* reveals a rich composition of bioactive compounds. Notably, it contains flavonoids (4.22%) and saponins, demonstrating its antioxidant potential[10]. The juice of *A. carambola* exhibits a strong antioxidant activity of 78.797 ppm, attributed in part to its vitamin C content of 1.232 mg/mL[11]. Additionally, the fruit contains gallic acid (0.96%), protocatechuic acid (0.05%), and quercetin (0.40%)[8]. Other essential nutrients include calcium, phosphorus, iron, sodium, potassium, zinc, vitamin C, and thiamin[7].

The pharmaceutical industry has witnessed significant growth, driven by the development of products that enhance health and prevent diseases. Nutraceuticals, derived from the words "nutrition" and "pharmaceutical," represent a class of functional foods with potential health benefits beyond their nutritional value[12]. These compounds have gained prominence as alternative or complementary therapies for various health conditions, including degenerative, non-degenerative, and neurodegenerative diseases. The appeal of nutraceuticals lies in their generally mild side effects and nutritional value, making them attractive options for health-conscious individuals seeking natural remedies[13].

Nutraceutical jelly candies offer a convenient and appealing delivery method for bioactive compounds. While they are susceptible to melting at room temperature, their attractive sensory properties, including color, smell, taste, and texture, make them suitable for all ages[14]. Previous studies have demonstrated the successful formulation of nutraceutical jelly candies using *C. moschata* as the active ingredient, with products meeting Indonesian National Standards (SNI 3547.2-2008)[15]. Similarly, *A. carambola* has been incorporated into jelly candies alongside pineapple fruit, resulting in favorable sensory evaluations[16]. Building upon these findings, this study aimed to develop nutraceutical jelly candies incorporating both *C. moschata* puree and *A. carambola* juice. The antioxidant properties of these ingredients were evaluated using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) method.

MATERIALS AND METHODS

Materials

Cucurbita moschata and *A. carambola* specimens were procured from Samarinda City and authenticated at the Dendrology Laboratory of the Faculty of Forestry, Universitas Mulawarman, Samarinda, East Borneo, Indonesia. The specimens were assigned the numbers 188/UN17.4.08/LL/2022 and 189/UN17.4.08/LL/2022, respectively. For experimental purposes, a blender, centrifuge, hotplate, mixers, and a UV-Vis spectrophotometer (Genesys 10S) were utilized. The materials employed included distilled water, DPPH, gelatin, carrageenan, methanol, sodium propionate, sorbitol, and *A. carambola* flavoring. **Figure 1** depicts the *C. moschata* and *A. carambola* specimens.

a **b**

Figure 1. *Cucurbita moschata* (**a**) and *A. carambola* (**b**).

Methods

Sample preparation

Cucurbita moschata fruits were peeled, deseeded, and washed under running water. The flesh was then cut into 2 x 2 x 2 cm cubes and steamed for 35 minutes at 45-50°C. The steamed flesh was subsequently mashed using a mixer. Ripe *A. carambola* fruits were peeled, deseeded, washed, and cut into small pieces before being blended. The resulting pulp was filtered using a filter cloth to remove any remaining solids.

Optimization of active substances

The main ingredients are combined and varied into three concentrations as shown in **Table I**. Then the antioxidant activity of each variation using the DPPH method with the aim of obtaining the optimal formula that has the strongest antioxidant activity. Stock solutions of the active substances were prepared by dissolving them in methanol to a concentration of 200 ppm in a 100 mL volumetric flask. Serial dilutions were then performed to obtain test solutions at concentrations of 20, 40, 60, 80, and 100 ppm in 5 mL volumetric flasks. Two milliliters of each test solution were transferred to aluminum foil-covered test tubes and mixed with 2 mL of a 40 ppm DPPH solution. The mixtures were incubated in the dark at room temperature for 30 minutes.

The absorbance of the samples was measured at the maximum wavelength of DPPH using a UV-Vis spectrophotometer. Measurements were performed in triplicate. The percentage of DPPH inhibition and IC_{50} values were calculated using the linear regression equation $y = bx + a$, where y represents the probit percent inhibition (5), x represents the concentration of the test solution, and IC_{50} is the antilog of the x-intercept. The equation for calculating percent inhibition is provided in **Equation 1**, and the equation for calculating IC_{50} is provided in **Equation 2**, in which a and b represent intercept and slope from the linear regression equation, respectively.

Table I. Variation in concentration of active ingredients.

Formula	*Cucurbita moschata* puree (g)	*Averrhoa carambola* juice (g)	Ratio
V1	1	3	1 : 3
V2	3	1	3 : 1
V3	2	2	1 : 1

$$\%inhibition = \frac{(absorbance_{control} - absorbance_{sample})}{absorbance_{control}} \text{x} 100\% \quad [1]$$

$$IC_{50} = \frac{5 - a}{b} \quad [2]$$

Optimization of jelly candy formula base

Simplex Lattice Design (SLD) using Design Expert software (version 13) was employed to optimize the jelly candy base formulation. Gelatin and carrageenan were selected as independent variables, while water content served as the response variable. The experimental design parameters were inputted into Design Expert, and the generated design matrix was used to prepare jelly candy base formulations with varying gelatin and carrageenan concentrations. The water content of each formulation was measured using a moisture analyzer. The resulting data were analyzed using Design Expert to determine the optimal jelly candy base formulation.

Jelly candy formulation

Gelatin and carrageenan, serving as gelling agents, were dissolved in hot water (80°C) under constant stirring to prepare the jelly candy base. Sorbitol, a sweetener and plasticizer, and sodium propionate, a preservative, were then incorporated into the base. The main ingredients, *C. moschata* puree and *A. carambola* juice, were added at an optimal concentration (previously determined) to the base at a temperature of 45-50°C to form the jelly candy dough. The dough was poured into molds and allowed to set at room temperature for 1 hour, followed by refrigeration for 24 hours. Finally, the jelly candies were removed from the refrigerator and allowed to stand at room temperature for 1 hour to obtain the finished product (**Figure 2**).

Antioxidant activity test of jelly candy

Jelly candy samples (1 g each) were dissolved in methanol p.a. and water, followed by centrifugation at 3500 rpm for 10 minutes. The supernatant was collected and diluted with methanol p.a. to a final concentration of 200 ppm, creating a stock solution. Serial dilutions were prepared from this stock solution to obtain concentrations of 20, 40, 60, 80, and 100 ppm in 5 mL measuring flasks. The antioxidant activity of the test solutions was assessed using the DPPH method. Two milliliters of each test solution and 2 mL of DPPH solution were mixed in a test tube, sealed, and shielded from light with aluminum foil. The absorbance of the samples was then measured at the maximum wavelength of DPPH.

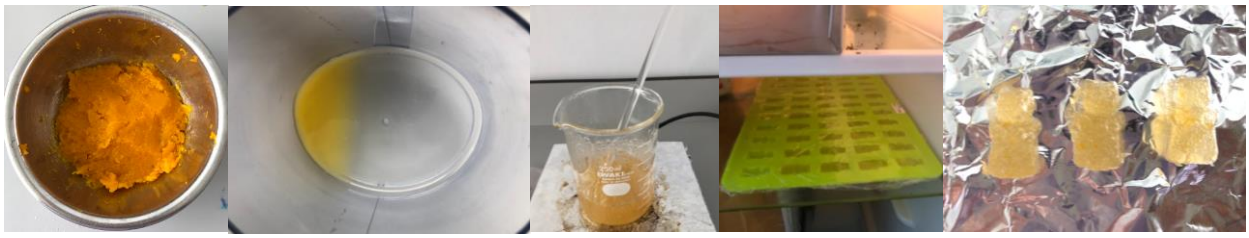


Figure 2. Representative images of the jelly candy preparation process. *Cucurbita moschata* puree (**a**), *A. carambola* juice (**b**), heated (**c**) and refrigerated jelly candy dough (**d**), and final jelly candy product (**e**).

Data analysis

Antioxidant activity was assessed using the DPPH method with three replicates. The percentage inhibition of DPPH and IC_{50} values were calculated using linear regression analysis, where the equation $y = bx + a$ was employed. The variable y was set to 5, and the antilog of x represented the IC_{50} value.

RESULTS AND DISCUSSION

The antioxidant activity of the combined *C. moschata* puree and *A. carambola* juice extracts were evaluated using the DPPH method. The selected *C. moschata* fruits were ripe, characterized by their orange skin and flesh, as previous research by Majid[17] demonstrated that mature *C. moschata* exhibits higher beta-carotene content (3.915 µg/g) compared to less ripe varieties (1.742 µg/g). The *A. carambola* fruits used were greenish-yellow, a color associated with higher vitamin C content (25.9 mg/100 g) compared to raw (25.2 mg/100 g) or fully ripe (23.4 mg/100 g)[18]. The IC_{50} values obtained from the combined extracts at various concentrations (V1= 1 : 3, V2= 3 : 1, V3=: 1 : 1) were 29.580±0.06, 38.905±0.03, and 55.463±0.08 ppm, respectively. Both V1 and V2 exhibited very strong antioxidant activity (<50 ppm), while V3 demonstrated strong antioxidant activity (50-100 ppm)[19]. A comparison of these IC_{50} values is presented in **Figure 3**.

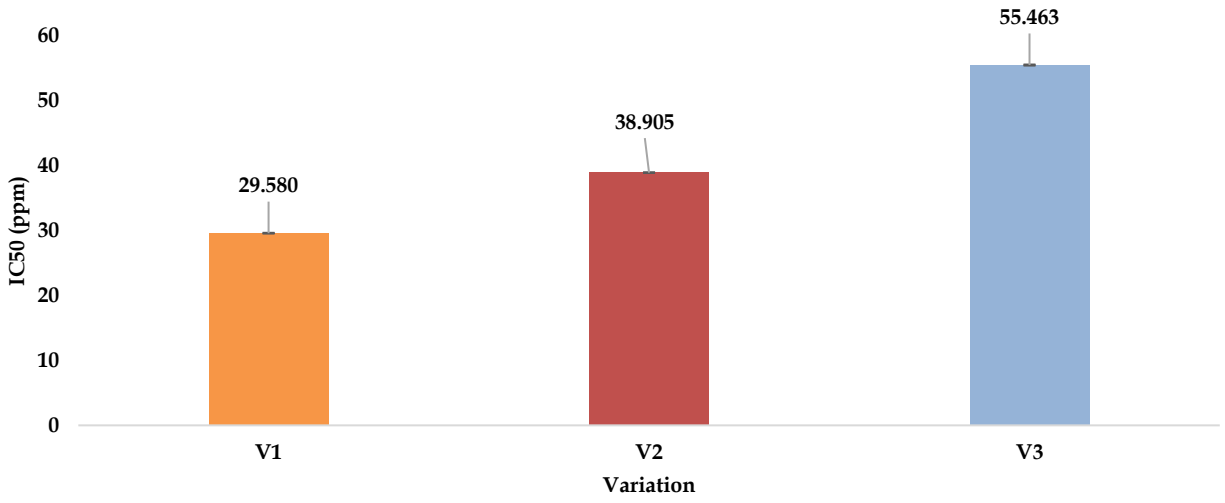


Figure 3. Antioxidant activity test of *C. moschata* puree and *A. carambola* juice extracts combination.

The DPPH method, a widely employed technique for evaluating antioxidant activity, was chosen due to its simplicity, rapidity, and ease of spectrophotometric measurement[20]. The method relies on the principle of DPPH free radicals scavenging electrons from antioxidant compounds, leading to a reduction in the characteristic purple color of DPPH. The IC_{50} value, representing the concentration of the test compound required to reduce DPPH free radicals by 50%, serves as a quantitative measure of antioxidant activity. A lower IC_{50} value indicates stronger antioxidant activity[21]. Based on our analysis, the optimal concentration ratio was determined to be V1. This formulation demonstrated the highest antioxidant activity as evidenced by the lowest IC_{50} value, suggesting its superior ability to scavenge free radicals.

The antioxidant activity of the *C. moschata* and *A. carambola* combination is attributed to the synergistic interaction of their respective phytochemical constituents. *Cucurbita moschata* contains a notable concentration of beta carotene (14.59%)[4] and various polyphenols (53.02 ± 1.56 mg GAE/g), including tannins (25.65 ± 0.08 mg CE/g), phenolic acids (quinic acid, p-coumaric acid, trans-cinnamic acid), and flavonoids (cirsiliol, luteolin)[6]. Similarly, *A. carambola* is characterized by a high content of flavonoids (4.22%)[10], vitamin C (1.232 mg/mL)[11], gallic acid (0.96%), protocatechuic acid (0.05%), and quercetin (0.40%)[8]. The combined presence of these antioxidant compounds in the two ingredients likely contributes to their enhanced free radical scavenging capacity.

Gelatin and carrageenan emerged as the optimal gelling agents in this study, contributing significantly to the jelly candy's texture and stability. Gelatin, a well-known gelling agent, forms a thermally reversible gel, meaning it can transition between sol and gel states depending on temperature[22,23]. This property makes gelatin preferable to irreversible gelling agents like pectin and gum Arabic[24]. While both gelatin and carrageenan can function as stabilizers, thickeners, and gel formers, carrageenan-based gels tend to be more brittle and less elastic. Therefore, combining gelatin and carrageenan can create a more desirable texture, with gelatin providing the necessary elasticity and carrageenan offering additional stability and structure[25].

Optimization of jelly candy formulation was conducted using Design Expert. Gelatin and carrageenan concentrations were identified as independent variables, while water content served as the dependent variable. Experimental formulations were prepared and analyzed for water content. The resulting data were evaluated using SLD method, yielding five optimal formulations presented in **Table II**.

Table II. Recommendations formula of jelly candy base.

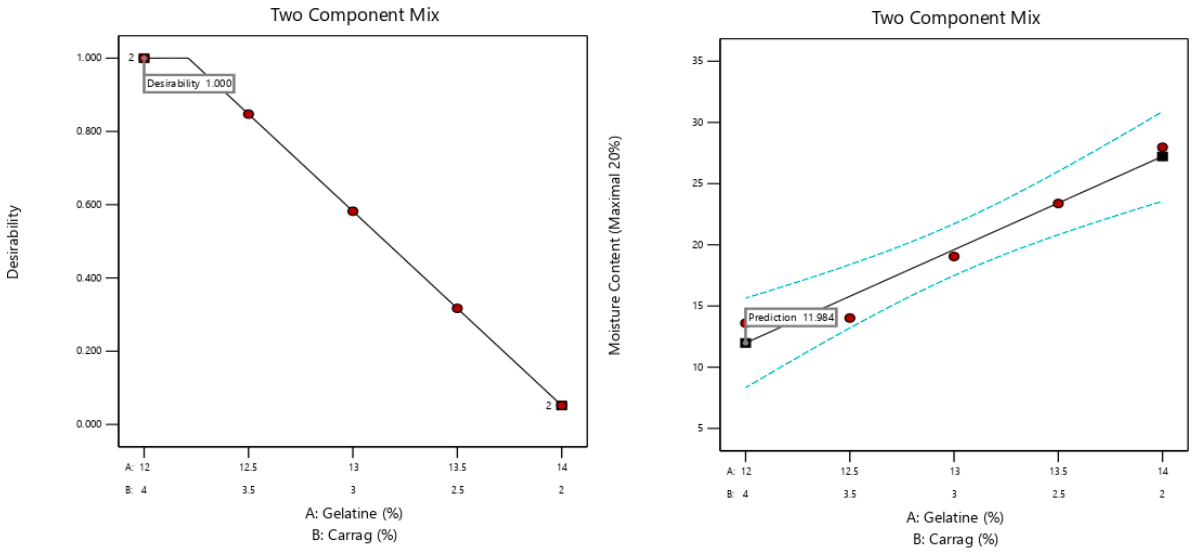
Material	Formula (%)					Material function
	B1	B2	B3	B4	B5	
Gelatin	14	13.5	13	12.5	12	Gelling agent
Carrageenan	2	2.5	3	3.5	4	Gelling agent
Sorbitol	10	10	10	10	10	Sweetener and plasticizer
Sodium propionate	0.3	0.3	0.3	0.3	0.3	Preservative
Averrhoa carambola flavour	0.25	0.25	0.25	0.25	0.25	Flavoring
Distilled water	ad 100	ad 100	ad 100	ad 100	ad 100	Solvent

Table III summarizes the water content analysis results for the various jelly candy formulations. Water content is a crucial factor in determining product quality and durability, as excessive moisture can lead to microbial growth and spoilage, while insufficient moisture can compromise texture and prevent microbial growth[26]. Two formulations, B1 and B2, were found to exceed the maximum water content limit of 20% as specified in SNI 3547.2-2008. This deviation was attributed to the higher gelatin content in these formulations compared to the others. The increased gelatin concentration likely facilitated the formation of a denser three-dimensional network within the gel matrix, capable of binding and retaining a greater amount of water[22].

Table III. Water content of jelly candy formula base.

Formula	Water content (%)			Average	Parameter
	R1	R2	R3		
B1	28.41	29.02	26.48	27.97±1.33	Max. 20%[27]
B2	25.99	21.35	22.79	23.38±2.37	
B3	19.08	19.04	19.03	19.05±0.03	
B4	13.61	14.1	14.34	14.02±0.37	
B5	13.46	13.59	13.74	13.60±0.14	

Optimization using SLD resulted in the counter-plot graphs depicted in **Figure 4**. The optimal emulgel formulation was identified as B5, containing 12% gelatin and 4% carrageenan. This formulation exhibited a desirability value of 1.00, indicating its optimal alignment with the desired response criteria. Desirability values range from 0 to 1, with values closer to 1 representing formulations that are closer to the desired target[28]. **Figure 4** illustrates the prediction results, demonstrating a direct correlation between increasing gelatin concentration and a corresponding increase in water content. The SLD-derived solution, consisting of 12% gelatin and 4% carrageenan, is presented in **Table IV**.



a b

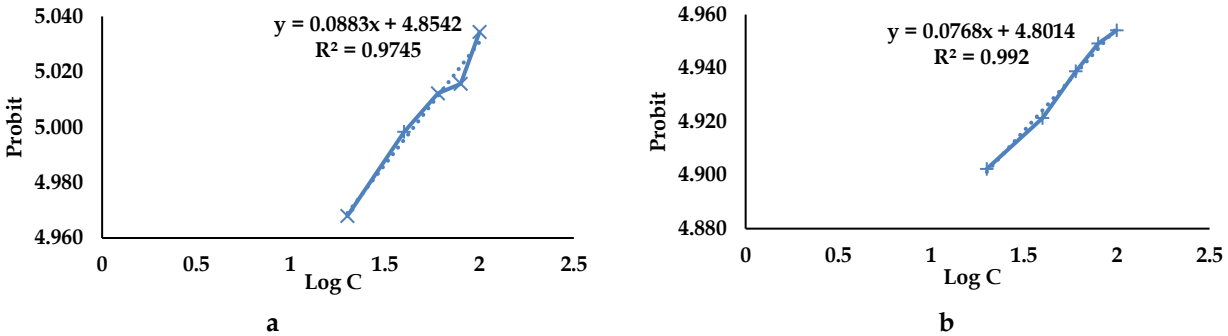
Figure 4. Desirability results from base optimization (**a**) and predicted moisture content results of jelly candy base using the SLD method (**b**).

Table IV. Optimal jelly candy base solution based on SLD method.

Gelatin (%)	Carrageenan (%)	Desirability	Decision
12	4	1.000	Selected

The antioxidant activity of the developed jelly candy formulations was evaluated using the linear regression equation depicted in **Figure 5**. The IC_{50} values, which indicate the concentration required to inhibit the DPPH radical by 50%, were determined for each formulation. The results demonstrated that the jelly candy formulations exhibited a strong antioxidant activity, with an IC_{50} value of 44.771 ppm. In contrast, the jelly candy base alone exhibited a very weak antioxidant activity, with an IC_{50} value of 385.478 ppm[19].

Interestingly, the IC_{50} values of the jelly candy formulations were slightly higher than those of the combined *C. moschata* puree and *A. carambola* juice (IC_{50} = 29.580 ppm). This suggests that the jelly candy base might have a modest inhibitory effect on the antioxidant activity of the combined ingredients. However, the jelly candy formulations still maintained a very strong antioxidant activity category.



a b

Figure 5. Desirability results from base optimization (**a**) and predicted moisture content results of jelly candy base using the SLD method (**b**).

CONCLUSION

Our findings demonstrate that *C. moschata* puree and *A. carambola* juice can be effectively combined to create nutraceutical jelly candies with potent antioxidant properties. The use of gelatin and carrageenan as gelling bases further enhances the formulation's stability and texture.

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AUTHORS' CONTRIBUTION

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Supervision: Andi Tenri Kawareng, Risna Agustina
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Visualization: -
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Writing - review & editing: Andi Tenri Kawareng, Risna Agustina

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Ethnomedicine Study of Medicinal Plants for Therapy of Elderly Sleep Disorders in Tengger Tribe

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Elderly
Plants
Sickness
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Abstract

Ethnomedicine offers valuable insights into plant-based therapies, potentially leading to the discovery of novel drugs. Sleep disturbances, including difficulty falling asleep, maintaining sleep, and early morning awakening, are prevalent among the elderly population and can significantly worsen Alzheimer's disease progression. This study explores the medicinal plants utilized by the Tengger tribe's elderly population for treating sleep disorders. Employing a mixed-methods approach, the study involved qualitative data collection through snowball sampling and in-depth interviews with 99 elderly participants and three traditional healers of the Tengger tribe. Quantitative data was obtained through questionnaires administered during field surveys. Participants were selected based on specific criteria: elderly individuals over 60 years of age, native Tengger tribe members with a history of using medicinal plants for sleep disorders; traditional healers were required to be native Tengger tribe members with knowledge passed down through generations. The study identified a total of 11 medicinal plants used for sleep disorders. Five plant species emerged as the most dominant based on the highest citation value (FC) analysis: kale (*Ipomoea reptans*), agarwood (*Aquilaria malaccensis*), sintok (*Cinnamomum sintoc*), Broadleaf plantain (*Plantago major*), and soursop (*Annona muricata*). The most commonly used plant parts were leaves, bark, and roots. Traditional preparation methods included boiling and burning the plant materials. Notably, knowledge of these medicinal plants is primarily transmitted orally within the community. Our findings highlight five medicinal plants employed by the Tengger elderly to manage sleep disorders, with limited documented evidence of their efficacy.

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INTRODUCTION

Indonesia's rich cultural diversity, shaped by its archipelago status and diverse ethnic groups, presents a unique advantage. Each ethnic group possesses distinct traditional knowledge rooted in their culture and heritage. This includes unique perspectives on health, illness, and the use of plant species in traditional medicine. These practices, often passed down through generations, reflect a deep-rooted cultural understanding of health and healing[1,2].

Ethnomedicine, the traditional use of plants for medicinal purposes, offers a valuable resource for discovering novel drug candidates[3]. However, many indigenous communities and healers maintain secrecy around their traditional knowledge, believing that sharing it may diminish their healing abilities. This has led to a significant portion of traditional medicinal knowledge remaining undocumented and at risk of being lost due to oral transmission practices[4]. Habitat degradation, loss

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of plant ecosystems, and cultural erosion further threaten the sustainability of these practices[5,6]. A systematic exploration of traditional medicine is therefore crucial to identify potential alternative treatments for sleep disorders.

Sleep disorders, including difficulty falling asleep (sleep onset insomnia), early morning awakening, and disrupted deep sleep (deep maintenance insomnia), are prevalent among the elderly[7]. Over 80% of individuals aged 60 and older report sleep disturbances, which can contribute to the early stages of neurodegenerative diseases like Alzheimer's and Parkinson's dementia[8]. Sleep disorders, characterized by insomnia, inadequate sleep duration, and frequent awakenings, can significantly impact overall health and contribute to the development of various neurological and non-neurological conditions.

In the elderly population, sleep disturbances occur in approximately 50-70% of individuals aged 65 and older[Error! Reference source not found.]. Globally, the incidence of sleep disorders in this age group is estimated to be between 13% and 47%[10]. In Indonesia, around 67% of senior citizens experience sleep difficulties[Error! Reference source not found.]. Three villages in the Poncokusumo District, namely Ngadas, Gubuk Klakah, and Pandansari, have a notably high proportion of elderly residents. According to medical records from the General Poly Poncokusumo Health Center on 2019, as many as 40.7% of these elderly individuals reported sleep disorders and other physical ailments.

Disordered sleep is recognized as one of the symptoms of a condition known as *kancilan/kancilen* within the ethnomedicine framework of the Tengger tribe. The Tengger tribe, residing in Poncokusumo District, Malang Regency, Indonesia, has a rich tradition of utilizing medicinal plants for various ailments[Error! Reference source not found.]. Given the significant elderly population and the long-standing use of traditional medicine within this community, there is a compelling need to investigate the ethnomedical knowledge related to sleep quality disorders. This study aims to document the traditional medicinal herbs employed by the elderly Tengger tribe to address sleep disturbances. Specifically, we will explore the application and preparation methods of these plants for treating sleep disorders. This research endeavors to preserve valuable traditional knowledge that might otherwise be lost over time[12].

MATERIALS AND METHODS

Materials

This ethnobotanical study was conducted in the Tenggerese villages of Ngadas, Gubuk Klakah, and Pandansari, located within the Poncokusumo District of Malang Regency, East Java, Indonesia. The region is characterized by a consistent climate and topography, situated between 112.1330° to 122.5455° East Longitude and 7.5890° to 8.6813° South Latitude as shown in **Figure 1**. Data collection spanned from September 2023 to January 2024, encompassing the following stages:

1. Participant Selection: Participants were recruited from the three selected villages.
2. Data Collection: Interviews and direct observations were conducted to gather information on plant usage for sleep disorders.
3. Plant Sample Collection: Plant samples were collected for identification and subsequent analysis.
4. Literature Review: Existing literature was reviewed to verify plant identities and scientific names.

The study methodology encompassed both qualitative and quantitative approaches. Qualitative data collection involved interviews and direct observations to understand the traditional knowledge and practices related to plant usage. Quantitative data was gathered through plant sample collection and subsequent literature reviews to verify plant identities and scientific names. This research was approved by the Health Research Ethics Committee of Universitas Airlangga (certificate No. 1140/HRECC.FODM/X/2023) prior to commencement.

Methods

Selection of informants

Purposive and snowball sampling techniques were employed to identify suitable informants for this study[14]. Inclusion criteria for the elderly community included being native Tengger tribesmen or descendants over the age of 60, having used medicinal plants for at least five years to address sleep disorders, possessing strong communication skills, and being willing to participate as research informants. Exclusion criteria for the elderly community were not being residents of Ngadas, Gubuk Klakah, or Pandansari villages and lacking knowledge of medicinal plants within a traditional medicine context.

Traditional healers were also included in the study. Inclusion criteria for traditional healers comprised being native Tengger tribesmen with a generational knowledge of traditional medicine and being recognized as trusted healers by the surrounding community for at least five years. Exclusion criteria for traditional healers mirrored those of the elderly community, specifically excluding individuals not residing in the designated villages and those lacking knowledge of medicinal plants within a traditional medicine context.

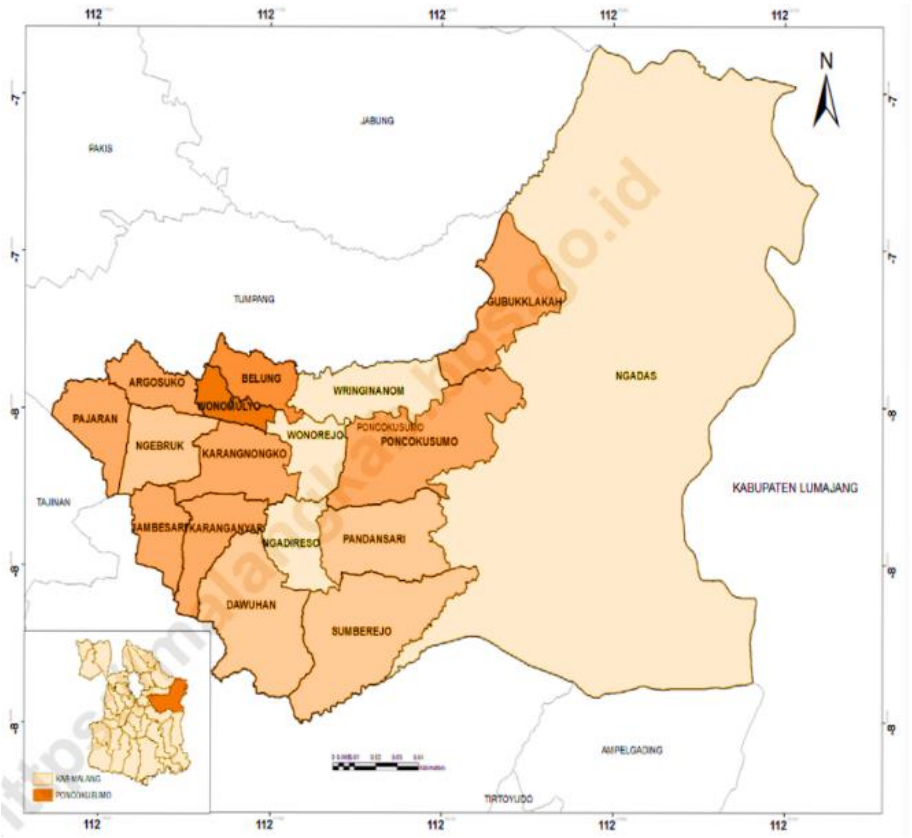

Figure 1. Ethnomedicine research location in the Tengger tribe, Poncokusumo District, Malang Regency (source: https://malangkab.bps.go.id/id).

Data collection

Data collection involved semi-structured interviews and observations to gather information about participants' demographics, knowledge of medicinal plants, processing methods, and the use of plants for treating sleep disorders. The questionnaire focused on eliciting details about the participants' health status, their understanding of sleep quality disorders, and their specific experiences with medicinal plants used to address these issues.

Data analysis

Data collected through field notes, interviews, questionnaires, and observations were systematically analyzed to identify medicinal plants used for treating sleep disorders, associated processing methods, and traditional practices for their application. To assess the prevalence of these medicinal plants in treating elderly patients' sleep problems, a quantitative analysis was conducted by calculating the citation frequency of each plant within our dataset.

RESULTS AND DISCUSSION

A total of 102 informants participated in the study, comprising 99 elderly community members and three traditional healers. Demographic characteristics are summarized in **Table I**. The majority of informants were female (86%), with the most common age group being 61-70 years old (68%). In terms of education, 49% of informants had a junior high school education or equivalent. The most prevalent religious backgrounds were Hinduism (45%), Islam (44%), and Buddhism (11%). The three traditional healers included in the study were all male (100%) with a majority having an elementary/middle school

education (67%). Additionally, 67% of the traditional healers identified as Hindu. The analysis of interview and questionnaire data yielded a comprehensive list of medicinal plants utilized by the community, along with their reported benefits and commonly used plant parts. This information is presented in **Table II** and **Figure 2**.

Table I. Demographic profile of informants (n=102).

Demographic profile	Group	Number of informants (elderly/healer)	
		N	%
Gender	Male	14/3	14/100
	Female	85/0	86/0
Age (years)	61-70	67/0	68/0
	71-80	26/2	26/67
	81-90	6/1	6/33
Education	Elementary school	24/2	24/67
	Secondary school	49/1	49/33
	High school	26/0	27/0
Routine activities	Workers	72/3	73/100
	Chicken farm worker	2/0	2/0
	Housewife	25/0	25/0
Religion	Islam	45/1	45/33
	Hindu	44/2	44/67
	Buddha	10/0	11/0

Table II. Medicinal plants based on plant parts used and processing methods.

Medicinal plant	Local name	Scientific name	Parts used	Processing method	Citation frequency (%)
Kale	*Kangkung*	*Ipomoea reptans*	Root	3-7 bunches of roots boiled	27
Agarwood and sintok	*Gaharu* and *sintok*	*Aquilaria malaccensis* and *Cinnamomum sintoc*	Stem bark	1 g of agarwood and 0.5 g of sintok barks for 1 inch of stem for burning	26
Broadleaf plantain	*Suri pandak*	*Plantago major*	Leaf	7-9 leaves boiled	18
Soursop	*Sirsak*	*Annona muricata*	Leaf	3, 5, or 7 leaves boiled	18
Pangotan	*Pangotan*	*Microsorum buergerianum*	Root	1-3 bunches of roots boiled	7
Tamarind perch	*Asam tengger*	*Radicula armoracia*	Shoots leaf	3, 5, or 7 leaves boiled	6
Mountain amethyst	*Kecubung gunung*	*Brugmansia candida*	Leaf	1-3 leaves boiled	8
Slender grape	*Ketirem/Tiyu*	*Cayratia clematidea*	Leaf	7 leaves boiled	5
Black nightshade	*Ranti*	*Solanum nigrum*	Leaf	3-5 leaves boiled	10
Garlic	*Bawang putih*	*Allium sativum*	Bulbs	1-3 cloves burned	3

a **b** **c** **d** **e**

Figure 2. Kale root (**a**), Broadleaf plantain leaf (**b**), soursop leaf (**c**), agarwood bark powder (**d**), and sintok bark (**e**).

Our ethnobotanical survey identified 11 medicinal plants used by the elderly Tengger community to address sleep disorders. These plants belonged to 10 distinct families, with *Convovulaceae*, *Malvaceae*, and *Lauraceae* being the most commonly represented (20% each). Other families included *Plantaginaceae* (15%), *Annonaceae* and *Solanaceae* (14% each), *Polypodiaceae* (6%), *Brassicaceae* (5%), *Vitaceae* (4%), and *Liliaceae* (2%) (**Figure 3**). Tengger elders demonstrated a nuanced understanding of medicinal plant usage, utilizing various plant parts, including leaves, bark, roots, leaf shoots, and bulbs. Consistent with previous ethnomedicinal studies, leaves emerged as the most commonly utilized plant part in traditional medicine practices among the elderly Tengger community with 40% (**Figure 4**)[12]. This preference is likely attributed to the relative ease of processing leaves through methods like boiling or pounding, making them readily accessible and adaptable for various medicinal applications[15]. Plant classification at the family level is a crucial factor in understanding the potential

medicinal value of plant species within local communities[5]. By examining the family-level classification of the identified medicinal plants, researchers can gain valuable insights into their pharmacological properties and potential therapeutic applications[16].

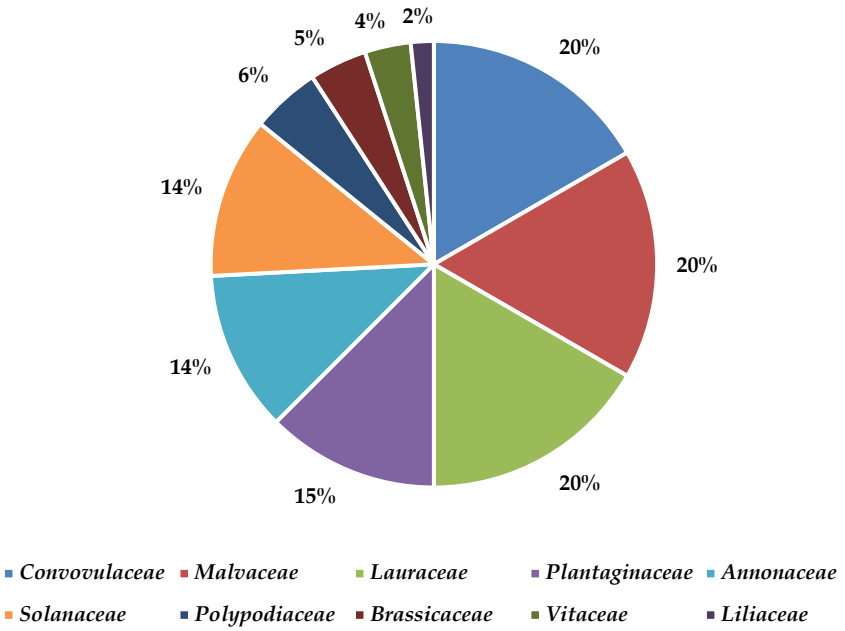

Figure 3. Families of medicinal plants used for therapy of elderly sleep disorders in Tengger Tribe.

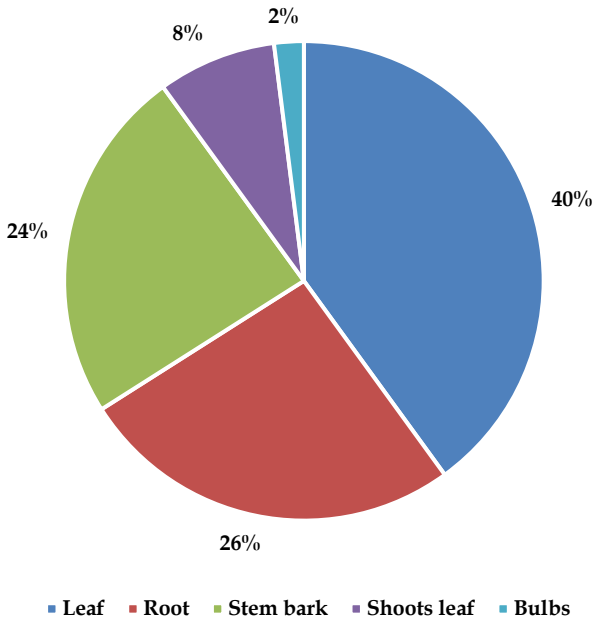

Figure 4. Parts of medicinal plants used for therapy of elderly sleep disorders in Tengger Tribe.

Ipomoea reptans contains notable levels of potassium and sodium[17]. These elements, often found as bromide salts, are known to exert a sedative effect by suppressing the central nervous system. Setiawan[18] proposed that the high potassium and sodium content in *I. reptans* binds with bromide to form bromide compounds. These bromide salts stimulate the inhibitory centers in the brainstem's reticular formation, leading to the opening of chloride channels and subsequent cell hyperpolarization[19]. This depolarization resistance contributes to the hypnotic and relaxing effects observed.

Quercetin, a flavonoid present in *I. reptans*, can also influence the central nervous system by stimulating the reticular formation's inhibitory centers. Additionally, quercetin modulates GABA receptors and ligand-gated ion channels, potentially inhibiting the conduction of nerve impulses and leading to a slower reaction time. Tissues containing quercetin were predominantly found in the roots (>50%), with some distribution in the stem and rhizome[20].

Aquilaria malaccensis, a medicinal plant traditionally used in cosmetics and aromatherapy, contains flavonoids, glycosides, tannins, and triterpenoids[21]. Previous research has demonstrated its potential neuroactive properties, including sedative effects[22]. Wang *et al*.[23] identified benzylacetone, alpha-gurjunene, and [+]-calarene as the major volatile components of *A. malaccensis* essential oil and reported its anesthetic effects in rats when inhaled. To further explore the sedative properties of benzylacetone, the study synthesized several derivatives and evaluated their sedative potency. The findings revealed that benzylacetone-like compounds exhibit sedative effects, with the strength of these effects influenced by the specific benzene ring substituents and carbon chain functional groups.

Plantago major, a member of the *Plantaginaceae* family, is a wild-growing medicinal plant commonly found in forests, fields, and damp areas. Previous studies have identified various bioactive compounds in P. *major* leaves, including phenolics, carboxylic acids, flavonoids, beta carotene, ascorbic acid, choline, and niacin[24]. Caro *et al*.[25] reported the traditional use of *Mentha spicata* and *P. major* tea for treating depression and insomnia in Colombian populations. These plants are believed to possess anxiolytic and hypnotic properties[26]. In Wistar rats, *P. major* extract (1000 mg/kg) was found to significantly prolong pentobarbital-induced sleep time[25].

Annona muricata fruits and leaves contain alkaloids like anonaine, nornuciferine, and asimilobine, which have known antidepressant properties[27]. Alkaloids can inhibit serotonin uptake in the brain, a neurotransmitter linked to mood regulation[28]. Previous studies on mice demonstrated that *A. muricata* leaf extract, when administered alongside a forced swimming test, exhibited antidepressant effects comparable to a positive control group receiving conventional antidepressants[29].

Furthermore, *A. muricata* leaves are known to contain flavonoids[30]. Flavonoids possess antioxidant properties due to their reactive hydroxyl groups. These compounds can neutralize free radicals, preventing oxidative stress-related diseases[31]. Flavonoids also interact with important enzymes in mitochondria and chelate divalent metal ions. Emerging evidence suggests that flavonoids may exert antidepressant effects by modulating monoamine neurotransmitter transmission in the brain[32]. While further research is warranted, the potential of *A. muricata* leaves as a natural alternative to conventional antidepressants warrants exploration, given their alkaloid and flavonoid content.

Five plants traditionally used by the Tengger Tribe for treating elderly sleep disorders were identified. Among these, only the leaves of *P. major* have been studied for their sedative and hypnotic properties[25]. The remaining four plants, *I. reptans*, *A. malaccensis*, *C. sintoc*, and *A. muricata*, have only been investigated for their sedative effects. This suggests a promising avenue for future research, particularly focusing on the hypnotic properties of these plants.

CONCLUSION

This ethnomedicinal study among the elderly Tengger people in Indonesia identified five prominent medicinal plants used to address sleep disorders: *I. reptans*, *A. malaccensis*, *C. sintoc*, *P. major*, and *A. muricata*. Leaves were the most commonly used plant part, and traditional processing methods often involved boiling and reciting mantras or prayers according to individual religious beliefs. Literature reviews support the potential of these plants for treating sleep disorders. The identified plants contain active compounds with sedative, antidepressant, and anxiety-reducing properties. Further research is warranted to validate these traditional uses and isolate the specific bioactive compounds responsible for their therapeutic effects.

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Funding acquisition: Aty Widyawaruyanti
Investigation: Devanus Lahardo
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Resources: Devanus Lahardo, Wiwied Ekasari, Aty Widyawaruyanti
Software: -
Supervision: Wiwied Ekasari, Aty Widyawaruyanti
Validation: Wiwied Ekasari, Aty Widyawaruyanti
Visualization: Devanus Lahardo
Writing - original draft: Devanus Lahardo, Wiwied Ekasari, Aty Widyawaruyanti
Writing - review & editing: Devanus Lahardo, Wiwied Ekasari, Aty Widyawaruyanti

DATA AVAILABILITY

The underlying data supporting the findings of this study are available upon request.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

Effects of Switching Dose, Dose Variation, and Warfarin Interaction on the Incidence of Stroke Recurrence in Stroke Patients with Atrial Fibrillation

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Keywords:
Atrial fibrillation
Recurrence stroke
Stroke
Warfarin

Abstract

Atrial fibrillation (AF) significantly increases the risk of stroke, necessitating anticoagulation therapy. Warfarin, a commonly prescribed anticoagulant regimen, requires careful monitoring to ensure patient safety. This study aimed to assess the impact of dose switching, dose variation, and potential interactions with warfarin on the incidence of stroke recurrence in stroke patients with AF. The study retrospectively analyzed the treatment records of stroke patients with AF in outpatient settings over one year. The subjects comprised 314 patients who received warfarin prescriptions at two Indonesian Hospitals from January 1, 2015, to December 31, 2019. Out of these patients, 50 had recorded data regarding dose adjustments, variations, and interactions. They were divided into two groups: a case group (n=11) with stroke recurrence and a control group (n=39) without recurrence. Statistical analysis, including chi-square tests and odds ratio calculations, revealed that both warfarin dose switching (OR=7.6) and dose variation (OR=6.6) significantly influenced the incidence of stroke recurrence. It implies that inconsistencies or alterations in warfarin dosing substantially elevate the likelihood of experiencing another stroke, potentially due to inadequate anticoagulation leading to clot formation. Interestingly, the analysis of drug interactions did not significantly impact stroke recurrence. In summary, the recurrence of stroke in patients with AF is notably influenced by warfarin dose adjustments and variations rather than drug interactions. This study highlights the critical importance of precise dosing strategies and vigilant monitoring to enhance the efficacy of anticoagulant therapy in this high-risk population.

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INTRODUCTION

Stroke, a neurological disorder characterized by impaired cerebral blood flow, imposes a significant burden on individuals and healthcare systems worldwide. Hemorrhagic stroke, in particular, requires extensive long-term treatment and incurs substantial costs, often leading to decreased patient productivity[1]. Globally, stroke remains a major health concern, affecting over 101 million people, with an annual incidence of 12.2 million new cases. Alarmingly, 6.5 million individuals succumb to stroke each year. While stroke traditionally impacted older populations, recent data indicate a concerning trend towards younger-onset strokes, with 63% of stroke cases occurring in individuals under 70 years of age in 2019[2]. In the United States, stroke ranks second only to ischemic heart disease as a leading cause of death, with approximately 795,000 individuals experiencing a stroke annually[3].

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Cardioembolic stroke, a type of ischemic stroke, occurs when blood clots (emboli) formed in the heart are carried to the brain, causing neurological deficits. Atrial fibrillation (AF), a cardiac rhythm disorder, is the most common cause of cardioembolic stroke, accounting for 45% of cases[4]. Strokes associated with AF carry a higher risk of recurrence, death, and dependence compared to those without AF, even after one year (42% vs 17.8%; 50% vs 36.1%; 32.2% vs 24.1%)[5,6]. This risk profile increases further in the third year of follow-up.

Anticoagulation therapy is a crucial strategy for reducing stroke risk in patients with AF. The Indonesian Heart Association recommends anticoagulants to mitigate hypercoagulation, prevent cardioembolic stroke, and reduce the risk of postoperative venous thrombosis and pulmonary embolism[7]. The anticoagulant landscape in Indonesia includes both vitamin K antagonists (VKAs) and the newer direct-acting oral anticoagulants (DOACs) like dabigatran, rivaroxaban, and apixaban. VKAs have historically been the mainstay of oral anticoagulation for stroke prevention[8]. However, DOACs have emerged as promising alternatives, offering several advantages in terms of convenience and safety profiles.

Anticoagulant therapy, particularly warfarin, is a cornerstone in preventing stroke events among individuals with a high risk of AF[9]. However, effective warfarin management necessitates careful monitoring due to its susceptibility to interactions with food and medications, as well as the presence of contraindications that can increase the risk of bleeding[10]. Individualized dosing of warfarin is crucial given the wide variability in bleeding rates associated with its use. The International Normalized Ratio (INR) serves as a key indicator for monitoring warfarin therapy, as deviations from the target range necessitate dose adjustments. Interactions between warfarin and other substances can pose significant risks to patients, potentially leading to adverse health outcomes and increased healthcare costs[11].

Drug-drug interactions (DDIs) and bleeding risks are significant concerns among inpatients receiving warfarin therapy in our institution. Clinicians must be vigilant in identifying potential DDIs and closely monitoring INR levels[12]. Previous research has consistently demonstrated the high prevalence of DDI during warfarin therapy, both in inpatients and outpatients[13,14]. To mitigate the risk of adverse events, strategies for identifying and managing warfarin-drug interactions are essential.

Although the absolute and relative risks of AF-associated ischemic stroke have decreased in the past two decades, AF remains a significant risk factor, contributing to approximately one in four ischemic strokes in 2020[15]. This underscores the substantial potential for improving stroke prevention among individuals with AF. Identifying potential drug interactions involving warfarin is crucial to minimizing adverse effects in patients with AF. The risk of adverse events increases with the number of medications administered concurrently with warfarin[16]. This study aimed to investigate the impact of dosage adjustments, variations, and drug interactions on the recurrence of stroke in AF patients receiving warfarin therapy. Two hospitals, a central hospital and a regional hospital, were selected for this study due to their high prevalence of AF-associated stroke cases.

MATERIALS AND METHODS

Materials

This study was conducted at Dr. Sardjito Hospital in Yogyakarta and Dr. Moewardi Hospital in Solo, Central Java, from August 2019 to March 2020. Data collection involved the retrospective review of patient medical records, which were summarized in a standardized data collection form. Data were collected using a standardized Case Report Form (CRF) designed to capture demographic information (medical record number, gender, date of birth, admission date), AF characteristics (anticoagulant medication, dosage, frequency, diagnosis of comorbidities, and in-hospital events), and laboratory data, medication usage, and nursing notes from the patient's medical records. Ethical approval for this study was obtained from the Faculty of Medicine, Universitas Gadjah Mada, and the Medical and Health Research Ethics Committee (MHREC) (Ethics Committee Approval Ref: KE/FK/002/EC/2018).

Methods

This study employed a retrospective case-control design to investigate the relationship between warfarin therapy and recurrent stroke in patients with AF. Data were collected from medical records of patients hospitalized at Dr. Sardjito General Hospital (218 patients) and RSUD Dr. Moewardi (96 patients) between January 1, 2015, and December 31, 2019. Inclusion criteria included patients aged 45 or older, outpatients, diagnosed with AF and stroke (ICD-10 codes I63 and I48),

and receiving warfarin therapy for at least six months and were observed for a year after the warfarin administration related to the frequency of examination and the therapeutic value of INR on the incidence of recurrent stroke,. Exclusion criteria encompassed patients who discontinued warfarin or switched to other anticoagulants, died during the observation period, or had a history of stage IV renal failure, pregnancy, or breastfeeding. A total of 50 patients met these criteria, divided into a case group (11 patients with recurrent stroke) and a control group (39 patients without recurrent stroke). The primary outcome was the incidence of recurrent stroke within one year of warfarin initiation, with consideration of examination frequency and INR therapeutic values.

Data analysis

To investigate the association between warfarin therapy factors (dose switching, dose variance, and drug interactions) and stroke recurrence, data analysis was performed using SPSS version 22. Statistical significance was set at a p-value of <0.05 and a 95% confidence interval. Bivariate analysis was conducted to identify variables associated with stroke recurrence. Chi-square tests were employed to analyze the relationships between predictor variables and the outcome variable. To assess the risk factors associated with AF and stroke recurrence, odds ratios (ORs) were calculated.

RESULTS AND DISCUSSION

As detailed in **Table I**, the study included patients with AF who had experienced a stroke and were receiving warfarin therapy. This retrospective study analyzed outpatient data from a one-year period to evaluate the association between warfarin treatment and stroke recurrence. Our analysis revealed that the timely administration of warfarin, along with appropriate supportive medications, was crucial in preventing stroke recurrence in patients with AF. Adherence to warfarin therapy and the use of additional preventive medications significantly reduced the risk of recurrent strokes. Both case and control groups consisted predominantly of patients over 60 years old (case: 45.5%; control: 71.8%) and a majority of female participants (case: 72.7%; control: 59%). All subjects in both groups received switching doses and utilized Indonesian health insurance for examinations (case: 90.9%, 90.9%; control: 56.8%, 86.8%). Notably, dose variations were observed in 81.8% of case subjects but in only 59.5% of control subjects. Congestive heart failure, hypertension, and diabetes were prevalent comorbidities in both groups.

Warfarin (brand names: Warfarin, Simarc, Notisil) is administered in 2 mg tablets. Dosage is individualized according to patient factors and regular INR monitoring. A review of patient medical records revealed a wide range of prescribed warfarin dosages, from 1 to 7 mg per day, as summarized in **Table II**. As depicted in **Table II**, the most frequent warfarin dosage in the stroke group was 1 mg/2 mg daily, indicating a biphasic dosing regimen with a higher dose on Mondays and Thursdays. In contrast, the non-stroke group exhibited a higher prevalence of 2 mg daily warfarin use. These findings align with the Oxford Haemophilia and Thrombosis Centre Protocols for Outpatient Oral Anticoagulation with Vitamin K Antagonists[17], which recommend a starting dose of 2 mg warfarin for patients with an INR within the target range of 2.6-2.7 after at least 7 days of treatment.

The analysis revealed that patients in this study frequently received non-warfarin medications to manage comorbidities such as congestive heart failure, diabetes mellitus, and hypertension. These medications can interact with warfarin, affecting its therapeutic efficacy and increasing the risk of bleeding events. It is essential to consider these interactions when administering non-warfarin drugs to patients on warfarin therapy. Bisoprolol, furosemide, and candesartan were the most frequently prescribed non-warfarin medications in both the case and control groups, accounting for 64%, 55%, and 44% of prescriptions, respectively. This aligns with the 2017 ACC/AHA/HFSA Focused Update on the 2013 ACCF/AHA Guideline for Management of Heart Failure[18], which recommends beta-blockers, ACE inhibitors or ARBs, and diuretics as a cornerstone of treatment for congestive heart failure.

Drug interactions are a common concern in polypharmacy. While this analysis did not explicitly assess drug interactions, it is important to note the potential for significant, moderate, or minor interactions among the prescribed medications. Careful monitoring and management of drug interactions are crucial to ensure optimal patient outcomes[19]. Drug interactions were classified based on their severity as major, moderate, or minor. Major interactions pose a significant health risk, potentially leading to life-threatening complications or prolonged/permanent damage. Moderate interactions, while not as severe, can

still cause substantial harm if left unaddressed. Minor interactions, although generally not harmful, may still result in some adverse effects[20].

Both the stroke recurrence and non-recurrence groups exhibited a high prevalence of drug interactions, as shown in **Table III**. Major interactions were observed in 81% of the recurrence group and 69% of the non-recurrence group. Moderate interactions were even more prevalent, occurring in 91% and 89% of patients, respectively. Minor interactions were also common, affecting 72% and 87% of patients. There were no statistically significant differences in the frequency of major, moderate, or minor interactions on recurrent stroke outcomes between the groups (p >0.05).

Table I. Patient characteristics.

Variable	Case group (recurrence stroke) N =11(22%)	Control group (non-recurrence stroke) N=39(78%)	p
Age (years)			
40-60	5(45.5)	9(23.1)	0.25
<40	1(9)	2(5.1)	
>60	5(45.5)	28(71.8)	
Sex			
Male	3(27.3)	16(41)	0.50
Female	8(72.7)	23(59)	
Switching dose			
Switching dose	10(90.9)	21(56.8)	0.04
No switching dose	1(9.1)	16(43.2)	
Dose variance			
Yes	9(81.8)	15(40.5)	0.03
No	2(18.2)	22(59.5)	
Health insurance			
Indonesian Social Security and Health	10(90.9)	33(86.8)	1.00
Non-Indonesian Social Security and Health	1(9.1)	5(13.2)	
Disease Risk Factor			
Diabetes mellitus			
Yes	2(18.2)	4(10.3)	0.60
No	9(81.8)	35(89.7)	
Hypertension			
Yes	2(18.2)	16(41)	0.28
No	9(81.8)	23(59)	
Congestive heart failure			
Yes	5(45.5)	16(48.7)	1.00
No	6(54.5)	23(51.3)	
Dyspepsia			
Yes	2(18.2)	6(15.4)	1.00
No	9(81.8)	33(84.6)	
Mitral valve insufficiency			
Yes	2(18.2)	14(35.9)	0.48
No	9(81.8)	25(64.1)	
Rheumatic tricuspid valve disease			
Yes	0(0)	7(17.9)	0.32
No	11(100)	32(82.1)	
Congenital malformation of aortic and mitral valve			
Yes	0(0)	4(10.3)	0.56
No	11(100)	35(89.7)	
Osteoarthitis			
Yes	1(9.1)	3(7.7)	1.00
No	10(90.9)	36(92.3)	
Chronic kidney disease			
Yes	0(0)	1(2.6)	1.00
No	11(100)	38(97.4)	
Disorder of lipoprotein metabolism and other lipidemia			
Yes	0(0)	2(5.1)	1.00
No	11(100)	37(94.9)	
Multiple sclerosis			
Yes	1(9.1)	0(0)	0.22
No	10(90.9)	39(100)	
Chronic ischemic heart disease			
Yes	1(9.1)	0(0)	0.22
No	10(90.9)	39(100)	

Table II. Warfarin dose in each group.

Oral warfarin dosage (daily dose)	Case (recurrence stroke) N =11(22%)	Control (non-recurrence stroke) N=39(78%)
1 mg	0(0)	6(15)
1mg/2 mg	4(36)	6(15)
2mg	0(0)	14(36)
2mg/3mg	2(18)	9(23)
2mg/4mg	0(0)	1(3)
3mg	3(27)	1(3)
4mg/6mg	1(9)	2(5)
6mg/7mg	1(9)	0(0)

Table III. Average patient drug interactions.

Drug interactions	Case (recurrence stroke) N =11(22%)	Control (non-recurrence stroke) N=39(78%)	p
Major	81	69	0.30
Moderate	91	89	0.49
Minor	72	87	0.36

Analysis of potential drug interactions is crucial for optimizing patient outcomes. **Table IV** presents the variables associated with recurrent stroke. However, statistical analysis revealed no significant correlation between the three investigated interactions and stroke recurrence (p-value >0.05). Warfarin interactions, categorized as major, moderate, or minor, were not found to be associated with stroke recurrence risk.

Table IV. Warfarin dose switching, variance and warfarin drug interactions in the incidence of recurrent stroke.

Variable	Case (reccurence stroke) N=11(22%)	Control (non-recurrence stroke) N=39(78%)	OR (95% CI)	p
Switching dose				
Yes	10(90.9)	21(56.8)	7.6(0.8-65.8)	0.04
No	1(9.1)	16(43.2)		
Dose variance				
Yes	9(81.8)	15(40.5)	6.6(1.2–34.9)	0.03
No	2(18.2)	22(59.5)		
Major interaction				
Yes	3(27.3)	8(20.5)	1.4(0.3-6.7)	0.68
No	8(72.7)	31(79.5)		
Moderate interaction				
Yes	6(54.5)	19(48.7)	1.2(0.3-4.8)	1.00
No	5(45.5)	20(51.3)		
Minor interaction				
Yes	6(54.5)	29(74.4)	0.4(0.1-1.6)	0.24
No	5(45.5)	10(25.6)		

Several previous studies have demonstrated the effectiveness of anticoagulant therapy in reducing stroke risk for patients with AF. Standard anticoagulants were associated with a 22% reduction in stroke risk compared to aspirin[21]. This aligns with the recommendation that most AF patients should receive long-term oral anticoagulants to prevent ischemic stroke and other stroke types[22]. Warfarin therapy has also been shown to reduce ischemic stroke risk by 43.5%[23]. Additionally, non-VKAs oral anticoagulants are considered safer and equally effective as warfarin in stroke prevention[24]. Our research differs from previous studies in its specific focus. While existing studies have generally examined the effectiveness of various anticoagulant therapies, including warfarin and non-VKAs oral anticoagulants, in reducing stroke risk for AF patients, this study aimed to investigate the relationship between warfarin treatment variables, such as dosage adjustments, dose variability, and drug interactions, and the incidence of stroke recurrence in this population.

The management of warfarin therapy requires careful consideration of dosage adjustments and potential drug interactions. Warfarin can interact with numerous medications, affecting the maintenance dose, particularly when interacting drugs are introduced or discontinued. These interactions can be pharmacokinetic or pharmacodynamic in nature[25]. For patients initiating warfarin therapy, the dose should be adjusted based on regular INR monitoring at specified intervals. The goal is to achieve a therapeutic INR range as quickly as possible, as excessively high INR levels can increase the risk of bleeding, while subtherapeutic INR levels may lead to recurrent stroke[26]. Given the risk of bleeding associated with warfarin therapy, routine monitoring of INR levels is essential. The target INR range is typically 2.0-3.0[27].

In this study, we observed that 90.9% of patients in the case group required routine warfarin dose adjustments within one year. Statistical analysis revealed a significant association between warfarin switching dose prescription and stroke

recurrence (OR = 7.6). This indicates that patients receiving variable warfarin doses had a 7.6-fold higher risk of recurrent stroke compared to those with consistent dosing. Such fluctuations in warfarin dosing can disrupt the delicate balance required for effective anticoagulation, leading to suboptimal blood thinning levels. These deviations create windows of vulnerability where clot formation is more likely, increasing the risk of subsequent strokes in patients relying on warfarin therapy.

Our analysis revealed a significant association between dose variation and stroke recurrence in patients with AF (**Table III**). Approximately 81.8% of patients received variable doses, highlighting the importance of consistent dosing for stroke prevention. Statistical analysis demonstrated that patients with inconsistent dosing had a six-fold higher risk of stroke recurrence (OR = 6.6). These findings underscore the critical need for meticulous monitoring of INR levels and timely dose adjustments to optimize warfarin therapy and minimize the risk of stroke recurrence. In this study, the earliest stroke recurrence occurred three months post-index stroke, while the longest was observed 15 months later. These findings align with previous research indicating a cumulative risk of cardioembolic stroke reaching 10% within the first year and increasing over time[28].

Analysis of drug interactions in stroke recurrence and non-recurrence groups revealed no significant difference in the severity of moderate and minor major interactions. However, both groups experienced a high incidence of drug interactions, with recurrence stroke patients reporting 81% major, 91% moderate, and 72% minor interactions, and non-recurrence stroke patients reporting 69% major, 89% moderate, and 87% minor interactions. To mitigate the risk of drug interactions, strategies such as replacing medications or adjusting dosing intervals should be considered. Consistent with the findings of previous studies, an increase in warfarin prescribing is strongly associated with a higher cumulative incidence of stroke/systemic embolic events (SEEs), major hemorrhage, gastrointestinal hemorrhage, fractures/falls, cardiovascular events, cardiovascular death, and mortality from other causes[29].

An analysis of drug interaction categories revealed no significant difference in the incidence of recurrent stroke for major, moderate, or minor interactions ($p > 0.05$). While statistically insignificant, the potential for drug interactions in stroke patients remains clinically relevant and underscores the risk of adverse drug reactions. The high incidence of drug interactions in this study can be attributed to the large number of medications prescribed to patients, particularly the elderly population, who often have multiple chronic health conditions[30,31].

This study, while offering valuable insights, has several limitations that warrant consideration. Firstly, the focus on two specific hospitals may limit the generalizability of the findings to a broader population. Future studies should consider a multicenter design with a more diverse patient sample to enhance external validity. Secondly, the retrospective nature of the study relies on the quality and completeness of medical records, which may introduce potential biases. While the study can generate hypotheses, it cannot definitively establish causation. Thirdly, the study should be mindful of potential confounding variables, such as age, comorbidities, and other medications, which could influence stroke recurrence risk. Incorporating appropriate statistical methods to control for these factors can strengthen the study's conclusions. Finally, a longer follow-up period might be necessary to capture all potential stroke recurrences, as these events can occur over an extended timeframe. In conclusion, while this study provides valuable insights, particularly regarding clinical relevance and the inclusion of a control group, these limitations should be acknowledged when interpreting the findings. Future research could be enhanced by expanding the sample size, addressing potential confounders, and adopting a multicenter design to improve the generalizability of the results.

Future research on stroke recurrence in patients with AF receiving warfarin therapy should focus on enhancing our understanding of the underlying factors and mechanisms involved. Prospective longitudinal studies with extended follow-up periods are necessary to capture the full spectrum of stroke recurrence patterns over time. Collaborative efforts among multiple medical centers and the implementation of randomized controlled trials can strengthen the evidence base for assessing the impact of various warfarin dosing strategies, including dose variation and switching. Additionally, incorporating pharmacogenomic studies to tailor warfarin dosing based on individual genetic factors can improve treatment efficacy. Comprehensive risk factor analysis, encompassing comorbid conditions, lifestyle variables, and concurrent medications, is essential for a more accurate risk assessment. Evaluating the quality of anticoagulation control, patient-reported outcomes, and the influence of drug interactions on warfarin therapy are crucial areas for future investigation.

CONCLUSION

Our analysis revealed that stroke recurrence in AF patients is significantly influenced by warfarin dosing and dose switching. Inconsistent or altered warfarin dosing increases the risk of recurrent stroke. While major, moderate, and minor DDIs did not exhibit significant differences in stroke recurrence rates, healthcare professionals must remain vigilant in assessing and managing these interactions to optimize anticoagulation therapy.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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Research Article

A Pre-Post Survey Analysis on Pharmacy Students' Perceptions of Pharmacist Roles in the Pharmaceutical Industry

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Abstract

The pharmaceutical industry is undergoing rapid evolution, characterized by a complex regulatory landscape and the need for diverse skill sets. This study aimed to assess pharmacy students' perceptions of the pharmaceutical industry and the impact of a dedicated seminar on their career aspirations and knowledge. A pre-post online survey was administered to 55 undergraduate pharmacy students at the National Pharmacy Seminar 2024, hosted by Jakarta Global University. Data were analyzed using descriptive statistics and the Wilcoxon signed-rank test ($p \leq 0.05$). Results indicate a strong preference for careers in state-owned pharmaceutical companies (63.6%) and research and development departments (34%). The seminar significantly enhanced participants' understanding of pharmacists' roles, industry complexities, drug development challenges, and regulatory requirements. Notably, 93% of participants reported that the seminar met their expectations and provided valuable insights for future career exploration. These findings underscore the importance of educational interventions in shaping pharmacy students' career trajectories and aligning their knowledge with the dynamic pharmaceutical industry.

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INTRODUCTION

The pharmaceutical industry, characterized by advancements in personalized medicine and gene therapies, operates within a complex regulatory landscape that demands stringent quality standards, extensive clinical trials, and careful navigation of drug pricing and vaccine development debates[1]. Success in this field requires a multifaceted skill set, encompassing pharmaceutical sciences, regulatory knowledge, effective communication, and practical experience[2]. Assessing the readiness of aspiring professionals in this area is crucial to addressing the evolving challenges and ensuring a successful career in the pharmaceutical industry.

The transition from academia to the pharmaceutical industry presents significant challenges for pharmacy graduates. Previous studies have highlighted knowledge gaps in regulatory compliance and industry intricacies, underscoring the need for targeted educational interventions[3,4]. Research on the evolving pharmaceutical landscape further emphasizes the importance of equipping students with industry-specific skills[5]. To address these challenges, educational initiatives such as guest lectures, industry talks, and national seminars are crucial. These interventions can bridge the gap between theoretical knowledge and practical industry requirements, fostering a smoother transition for pharmacy graduates[6].

National pharmacy seminars serve as valuable educational platforms for pharmacy students, offering insights into the multifaceted roles and challenges of the pharmaceutical profession[7]. Despite the growing importance of professional

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development seminars, research exploring pharmacy students' perceptions and expectations of these programs remains limited. This study aimed to delve into the perceptions and expectations of pharmacy students who participated in such a seminar, shedding light on their understanding of the industry and their aspirations for future careers. By understanding their perspectives, we can identify areas for improvement in pharmacy education to ensure that students are well-prepared to navigate the complexities of the pharmaceutical field.

MATERIALS AND METHODS

Materials

This study involved 63 undergraduate pharmacy students from Jakarta Global University, Depok, Indonesia, who participated in the "National Pharmacy Seminar 2024: Shaping the Future in Industry, Hospital, and Entrepreneurship," held on January 20, 2024. A purposive sampling method was employed to recruit students who attended the seminar. Of the initial 63 participants, 55 completed both the pre- and post-seminar surveys. The seminar focused on exploring the professional roles of pharmacists in three distinct domains: the pharmaceutical industry, hospital settings, and herbal entrepreneurship. The primary data collection tools for this study were online surveys administered through Google Forms. The pre-seminar survey link is available at: https://s.jgu.ac.id/pre-seminar and the post-seminar survey link is available at: https://s.jgu.ac.id/post-seminar.

Methods

To assess the impact of the seminar on students' knowledge and career interest, an online survey was administered before and after the event. The survey instrument was developed through a review of existing literature and consultations with practicing pharmacists[8]. A pilot test with a small group of pharmacy students was conducted to refine the survey questions for clarity and reliability. The survey consisted of questions related to participants' knowledge of the pharmacist's role in the pharmaceutical industry, career interest in the industry, and demographic information such as gender, class category, and academic year[9]. All survey responses were collected using a four-point Likert scale.

Data analysis

Data analysis was conducted using IBM SPSS version 26. Quantitative data collected from the survey were entered into Excel and subsequently uploaded to SPSS 26 for statistical analysis. Descriptive statistics were calculated to summarize the relevant variables. To assess changes in mean scores between the pre- and post-seminar surveys, the non-parametric Wilcoxon signed-rank test was employed. A p-value of ≤0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The examination of survey demographic data, involving a participant cohort of 55 individuals, revealed distributions across key variables as illustrated in **Table I**. In evaluating the student's interest in the pharmaceutical industry, participants were queried about their future career preferences within pharmaceutical companies. Four students (7.3%) expressed interest in local companies, 16 (29.1%) in multinational enterprises, and 35 (63.6%) in state-owned companies. Additionally, their departmental preferences were assessed, with 34% favoring research and development, followed by 16% and 15% for production and quality control, respectively, among the 67 selections made by the 55 participants. The distribution of interest in other departments is visually represented in **Figure 1**.

The analysis extended to examine students' understanding of the role of pharmacists in the pharmaceutical industry, as detailed in **Table II**. Overall, after the seminar, there was a significant increase in their comprehension with all p-values indicating significance levels below 0.05. Specifically, the post-survey revealed that the three questions of the survey (Q_{1-3}) highlighted a notable enhancement in students' overall understanding of the complexities and highly regulated nature of the pharmaceutical industry (p-value <0.05). In terms of percentages, a total of 42 students (76.4%) demonstrated a heightened familiarity with the intricacies of the pharmaceutical industry (Q_1), while 46 students (83.6%) acquired a better understanding of the challenges and considerations involved in drug development and manufacturing (Q_2). Additionally, 44 students (80.0%) became familiar with the regulatory requirements and compliance standards associated with drug

manufacturing (Q_3) after attending the seminar. This highlights a substantial advancement in students' awareness of the crucial aspects related to drug quality assurance and regulatory adherence, reinforcing the positive impact of the seminar on their knowledge acquisition[10].

The consistent pre- and post-seminar interest levels among participants in pharmaceutical industry employment, as evidenced by the non-significant mean difference, underscore the seminar's effectiveness (Q_{12}). Post-seminar, 54 students demonstrated increased or sustained interest in pursuing a career in the pharmaceutical industry. Notably, only one student reported persistently low interest (pre=4), while none indicated no interest (pre=1) in pursuing a career in the pharmaceutical industry after attending the seminar. These nuanced insights into the participants' interests provide valuable context to the broader assessment of their preferences within the pharmaceutical sector.

Table I. Demographic distribution of survey participants.

Demographic Variable	Options	Number of Participants	%
Gender	Male	7	12.7
	Female	48	87.3
Class category	Evening classes	17	30.9
	Regular classes	38	69.1
Age (years)	18-22	47	85.5
	>22	8	14.5
Study year	1st	29	52.7
	2nd	12	21.8
	3rd	7	12.7
	4th	5	9.1
	5th	2	3.6

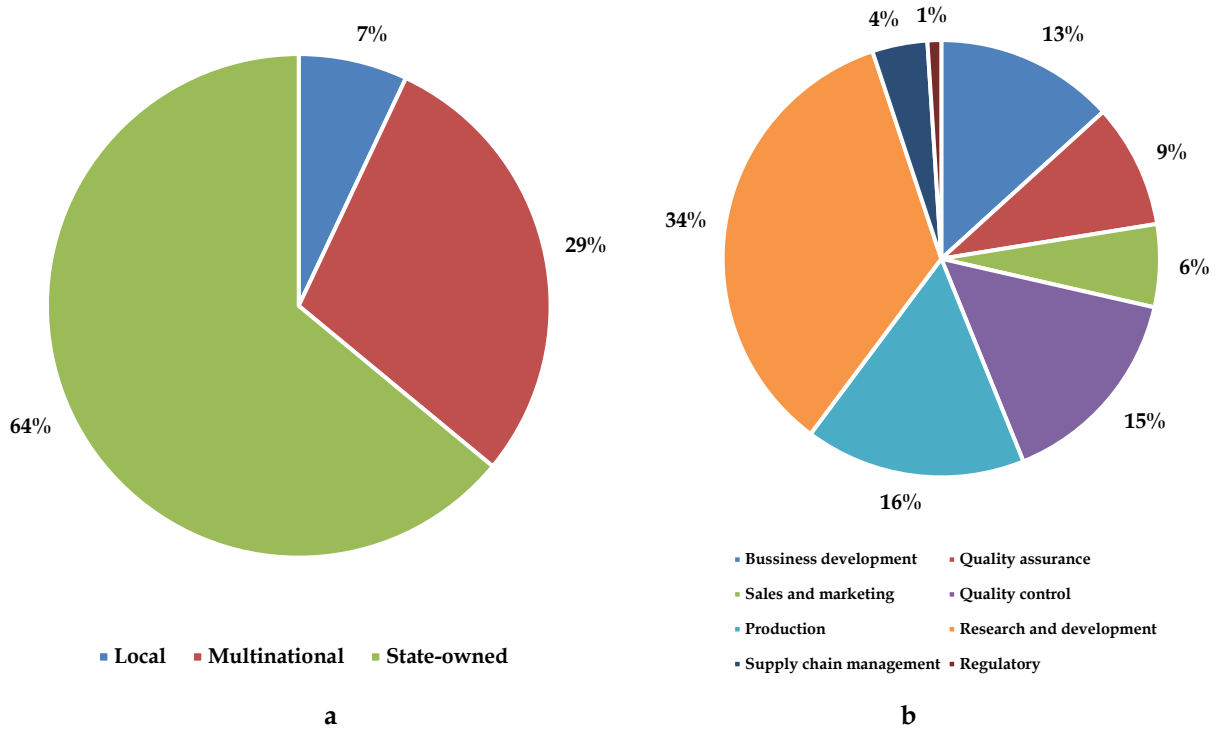

Figure 1. Distribution of participants' preferences for company types (**a**) and various departments in future pharmacy careers (**b**).

The subsequent dataset (**Table II**) comprised seven questions aimed at gauging students' comprehension of the pharmacist's role and responsibility in ensuring drug quality during manufacturing ($Q_{4\text{-}10}$). Post-seminar, there was a notable and statistically significant increase in students' understanding, with p-values consistently below 0.05. Importantly, the majority of students demonstrated a very well understanding, and no students exhibited poor knowledge of the subject. Notably, there was a significant improvement in students' comprehension of the pharmacist's role in ensuring regulatory compliance within the pharmaceutical industry. These findings underscore the positive impact of the seminar on advancing students' comprehension of their prospective roles within the pharmaceutical sector[11,12].

Table II. Pre-post survey analysis of student understanding of seminar topics on the role of pharmacists in the pharmaceutical industry.

No.	Question statement	4 (Pre/Post)	3 (Pre/Post)	2 (Pre/Post)	1 (Pre/Post)	Pre-mean (SD) N=55	Post-mean (SD) N=55	Z-value	*p*-value
Q_1	How well were you acquainted with the intricacies of drug manufacturing processes within a pharmaceutical plant?	Very familiar (1/8)	Moderately familiar (23/34)	Slightly familiar (23/13)	Not familiar at all (8/0)	2.31 (0.74)	2.91 (0.62)	4.262	0.000
Q_2	How familiar were you with the challenges and considerations related to drug formulation and production in a pharmaceutical plant?	Very familiar (2/8)	Moderately familiar (25/38)	Slightly familiar (23/9)	Not familiar at all (5/0)	2.44 (0.71)	2.98 (0.56)	4.316	0.000
Q_3	How familiar were you with the regulatory requirements and compliance standards specific to drug manufacturing in the pharmaceutical industry?	Very familiar (0/8)	Moderately familiar (16/36)	Slightly familiar (27/11)	Not familiar at all (12/0)	2.07 (0.72)	2.95 (0.59)	5.197	0.000
Q_4	How well did you understand the pharmacist responsibilities in ensuring the efficiency and effectiveness of drug manufacturing operations?	Well (8/21)	Moderately (32/34)	Poorly (13/0)	Very poorly (2/0)	2.84 (0.71)	3.38 (0.49)	4.499	0.000
Q_5	How confident were you in understanding the role of a pharmacist in ensuring the quality and consistency of pharmaceutical drug manufacturing processes?	Very confident (6/22)	Moderately confident (27/31)	Slightly confident (16/2)	Not confident at all (6/0)	2.60 (0.83)	3.36 (0.56)	4.945	0.000
Q_6	How well did you understand the pharmacist involvement in overseeing the manufacturing of different drug forms, such as tablets, capsules, and injectables?	Well (10/21)	Moderately (37/32)	Poorly (8/2)	Very poorly (0/0)	3.04 (0.58)	3.35 (0.55)	2.959	0.003
Q_7	To what extent is your understanding of the challenges faced by pharmacists in drug manufacturing in the pharmaceutical industry?	Well (2/12)	Moderately (28/43)	Poorly (23/0)	Very poorly (2/0)	2.55 (0.63)	3.22 (0.42)	5.049	0.000
Q_8	How well did you understand the pharmacist role in	Well (4/30)	Moderately (21/23)	Poorly (25/2)	Very poorly (5/0)	2.44 (0.76)	3.51 (0.57)	6.050	0.000

	optimizing manufacturing processes to meet production targets while ensuring product quality?								
Q_9	How confident were you in your understanding of the plant manager's role in managing resources and personnel to ensure efficient drug manufacturing?	Very confident (3/13)	Moderately confident (18/39)	Slightly confident (28/3)	Not confident at all (6/0)	2.33 (0.75)	3.18 (0.51)	5.201	0.000
Q_{10}	To what extent is your understanding of the pharmacist's contribution to ensuring compliance with regulations and regulatory standards in the drug manufacturing process in the pharmaceutical industry?	Well (5/24)	Moderately (23/28)	Poorly (23/3)	Very poorly (4/0)	2.53 (0.77)	3.38 (0.59)	5.425	0.000
Q_{11}	To what extent is your interest in the expected insights about the responsibilities and challenges faced by pharmacists in drug manufacturing in the pharmaceutical industry?	High interest (24/29)	Moderate interest (26/26)	Low interest (3/0)	No interest (2/0)	3.31 (0.74)	3.53 (0.50)	2.355	0.019
Q_{12}	To what extent is your interest in working in drug manufacturing in the pharmaceutical industry?	High interest (28/24)	Moderate interest (21/30)	Low interest (4/1)	No interest (2/0)	3.36 (0.78)	3.42 (0.53)	0.529	0.597

The seminar evaluation focused on students' expectations (Q_{11}). A significant majority (90.9%, N=55) attended the seminar to gain insights into the challenges and responsibilities of pharmacists in drug manufacturing. Post-seminar evaluations confirmed that all students remained interested in exploring these aspects further. Moreover, **Table III** highlights suggested topics for future seminars, reflecting students' preferences for upcoming events. This culmination of results underscores the overall positive impact of the seminar, as evidenced by the participants' heightened understanding, sustained interest, and favorable reception of the event[13]. This comprehensive analysis of the survey data provides valuable insights into students' perceptions and knowledge development in the pharmaceutical industry.

Table III. Requested seminar topics related to career opportunities in the pharmaceutical industry as suggested by students.

No.	Topic statement
1	Strategies for attaining desired positions
2	Becoming a pharmacist and successful entrepreneur in the pharmaceutical field
3	Detailed explanation of necessary skills, preparations, tips, and tricks for entering the pharmaceutical industry
4	Improving access and drug distribution
5	Enhancing personal quality as a pharmacist within the industrial environment with a focus on the latest insights emphasizing creativity and innovation
6	Business understanding in the pharmaceutical industry, including explanations of marketing strategies, and sales.
7	Knowledge related to jobs in each department associated with pharmacy

The analysis of students' post-seminar interest in the pharmaceutical industry not only revealed a positive impact but also provided insights into the theoretical underpinnings guiding this transformation. The heightened and sustained interest among pharmacy students in pursuing careers in the pharmaceutical industry post-seminar can be dissected through the lens of the "Expectancy-Value Theory," shedding light on the perceived value and anticipated success associated with working in the pharmaceutical sector[14]. For pharmacy students, the pharmaceutical industry offers a plethora of valuable opportunities. Firstly, it provides a dynamic and evolving professional landscape, where individuals can actively contribute to advancements in healthcare, drug development, and patient outcomes. The industry fosters an environment conducive to continuous learning and innovation, offering pharmacists the chance to stay at the forefront of scientific and technological advancements[1,15]. Additionally, a career in the pharmaceutical industry often involves collaboration with multidisciplinary teams, providing pharmacists with the chance to apply their clinical knowledge in a collaborative and impactful manner[16]. Moreover, the industry presents opportunities for career growth, leadership development, and the potential to make a tangible difference in public health. Overall, the pharmaceutical sector, with its emphasis on research, innovation, and patient-centered care, emerges as a valuable and rewarding avenue for pharmacist students keen on contributing to the advancement of healthcare[17,18].

The substantial increase in students' comprehension of the complexities, regulations, and challenges within the pharmaceutical industry post-seminar aligns seamlessly with the principles of the "Constructivist Learning Theory." This theory posits that individuals actively construct knowledge based on their experiences[19]. The seminar, functioning as an experiential learning opportunity, acted as a catalyst for students to engage actively with the intricacies of the pharmaceutical industry, facilitating the internalization of crucial information about their future roles[20]. This active engagement enabled students to not only grasp theoretical concepts but also to contextualize and apply their knowledge within the dynamic and multifaceted landscape of the pharmaceutical sector.

This heightened understanding corresponds directly with the competencies essential for pharmacists operating in this complex industry. It underscores the importance of regulatory acumen, a nuanced understanding of industry complexities, and the ability to navigate challenges inherent to drug development and manufacturing[21]. In alignment with Kolb's Experiential Learning Theory[22], which posits that learning is most effective when it involves a cycle of concrete experience, reflective observation, abstract conceptualization, and active experimentation, the seminar offered a platform for students to engage in this iterative learning process[23]. The increased understanding resonates with the competencies pharmacists need, emphasizing their ability to navigate the intricate regulatory landscape, contribute to quality assurance processes, and effectively address challenges within the pharmaceutical manufacturing domain.

Building upon the students' enhanced understanding of the pharmaceutical industry, their seminar topic suggestions create a strategic roadmap that seamlessly aligns with their aspirations. The emphasis on business understanding, regulatory compliance, and department-specific roles not only mirrors their deepening comprehension of the pharmaceutical landscape but also strategically aligns with competencies crucial for industry success[24]. This proactive approach reflects an intention to equip themselves with the essential knowledge and skills for seamless integration into the multifaceted pharmaceutical sector. Furthermore, this alignment between their expectations and the intricate demands of the pharmaceutical field highlights their awareness of the competencies required for success in this dynamic sector showcasing a commitment to acquiring the necessary skills for a meaningful contribution to the pharmaceutical industry.

Finally, while this study has provided valuable insights into the perceptions and aspirations of pharmacy students regarding the pharmaceutical industry, it is crucial to acknowledge its limitations. The small sample size may restrict the generalizability of the findings, and the use of survey instruments, although informative, may have inherent limitations in capturing the nuanced intricacies of student perspectives[25]. Additionally, the potential influence of social desirability response bias, pre-existing interest in evidence-based learning strategies, and the utilization of a four-point Likert scale could have impacted the students' survey responses and subsequent outcomes. Recognizing these limitations, future research endeavors could employ more extensive sampling methodologies and diversified assessment tools to enhance the robustness and applicability of findings in understanding the complex dynamics of pharmacy students' perceptions in the ever-evolving landscape of the pharmaceutical industry.

CONCLUSION

As we navigate the 21st century, the education of pharmacy students is pivotal in ensuring the continued advancement of the pharmaceutical industry. This survey analysis offers valuable insights into the interests and perceptions of pharmacy students, shedding light on the potential impact of educational interventions. By understanding the evolving needs and expectations of students, educators, policymakers, and industry stakeholders can refine educational strategies, curricula, and industry engagement initiatives. This analysis contributes to the ongoing dialogue on aligning pharmacy education with the dynamic pharmaceutical landscape, fostering a cadre of well-prepared professionals equipped to address the challenges and opportunities of the evolving industry.

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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