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Original Article

In-silico Prediction of Azadirachta indica Compounds as Potential Therapeutic Inhibitor of Lysyl Oxidase to Suppress Canine Mammary Tumor Proliferation

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ABSTRACT

Canine mammary tumor (CMT) is one of the leading causes of death in female dogs, mainly due to the unavailability/expensive treatment, adverse and untargeted nature of the contemporary therapeutics. **Objectives:** To discover a biological mediator from the *Azadirachta indica* extracts by targeting Lysyl Oxidase (LOX), which is one of the enzymes responsible for accelerating the development of tumors and altering cellular microenvironment in mammals is considered to be suitable targets for anti-cancerous drugs. **Methods:** Current study utilized computer-aided drug designing (CADD) to investigate 33 phytocompounds derived from this plant to check their potential inhibition properties against LOX protein. The phytochemicals were docked onto the protein and the ligands with the lowest binding energies were evaluated over the several parameters using PyRx software. Molecular dynamic simulation was also performed to further investigate the stability and conformational changes of the resultant ligand-protein complex by analyzing RMSD & RMSF values, H-bond graphs and Heat maps through VMD/NAMD softwares. **Results:** The results revealed that Azadirachtin to be the most pertinent agent in LOX inhibition with a docking score of -12.6 kcal/mol and showed promising in-silico stability as well. Drug likeliness potential was further assessed based on Lipinski's rule of five which reflect the safer nature of this drug agent. **Conclusions:** Moreover, wet-lab in-vitro experiments followed by clinical trials are still needed to attest the validity of this virtually piloted phytocompound against LOX protein for CMT cure.

INTRODUCTION

Canine mammary tumor (CMT), the second most commonly identified type of cancer in female dogs, is stated to be their leading cause of death [1]. While a range of causes have been reported for the occurrence of CMT such as genetic mutations in the associated genes, damaged DNA repair system, imbalanced sex hormones and epigenetic modifications including age, diet and high body fat content [2, 3]; a correlation between type of breed and incidence of CMT has also been observed [4]. Smaller breeds are at higher risk of developing the disease in both the benign and malignant forms [5]. Benign neoplasia can be treated with abscission and have a decent prognosis however, malignant cases are threatening as they can metastasize to neighboring lymph nodes and far off interior organs [6, 7]. In addition, 35-70% bitches experience recurring cancers and have to be subjected to radio- and chemotherapy [8]. A plethora of studies on human breast cancer have been reported using mice models paired with material extracted from patients or xenografts of cancer cell lines. Although these have aided in the progress of

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breast cancer diagnosis and treatment, they are hindered by limitations of molecular differences in the model and humans as a result, more precise alternatives are needed to make substantial advances in the field [9]. Genes encoding mammaglobin-B, a protein classified as a secretoglobin, are often over-expressed causing accumulation of the protein and ultimately resulted as CMT [10]. Similarly, mammaglobin in humans has been identified as a marker for breast cancer making female dogs suitable translational models for further clinical experiments, research and treatment options [11]. The LOX is an enzyme also known as protein-lysine 6-oxidase belonging to the oxide reductase group of catalysts and is upregulated in mammals suffering from CMT [12]. Since the primary function of this protein is to mediate collagen cross linking they serve as molecular markers for CMT as samples of tumor cells most often indicate increased cross linking [13]. Furthermore, they accelerate tumor development and metastasis by actively remodeling the tumor microenvironment [14]. Inhibiting their function can greatly decrease the chances of the initiation of oncogenesis. The extracts of *Azadirachta indica*, more commonly known as "Neem" in local language, contain a variety of secondary metabolites, referred as phytocompounds that naturally possess anti-cancerous properties [15]. Some of the therapeutic phytochemicals include azadirachtin, catechins, glycoproteins, nimbins, gallic acid, tannins, phenols, flavonoids, limonoids, and triterpenes [16]. Additionally, they house the advantages of being safe to use, cost effective, eco-friendly and extremely efficient in terms of affect and response [17]. In the current study molecular docking and dynamic simulations approaches are being adopted to investigate the inhibitory effects of *Azadirachta indica* phytocompounds as potential drug agents by targeting the LOX protein as a treatment option for CMT.

METHODS

Selection of Medicinal Herb (*Azadirachta indica*)

Azadirachta indica, commonly referred to as "Neem", which contain a range of phytocompounds that display potent anti-cancerous properties against various cell lines [18]. It is mostly cultivated in Pakistan/India. With a phytochemical profile of almost 135 compounds with distinct chemical structures and therapeutic uses, polyphenols are considered most pertinent with reference to anti-tumor activity as they induce apoptosis in cancer cells by mobilizing the copper ions attached to the chromatin resulting in fragmentation of the DNA [19, 20]. Some of the chemical structures of phytocompounds of Neem are given in Figure 1.

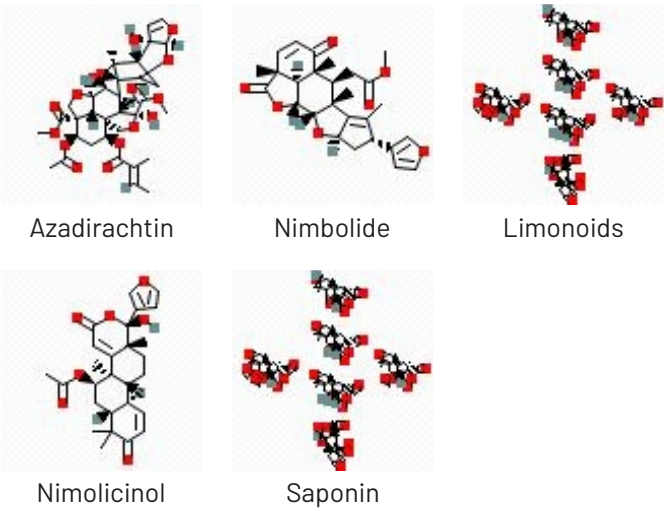

Figure 1: Chemical structures of some of the *Azadirachta indica* phytocompounds

Below (Table 1) contains the chemical details of 33 ligands present in *Azadirachta indica* that were further analyzed during the course of this research endeavor. Azadirachtin, Nimbolide and Saponins are some of the majorly extracted phytocompounds that are often used as insecticides, herbicides and pesticides respectively.

Table 1: Physicochemical profile of different phytocompounds of *Azadirachta indica*

Ligands	Molecular Weight (g/mol)	PubChem CID	LogP	Molecular Formula
Azadirachtin	720.7	5281303	1.09	$C_{35}H_{44}O_{16}$
Salannin	596.7	6433066	3.9	$C_{34}H_{44}O_{9}$
Nimbin	540.6	108058	2.3	$C_{30}H_{36}O_{9}$
Nimbolide	466.5	12313376	2.2	$C_{27}H_{30}O_{7}$
Gedunin	482.6	12004512	4.2	$C_{28}H_{34}O_{6}$
Quercetin	302.23	5280343	1.5	$C_{15}H_{10}O_{7}$
Epoxyazadiradione	466.6	49863985	4.2	$C_{28}H_{34}O_{6}$
DPPH	790.7	86650676	-	$C_{36}H_{26}N_{10}O_{12}$
Palmitic acid	256.42	985	6.4	$C_{16}H_{32}O_{2}$
Stearic acid	284.5	5281	7.4	$C_{18}H_{36}O_{2}$
Oleic acid	282.5	445639	6.5	$C_{18}H_{34}O_{2}$
Nimolicinol	482.6	184937	3.9	$C_{28}H_{34}O_{7}$
Saponin	1223.3	198016	-2.7	$C_{58}H_{94}O_{27}$
Tannin	952.7	44144428	2.5	$C_{42}H_{32}O_{26}$
Anthraquinone	208.21	6780	3.4	$C_{14}H_{8}O_{2}$
Alkaloids	542.7	265028	6	$C_{34}H_{42}N_{2}O_{4}$
Scopoletin	192.17	5280460	1.5	$C_{10}H_{8}O_{4}$
Limonoids	2831.3	71597583	-	$C_{163}H_{200}O_{42}$
Catechins	290.27	1203	0.4	$C_{15}H_{14}O_{6}$
Lupeol	426.7	259846	12.1	$C_{30}H_{50}O$
Campesterol	400.7	173183	-	$C_{28}H_{48}O$
Naheedin	528.7	129754	5.3	$C_{32}H_{48}O_{6}$
Azadirone	436.6	10906239	-	$C_{28}H_{36}O_{4}$
Azadironolide	468.6	10814144	4.4	$C_{28}H_{36}O_{6}$
Mahmoodin	526.6	126566	3.7	$C_{30}H_{38}O_{8}$
Azadiradione	450.6	12308714	4.8	$C_{28}H_{34}O_{5}$

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Ligands	Molecular Weight (g/mol)	PubChem CID	LogP	Molecular Formula
Deacetylnimbin	498.6	10505484	1.7	$C_{28}H_{34}O_8$
Azadiradionolide	466.6	11798426	4.1	$C_{28}H_{34}O_6$
Meliacinol	538.7	101026859	4.1	$C_{32}H_{42}O_7$
Zafaral	484.6	101355583	4	$C_{29}H_{40}O_6$
Epicatechin	290.27	72276	0.4	$C_{15}H_{14}O_6$
Luteolin	286.24	5280445	1.4	$C_{15}H_{10}O_6$
Kaempferol	286.24	5280863	1.9	$C_{15}H_{10}O$

Selection of Targeted Protein

Lysyl oxidase (LOX), a protein that is the chief contender involved in canine mammary tumors was targeted in this study. Abnormal expression of LOX can result in alterations of the micro-environment of the cells that may lead to tumor initiation and metastasis. Although the basic function of the enzyme is to cross link elastin molecules and collagen, it interacts with histone H1 and H2 in the cell causing cancer [21]. However, the mature form of the LOX pro-peptide is involved in cancer progression that is intended to be inhibited in this study to stop cancer development [22].

Homology Modeling of Protein using I-TASSER

The sequence retrieved from UNIPROT was then used to design a complete protein structure via I-TASSER. The pipeline of this software consists of three main steps where first the template is identified then full-length structure-based assembly is visualized and lastly its structural based functional annotation is done and a similar protein structure from that sequence can be obtained using this software tool [23].

Molecular Docking Studies using PyRx

PyRx, a software designed to aid in the discovery of new therapeutic drugs, was used for the molecular docking of *Azadirachta indica* compounds. It facilitates the visualization of protein-ligand interactions and determines using models and simulations, the potential drug-like properties of a compound. In order to validate the predictions, the results were cross-checked using the MOE software.

Preparation of Ligands

Several databases are available to obtain the desired ligand e.g., DrugBank, Zinc, PubChem, Asinex ChEMBL, Merck, Enamine etc. These ligands can either be downloaded in SDF format or can be sketched in MOE interface by using Builder Mode. After sketching, the partial charges were added by computing it in MOE. Once the charges were added, the prepared ligand was then saved as an MDB file.

Preparation of Targeted Protein

The targeted protein was derived from UNIPROT and further prepared for evaluation using PyRx software. With the aim of exposing the active site for optimal ligand interaction during the docking process, water molecules were removed from the surface of the protein, repeating chains of Mpro were eliminated, and other inhibitors near the active site were also removed. Moreover, I-TASSER was utilized to align and correct the 3D model of the protein structure while the sequence of the most appropriate active site was chosen based on the chain with the most energetic amino acid residues (Figure 2).

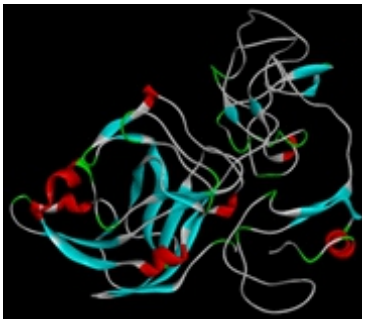

Figure 2: Prepared structure of LOX protein

Docking and Surface Mapping

The objective of ligand-protein docking is to forecast the best way for a ligand to attach to a protein with respect to its known three-dimensional structure. To fulfill this, the prepared ligand and protein molecules were uploaded to PyRx in the SDF format and parameters were adjusted. For energy minimization of the ligand, the UFF force field was selected which was set to run at 500 steps and the ligand-protein complex thus obtained was converted to pdbqt format. Thereafter, the Autodock Vina wizard command was selected and grid-box was adjusted on the ligand-protein complex that led to the initiation of the docking process and subsequent binding affinity scores.

Lipinski's Rule of Five

Lipinski's rule, also termed as the Pfizer rule of five for drug likeliness, are a standard set of parameters that aid in the evaluation of a compounds pharmacology and oral bioavailability [24]. Phytocompounds of *Azadirachta indica* were selected for dynamic simulation by applying Lipinski's rule on those with top binding affinity and the subsequent results were statistically analyzed using ADMET tool.

Molecular Dynamic Simulation using VMD/NAMD Softwares

Based on the principles of interatomic interactions, molecular docking simulation help predict the mobility dynamics of a molecular system. Parameters including conformational anomalies, fluctuation, and stability of the protein-ligand complex can be investigated using inherent tools of VMD and NAMD software. The docked complex with the highest score, Azadirachtin with LOX, was used for molecular dynamic simulation and the results were further interpreted to analyze the stability of the complex by plotting graphs of RMSD, RMSF, Hydrogen bonds, and heat maps.

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Defining Parameters & Executing Simulation Process

The structure of the protein-ligand complex was saved in the PSF file format using VMD and further parameterized via CHARMM-GUI input generator. The topologies of both the protein and ligand were merged and solvation was performed to get the cubic water box around the complex. The process of molecular docking simulation was initiated for 1ns which is 500,000 steps and energy minimization was performed for 1000 steps via conjugate gradient method. In order to establish the periodic boundary conditions for the complex, a constant temperature of 310K and pressure of 1 atm was maintained for the simulation process. After defining the pertinent factors, the ultimate command of simulation was run on windows power shell by using NAMD software.

RESULTS

Docking Scores of *Azadirachta indica* Compounds

LOX protein was docked with 33 phytocompounds of *Azadirachta indica* and their respective binding energies were obtained through PyRx. The general principle states that the efficiency of a ligand as a drug increases as its binding energy decreases. A list of the docking scores of the phytocompounds is given below in (Table 2) according to which Azadirachtin gave the best scores with the binding energy of -12.6 kcal/mol which was followed by Limonoids, Saponin, Nimbolide, Nimolicinol and Meliacinol having energies of -12.1, -9.3, -9.0, -8.7 and - 8.7 kcal/mol respectively.

Table 2: Docking scores of *Azadirachta indica* with LOX protein

Ligands	Docking Score
Azadirachtin	-12.6
Salannin	-7.5
Nimbin	-7.5
Nimbolide	-9.0
Gedunin	-8.2
Quercetin	-7.4
Epoxyazadiradione	-8.5
DPPH	-7.1
Palmitic acid	-4.7
Stearic acid	-5.1
Oleic acid	-5.4
Nimolicinol	-8.7
Saponin	-9.3
Tannin	-8.1
Anthraquinone	-6.3
Alkaloids	-8.5
Scopoletin	-5.7
Limonoids	-12.1
Catechins	-6.9
Lupeol	-8.4
Campesterol	-7.5
Naheedin	-8.1
Azadirone	-7.7
Azadironolide	-8.3
Mahmoodin	-8.2
Azadiradione	-8.1
Deacetylnimbin	-7.0
Azadiradionolide	-8.4
Meliacinol	-8.7
Zafaral	-12.6
Epicatechin	-7.4
Luteolin	-7.8
Kaempferol	-7.1

2D/3D Interactions of the Best Docked Ligands with LOX

Out of the 33 phytocompounds, complex formation of compounds with the 6 best docking scores is illustrated in Figure 3.

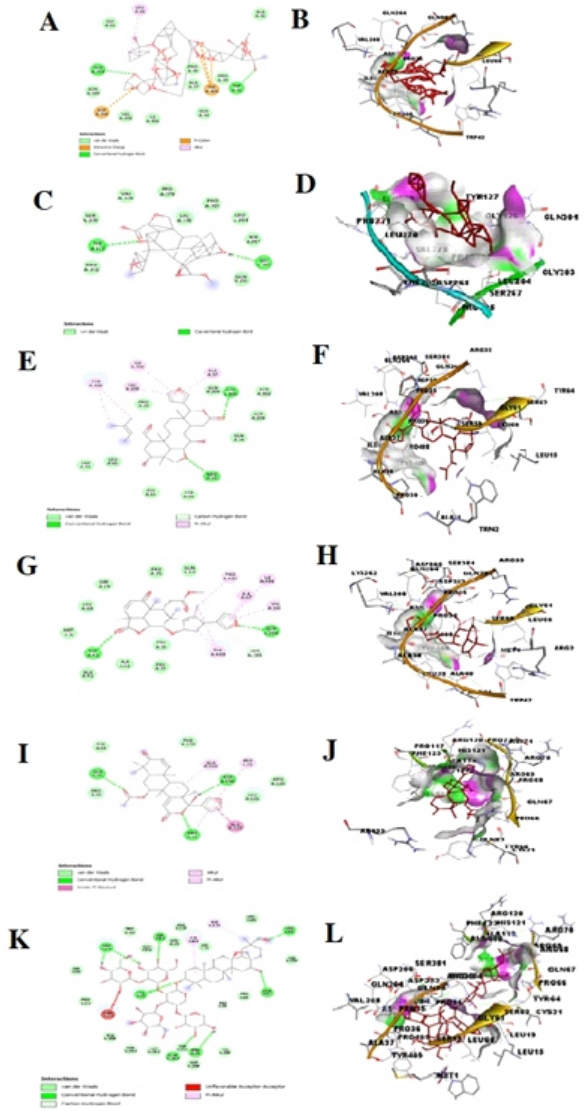

Figure 3: 2D/3D interactions of Azadiarachtin (A & B), 2D/3D interactions of Limonoids (C & D), 2D/3D interactions of Malicinol (E & F), 2D/3D

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interactions of Nimbolide (G & H), 2D/3D interactions of Nimocilinol (I & J), 2D/3D interactions of Saponin (K & L)

Interpretation of Ligands in Terms of Lipinski's Rule

Corresponding to Lipinski's rule, the ligand which fulfills two or more pre-defined parameters out of the rule of five can be considered as a potentially viable drug [25]. The assessment of absorption, digestion, metabolism, excretion, and toxicity (ADMET) [26] of the compounds with the best docking score, complying with the criteria of the Lipinski's rule, are stated in (Table 3).

Table 3: Details of *Azadirachta indica* phytocompounds according to Lipinski's rule

pubChem ID	Ligands	Molecular Weightg/mol (MW ≤ 500)	LogP (≤ 5)	H-Bond donors (≤ 5)	H-Bond acceptors (≤ 10)	TPSA (Å)^2	Violations
5281303	Azadirachtin	720.71	3.90	3	16	215.34	2
71597583	Limonoids	2831.3	5.00	5	6	-	1
101026859	Meliacinol	538.67	3.3	2	7	106.2	1
12313376	Nimbolide	466.52	3.51	0	7	92.04	0
184937	Nimolicinol	482.57	3.38	1	7	103.04	0
198016	Saponin	1223.35	4.37	15	27	422.05	3
101355583	Zafaral	484.62	3.06	0	6	86.74	0
173183	Campesterol	400.68	4.92	1	1	20.23	0
44144428	Tannin	952.62	0.33	15	26	452.03	3
12004512	Gedunin	482.57	3.19	0	7	95.34	0

Molecular Dynamic Simulation Analysis

Once a bioactive compound binds to its respective target, it causes conformational changes in the primary and secondary structure of the target, consequently altering its fundamental functionality [27]. Thus, molecular dynamic simulation analysis was conducted on Azadirachtin as it demonstrated the lowest E-score, in order to investigate the structural perturbations induced in LOX upon the formation of *Azadirachtin*-LOX complex.

Root Mean Square Deviation (RMSD) Analysis

With the aim of assessing the degree of structural shift in the initial protein molecule and its final conformation, RMSD analysis was computed utilizing the built-in trajectory tool in VMD, which is the distance between the coordinates of a group of atoms and the results were subsequently presented in the form of a graph given in figure 4. A stable ligand-protein complex tends to give RMSD values ranging from 2-3Å, however, if it is more than 4Å**,** the resultant complex may be precarious [28]. The graph deviation shows the fluctuation from 1.2Å to 1.8Å between its lengths which indicates the stability of the *Azadirachtin*-LOX complex(Figure 4).

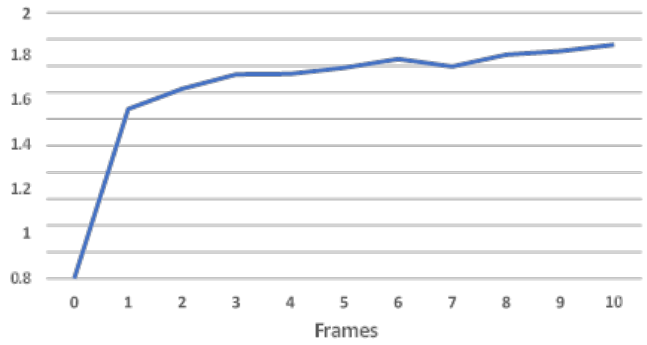

Figure 4: RMSD plot of Azadirachtin-LOX complex

Root Mean Square Fluctuation (RMSF) Analysis

To further investigate the segment of the ligand-protein complex that has caused the deviation from the primary structure, the RMSF value is computed. While the RMSD value gives similar information, RMSF aids in interpreting the flexibility of individual portions of the protein by estimating the time average of the RMSD [29]. The higher the value of RMSF obtained, the more the flexibility of the protein, but values exceeding 3.4Å are considered discordant. (Figure 5) demonstrates the RMSF plot of Azadirachtin-LOX complex that indicates stability throughout all frames as the values remain less than the threshold of 3.4Å.

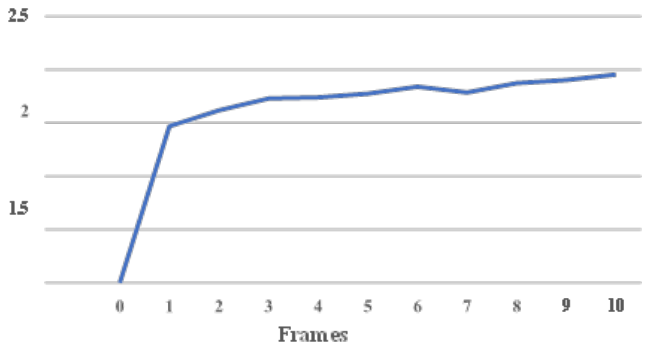

Figure 5: RMSF plot of Azadirachtin-LOX complex

Hydrogen Bonding Analysis

Hydrogen bonds are vital determinants of the stability of proteins and the specificity of ligand-protein interaction [30], thus the hydrogen bonding analysis was carried out to ascertain the number of H-bonds in the Azadirachtin-LOX complex and establish its stability. The results, plotted in the form of graphs (Figure 6) indicate the formation of hydrogen bonds over a range of frames suggesting sufficient stability of our desired complex.

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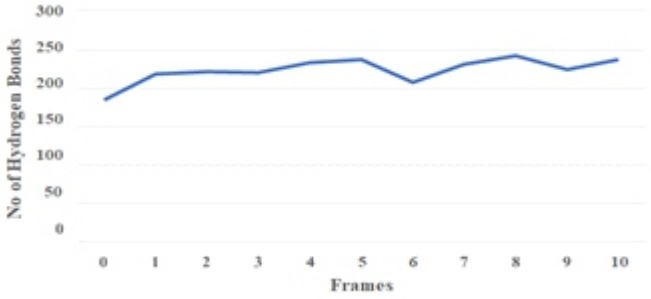

Figure 6: Hydrogen Bond Analysis of *Azadirachtin*

Heat Map Analysis

The RMSD values of the Azadirachtin-LOX complex were also depicted in the form of a heat map (Figure 7) which indicated exceptional stability of the complex during molecular docking simulation.

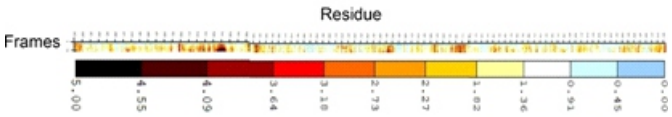

Figure 7: Heat map of *Azadirachtin*-LOX complex

DISCUSSION

Despite the availability of several treatment plans for breast cancer, patients still have to face the uncertainty of the likelihood of their survival or the danger of reoccurrence. Moreover, the cytotoxicity associated with chemotherapy, radiotherapy and mastectomy tend to complicate the situation even more [31], and thus the need of an alternative management technique arises. Plant extracts contain a myriad of phytocompounds that have the ability to mediate biological activities including anti-cancerous, anti-oxidant, anti-microbial and anti-inflammatory properties [32]. *Azadirachta indica* for instance, commonly called divine life plant or the home-pharmacy, has been reported to visibly ameliorate breast cancer in the MCF7 cell line by the compounds present with in it [33]. Further studies have elaborated on the mechanism of action of the compounds stating that they can influence monostatic dormancy through various methods such as suppressing the pathway of NF-κβ, raising the expression of p53 and pTEN tumor suppressor proteins, causing a decrease in c-Myc oncogenes or the expansion in programmed cell death of tumor suppressing cells [34]. Azadirachtin, a liminoid phytocompound extracted from *Azadirachta indica* displayed the lowest binding energy of -12.6 kcal/mol; along with a structurally stable and flexible protein-ligand complex with the active site of the LOX enzyme. 2D and 3D interactions between the ligand and protein, obtained through computer aided drug design technologies including the processes of docking and molecular simulation revealed the presence of strong hydrogen bonds that impart the complex its stability. The results suggest Azadirachtin to be a possible candidate for further research as a drug that can be used against canine mammary tumor and in the future, optimized as a form of medication for breast cancer in humans. Currently, a variety of drugs, conjugated with chemotherapy and radiotherapy, are commercially used to help treat breast cancer which include Olaparib, Talazoparib, Veliparib, and Rucaparib [31]. Moreover, management of canine mammary tumors also utilizes similar drugs such as Cytxan and Adriamycin [10]. Despite being approved by the FDA, these treatments cause severe fatigue, muscular pain and damage, alopecia, cytopenia, and cardiotoxicity in patients largely due to their non-specific targeting of receptors and cells in the body [35]. Subsequently, the need for a biologically compatible cure still exists and *Azadirachta indica* derived Azadirachtin can fulfill this requirement. Other than promising RMSD and RMSF values indicating positive protein-ligand formation, *Azadirachta* was confirmed to be non-toxic under the Lipinski's rule and ADMET analysis. In depth analysis and more simulations of Azadirachtin as an inhibitor of LOX protein can provide better understanding of the long-term stability of the conjugate. Furthermore, even though mammary organs of female dog, are the closest experimental model to human breasts, insight into even more complementary models can aid in achieving more accurate simulations that can consequently establish in vivo experimental models and clinical trials in the future.

CONCLUSIONS

The focus of this study was to determine potential drug agents from *Azadirachta indica* against LOX protein, an enzyme actively responsible for the proliferation of canine mammary tumors. In-silico analysis by molecular docking and molecular dynamic simulation revealed that Azadirachtin exhibited the best docking score and highest binding affinity with LOX. In addition, the protein-ligand complex formed displayed remarkable stability proposing that Azairachtin can be a promising inhibitor of the tumor hastening LOX enzyme.

Authors Contribution

Conceptualization: RS, IA
Methodology: MF
Formal analysis: MF
Writing-review and editing: RS, MF, IA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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