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RESEARCH ARTICLE

A network pharmacology-based approach to explore the potentials of saluang belum (*Luvunga sarmentosa)* in the male human reproductive system

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Keywords
Luvunga sarmentosa
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Abstract

Background: Saluang belum's root (*Luvunga sarmentosa*) is one of the local plants in Central Kalimantan and is used by the Dayaknese to increase sexual activity. In previous research, *L. sarmentosa* root extract compounds have been characterised, but the bioactivity of each on male fertility has not been studied. **Objective**: To use bioinformatics to study the effects and mechanisms of molecular compounds of L. *sarmentosa* extract on the biological system of the male fertility complex. **Methods:** Network pharmacology methods were used to determine target essentially functional proteins in biological system networks. The molecular mechanism of *L. sarmentosa* compound activity was analysed using molecular docking and dynamic simulation software such as Autodock 4 and MOE. **Result:** The network pharmacology analysis showed that the Pregnane X receptor (PXR) protein contributed essentially to male reproductive biological functions. Based on the molecular docking simulations of PXR, 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one was the best. Pharmacological and toxicity parameters also showed that this compound had good characteristics. In addition, the molecular dynamics simulations showed that the best compounds could maintain complex interactions with PXR based on the resulting RMSD values. **Conclusion:** The compound 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one had the most potential as a PXR inductor. This compound also had a better activity than the standard inductor.

Introduction

Infertility occurs in approximately 16% of couples globally (Satouh *et al.*, 2018). About 36% of the total cases of infertility are caused by male factors such as bad quality and low quantity of spermatozoa (Valzon *et al*., 2018). According to the World Health Organization, sperm motility and viability are important parameters that determine the quality of spermatozoa and male fertility (Campbell *et al.*, 2021).

Efforts to improve the quality of spermatozoa are continuing (Stephens *et al*., 2013). So far, it is known that improving the quality of spermatozoa using hormonal therapy is not optimal and has side effects, including damage to the germinal epithelium and fluctuations in emotional state (Martinez *et al*., 2020).

Therefore, safe herbal drug compounds may be considered to improve the quality of spermatozoa. Various herbal compounds are known to help improve the quality of spermatozoa, one of which is the saluang belum root (*Luvunga sarmentosa*) (Syarif *et al*., 2016).

The roots of *L. sarmentosa* are one of the local plants of Central Kalimantan in Indonesia, traditionally used by the Dayaknese people to increase sexual activity and treat sexual dysfunction and male fertility. Based on the results of its phytochemical screening using thin-layer chromatography, it was discovered that the roots of saluang belum contain flavonoids and steroid

compounds, which tend to increase sexual activity in rodents (Anggriani, 2018; Wati *et al.*, 2018). With the discovery of the classes of these secondary metabolites, it is necessary to carry out further research to identify and isolate pure compounds from saluang belum root extract.

L. sarmentosa root extract has been shown to positively affect the viability and motility of rat spermatozoa (Syarif *et al*., 2016). However, little is known about the same effect on human spermatozoa, especially the mechanisms at the molecular level. Further research is therefore required to determine the role of active compounds in the roots of *L. sarmentosa* in the regulation of human spermatozoa. The active compounds of *L. sarmentosa* can improve the physiological functions of spermatozoa, such as motility (kinetics) (Permatasari *et al*., 2023). Spermatozoa motility depends on several metabolic pathways and regulatory mechanisms. The exact mechanism by which the compounds in L. *sarmentosa* extract can influence the steroidogenesis signalling pathway as key to increasing the testosterone level in men is yet to be known. Thus, this research was conducted to analyse the active compounds from *L. sarmentosa* root on male fertility. Therefore, this research was conducted to analyse the effects of active compounds from *L. sarmentosa* roots on male fertility.

Research conducted by Syarpin *et al*. (2023) identified the phytochemical components contained in the *L. sarmentosa* fraction using n-hexane, chloroform, ethyl acetate, and methanol as solvents. The highest percentage of compounds were alkaloids and amino acid derivatives. This research also reported that the compound content yielded the best antioxidant activity compared to other fractions (Syarpin *et al*., 2023).

The active compound produced by *L. sarmentosa* has not been tested for its structural dynamics when it binds to several proteins related to its mechanism of increasing fertility using bioinformatics. Therefore, this research aimed to visualise the effects of *L. sarmentosa* extract compounds on the complex biological system of male human reproduction using bioinformatics analysis. This research can be used as a reference for future male fertility drug candidates.

Methods

Identification of protein targets associated with male fertility

Potential protein targets associated with male fertility were identified using SwissTargetPrediction (Daina *et al*. 2019). The standard compound used to determine potential targets is ginsenoside. This compound improves male reproductive function, especially by increasing fertility and libido (Park *et al.,* 2017; Leung & Wong, 2013). Standard compounds were selected to increase accuracy in selecting potential target proteins. The selected protein target has a probability value greater than zero.

Construction of protein-protein interaction (PPI) networks and important subnetworks

The STRING 11.0 database web server (http://string-db.org) was used to create a target protein-protein interaction network obtained from SwissTargetPrediction results (Szklarczyk *et al*., 2023). *Homo sapiens* parameters were used as filters in PPI construction. The obtained protein interaction network was then further analysed to assess important subnetworks using the Cytohubba Plug-in in Cytoscape 3.10.1 software (Xiang *et al.,* 2022).

Pathway-enrichment analyses

Important proteins related to male fertility were further analysed to produce information about the biological processes (BPs) influenced by these proteins. Cytoscape 3.10.1 software via the ClueGO Plug-in is used to analyse functional groups in biological networks (Xiang *et al*., 2022). The top hub targets that connected more with other key targets were selected as potential target proteins.

Preparation of 3D structures of potential ligands and proteins

The results of Syarfin (2023) were inventoried, and the 3D structure was downloaded from PubChem in SDF file format (Syarpin *et al*., 2023). Structure preparation was carried out before it was used in molecular docking simulations. In total, 34 3D structures of chemical compounds were optimised to have a 3D shape with minimal energy using Avogrado. The type of force field used in the energy minimisation process is MMFF94. At this stage, an optimised 3D chemical compound structure will be obtained. The 3D structures of important proteins from the previous stages are downloaded from the Protein Data Bank Web Server in the PDB file format. Protein preparation was carried out by repairing the 3D structure of the protein and adding missing hydrogen atoms using Autodock Tools and Discovery Studio 2021 software. Water molecules and ligands unrelated to the research parameters were removed from the 3D structure of the protein. Measurement of the XYZ active site coordinates of the protein was also carried out in this protein preparation stage (Xiang *et al*., 2022; Sharma *et al*., 2021; Seah *et*

al., 2015). A 3D protein structure was obtained, prepared, and then stored in the database at this stage.

Screening of pharmacology and toxicity properties of potential compounds

The pharmacology and toxicity properties of compounds of *L. sarmentosa* were described using Osiris Data Warrior, PubMed and SwissAdme. At this stage, the compounds with the best properties were selected to analyse their molecular mechanism using docking and dynamic simulation.

Molecular docking and dynamics simulation

This simulation aimed to validate the network pharmacology study. Important constituents derived from network pharmacology results are docking and molecular dynamics targets. Molecular docking simulations were performed using the best compounds based on the previous stage in the XYZ coordinates of the protein's active site. The software used in the simulation process was Autodok 4.2.6, which was integrated into AMDock (Valdés-Tresanco *et al.*, 2020). Chemical compounds resulting from molecular docking with RMSD < 2 Å are successful. At this stage, the best compounds from each molecular docking with the lowest binding affinity values and best ligand efficiency will be selected for the molecular dynamics simulation. Molecular dynamics simulations were done using the NPA algorithm on the AMBER10:ETH force field (Khelfaoui *et al.*, 2021). This simulation was carried out at 300 K. The protein and ligand were flexible. The value of the neighbour search will be set at 150 ps. At this stage, the stability of protein-ligand complexes will be explained by analysing their RMSD over time.

The selection of candidate compounds with potential as male fertility drug compounds was done by analysing data from pharmacology and toxicity characteristics, binding energy from molecular docking simulation results, and stability of ligand-protein complexes from molecular dynamics simulation results.

Results

Determination of candidate protein targets

The determination of candidate target proteins was done using SwissTargetPrediction. The ginsenoside compound from the ginseng plant is used as a standard compound. The analysis results using SwissTargetPrediction show that 46 proteins can be targets of ginsenoside compounds. The protein gene was then analysed further to find out its group. The results of the analysis are shown in Figure 1.

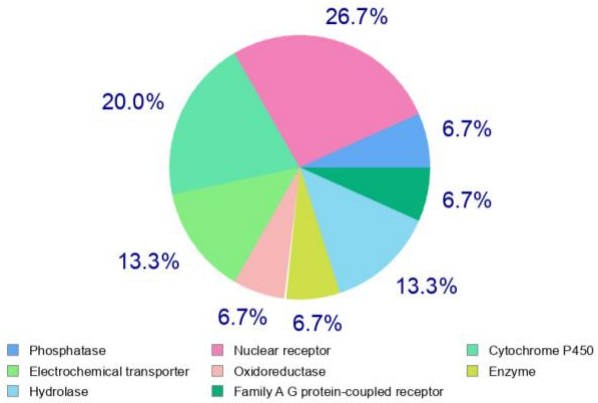

Figure 1: Percentage of potential protein target classes from bioactivity analysis of ginsenosides

Acquisition of primary target proteins via Protein-Protein Interaction (PPI) analysis

The Protein-Protein Interaction (PPI) network was created with STRINGDB. A total of 46 protein targets were imported into STRINGDB for correlation analysis between one another. The complex PPI network is shown in Figure 2.

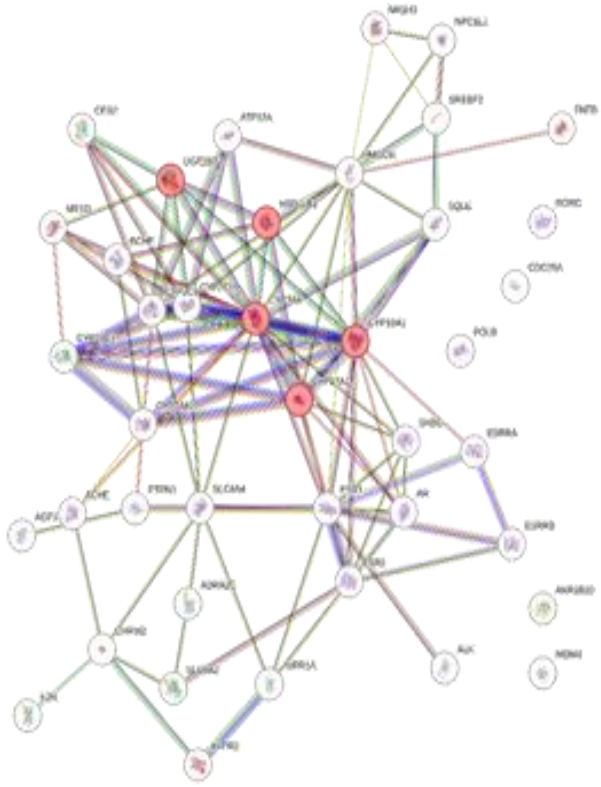

Figure 2: The protein-protein network of 49 potential targets according to the STRING database. The red circle indicates the query proteins and the first shell of interactors.

Cytoscape software was used to re-visualise and analyse PPI complexes to determine the degree of centrality between proteins. The result of this step is shown in Figure 3.

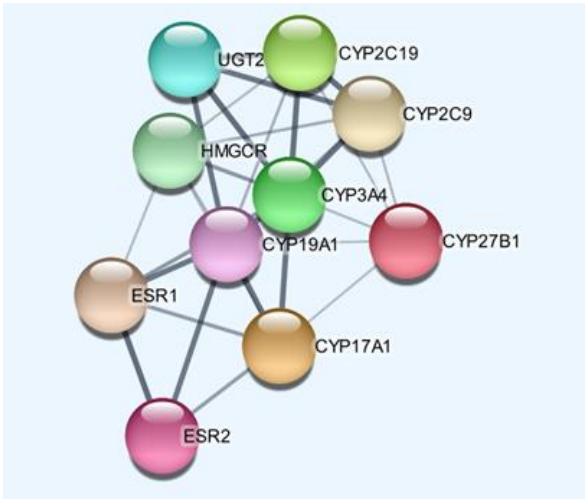

Figure 3: The network of ten proteins with the highest degree of centrality values

Pathway-enrichment analyses

The selected protein candidates were then analysed further to understand their gene ontology (GO) function using the Cytoscape *plugin*, namely ClueGO. The results of this analysis will obtain functional linkages in biological networks. The results of the GO-enrichment analysis are shown in Figure 4. The results of the GO-enrichment analysis showed that CYP3A4 had the highest degree in male reproductive systems, such as androgen and estrogen systems. Seah (2015) states that the protein pregnane X receptor (PXR) regulated CYP3A gene expression. The up-regulation of glutathione s-transferase is another biological function of PXR in the male reproductive system (Zeng *et al*., 2022; Fafula *et al*., 2019; Falkner *et al.*, 2001).

Visualisation and preparation of PXR receptors

The 3D structure crystal PXR with ID 1M13 was downloaded from the Protein Data Bank. As shown in Figure 5, these proteins were visualised using the Biovia Discovery Studio 2021 client.

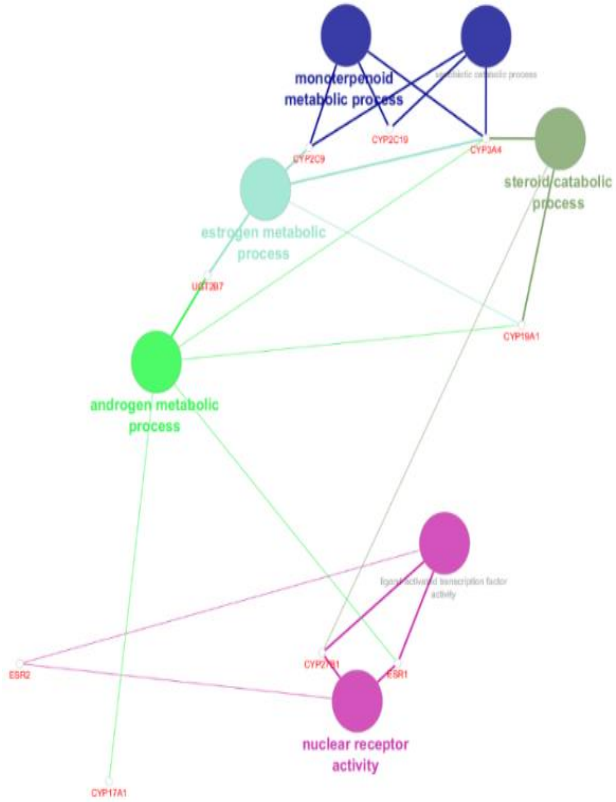

Figure 4: Result from The GO function analysis of biological function from potential protein target

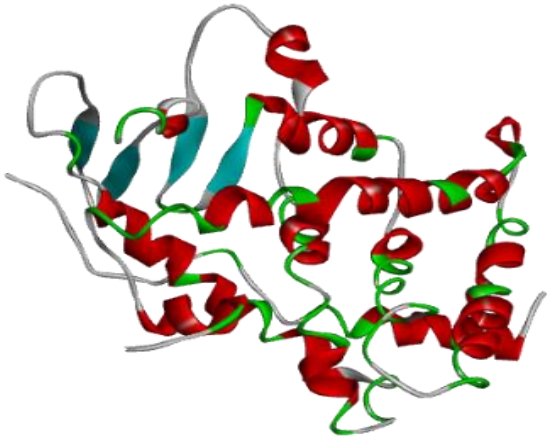

Figure 5: Visualisation of 3D structure from protein target Pregnane X Receptor (PXR)

Results of selection of L. sarmentosa compounds based on toxicity and pharmacological properties

The toxicity and pharmacological properties of 34 L. *sarmentosa* compounds were analysed to select candidate compounds for molecular docking simulations. Table I shows the best six compounds.

Table I: Best compound result of analysis of toxicity and pharmacology parameters

No	Compound name	Toxicity			Lipinski's rule of five	Drug-likeness value
		Carcinogenicity	Immunogenicity	Tumorigenic		
1	Ostruthin	Not active	Not active	Not active	Fulfil	-4.619
2	p-Hydroxyketorolac	Not active	Not active	Not active	Fulfil	2.593
3	N-feruloylglycine	Not active	Not active	Not active	Fulfil	-4.601
4	Dimethyltryptamine	Not active	Not active	Not active	Fulfil	3.799
5	Tolpropamine	Not active	Not active	Not active	Fulfil	3.454
6	6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one	Not active	Not active	Not active	Fulfil	3.403

Molecular docking ligand-PXR simulation

Molecular docking simulation is a computational method to predict ligand activity from *L. sarmentosa* and PXR extracts. This simulation was also carried out to determine the best ligand, which has a better activity value than the natural PXR ligand, hyperforin. Comparative ligands such as In this research, molecular docking simulations were carried out using Autodock 4, which is integrated with AMDock (Valdés-Tresanco *et al*., 2020). The results of the simulation are shown in Table II.

Table II: Result of molecular docking simulation using Autodock

No	Compound name	Affinity energy (kcal/mol)	Interaction			Ligand efficiency
			Hydrogen bond	van der Waals	Other interactions	
1	Ginsenosides	-10.43	GLN285	LEU234;SER247;ARG410;PHE420;HIS327	TYR306;MET323;MET243;LEU209;CYS284;LEU240;PHE281;LEU206;ILE414;LEU411;PHE288;MET246;CYS301;TRP299	-0.33
2	6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one	-8.59	LYS210	PHE166;GLN285;TRP299;LEU324;LEU308;LEU209;VAL211;MET243	CYS301;MET246;TYR306;PHE288;HIS327;MET323	-0.45
3	Ostruthin	-8.56	LYS210;Val211	ALA244;SER247;MET243;PRO227;ILE236;PRO228	LEU411;PHE420;MET425;ILE414;LEU240;LEU206;LEU209;LEU239	-0.39
4	p-Hydroxyketorolac	-8.33	VAL211;PRO288;	ILE236;ALA229;GLU235	MET243;LEU209;LEU239;PRO227;LYS210;LEU206	-0.42
5	Rifampicin	-7.25	LYS252;ASP245	PHE420;PHE172;ASN171;PRO419;LYS170TYR249;HIS168;THR248;THR422	PHE169;ARG173	-0.12
6	N-feruloylglycine	-6.81	LYS210;PRO228;TYR225	SER208;LEU308;TRP299;LEU324;LEU206;ILE236;LEU209;VAL211;MET243;LEU239;SER238;PRO227		-0.38
7	Dimethyltryptamine	-6.65	LEU206	LEU209;VAL211;LYS210;GLU235;PRO227;LEU240;LEU239;MET243	ILE236;PRO228	-0.48
8	Tolpropamine	-6.63	-	LEU215;ASN224;GLY176	TYR225;LEU213;PRO175;LEU174;TRP223;VAL177	-0.35
9	Hyperforin	-6.57	TYR249;SER256	HIS168	PHE162;MET250;ILE255;LYS252	-0.17

Figure 6 shows the docking model of the ligand 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one and PXR complex. The 2D visualisation interactions showed the van der Waals interaction and hydrogen bond in the complex.

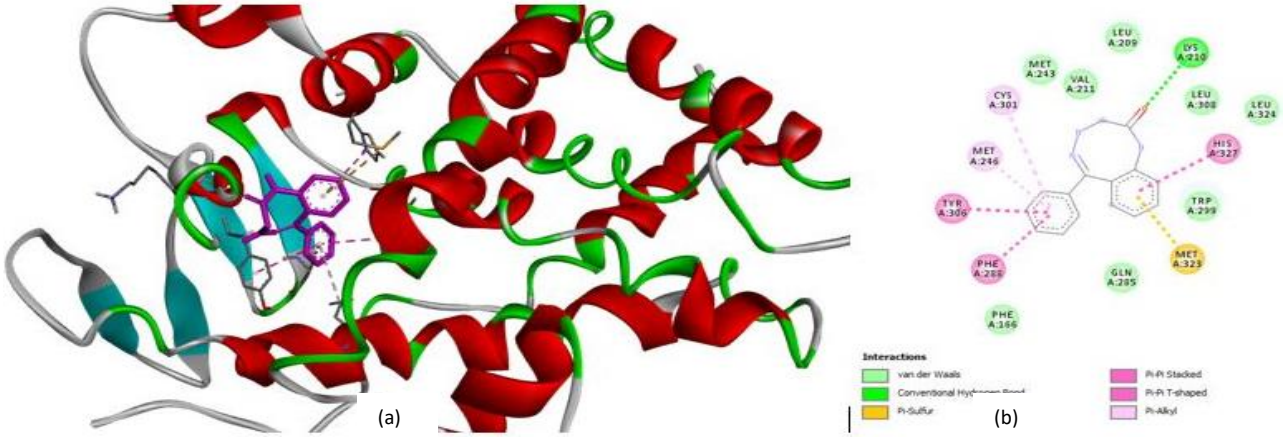

Figure 6: Docking model of ligand 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one and PXR complex (a) 3D Visualisation of the molecular interactions; (b) 2D visualisation interactions showed the van der Waals interaction and hydrogen bond in 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one and PXR complex.

Best molecular dynamic simulation of ligand complexes

The potential ligand chosen to see its activity in molecular dynamics simulations was 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one. This selection was based on the fact that this ligand is an L. sarmentosa extract ligand with the highest affinity value and a higher ligand efficiency value than ginsenosides. Not only that, this ligand also had the same interaction as ginsenosides. Molecular dynamics simulations were used to predict the conformational stability of the PRX complex and the observed ligand over comparable periods. Thus, these simulations allow enzyme–ligand complexes to implement model-induced fits, making them more accurate and reliable than docking simulations. In this research, molecular dynamics simulations were carried out using the NPA algorithm on the AMBER10:ETH force field. At a temperature of 300 K, the molecular dynamics simulation is generally divided into three stages, namely the initialisation, balance and production stages. When the production stage was complete, a trajectory simulation was produced, showing changes in the conformation of the ligand and PXR complex. The results of the molecular dynamics simulation of the ligand 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one and PXR are shown in Figure 7.

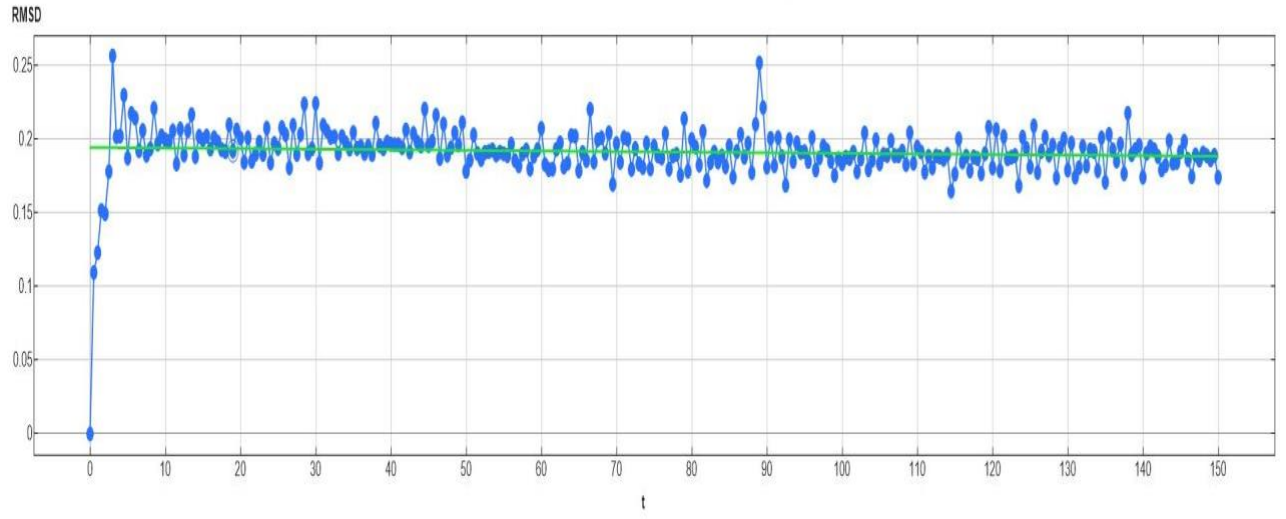

Figure 7: RMSD curve of 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazosin-2-one complex with PXR. An rmsd value of no more than 2 Å during the simulation indicated a stable ligand-protein complex.

Discussion

Figure 1 shows that 26.7% is the cytochrome P450 group, and 6.7% is the nuclear receptor protein group. Proteins in this group are generally involved in metabolising sex hormones such as endogenous and androgen receptors. PPI is carried out to see the function of the biological relationship between the target proteins obtained. STRINGDB analysis shows that the PPI network complex produces 41 nodes and 106 edges. All protein targets were rearranged to determine ten proteins with high degree centrality values that have important roles in the PPI network. This assessment was carried out with a plugin from Cytoscape, namely CytoHubba. The top ten protein targets were CYP3A4, CYP19A1, UGT2, CYP2C19, CYP2C9, CYP27B1, CYP17A1, ESR2, ESR1 and HMGCR.

The results of GO-Enrichment analysis show that CYP3A4 has the highest degree of association with biological functions in androgen metabolic, estrogen metabolic and steroid catabolic processes. These three processes play an important role in the biological function of male fertility. According to Seah (2015), nuclear receptors such as the pregnane X receptor (PXR) regulate CYP3A gene expression and are also known to have a major role in the transcriptional induction mechanism of CYP3A4. This protein is a target to determine the bioactivity of the *L. sarmentosa* compound as an inductor for this protein. The PXR itself have function to up regulates the expression of Glutathione S-transferase (GST) in human. GST is important in the male reproductive tract and sperm physiology system. The activation of PXR could increase GST expression and is expected to improve the male reproductive system (Zeng *et al*., 2022; Falkner *et al*., 2001; Fafula *et al*., 2019).

The obtained PXR structure shown in Figure 5 has a resolution of 2.15 Å, which shows that the structure of this protein is good. The grid box coordinate analysis results using Biovia Discovery Studio 2021 based on the position of the ligand attached to PXR were at position X: 10.096, Y: 76.853, and Z: -1.643 with dimensions of 60 x 60 x 60. Based on Table I, the six potential compounds to be developed as male fertility drug candidates were selected based on parameters such as toxicity properties, pharmacological properties of compound sub-structures, BRENK analysis and Lipinski Rule of Five. This research was conducted to ensure that the candidate compounds had good pharmacological properties and would not cause negative health effects.

Table II revealed that all ligands from L. sarmentosa extract have a lower affinity energy value than the natural compound, namely Hyperion. All L. sarmentosa compound extract ligands have better activity than natural ones. The rifampicin ligand has long been known as a PXR inductor, and based on simulation results, it showed that three compounds have lower affinity values, namely 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one, Ostruthin, and *p*-Hydroxyketorolac. Even though the affinities of these three compounds were not better than those of ginsenosides, these three compounds have better ligand efficacy values. This showed that these three ligands could be developed as PXR inductors. The ligand 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one had a similar interaction with ginsenosides, i.e. the interaction with CYS301.

Meanwhile, during molecular dynamics simulations, Root Mean Square Deviation (RMSD) was used to describe the conformational changes between two atomic coordinates. Therefore, the stability status of the ligand complex and PXR enzyme could be determined when the temperature increased. The RMSD value in Figure 7 showed that the ligand 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one had a stable complex with PXR because it had a low RMSD value (1.5 - 1.8 Å) at 300 K.

Conclusion

This research showed that the pregnane X receptor (PRX) was one of the main targets because of its biological function in increasing male fertility. Proteins are involved in many signalling pathways and biological processes in the body. Docking and molecular dynamics studies showed that the *Luvunga sarmentosa* extract compound could be a PXR inducer, as seen from its better binding affinity than the native ligand hyperforin. The compound 6-phenyl-3,4-dihydro-1H-1,4,5-benzotriazocin-2-one is a potential candidate as a PXR inductor based on the results of bioinformatics analysis. In summary, the findings in this study may provide new insights into developing potential male fertility drugs in future. Therefore, research on Luvunga sarmentosa compounds needs to be further studied *in vitro* and *in vivo*.

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