

The effectiveness of *Saluang Belum* (*Luvunga sarmentosa*) n-hexane Fractination Compounds in Enhancing Male Fertility via ESR1 and PI3K/Akt Pathway Modulation

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Abstract

Infertility has become a significant global health issue, including in Indonesia, where 40% of cases are attributed to male factors. An increasing number of studies report the effectiveness of compounds from *Saluang Belum* (*Luvunga sarmentosa*/*L. sarmentosa*) in improving spermatozoa survival. However, the therapeutic effect and mechanisms of action of this compound on male reproduction are not yet been verified. Therefore, this study aimed to identify potential protein targets and to validate ligand-protein interactions through molecular docking, in order to determine the therapeutic effects and mechanisms of *L. sarmentosa* compounds in pathways related to male infertility. The methodology of this study was an in silico design, in which potential target proteins were identified from the overlapping of 10 *L. sarmentosa* compounds with male infertility-related protein using network pharmacology. Subsequently, the ligand-protein interaction were validated through molecular docking simulations, and the pharmacokinetic properties (ADMET) of the compounds were analyzed. The network pharmacology analysis reveals that vital proteins associated with pathogenetic pathway of male infertility can be modulated by *L. sarmentosa* such as ESR1 and PIK3CA. Docking simulation identifies two promising compounds, ostruthin and 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one, with more negative binding affinity scores (-8.50 and -7.70 kcal/mol). These compounds form stable conventional hydrogen bonds with the shortest interaction distance and predicted to have favorable ADMET properties. In conclusion, both compounds are potentially exhibit anti-infertility activity against male infertility in silico and were predicted to exhibit favorable pharmacokinetic profiles.

Keywords: Saluang Belum (*Luvunga sarmentosa*); Male Infertility; In Silico; Molecular Docking; Network Pharmacology.

Abbreviations: *Luvunga sarmentosa* (*L. sarmentosa*); protein-protein interactions (PPI); Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET).

INTRODUCTION

Infertility is defined as the inability of a married couple to conceive a child after 12 months of regular, unprotected sexual intercourse. The World Health Organization (WHO) estimates that the prevalence of infertility affects 50-80 million couples globally, with approximately 2 million new infertile couples added each year.(Rusanova, 2024) According to population census data, there are 3 million, or about 12%, infertile couples distributed across both countryside and metropolitan areas in Indonesia. Currently, 40% of these cases are attributed to male factors.(Iskandar et al., 2023)

Spermatozoa are produced through the process of spermatogenesis in the testes, specifically within the seminiferous tubules. The produced spermatozoa undergo maturation in the epididymis to acquire motility and the ability to fertilize an ovum.(Agustinus et al.,

2018) Until now, 40-50% of male infertility cases remain idiopathic.(Bhattacharya et al., 2024) However, factors such as low sperm concentration, poor motility and viability, impaired capacitation, high levels of apoptosis in spermatozoa, and hypogonadism are suspected to contribute to infertility.(Aitken et al., 2015; Brenda C., 2022) Spermatozoa apoptosis becomes abnormal with the aging of the testes resulting in a decline in germ cells. This is caused by a decrease in androgen levels or an increase in oxidative stress. Additionally, the lack of surface cell receptors allows specific factors to directly penetrate cells and activate apoptosis pathways.(Asadi et al., 2021) The parameters established by the WHO for assessing male fertility are sperm motility and viability.(Yadav et al., 2021)

Dayaknese people in Central Kalimantan used the root of *Saluang Belum* (*Luvunga sarmentosa*/*L. sarmentosa*) as an alternative herbal therapy with

positive effects on enhancing stamina, sexual drive, and male fertility. Phytochemical screening of *L. sarmentosa* using vacuum chromatography column with chloroform and methanol as the eluent revealed the presence of terpenoids, flavonoids, steroids, alkaloids, saponin, and tannins. Between both eluent, the sperm motility increased significantly in the methanol group.(Permatasari et al., 2023)

Furthermore, a study by Syarpin identified phytochemical components through LCMS/MS testing of the *L. sarmentosa* fraction, using n-hexane as one of the solvents, producing ten active compounds. However, molecular docking studies of these compounds with potential target proteins involved in male fertility mechanisms have not yet been conducted.(Syarpin et al., 2023)

The network pharmacology bioinformatics method can identify potential compounds against male infertility by analyzing protein-protein interactions (PPI) to determine potential target proteins that may be involved in the action of *L. sarmentosa* root compounds. Subsequently, the interactions between the plant compounds and proteins will be validated by finding the optimal geometry and interaction energy values using molecular docking.(Noor et al., 2022; Pinzi & Rastelli, 2019)

Until now, there have been few studies reporting the mechanisms of action of the n-hexane fraction of *L. sarmentosa* compounds on male infertility, particularly at the molecular level. This study aims to analyze the mechanisms of the bioactive compounds in n-hexane *L. sarmentosa* that may impact the male reproductive system using in silico studies.

MATERIALS AND METHODS

Materials

This study was conducted on ten compounds from the n-hexane fraction of *L. sarmentosa*, namely ostruthin, (E, E)-alpha-farnesene, cryptotanshinone, curcumin, benzyl butyl phthalate, 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one, trioxsalen, tolpropamine, 3,4-dihydrocadalene, and naproxen. These compounds were retrieved from PubChem database. The potential target proteins will be identified through network pharmacology and then downloaded from the protein data bank. The equipment used in this study was an Acer TravelMate device with the following specifications: 16 GB DDR 4 RAM, 1TB SSD hard drive, Intel®Core™ i7 processor, and Windows 11 Home operating system. The device was equipped with several software programs, including ChemDraw, Chem3D, Cytoscape, MGL Tools with AutoDock4 and AutoDock Vina, Java with Osiris DataWarrior, and BIOVIA Discovery Studio Visualizer.

Methods

Extraction of the n-hexane fraction of L. sarmentosa compounds

The extraction of bioactive compounds from the n-hexane fraction of *L. sarmentosa* root was performed using the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) by inputting the compound names in the search column. (Burley et al., 2023)

Target protein analysis of male infertility and n-hexane fraction of L. sarmentosa compounds

The search for proteins associated with male infertility was conducted using the GeneCard database (<https://www.genecards.org/>)(Safran et al., 2022; Stelzer et al., 2016), in conjunction with the OMIM database (<https://www.omim.org/>)(Amberger et al., 2015; Hamosh et al., 2021), by entering the keyword “Male Infertility”. Following this, potential target proteins of the bioactive compounds from the n-hexane fraction of *L. sarmentosa* root were screened using the Swiss Target Prediction database (<http://www.swisstargetprediction.ch/>) by entering the query molecule as SMILES. Target proteins with a probability value >0 were selected.(Daina et al., 2019; Huang et al., 2022) To further validate the protein data, the PharmMapper website (<https://www.lilab-ecust.cn/pharmmapper/>) was used, with a Z-score value >1.5.(Wu et al., 2023) A Venn diagram, created with the Venny 2.1.0 website (<https://bioinfogp.cnb.csic.es/tools/venny/>), was used to intersect proteins associated with infertility and target proteins from the bioactive compounds of the n-hexane fraction of *L. sarmentosa* root. (Heberle et al., 2015; Jia et al., 2021)

Protein-protein interaction network

Protein networking was performed using the STRING website (<https://string-db.org/>) with a required score set to the highest confidence (0.900). Furthermore, PPI analysis was carried out using the Cytoscape application. Prior to this, the exported protein network was cleaned to remove disconnected nodes from the main network. (Doncheva et al., 2023; Szklarczyk et al., 2023)

Identification of potential target proteins

Using the CytoNCA plug-in in Cytoscape, centrality parameter analysis without weight, including Degree, Eigenvector, Betweenness, and Closeness, was conducted.(J. Liu et al., 2022; Tang et al., 2015) The top ten proteins with the highest values for each parameter were identified and intersected using Venny 2.1.0. The main protein network results were then visualized again using Cytoscape.

Enrichment analysis of KEGG pathway and Gene ontology

KEGG pathway and Gene Ontology analysis of potential target proteins were conducted to determine biological

networking, utilizing the ShinyGo database website (<https://bioinformatics.sdstate.edu/go/>) with a significance threshold of $p < 0.05$ and top 10 pathways to show. (Xijin Ge et al., 2020)

Druglikeness profiling of *L. sarmentosa* compounds

Drug-likeness analysis was performed to screen bioactive compounds from the n-hexane fraction of *L. sarmentosa* roots based on Lipinski's Rule of Five (RO5). This analysis was carried out via the SwissADME (<http://www.swissadme.ch/>) website by inputting SMILES. (Ferreire & Andricopulo, 2019; Kar & Leszczynski, 2020) Compounds meeting the five RO5 criteria, such as $\log P < 5$, molecular weight < 500 , hydrogen bond donors < 5 , hydrogen bond acceptors < 10 , and molar refractivity between 40-130, were subsequently analyzed using molecular docking. (X. Chen et al., 2020; Ivanović et al., 2020)

Molecular docking simulation

Molecular docking examines the interaction between ligands from *L. sarmentosa* compounds and target protein receptors by analyzing active sites that best match the geometry and calculating ligand interaction energies to identify potential compounds that effectively inhibit or enhance biological functions. (Pinzi & Rastelli, 2019)

Potential target proteins identified through network pharmacology analysis were downloaded from the Protein Data Bank website and prepared using AutoDock Tools by separating the receptor from its native ligand. (Bitencourt-Ferreira et al., 2019) Validation testing between the native ligand and target protein was performed to analyze the active site with the best geometry. This validation stage involved 100 redocking simulations using AutoDock Vina, where receptors suitable for molecular docking had an RMSD value < 2 Å. Additionally, bioactive compounds from the n-hexane fraction of *L. sarmentosa* roots, as well as tamoxifen and pentoxifylline (control compounds for male infertility drugs), were downloaded and prepared with their 3D structures.

Molecular docking simulations between the ligands of *L. sarmentosa* compounds and the control compounds with potential target proteins were conducted using AutoDock Vina. (Vieira & Sousa, 2019) The docking results were visualized using BIOVIA Discovery Studio software to examine the 2D and 3D structures, bonds formed, and interaction distance between ligands and target proteins.

ADMET property prediction for *L. sarmentosa* compounds

Screening for absorption, distribution, metabolism, excretion, and toxicity (ADMET) of bioactive compounds from the n-hexane fraction of *L. sarmentosa* roots was carried out using PkCSM webserver (<https://biosig.lab.uq.edu.au/pkcsfm/>).³⁴ Additionally,

Osiris DataWarrior software was used to evaluate the toxicity properties of the compounds, including tumorigenicity, immunogenicity, and mutagenicity. (Sander et al., 2015)

Data analysis

The activity of bioactive compounds from the n-hexane fraction of *L. sarmentosa* roots is considered favorable if they are able to bind or interact with the target proteins at the active site, form conventional hydrogen bonds, and produce a binding free energy value (ΔG). The data obtained from this study will be tabulated using Microsoft Excel and visualized using BIOVIA Discovery Studio Visualizer.

RESULTS AND DISCUSSION

Potential targets of the *L. sarmentosa* compound in male infertility

Data on proteins and genes involved in male infertility were retrieved from databases such as GeneCard and OMIM, identifying 2,773 proteins associated with male infertility. Meanwhile, the search for potential target proteins of bioactive compounds from the n-hexane fraction of *L. sarmentosa* root extract using the SwissTargetPrediction and PharmMapper websites identified 354 target proteins present in the bioactive compounds of the n-hexane fraction of *L. sarmentosa* root extract. The intersection of these two datasets revealed 103 proteins involved in targeting male infertility through the bioactive compounds of the n-hexane fraction of *L. sarmentosa* root extract (Figure 1).

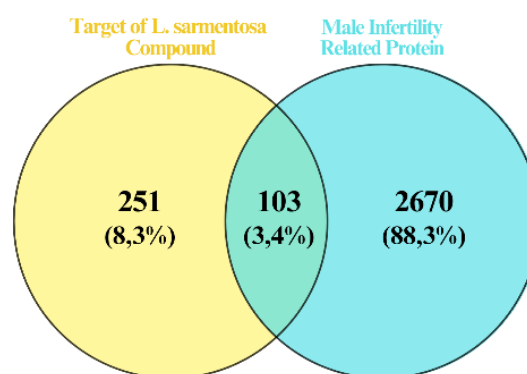


Figure 1. Intersection of male infertility related protein with *L. sarmentosa* compound target using Venn diagram analysis.

Network construction and potential target protein selection

Subsequently, network construction analysis of the 103 target proteins was performed using the STRING website with a required score set to the highest confidence (0.900). The resulting PPI network was then imported into Cytoscape for further PPI analysis. From the initial network visualization, some proteins were observed to be

disconnected from the main network. These proteins were removed using Cytoscape, resulting in 62 proteins

that interact within the main network (Figure 2A-B).

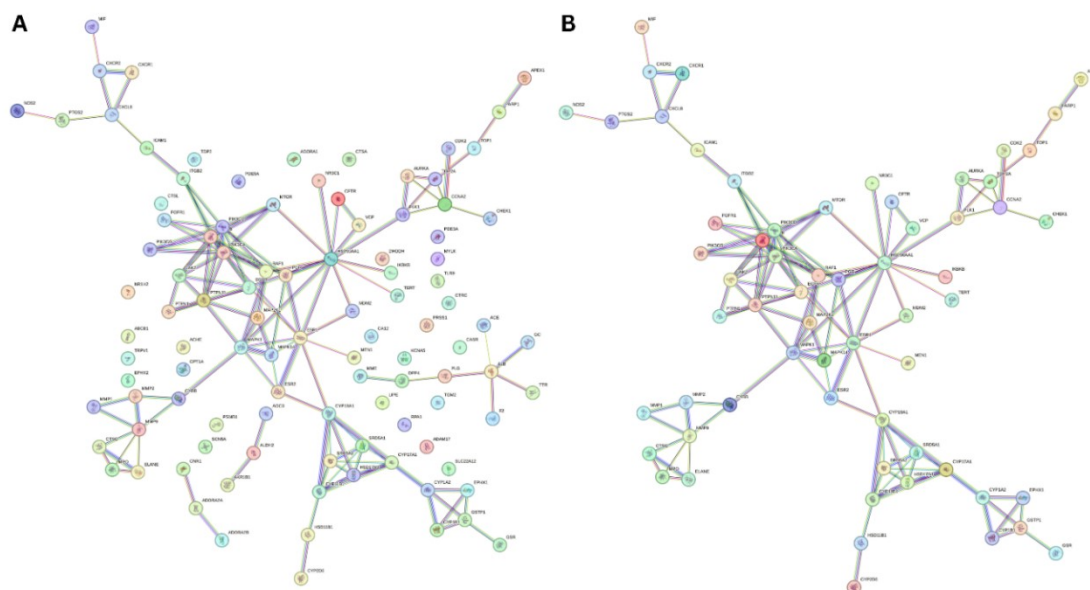


Figure 2. Protein-protein interaction network of the *L. sarmentosa* compound target in male infertility. A The PPI network of 103 protein targets of *L. sarmentosa* compound targets in male infertility according to STRING; B The PPI network of 62 protein targets of *L. sarmentosa* compound target in male infertility after clearing non interaction proteins from the main network.

The cleaned protein network was then analyzed to identify the most promising proteins using the CytoNCA tool in Cytoscape. For this analysis, the top 10 proteins were selected based on four key centrality parameters: Degree, Eigenvector, Betweenness, and Closeness. The

results from these four parameters were further intersected using the Venny 2.1.0 website. This analysis identified three potential proteins in the main network, with two primary proteins selected: ESR1 and PIK3CA (Figure 3A-E).

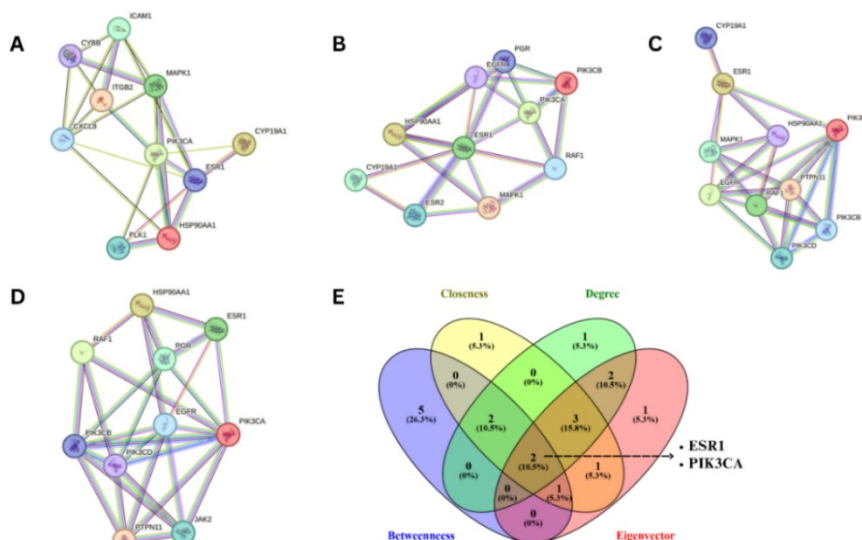


Figure 3. Top 10 target proteins in four centrality based on the highest score. A Betweenness; B Closeness; C Degree; D Eigenvector; E Intersection of top 10 proteins of each centrality using Venn diagram analysis including ESR1 and PIK3CA.

Following the identification of these two key target proteins from the main network, the visualization was

updated by integrating the proteins, the main network, and compounds from *L. sarmentosa* using Cytoscape.

This final visualization illustrates the relationship between the proteins associated with male infertility and the *L. sarmentosa* compounds (Figure 4).

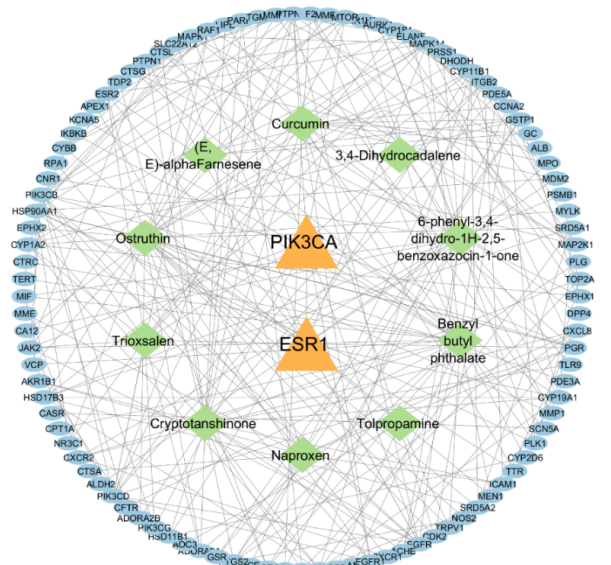


Figure 4. Final Visualization of 2 Potential Target Proteins, n-Hexane *L. sarmentosa* Compounds, and Main Network Interactions.

Enrichment analysis of Gene Ontology and KEGG pathway

The KEGG and Gene Ontology (GO) analyses were performed using the ShinyGo website. This study utilized 62 male infertility-related proteins associated with *L. sarmentosa* compounds within the main network. KEGG signalling pathway analysis identified 189 pathways involved with these 62 proteins. Among the top 10 pathways based on significant enrichment, the estrogen signaling pathway and phospholipase D signaling pathway were highlighted as particularly important.

GO provides a representation of gene function across three key aspects: molecular function, cellular component, and biological process. The GO analysis revealed 363 molecular functions, with the most significant being protein serine/threonine/tyrosine kinase activity and enzyme binding. Furthermore, 187 cellular components, with the most important being the phosphatidylinositol 3-kinase class IA complex and mitochondrion. Letter, 1,000 biological processes, with the most significant being cellular response to oxygen-containing compounds and cellular response to chemical stimulus (Figure 5A-D).

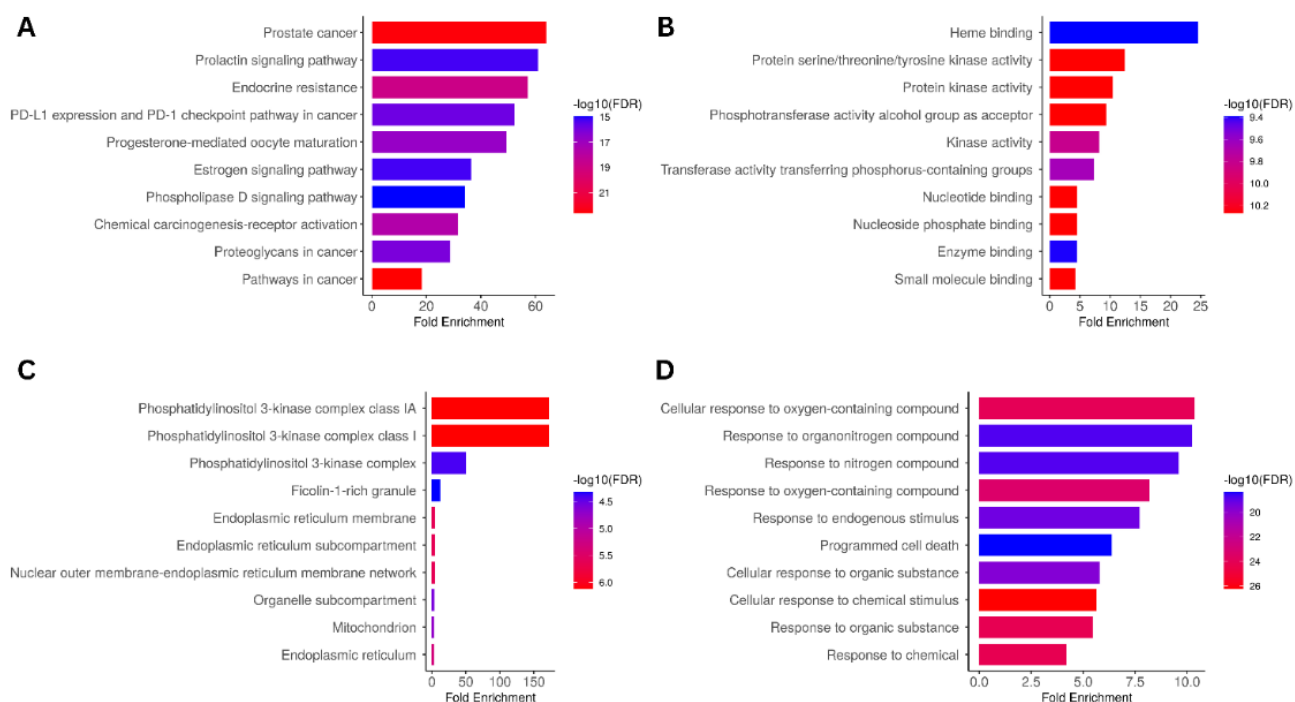


Figure 5. KEGG pathway enrichment and Gene Ontology analysis of 62 male infertility-related proteins associated with *L. sarmentosa* compounds. A Top 10 KEGG pathway analysis; B Top 10 GO molecular function analysis; C Top 10 GO cellular component analysis; D Top 10 GO biological process analysis.

Prediction of druglikeness profiles of *L. sarmentosa* compounds

Drug-likeness analysis is used to predict the similarity of a compound's pharmacological and biological activity to

that of a drug when administered to humans via the oral route. This analysis was conducted on ten compounds from the n-hexane fraction of *L. sarmentosa*, namely ostruthin, (E, E)-alpha-farnesene, cryptotanshinone,

curcumin, benzyl butyl phthalate, 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one, trioxsalen, tolpropamine, 3,4-dihydrocadalene, and naproxen.

The result showed that one control compound, tamoxifen, and the test compound (E, E)-alpha-farnesene each had one violation of the RO5 (Table I). However, according to the RO5 guidelines, compounds predicted to

have good bioavailability can have up to one violation. (Ivanović et al., 2020) Thus, all compounds, including both the test compounds and the control compounds, are predicted to be active as oral drugs and can proceed to molecular docking analysis to determine the interaction between the test compound ligands and the target proteins

Table 1. Druglikeness profiles of *L. sarmentosa* compounds.

	MlogP	MW	HBD	HBA	MR	Violation
Control						
Tamoxifen	5.9961	371.524	0	2	119.71	1
Pentoxifylline	0.1930	278.312	0	7	76.89	0
Compound						
Ostruthin	4.7338	298.382	1	3	91.79	0
(E,E)-alpha-Farnesene	5.2015	204.357	0	0	72.32	1
Cryptotanshinone	3.4433	296.366	0	3	85.13	0
Curcumin	4.8449	202.341	0	0	69.55	0
Benzyl Butyl Phthalate	4.0005	312.365	0	4	87.91	0
6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one	2.6944	251.285	0	3	76.88	0
Trioxsalen	3.4644	228.247	0	3	67.15	0
Tolpropamine	4.0786	253.389	0	1	82.98	0
3,4-Dihydrocadalene	4.5416	200.325	0	0	68.39	0
Naproxen	3.0365	230.263	1	2	66.79	0

Docking analysis of ligand-protein interaction

Prior to the molecular docking simulation, validation tests were conducted between each native ligand and the target proteins, ESR1 and PIK3CA. The RMSD values obtained from the validation were 1.08 Å and 1.19 Å, respectively, indicating that the grid box used

represented the optimal geometry. Furthermore, the results showed that several compounds interacted well with the target protein ESR1 (Table 2) and PIK3CA (Table 3), as evidenced by the comparison of Gibbs free energy binding (ΔG) values between the positive control compounds and *L. sarmentosa* compounds.

Table 2. Results of molecular docking analysis for the ESR1 target protein.

	Free Energy Binding (kcal/mol)	Conventional hydrogen bond	Other bond	Shortest interaction distance (Å)
Control				
Tamoxifen	-9.8	-	LEU 346 ^a , MET 421 ^a , PHE 404 ^a , ALA 350 ^a , LEU 525 ^a , LEU 346 ^a , LEU 387 ^a , LEU 391 ^a , MET 421 ^a , LEU 525 ^a	3.69
Compound				
Ostruthin	-8.50	HIS 524	ALA 350 ^a , LEU 525 ^b , LEU 387 ^a , MET 388 ^a , LEU 346 ^a , MET 421 ^a , LEU 349 ^a , MET 343 ^c , PHE404 ^a , LEU 428 ^a , LEU 384 ^a	2.35
(E,E)-alpha-Farnesene	-6.60	-	ALA 350 ^a , MET 343 ^a , LEU 346 ^a , LEU 525 ^a , LEU 384 ^a , LEU 387 ^a , LEU 346 ^a , MET 388 ^a , LEU 391 ^a , LEU 428 ^a , ILE 424 ^a , PHE 404 ^a , HIS 524 ^a	3.69
Cryptotanshinone	-8.80	-	GLY 420 ^x , MET 343 ^c , LEU 346 ^a , LEU 349 ^a , ALA 350 ^a , LEU 387 ^a , LEU 391 ^a , LEU 525 ^a , PHE 404 ^a , HIS 524 ^a	3.79
Curcumin	-7.00	-	LEU 525 ^b , MET 343 ^b , ALA 350 ^a , LEU 384 ^a , ILE 424 ^a , LEU 387 ^a , MET 388 ^a , LEU 391 ^a , LEU 428 ^a , LEU 346 ^a , MET 421 ^a , TRP 383 ^a , PHE 404 ^a , HIS 524 ^a	3.70
Benzyl Butyl Phthalate	-7.60	-	GLU 353 ^d , MET 421 ^c , ALA 350 ^a , LEU 525 ^a , LEU 346 ^a , LEU 349 ^a , LEU 391 ^a	4.16
6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one	-8.60	-	LEU 525 ^b , MET 343 ^c , LEU 384 ^a , LEU 346 ^a , ALA 350 ^a	3.87
Trioxsalen	-8.50	-	MET 343 ^c , LEU 346 ^a , LEU 387 ^a , MET 388 ^a , LEU	4.05

	Free Energy Binding (kcal/mol)	Conventional hydrogen bond	Other bond	Shortest interaction distance (Å)
Tolpropamine	-8.30	-	391 ^a , LEU 525 ^a , HIS 524 ^a , ALA 350 ^a , LEU 384 ^a , ALA 350 ^a , LEU 525 ^a , TRP 383 ^a , LEU 346 ^a , LEU 391 ^a	4.02
3,4-Dihydrocadalene	-7.70	-	LEU 346 ^a , ALA 350 ^a , LEU 384 ^a , LEU 349 ^a , MET 388 ^a , MET 421 ^a , ILE 424 ^a , LEU 428 ^a , LEU 525 ^a	4.00
Naproxen	-7.90	-	GLY 420 ^x , LEU 346 ^b , LEU 349 ^a , LEU 525 ^a , PHE 404 ^a , HIS 524 ^a , MET 421 ^a	3.79

^xOther hydrogen bond, ^aAlkyl/Pi-Alkyl interaction, ^bPi-Sigma/Pi-Pi T-Shaped, ^cPi-Sulfur/Sulfur-X interaction, ^dPi-Anion

Table 3. Results of molecular docking analysis for PIK3CA target protein.

	Free Energy Binding (kcal/mol)	Conventional hydrogen bond	Other bond	Shortest interaction distance (Å)
<i>Control</i>				
Pentoxifylline	-6.70	LYS 802, SER 854, TYR 836	ASP 810 ^d , ASP 933 ^a , ILE 800 ^a , ILE 848 ^a , ILE 932 ^a	3.69
<i>Compound</i>				
Ostruthin	-8.80	-	SER 774 ^x , ILE 848 ^b , TYR 836 ^b , MET 772 ^c , ILE 932 ^a , VAL 851 ^a , TRP 780 ^a , PHE 930 ^a , PRO 778 ^a , ILE 800 ^a , LYS 802 ^a	2.35
(E,E)-alpha-Farnesene	-6.40	-	PRO 778 ^a , LYS 802 ^a , ILE 848 ^a , ILE 932 ^a , ILE 800 ^a , MET 922 ^a , VAL 851 ^a , TYR 836 ^a , PHE 930 ^a	3.69
Cryptotanshinone	-9.10	-	ILE 932 ^b , TYR 836 ^b , PRO 778 ^a , LYS 802 ^a , VAL 851 ^a , MET 922 ^a , PHE 930 ^a , ILE 800 ^a , ILE 848 ^a	3.79
Curcumin	-7.00	-	ILE 848 ^b , ILE 800 ^a , ILE 932 ^a , LEU 807 ^a , VAL 851 ^a , MET 922 ^a , VAL 850 ^a , TYR 836 ^a	3.70
Benzyl Butyl Phthalate	-7.60	-	ILE 932 ^b , TRP 780 ^b , TYR 836 ^b , ILE 848 ^a , MET 922 ^a	4.16
6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one	-7.70	VAL 851	VAL 851 ^x , MET 922 ^c , ILE 848 ^b , ILE 932 ^b , TYR 836 ^b , ILE 800 ^a , VAL 850 ^a	3.87
Trioxsalen	-8.50	ASP 933	ILE 932 ^b , TYR 836 ^b , MET 922 ^c , ILE 848 ^a , VAL 850 ^a , TRP 780 ^a , ILE 800 ^a	4.05
Tolpropamine	-7.30	-	TYR 836 ^x , ILE 932 ^b , TYR 836 ^b , VAL 850 ^a , VAL 851 ^a , MET 922 ^a , ILE 800 ^a	4.02
3,4-Dihydrocadalene	-7.40	-	ILE 848 ^a , ILE 800 ^a , ILE 932 ^a , CYS 838 ^a , LYS 802 ^a , TYR 836 ^a	4.00
Naproxen	-8.00	VAL 851, ASP 933, ASP 810	ILE 932 ^b , MET 922 ^c , TYR 836 ^b , ILE 848 ^a , VAL 850 ^a	3.79

^xOther hydrogen bond, ^aAlkyl/Pi-Alkyl interaction, ^bPi-Sigma/Pi-Pi T-Shaped, ^cPi-Sulfur/Sulfur-X interaction, ^dPi-Anion

The best ligand interaction for *L. sarmentosa* with ESR1 was ostruthin. When compared to the control ligand tamoxifen, ostruthin and tamoxifen showed docking result with ΔG values of -9.8 and -8.50 kcal/mol. Notably, ostruthin formed a conventional hydrogen bond with HIS 524 at an interaction distance of 2.35 Å (Figure 6A-B). This suggests that ostruthin exhibits better potential activity against the ESR1 protein.

For the interaction of *L. sarmentosa* ligands with the target protein PIK3CA, the three best compounds were trioxsalen, naproxen, and 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one, with ΔG values of -8.50, -8.00, and -7.70 kcal/mol. As shown in Fig. 6D-F, each compound

formed conventional hydrogen bonds with short interaction distance: ASP 933 (2.38 Å), VAL 851 (1.78 Å), and VAL 851 (2.01 Å). These interactions were better compared to the control compound pentoxifylline (PTX), which had ΔG values of -6.70 kcal/mol and formed a conventional hydrogen bond with LYS 820 at an interaction distance of 2.14 Å (Fig. 6C).

Based on the three test parameters, Gibbs free energy binding (ΔG), conventional hydrogen binding, and interaction distance ¹², it was concluded that the test compounds exhibited better interaction with each target protein ESR1 and PIK3CA compared to their control compounds.

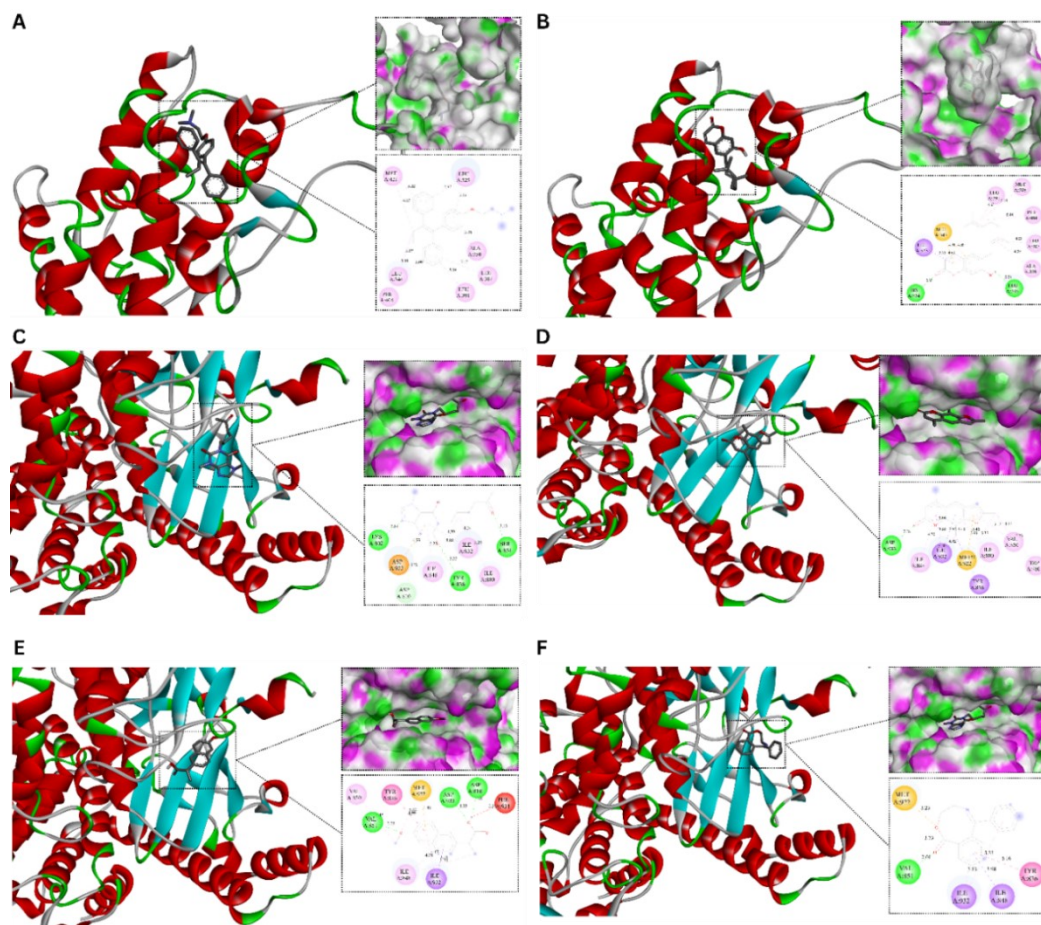


Figure 6. 2D and 3D visualization of molecular docking result of best protein-ligand complexes. A ESR1-Tamoxifen (control) B ESR1-Ostruthin C PIK3CA-Pentoxifylline (control) D PIK3CA-Trioxsalen E PIK3CA-Naproxen F PIK3CA-6-phenyl-3,4-dihydro-1H-2,5- benzoxazocin-1-one.

Assessment of ADMET profiles of *L. sarmentosa* compounds

Pharmacokinetic profile analysis is a bioinformatics method used to determine the therapeutic efficacy of potential drug candidate compounds by evaluating several parameters, including absorption, distribution, metabolism, excretion, and toxicity (ADMET).

The absorption parameters in this study include water solubility, Caco-2 permeability, intestinal absorption, and skin permeability. Based on the absorption analysis, while both the test and control compounds are lipophilic, the absorption properties of the test compounds for other components indicate better intestinal mucosal absorption compared to the control compounds (Table 4).

Table 4. Absorption properties of the pharmacokinetic profile analysis of the main compound of *L. sarmentosa*.

	Water solubility (log mol/L)	Caco2 permeability (log Papp in 10 ⁻⁶ cm/s)	Intestinal absorption (%)	Skin permeability (log Kp)
<i>Control</i>				
Tamoxifen	-5.929	1.065	96.885	-2.737
Pentoxifylline	-0.634	0.424	100.000	-2.977
<i>Compound</i>				
Ostruthin	-5.455	1.440	94.304	-2.605
6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one	-4.057	1.503	100.000	-2.547
Trioxsalen	-4.038	1.351	97.829	-2.317
Naproxen	-3.591	1.311	95.867	-2.631

The distribution parameters analyzed include volume distribution at steady state (VD_{ss}), blood-brain barrier (BBB) permeability, and central nervous system (CNS)

permeability. The distribution analysis revealed that all compounds have similar distribution profiles, with

moderate potential to penetrate the BBB and CNS (Table 5).

For metabolism, the parameters analyzed included cytochrome P450 components, specifically CYP3D6 and CYP3A4 substrates and inhibitors. The analysis showed that only tamoxifen acts as both a substrate and an inhibitor of cytochrome P450, indicating that the other

compounds are less likely to interfere with cytochrome P450 metabolism (Table 5).

The excretion parameter analyzed was total clearance, which measures the overall elimination of a compound from the body and serves as a reference for determining drug dosage. Among all compounds, only the test compound naproxen exhibited poor excretion (Table 5)

Table 5. Distribution, metabolism, and excretion properties of the pharmacokinetic profile analysis of the main compound of *L. sarmentosa*.

	Distribution			Metabolism				Excretion
	VD _{ss} (human) (log L/kg)	BBB permeability (log BB)	CNS permeability (log PS)	CYP2D6 substrate	CYP3A4 substrate	CYP2D6 inhibitor	CYP3A4 inhibitor	Total clearance (log ml/min/kg)
Control								
Tamoxifen	0.830	1.329	-1.473	No	Yes	Yes	No	0.556
Pentoxifylline	1.669	-0.950	-3.058	No	No	No	No	0.735
Compound								
Ostruthin	0.044	0.136	-1.753	No	No	No	No	0.989
6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one	-0.228	0.317	-1.060	No	No	No	No	0.762
Trioxsalen	-0.199	0.288	-1.340	No	No	No	No	0.747
Naproxen	-0.670	0.164	-1.363	No	No	No	No	0.219

The final parameter in the pharmacokinetic analysis was toxicity, which included components such as oral rat acute toxicity (LD₅₀), hepatotoxicity, tumorigenicity, immunogenicity, and mutagenicity. The analysis showed

that the non-toxic test compounds were ostruthin and 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one, whereas the control compound PTX tended to exhibit toxic properties (Table 6).

Table 6. Toxicity properties of the pharmacokinetic profile analysis of the main compound of *L. sarmentosa*.

	Oral rat acute toxicity (LD ₅₀) (mol/kg)	Hepatotoxicity	Tumorigenicity	Immunogenicity	Mutagenicity
<i>Control</i>					
Tamoxifen	2.285	No	Inactive	Inactive	Inactive
Pentoxifylline	2.436	Yes	Inactive	Active	Active
<i>Compound</i>					
Ostruthin	2.333	No	Inactive	Inactive	Inactive
6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one	2.144	No	Inactive	Inactive	Inactive
Trioxsalen	2.173	No	Active	Inactive	Inactive
Naproxen	2.171	No	Inactive	Inactive	Active

Based on the pharmacokinetic analysis, the best bioactive compounds from the n-hexane fraction of *L. sarmentosa* binding to the target proteins ESR1 and PIK3CA were ostruthin and 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one. These two compounds

demonstrated superior pharmacokinetic properties compared to their controls. The control compound tamoxifen showed poor metabolism in the body, while PTX exhibited a tendency to be toxic.

Discussion

To date the exploration of natural compounds as drug candidates continues, including efforts to address male infertility. Male infertility is characterized by abnormal spermatozoa function, such as low sperm concentration, reduced motility or viability, or impaired ejaculation processes.(Aitken et al., 2015; Assidi, 2022) In this study, a natural compound endemic to Central Kalimantan, the root of Saluang Belum (*L. sarmentosa*) was selected.(Permatasari et al., 2023) Secondary bioactive compounds identified through LCMS/MS analysis of the n-hexane fraction of *L. sarmentosa* root extract include ostruthin, (E, E)-alpha-farnesene, cytotanshinone, 3,4-dihydrocadalene, curcumin, benzyl butyl phthalate, 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one, tolpropamine, trioxsalen, dan naproxen.(Syarpin et al., 2023) Subsequent testing was conducted on these secondary metabolites against potential target proteins associated with male infertility to identify the most promising candidates for infertility treatment.

The first stage of this study involved identifying potential proteins implicated in the mechanism of male infertility using a network pharmacology approach. This method allows for the analysis of synergistic relationships between each compound and its biological targets, facilitating the exploration of mechanisms of action of bioactive compounds within a biological network.(Azzahra et al., 2024) In this study, 103 target proteins were identified as being involved in the interaction with the bioactive from the n-hexane fraction of *L. sarmentosa* root extract and male infertility-related proteins. From this protein network, two key potential targets were identified: ESR1 and PIK3CA.

The estrogen receptor (ESR) functions as a mediator for estrogen hormones to elicit physiological responses. ESR consists of two subtypes: ER α and ER β . Among these isoforms, ER α is composed of 595 amino acids, also known as ESR1, and is located on chromosome 6q25. Meanwhile, ER β is composed of 530 amino acids, also known as ESR2, and is located on chromosome 14q23-24. Both receptors are expressed in human testicular germ cells during spermatogenesis and play a role in reproduction through their interaction with estrogen.(Qamar et al., 2025)

An *in vivo* study investigating the effects of ESR1 deletion in male mice revealed a significant reduction in daily sperm production, epididymal sperm motility, and dilated seminiferous tubules with impaired spermatogenesis. This highlights the role of ESR1 in male reproduction.(Cooke et al., 2017)

PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) encodes a critical component of the lipid kinase phosphatidylinositol 3-kinase (PI3K) involved in the PI3K/Akt pathway, which supports cell survival, motility, and proliferation. Lipid kinase PI3K is thought to regulate various cellular

activities through the production of phosphatidylinositol (3,4,5)-triphosphate (PIP3). The activity of class I PI3K is tightly controlled by signals from receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), insulin receptor, and G-protein-coupled receptor (GPCR). When class I PI3K, including PIK3CA, is activated by RTK signals, PIP3 is produced through the phosphorylation of PIP2. The accumulation of PIP3, localized at the plasma membrane, subsequently activates protein kinase B (PKB), also known as Akt, which regulates various cellular activities, including proliferation, apoptosis, and autophagy. (Lai et al., 2015; Niu et al., 2022)

Spermatozoa apoptosis can become abnormal with increasing testicular age due to a decline in androgen levels or an increase in oxidative stress. Additionally, the lack of surface cell receptors allows specific factors to penetrate cells directly and activate apoptotic pathways. (Asadi et al., 2021) There are two distinct signalling pathways for initiating apoptosis: the extrinsic pathway and the intrinsic pathway. The intrinsic pathway is initiated when cytochrome C and other apoptotic factors are released from the mitochondria due to chemical compound induction, causing an imbalance in the proportion of Bcl-2 family proteins. Simultaneously, there is an increase in the permeability of the outer membrane of the cell organelle. This process is regulated by pro-apoptotic proteins, such as Bax, Bak, and Bok, which are activated by BH3-only pro-apoptotic proteins like Bad, Bim, Bid, Noxa, and others. (Nakidkina & Kuzmina, 2019; Ojo et al., 2023)

Therefore, the regulation of cell survival, which acts as an anti-apoptotic mechanism, is crucial. Akt is one of the cell survival proteins. The activation of Akt is triggered by extracellular stimulation that activates PI3K. Akt functions by mediating the phosphorylation of the Bad protein, binding it to the 14-3-3 proteins, and isolating it into the cytosol. This prevents Bad from forming heterodimers with anti-apoptotic proteins Bcl-1 and Bcl-XL, thereby nullifying its pro-apoptotic activity. (Stickles et al., 2015) A study by Humairoh found that activation of the Akt/PKB protein via the PI3K/Akt pathway, including by PIK3CA, exhibited anti-apoptotic effects and preserved spermatozoa viability by preventing their entry into the apoptosis cascade. (Humairoh, 2021)

KEGG analysis of male infertility-related proteins associated with *L. sarmentosa* compound revealed significant pathway enrichment, including 10 key pathways such as the estrogen signaling pathway and the phospholipase D signaling pathway. (Kanehisa et al., 2022) Through the estrogen signaling pathway, either directly (genomic) or indirectly (non-genomik), estrogen can diffuse into cells and interact with intracellular ESR1 or ESR2. (Fuentes & Silveyra, 2019; Li et al., 2015) Research has shown that the absence of membrane-bound or intracellular ESR1 in the testes of mice results

in reduced sperm production and poor sperm motility. This highlights the critical role of the estrogen signaling pathway via ESR1 in regulating spermatogenesis in men. (Hammes & Levin, 2019) Additionally, activation of the phospholipase D signaling pathway enhances sperm capacitation, improving spermatozoa's ability to survive within the female reproductive tract. (Zou et al., 2022)

GO analysis of male infertility-related protein linked to *L. sarmentosa* compounds identified 10 top-ranked genes based on their enrichment scores for specific functions. In the molecular function category, which describes the molecular level activity of proteins, the study identified serine/threonine/tyrosine kinase activity. This protein activity induces the PI3K/Akt signaling pathway, allowing Akt protein to play roles in biological processes such as cell proliferation, survival, differentiation, and apoptosis. (K. Q. Chen et al., 2022; Y. Liu et al., 2024)

Furthermore, the cellular component analysis in GO describes the structure, part, or cellular locations where protein functions, either inside or outside the cell. In this study, the identified cellular component was the phosphatidylinositol 3-kinase complex class IA (PI3K class IA), a catalytic subunit within the PI3K family. One of its functions is to act as an anti-apoptotic protein for spermatozoa. (Lai et al., 2015; Niu et al., 2022)

The biological process category represents a series of biological activities carried out by genes or proteins. In this analysis, the identified process was the cellular response to oxygen-containing compounds, which describes cellular reactions to oxygen-containing substances. While oxygen plays essential roles in organisms, it can also trigger adverse effects such as oxidative stress-induced cellular damage. In response, cells may activate survival mechanisms through anti-apoptotic proteins to counteract these effects. (Kocherova et al., 2019)

A significant number of agents are used to treat male infertility, one of which is selective estrogen receptor modulators (SERMs). These non-steroidal compounds act as agonists or antagonist ligands for estrogen receptors depending on the target tissue. In the testes, SERMs bind to ESR1 located in the nucleus of Leydig cells, enhancing sensitivity to LH and promoting testosterone synthesis. One of the SERMs used for decades is tamoxifen, which is utilized in this study as the control compound for the ESR1 protein. Although the use of SERMs has proven effective in the empirical treatment of male infertility, several long-term side effects have been reported, including an increased risk of pulmonary embolism, deep vein thrombosis, and stroke. (Cannarella et al., 2019; Sethumadhavan, 2021)

Various studies have also been conducted to improve testicular spermatozoa quality *in vitro*, including interventions using Pentoxifylline (PTX). (Dhulqarnain et al., 2021) PTX provides significant protection against

testicular damage by increasing the expression of anti-apoptotic genes, Akt and PI3K, and reducing apoptotic expression (Caspase 3). In this study, PTX is used as the control compound for the PIK3CA protein. Although PTX significantly enhances sperm motility, it has been reported to exhibit embryotoxic effects and potentially deplete the metabolic energy sources of sperm, which may render sperm immotile permanently. (Sabz et al., 2024)

Druglikeness analysis using Lipinski's Rule of Five (RO5) is employed to predict the drug-like properties of compounds and serves as a preliminary screening step before molecular docking is conducted. The lipophilicity parameter (MlogP) represents the molecule's ability to be absorbed. The molecular weight (MW) parameter correlates with compound size, as molecules with larger weights (>500) may impede absorption in the intestinal lumen. The number of hydrogen bonds is based on their correlation with compound interaction with water and the reduction of permeability across the lipid bilayer membrane. The molar refractivity (MR) parameter reflects intestinal absorption and oral bioavailability. (Agoni et al., 2020; Ivanović et al., 2020) Based on this study, all compounds, including both test and control compounds, show potential as oral drug candidates.

In molecular docking simulations Gibbs free energy binding (ΔG) is a thermodynamic measure used to predict the spontaneity of a chemical reaction at equilibrium under constant pressure and temperature. The more negative the ΔG value, the stronger and more stable the protein-ligand conformational binding activity of a compound. Another parameter is the conventional hydrogen bond, which plays as crucial role in maintaining the molecular bonding complexity and can trigger biological functions on the target protein. However, hydrogen bonds are considered stable and strong when the bond length is $<2.7 \text{ \AA}$. (da Fonseca et al., 2024; Pinzi & Rastelli, 2019)

The first protein tested, ESR1, was analyzed against the compounds of *L. sarmentosa*. The results showed that while the ΔG value of ostruthin was not lower than that of the control compound tamoxifen, ostruthin exhibited shorter hydrogen bond distances compared to tamoxifen. This indicates that the binding between ostruthin and the ESR1 receptor is more stable. These findings confirm the mechanisms of action of *L. sarmentosa* extract on male reproduction, leading to increased motility, sperm viability, and spermatogenic potential in mice. (Permatasari et al., 2024)

An ADMET pharmacokinetic analysis was conducted. Based on the results, the best bioactive compound from the n-hexane fraction of *L. sarmentosa* that binds to the ESR1 protein target is ostruthin. This compound exhibits better pharmacokinetic properties compared to its control compound, tamoxifen, which is relatively difficult for the body to metabolize.

The second potential protein, PIK3CA, was then tested against the compounds of *L. sarmentosa*. The

results identified three test compounds, trioxsalen, naproxen, and 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one, that met two key parameters from the molecular docking analysis for the PIK3CA protein target. These three compounds also demonstrated lower ΔG values compared to the control compound, PTX, and exhibited shorter protein-ligand interaction distances. This suggests that these compounds have the potential to replace PTX, offering better and more stable effects.

These findings confirm the study by Humairoh, which reported the mechanism of action of *L. sarmentosa* extract in enhancing sperm motility through processes related to apoptosis and cell survival. This was evidenced by reduced caspase-3 levels and increased Akt protein expression and tyrosine phosphorylation. (Humairoh, 2021)

Subsequently, an ADMET pharmacokinetic analysis was conducted on the three compounds binding to PIK3CA, identifying 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one as the best compound. Unlike the other two test compounds, this compound is neither tumorigenic nor mutagenic. Furthermore, its ADME characteristics are similar to those of the control compound. However, when considering toxicity parameters, this compound is safer than PTX, which tends to exhibit higher toxicity.

CONCLUSIONS

The network pharmacology analysis of bioactive compounds from the n-hexane fraction of *L. sarmentosa* root, involved in the mechanism of male infertility, identified 103 target proteins. These were then filtered using four centrality parameters, resulting in two significant potential protein targets: ESR1 and PIK3CA. Based on molecular docking analysis of the test ligands from the n-hexane fraction of *L. sarmentosa* root against the target proteins, followed by pharmacokinetic profiling, the best compound binding to ESR1 was identified as ostruthin, while the best compound binding to PIK3CA was 6-phenyl-3,4-dihydro-1H-2,5-benzoxazocin-1-one. In future research, it is recommended to develop research methods to evaluate the pharmacokinetics, safety, and efficacy of both compounds using experimental models, including *in vitro*, *in vivo*, and clinical trials. This is expected to expand the potential application of these compounds as a more comprehensive alternative treatment for male infertility.

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