UNIVERSITY OF CAPE COAST
COLLEGE OF AGRICULTURE AND NATURAL SCIENCES
SCHOOL OF BIOLOGICAL SCIENCES
DEPARTMENT OF BIOCHEMISTRY

IN-SILICO STUDIES OF 20 BIOACTIVE COMPOUNDS OF *Azadirachta indica* (NEEM) AGAINST PHOSPHOLIPASE A2 AS POTENTIAL TARGET FOR SNAKEBITE TREATMENT

BY
NANCY DIAH
EUGENE KONADU YIADOM
KENNETH KWANSA-AIDOO
MAAME SERWAAH BONSU

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY OF
THE SCHOOL OF BIOLOGICAL SCIENCES, UNIVERSITY OF CAPE COAST, IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A BACHELOR OF SCIENCE (HONOURS) DEGREE IN BIOCHEMISTRY

NOVEMBER, 2022

DECLARATION

We, hereby declare that this dissertation is the result of our own original work and that no part of it has been presented for another degree in this university or elsewhere.

Candidates:

BS/BCH/17/0070

BS/BCH/18/0004

BS/BCH/18/0086

BS/BCH/18/0096

Candidates' Signature: ……

Date: ……...

Supervisor: Prof. Michael Buenor Adinortey

Supervisor's Signature: ……

Date: ……

ABSTRACT

Snakebite envenoming is a neglected tropical disease that claims over 100,000 human lives each year around the world. Although some medicinal plants are used to treat snakebite envenomation, little is known about their pharmacological and biochemical efficacy. In this study, twenty bioactive compounds (ligands) of *Azadirachta indica* (neem) were analysed for their inhibitory effects on Phospholipase A2 of Naja melanoleuca venom. The twenty ligands were docked against Phospholipase A2 protein of the snake venom retrieved from PDB via Pyrx using AutoDock Vina. All of the ligands had binding affinities for Phosphlipase A2, but 12 of them had stronger binding affinities with values ranging from -7.0 to 9.0 kcal/mol. The interactions between the ligands and the protein were assessed using BioVia. Also, the pharmacological activities of the ligands were assessed using SwissAdme. Seven out of the twelve ligands showed drug-likeness, however, some had low GI absorption, showed blood-brain barrier permeability, were P-glycoprotein substrates and inhibited two or more of the Cytochrome P450 isoenzymes. For example, 17-Hydroxyazadiradione, which had the highest binding affinity of -9.0, exhibited drug-likeness; however, it was a P-glycoprotein substrate and inhibited CYP3A4. Ellagic acid, with a binding affinity of -8.7, also showed drug-likeness but only inhibited CYP1A2 of the Cytochrome P450 isoenzymes. It was then concluded that ellagic acid is a potential ligand with inhibitory effects on the snake venom Phospholipase protein. It was recommended that in-vitro and in-vivo experimental studies be conducted to validate the findings made as a result of the computational method used.

ACKNOWLEDGEMENTS

Our heartfelt gratitude goes to our families and loved ones for their unwavering support throughout our four-year degree program. We are extremely grateful to Prof. Michael B. Adinortey for his guidance, criticisms, and corrections. We also acknowledge the assistance of Mr. Sadique Ibrahim Amfoh and express our heartfelt gratitude to him for his help throughout the research.

DEDICATION

To our beloved parents.

TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGES**

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Background

Snakebite envenoming is a neglected tropical disease that claims over 100,000 human lives each year around the world (Gutiérrez et al., 2017). It is a serious medical problem and a health hazard that also contributes to high mortality rates in many parts of the world, especially in tropical countries. In sub-Saharan Africa alone over 30,000 deaths occur each year as a result of the severe morbidity and mortality caused by snakebite envenoming (Chippaux et al., 2011; Mender et al., 2022). In West Africa, many times, snakebite victims must travel many hours to the nearest medical facility, which is frequently underequipped to handle these urgent medical situations (Habib et al., 2015). Many also die within the shortest possible time because of the high toxicity of the venom. During a snake bite, their venom is injected into the human victim either subcutaneously or intramuscularly (Singh, 2010). The snake venom is a complex mixture of proteins and enzymes such Phospholipase A_2 (PLA_2), hyaluronidase, phosphomonoesterase, L-amino acid oxidase, acetylcholinesterase, glycoprotein, low molecular weight polypeptide, metal ions, and these molecules function as

neurotoxins, myotoxins, cardiotoxins and cytotoxins (Soares et al., 2005; Bala et al., 2018).

Snakes of the genus Naja are commonly referred to as cobras. Cobras belong to the Elapidae family and can be found in Africa and Southern Asia (Leong et al.,2012). In West Africa, the black cobra, Naja melanoleuca, is one of the deadliest cobra species. The black forest cobra is about 3m long and can produce venom yields of more than 1g per milking. Phospholipase A2 is one of the venom compositions of the N. melanoleuca. Phospholipase A2 protein is very abundant in Naja melanoleuca venom and catalyses the hydrolysis of glycerophospholipids at the sn-2 position, releasing lysophospholipids and fatty acids (Carvalho et al. ,2013).

Medicinal plants have been used as folk medicine for treatment of snakebite. People living in rural areas as well as tribal areas mainly depend on medicinal plants to treat snakebites. Local healers and traditional medicine practitioners solely rely on medicinal plants because of their safety, effectiveness, availability and affordability. One of such plants used to treat snakebite is the Neem plant. According to Pramely and Leon (2012) each part of the Neem tree possesses some medicinal property. Also, several parts of the Neem tree such as the leaves, bark, fruit, flowers, oil and gum are used to treat certain medical conditions including cancer, hypertension, heart diseases and diabetes (Islas et al., 2020).

With the current advancement in science and modern technology, the identification and modification of specific properties and interactions of medicinal plants is now possible. This has enabled the identification of plant parts

that are effective against certain conditions and diseases. Also, understanding the principles by which bioactive compounds in plants recognize and interact with proteins is of vital significance in pharmaceutical research and drug development (Blaney, 2012). The identification and recognition of plant parts that are effective against certain conditions and diseases involves analysis of molecular recognition events including binding energetics, molecular interactions and induced conformational changes through the use of molecular docking studies, structure-based virtual screening and molecular dynamics (Leonardo et al., 2015). The interaction between the bioactive compounds in the neem plants with the phospholipase green mamba venom through the use of the molecular docking studies, molecular dynamics and the structure-based virtual screening can help predict the antivenom potential of neem plant for further in vitro and in vivo studies.

1.2 Justification

Many plants are traditionally used by local healers and tribes in Ghana to treat various diseases and ailments. Several plants are also known to have snake antivenom properties, but their efficacy is frequently unproven. One such plant is *Azadirachta indica* (neem), which local healers claim has antivenom properties. Although anticancer, antioxidant, and antimicrobial properties of A. indica have been reported in scientific literature, no literature has been reported on its antivenom potential and properties. The goal of this study is to figure out how bioactive compounds in neem plants interact with phospholipase A2.

1.3 Main Aim

To perform in *silico studies* of some selected bioactive compounds of *Azadirachta indica* (Neem) against Phospholipase A2 as potential target for treatment of snakebite.

1.4 Specific Objectives

1. To use the software Pyrx 0.8.0.0 to perform molecular docking
2. To use the online databases SwissADME to carry out pharmacological assessment

CHAPTER TWO

LITERATURE REVIEW

2.1 Naja species

Cobras are the common name for snakes of the genus Naja. They are members of the Elapidae family and are found in Africa and Southern Asia. Different species of cobra have been identified worldwide. These species of cobra are found in variety of habitats and climate zones. The most common Naja species that endanger human health are Naja kaouthia, Naja oxiana, Naja naja, Naja atra, Naja sputatrix, Naja siamensis, Naja philippinesis, Naja sumatrana and Naja melanoleuca (Warrel, 1999; Shabbir et al.,2014; Tan et al., 2019). Snakes from this genus demonstrate similarities with respect to biochemical, toxicological and antigenic properties in terms of their venom composition (Casasola et al.,2009). Venoms of elapids of the genus Naja (cobras), both African and Asian, have been thought to be mostly active at two distinct levels: a neurotoxic level caused primarily by post-synaptic toxins acting at neuromuscular junctions, and a less well-understood cytotoxic level causing tissue damage. Phospholipase A2 (PLA2) is abundant in the venom of Naja species In Africa,

Naja melanoleuca which is the black cobra is one of the deadliest cobra species and in this work, much attention is given to its venom composition and toxicity.

2.2 African forest cobra (Naja melanoleuca)

The Forest Cobra, Naja melanoleuca, is a common forest elapid. This species of cobra which is also referred to as the black cobra, is an extremely venomous member in the elapidae family (Lauridsen et al., 2017). The black forest cobra has a length of about 3m and has the capacity to provide venom yields above 1g per milking (Mirtschin et al., 2006). The distribution of the species is primarily tropical, confined to Africa's tropical, subtropical, and adjacent bushes. According to (Luiselli and Angelici 2000; Shine et al., 2007), N. melanoleuca is the largest of the African cobra species and it is known to live in river areas, primary and secondary forest and suburban habitats in Western, Central, and Southern Africa. The black forest cobra mostly feeds on mammals, frogs and fishes. It has varying colouration and is active during the day.

2.3 Composition and toxicity of the venom of Naja melanoleuca

Due to the severity of envenoming of N. melanoleuca, it becomes relevant to identify and know the composition of the venom so that appropriate antivenom treatment or therapy can be administered during envenomation. Lauridsen et al., (2017) carried out a toxicovenomic investigation of the venom of the forest cobra. The results from their proteome analysis showed the presence of 52 proteins. These proteins mainly consist of the three-finger toxins (3FTx), which include

post-synaptically active α-neurotoxins, and are the most abundant proteins in the venom (57.1 wt%). The second most abundant proteins in the venom are the phospholipase A2 (12.9 wt%), followed by snake venom metalloproteinases (SVMPs) (9.7 wt%), cysteine-rich secretory proteins (CRISPs) (7.6 wt%), and Kunitz-type serine proteinase inhibitors (3.8 wt%). The venom also contains other additional proteins which are less than 3 wt% (Lauridsen et al., 2017).

The toxicity associated with N. melanoleuca envenomation is a progressive descending paralysis that begins with palpebral ptosis and, in severe cases, results in respiratory arrest and death. Victims of N. melanoleuca experience a descending progressive paralysis that begins with ptosis, external ophtalmoplegia, and weakness of cranial nerve innervated muscles, with victims having difficulty swallowing and speaking (Wilkins et al., 2018).

Fig 2.1 *The Central African forest cobra (N. melanoleuca). Image by Jean-Francois Trape.*

2.3.1 Phospholipase A2 (PLA2) of Naja melanoleuca

The enzyme phospholipase A2 (PLA2) is abundant in Naja melanoleuca venom. The molecular weight of the protein ranges from 11,000 Da and 15,000 Da and has amino acids between 119 and 134. PLA2's biochemistry reveals a catalytic site preserved in a tetrad His 48, Asp 49, Tyr 52, and Asp 99, as well as a Ca2+-binding loop containing Tyr 28, Gly 30, Gly 32, and Asp 99. The Asp 49 present in the catalytic site is responsible for the high catalytic activity. PLA2 of N. melanoleuca catalyses the hydrolysis of glycerophospholipids at the sn-2 position, releasing lysophospholipids and fatty acids (Carvalho et al. ,2013). PLA2 can cause neurotoxicity, cytotoxicity, cardiotoxicity, and haematological disorders via the leukotriene pathway and prostaglandins (Trento et al., 2019). It is also involved in the inflammatory process, which is characterised by increased microvascular permeability, edoema formation, leukocyte recruitment in tissues, nociception, and the release of inflammatory mediators, all of which cause a variety of local and systemic inflammatory disorders (Carvalho et al. ,2013).

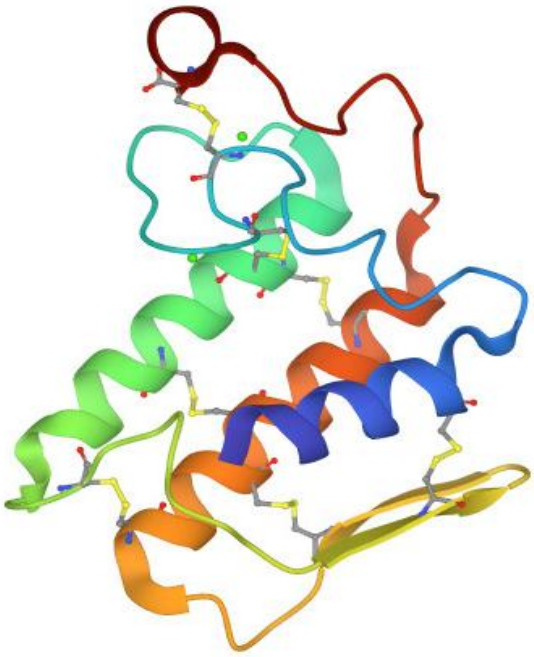


Fig 2.2 Crystal structure of Phospholipase A2 retrieved from PDB

2.4 Azadirachta indica (neem)

The neem plant is a non-leguminous multi-purpose tree which belongs to the Meliaceae family (Adjorlolo et al., 2016). The first discovery of the plant was made about 4,500 years ago in India (Patel et al., 2016). *Azadirachta indica*, the scientific name for neem was taken from the Persian language and literally means "the free tree of India (Kumar and Navaratnam 2013). Various parts of Neem tree, including leaves, flowers, fruits, seeds and bark possess some medicinal property (Drabu and Babu, 2012; Pramely and Leon, 2012). As a result of its immense therapeutic potential, it is also known as "Village pharmacy", "Tree of the 21st century" and "A tree for solving global problems" (Paul et al.,2011)

Currently, neem tree can be found in almost every continent including Asia, Australia, North and South America and Africa. It has, however, been introduced in a number of countries around the world, including 46 African countries (Orwa et al., 2009).

According to Streets (1962) the plant was introduced in Ghana in 1915. Since then, the plant has spread throughout the country. The tree grows in most parts of the country and the dominant tree species are found in Central, Western and Eastern regions (Timpong-Jones 2011). In Ghana here, the neem tree is known to be a drought tolerant plant that thrives in areas with long dry seasons and rainfall as low as 130mm per annum (Gowda and Sastry, 2000). It is also known to grow in any type of soil and tolerate wide range of pH from 4 to 10 (Girish and Shankara, 2008). The tree (Fig 2.3) can grow to a height of about 15-20 metres and live for approximately 200 years (Kumar and Navaratnam 2013). The branches of the tree spread out widely and form an oval crown as the tree develops a deep and strong tap root. The leaves (Fig 2.4) are pinnate and green, but when the plant is young, the leaves turn purple-red. The bark is brown and fissured vertically. The fruit is small, yellow and edible (Tomar et al., 2008).

Fig. 2.3 Neem tree located on University of Cape Coast Campus

Fig. 2.4 Leaves of Neem tree.

2.5 Medicinal Properties of Neem

Neem has therapeutics implication in either treating or preventing diseases. In recent years, neem phytoconstituents have been shown to have a wide range of biological and pharmacological activities. These activities include, but are not limited to, anti-inflammatory, antipyretic, antihistamine, antifungal, antibacterial, anti-ulcer, analgesic, anti-tubercular, anti-malarial, diuretic, spermicide, anti-arthritic, anti-protozoal, insect repellant, anti-feedant, and anti-hormonal properties (Kumar and Navaratnam, 2013; Tiwari et al., 2014). Azadirachta indica extract is an important source

of compounds having anti-cancer, anti-oxidant, anti-inflammatory, anti-malarial, anti-microbial and among others.

2.5.1 Anticancer properties of Neem

The multifaceted nature of cancer makes it a huge global health issue. Changes in molecular and genetic pathways contribute to the onset and spread of cancer. Through the modification of molecular pathways, neem and its phytoconstituents contribute to the prevention of cancers (Alzohairy, 2016). The bioactive compounds isolated from neem plant have been shown unequivocally to induce apoptosis in various types of cells such as tumour cells, as well as to organise and prepare the immune system for take on cancer cells via cross priming (Rajkumar et al., 2011). Neem phytochemicals inhibit cancer cell proliferation and growth, induce cell cycle arrest and apoptosis, disrupt growth

factor signalling, inhibit angiogenesis, and reduce tumour cell invasion and migration (Patel et al., 2016)

2.5.2 Antioxidant properties of Neem

One of the primary causes of the development of many diseases is the free radicals or reactive oxygen species. Free radicals are, however, stabilized by antioxidants before they cause damage to biological cells (Nunes et al., 2012). Neem plants play a role in free radical scavenging activity and has been reported to possess antioxidant properties. The leaves, seeds, bark, and oil of neem are high in antioxidants. For instance, leaf and bark extracts of neem analysed by (Sithisarn et al., 2005) clearly showed that neem possesses antioxidant properties.

2.5.3 Anti-inflammatory properties of Neem

Inflammation is a pathophysiological condition that is involved in a variety of diseases such as cancer and diabetes, as well as other states such as alcohol consumption and food digestion (Islas et al., 2020). An important property found in Neem extracts is their ability to work as anti-inflammatory agents.

2.5.4 Antimalaria properties of Neem

Extracts of neem leaves have been shown to be effective against malarial parasites. Components of the alcoholic extract of leaves have been found to be effective against both chloroquin-resistant and chloroquin-sensitive strains of the malaria parasite (Subapriya et al., 2005; Udeinya et al., 2008)

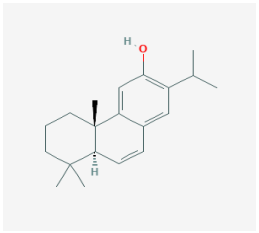
2.5.5 Antimicrobial properties of Neem

The oil extracted from neem leaves has been shown to have antibacterial activity against a variety of Gram-negative and Gram-positive microorganisms, including M.tuberculosis and Streptomycin-resistant strains (Asif, 2012). In vitro, neem leaf extract has been shown to inhibit Vibrio cholerae, Klebsiella pneumoniae, Mycobacterium tuberculosis, and Mycobacterium pyogenes (Vashist and Jindal, 2012).

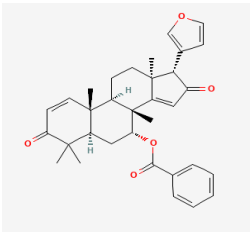
2.6 Bioactive compounds of Neem

Azadirachta indica is rich in a wide range of compounds, of which several have pharmacological potential. According to (Subapriya and Nagini, 2005) neem contains more than 140 bioactive compounds. These bioactive compounds are classified into two groups namely isoprenoids and nonisoprenoids. Diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type compounds, and C-secomeliacins such as nimbin, salanin, and azadirachtin are examples of isoprenoids. Nonisoprenoids include proteins or amino acids, carbohydrates (polysaccharides), sulphur compounds, polyphenolics like flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, and so on. Triterpenes are the most commonly used therapeutic compounds among all of these compounds (Pramely and Raj, 2012).

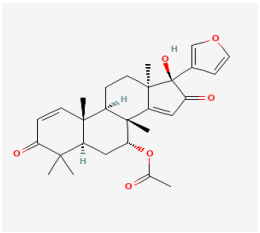
The leaves are known to contain compounds such as nimbin, nimbanene, 6adesacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n hexacosanol and various amino acids, and nimbiol, among others. Nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and amino acid, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione, and nimbiol are all found in the leaves (Kumari et al., 2020).



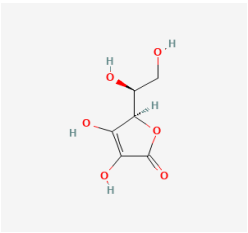
6,7-Dehydroferruginol acid



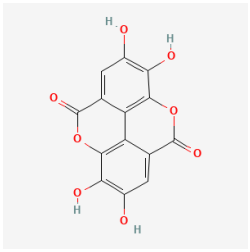
7-Benzoylnimbocinol



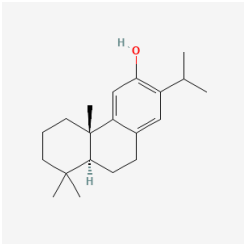
17-Hydroxyazadiradione



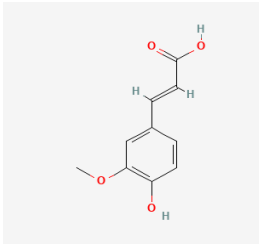
Ascorbic



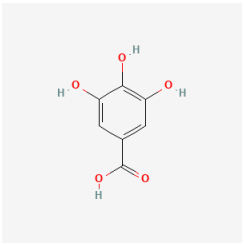
Ellagic acid



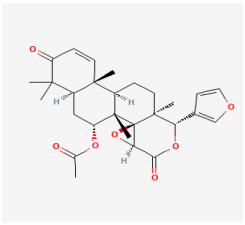
Ferruginol



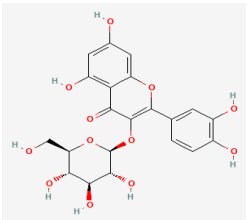
Ferulic acid



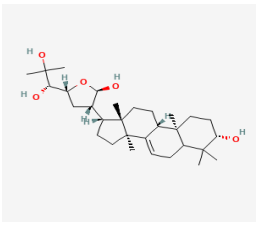
Gallic acid



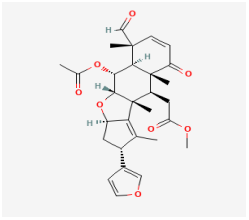
Gedunin



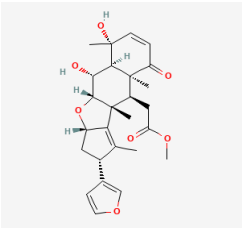
Isoquercitrin



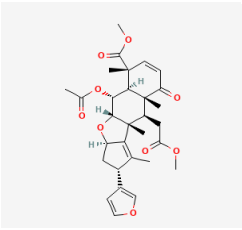
Meliantriol



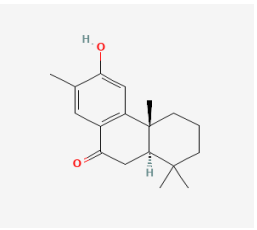
Nimbanal



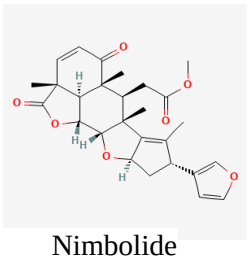
Nimbandiol



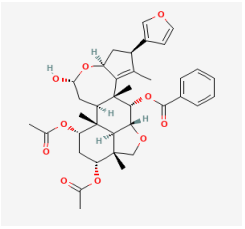
Nimbin



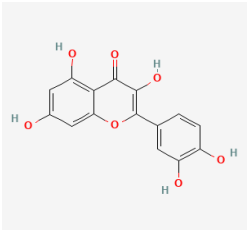
Nimbiol



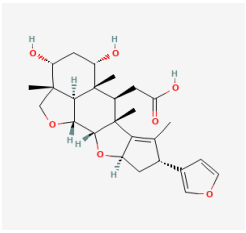
Nimbolide



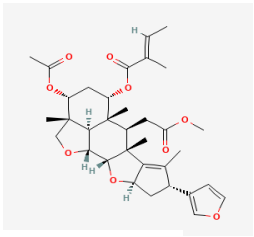
Nimbolinin



Quercetin



Salannic acid



Salannin

Fig. 2.5 Some selected structural compounds found in Neem

CHAPTER THREE

MATERIALS AND METHODS

3.1. Retrieval of the 3D Structure of Phospholipase A2 from PDB

The 3D crystal structure of Naja atra phospholipase A2 with PDB ID 1POA was retrieved from the Protein Data Bank (PDB). The resolutions of the structures in the PDB files served as the criterion in selecting the protein. The quality of a crystal structure was evaluated based on its resolution, as better resolution has a lower value of angstrom and provides a more accurate data on the location of atoms in the protein structures. The resolution of the phospholipase selected had X-RAY DIFFRACTION 1.5 Å

3.2 Collection of Bioactive Compounds (Ligands)

SDF files in the 3D conformer of 20 biactive compounds; 6,7-Dehydroferruginol, 7-Benzoylnimbocinol, 17-Hydroxyazadiradione, Ascorbic acid, Ellagic acid, Ferruginol, Ferulic acid, Gallic acid, Gedunin, Isoquercitrin, Meliantriol, Nimbanal, Nimbandiol, Nimbin, Nimbiol, Nimbolide Nimbolinin, Quercetin, Salannic acid, Salannin were downloaded from Pubchem Database and saved.

3.3 Protein Preparation by BIOVIA Discovery Studio.

The downloaded phospholipase protein from PDB database was prepared by deleting water and other molecules which were not needed in BIOVIA software. The protein was then saved in pdb format.

3.4 Molecular docking

PyRx 0.8.0.0 was used to assess all of the bioactive compounds (ligands) for molecular docking using AutoDock Vina. During the preparation, all ligand SDF files were converted to PDB format as accepted by AutoDock Vina, and the phospholipase A2 molecular structure was minimised using the universal force field and then converted to PDB format. The 20 bioactive compounds and the prepared phospholipase protein were uploaded in PyRx 0.8.0.0 and run for molecular docking.

3.5 Pharmacological assessment

SwissADME was used to assess and to determine the pharmacokinetics and the drug-likeness of each of the ligands. This was carried out by downloading the Canonical SMILE format of the ligands from PubChem. The SwissADME was then used to run the SMILE format to determine their pharmacological and drug-likeness.

CHAPTER FOUR

RESULTS

Ribbon representation of Phospholipase A2 from PDB after it had been prepared and viewed using BioVia.

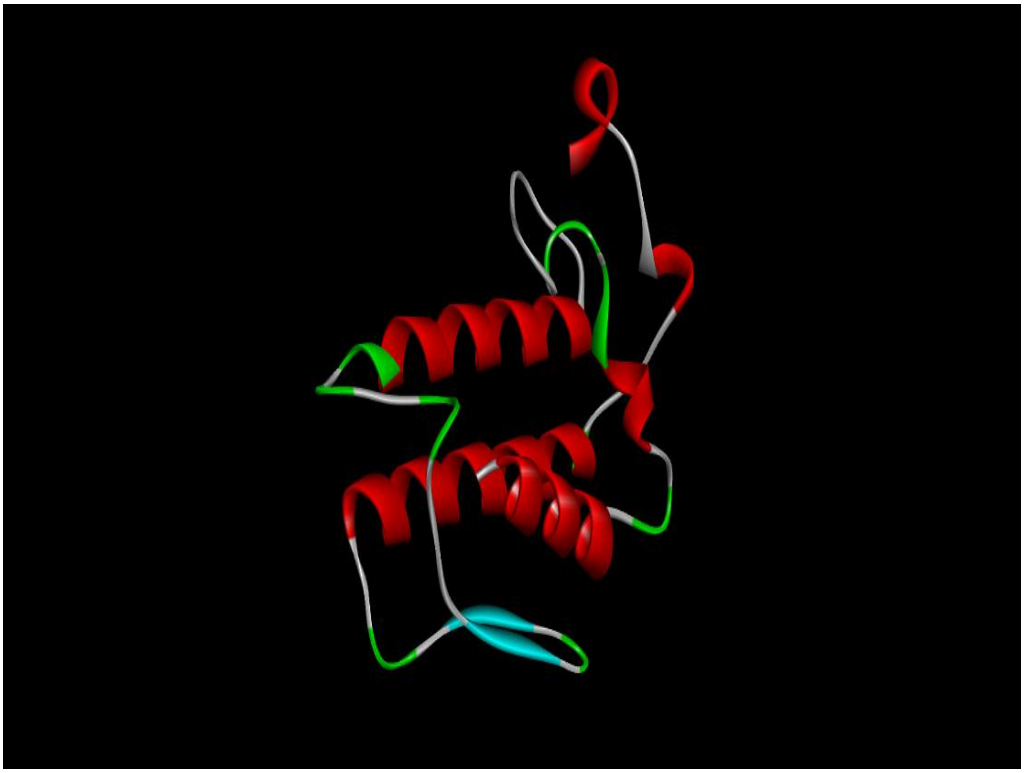


Fig 4.1: Ribbon representation of Phospholipase A2 after preparation.

Table 1 discusses binding affinities of ligands of Azadirachta indica and the phospholipase A2 indicating their respective binding affinities from -7.0, retrieved from PyRx 0.8.0.0

Table 1: Docking results of Phospholipase A2 and ligands with binding affinities ranging from -7.0 to 9.0

Ligand	Binding Affinity
6,7-dehydroferruginol	-8.6
7-desacetyl-7-benzoylazadiradione	-9
17-Hydroxyazadiradione	-9
Ellagic acid	-8.7
Ferruginol	-8.1
Gedunin	-8.6
Isoquercitrin	-8.1
Meliantriol	-7.9
Nimbin	-7.3
Nimbiol	-8.6
Nimbolide	-7.6
Quercetin	-8

3D visualization of the interaction of 7-desacetyl-7-benzoylazadiradione with the highest binding affinity (-9.0) at the binding site of Phospholipase A2 using BioVia.

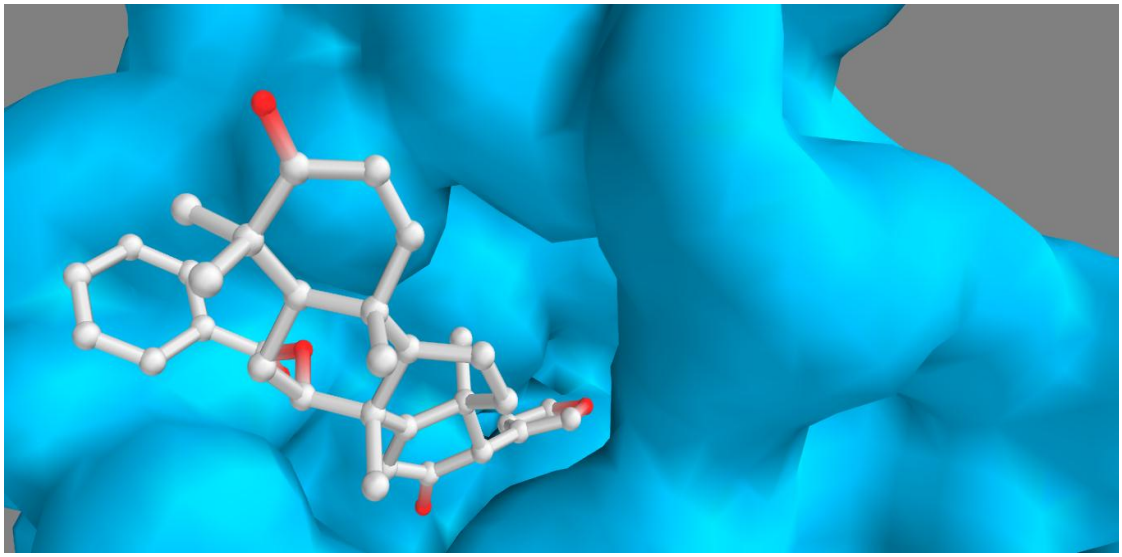


Fig 4.2: 3D representation of 7-desacetyl-7-benzoylazadiradione at the binding site of Phospholipase A2

2D visualization of the interaction of 7-desacetyl-7-benzoylazadiradione with the highest binding affinity (-9.0) at the binding site of Phospholipase A2 using BioVia.

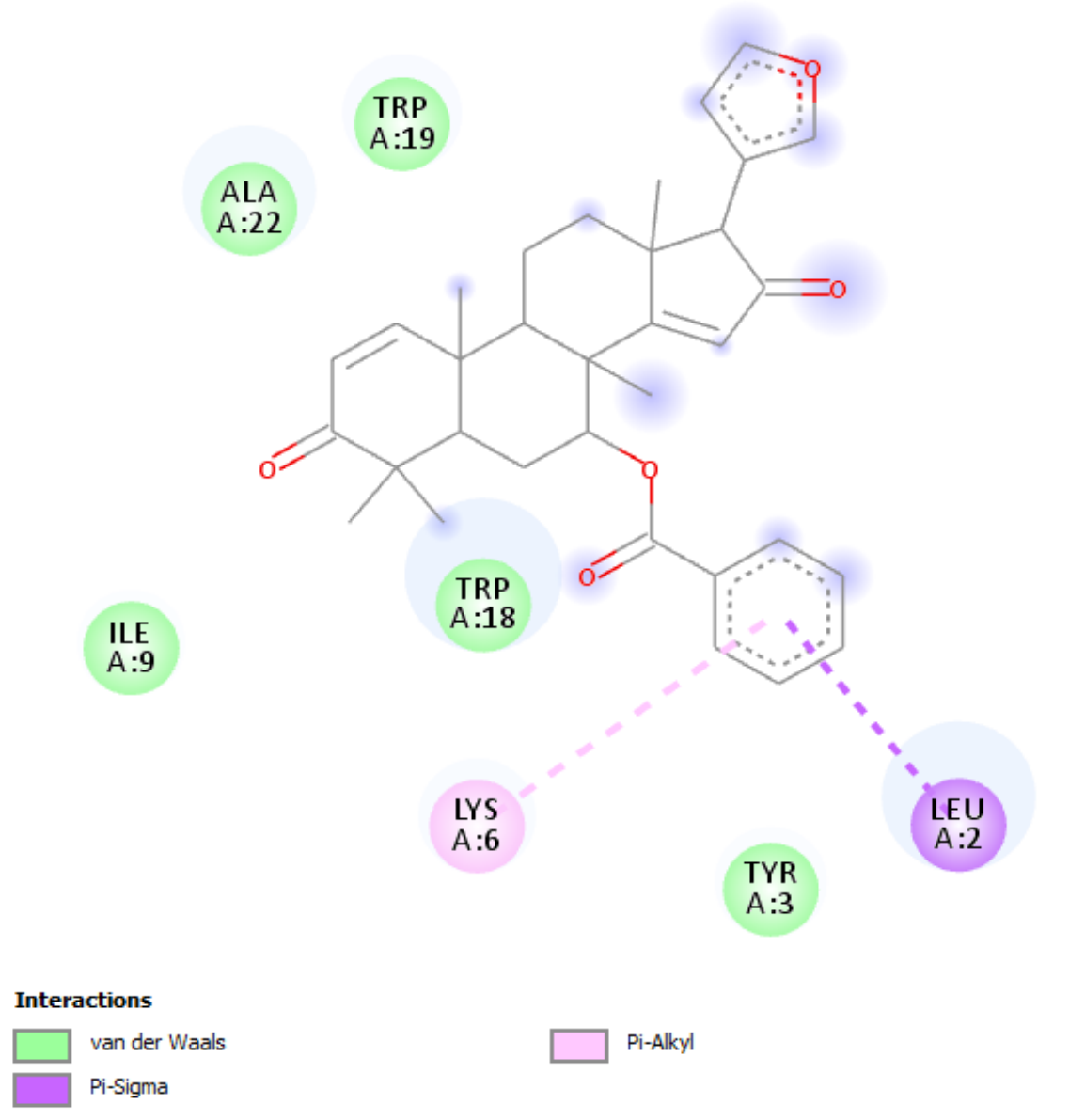


Fig 4.3: 2D representation of interaction of 7-desacetyl-7-benzoylazadiradione at the binding site of Phospholipase A2.

3D visualization of the interaction of 17-Hydroxyazadiradione with the highest binding affinity (-9.0) at the binding site of Phospholipase A2 using BioVia

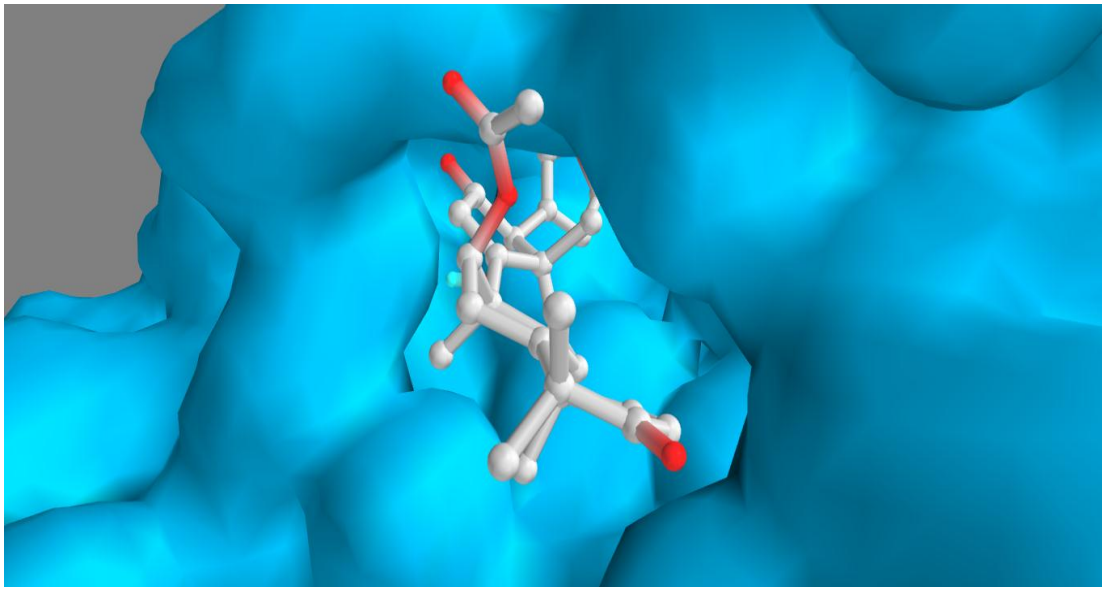


Fig 4.4: 3D representation of interaction of 17-Hydroxyazadiradione at the binding site of Phospholipase A2.

2D visualization of the interaction of 17-Hydroxyazadiradione with the highest binding affinity (-9.0) at the binding site of Phospholipase A2 using BioVia.

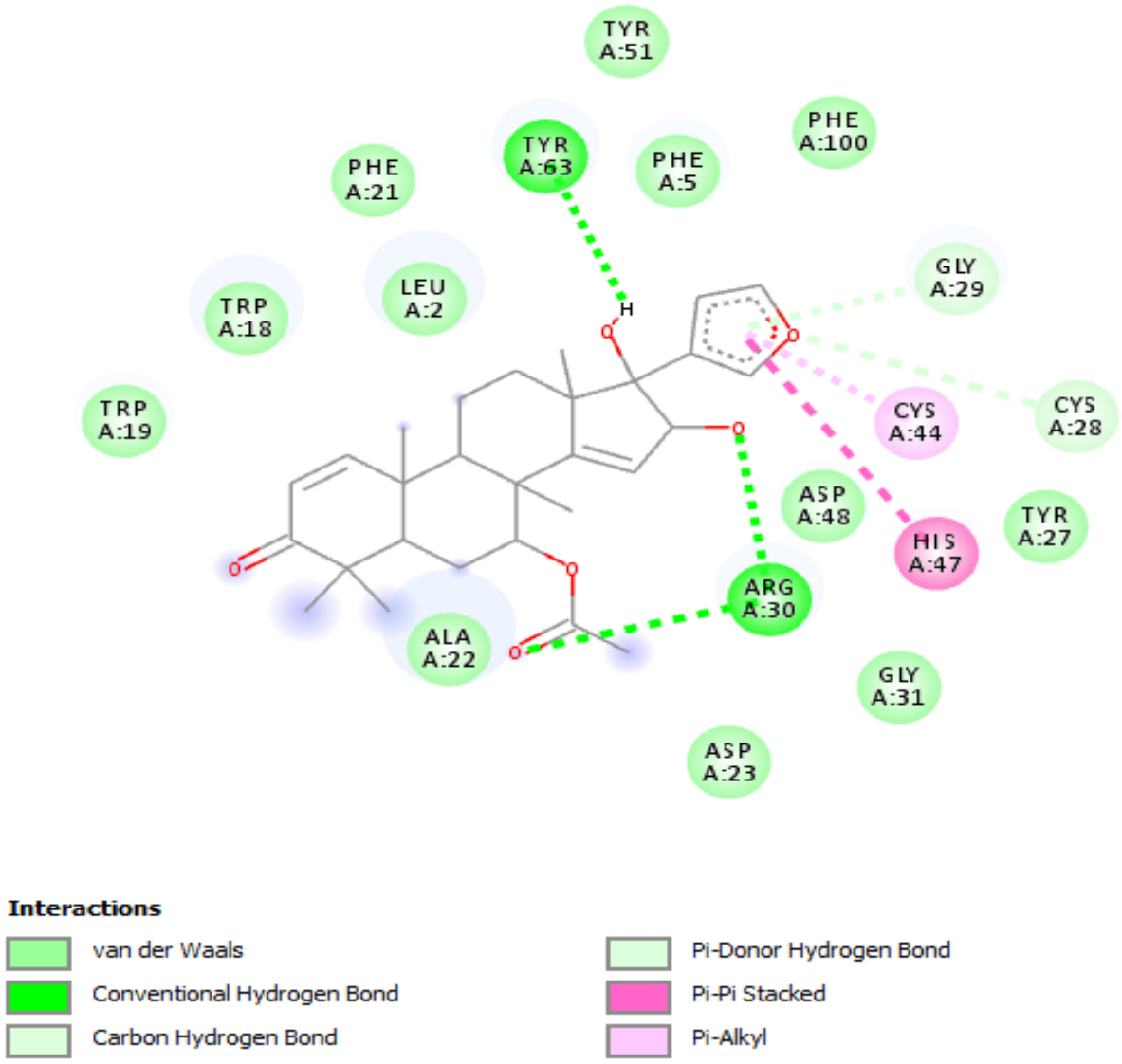


Fig 4.5: 2D representation of interaction of 17-Hydroxyazadiradione at the binding site of Phospholipase A2

Table 2 discusses the pharmacological evaluation of the ligands using the software SwissADME. Pharmacokinetic properties are assessed based on gastrointestinal (GI) absorption blood-brain barrier permeability and P-glycoprotein

Table 2: Pharmacokinetics of ligands from Azadirachta indica using SwissADME

Ligands	GI absorption	BBB permeant	Pgp substrate
6,7-dehydroferruginol	High	Yes	No
7-desacetyl-7-benzoylazadiradione	Low	No	No
17-Hydroxyazadiradione	High	No	Yes
Ellagic acid	High	No	No
Ferruginol	High	Yes	No
Gedunin	High	No	Yes
Isoquercitrin	Low	No	No
Meliantriol	High	No	Yes
Nimbin	High	No	No
Nimbiol	High	Yes	No
Nimbolide	High	No	Yes
Quercetin	High	No	No

Table 3 discusses the pharmacological evaluation of the ligands using the software SwissADME. Pharmacokinetic properties are assessed based on the Cytochrome P450 isoenzymes.

Table 3: Phamarcokinetics of ligands from Azadirachta indica using SwissADME

Ligands	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
6,7-dehydroferruginol	No	Yes	Yes	No	No
7-desacetyl-7-benzoylazadiradione	No	No	Yes	No	Yes
17-Hydroxyazadiradione	No	No	No	No	Yes
Ellagic acid	Yes	No	No	No	No
Ferruginol	No	Yes	Yes	Yes	No
Gedunin	No	No	No	No	No
Isoquercitrin	No	No	No	No	No
Meliantriol	No	No	No	No	No
Nimbin	No	No	No	No	No
Nimbiol	No	Yes	Yes	Yes	No
Nimbolide	No	No	No	No	No
Quercetin	Yes	No	No	Yes	Yes

Table 4 highlights the drug-likeness of the ligands using SwissADME. The drug-likeness are assessed based on the Lipinski rule. Based on the rules 7 out of the 12 ligands showed drug-likeness.

Table 4: Drug-likeness of ligands of Azadirachta indica using SwissADME

Ligands	Lipinski (Druglikeness)	#violations	Bioavailability Score
6,7-dehydroferruginol	No	1 Violation(s)	0.55
7-desacetyl-7-benzoylazadiradione	No	2 Violation(s)	0.17
17-Hydroxyazadiradione	Yes	0 Violation(s)	0.55
Ellagic acid	Yes	0 Violation(s)	0.55
Ferruginol	No	1 Violation(s)	0.55
Gedunin	Yes	0 Violation(s)	0.55
Isoquercitrin	No	2 Violation(s)	0.17
Meliantriol	Yes	0 Violation(s)	0.55
Nimbin	No	1 Violation(s)	0.55
Nimbiol	Yes	0 Violation(s)	0.55
Nimbolide	Yes	0 Violation(s)	0.55
Quercetin	Yes	0 Violation(s)	0.55

CHAPTER FIVE

DISCUSSION

Naja melanoleuca envenomation is a tropical disease with alarming clinical manifestations at high occurrence rates in Ghana. However, several plants are known to inhibit the venom activities of Naja melanoleuca of which Azadirachta indica plays a prominent role. Out of 20 bioactive compounds present in Azadirachta indica, 12 bioactive compounds inhibited phospholipase A2 protein in the venom of Naja melanoleuca. These compounds are 6, 7-dehydroferruginol, 7-desacetyl-7-benzoylazadiradione, 17-Hydroxyazadiradione, Elagic acid, Ferruginol, Gedunin, Isoquercitrin, Meliantriol, Nimbin, Nimbiol, Nimbolide and Quercetin. These compounds were identified based on results from protein-ligand interactions by virtual molecular screening in PyRx 0.8.0.0. This screening was performed to identify the ligands having the desired function to inhibit phospholipase A2 in the venom of Naja melanoleuca. Values of the binding affinities, which indicated the strength of the binding interactions between the protein and the various ligands, aided in the identification of these ligands as potent drug molecules from Azadirachta indica that can bind and inhibit the protein. Binding affinities ranging from -7.0 to -9.0 implied stronger protein-

ligand interactions (Table 1). 7-desacetyl-7-benzoylazadiradione and 17-Hydroxyazadiradione showed the strongest binding affinity of -9.0 to phospholipase A2.

The amino acids of Phospholipase A2 which comprises Lys 6, Leu 2, Tyr 3, Trp 18, Ile 9, Ala 22 and Trp 19 which reacted with the ligand, 7-desacetyl-7-benzoylazadiradione. Leu 2 interacted with the ligand through C-H-π interaction. The C-H-π interaction is a non-covalent interaction between C-H group of Leu2 and the face of an electron-rich π system of the aromatic ring of the ligand, 7-desacetyl-7-benzoylazadiradione. The alkyl group of Lys 6 interacted with the pi-electron cloud over the aromatic group of the ligand, 7-desacetyl-7-benzoylazadiradione through pi-alkyl interaction. Tyr 3, Trp 18, Ile 9, Ala 22 and Trp 19 interacted with the ligand, 7-desacetyl-7-benzoylazadiradione through weak van der Waals interactions. Also, the amino acids of Phospholipase A2 which comprises Phe 5, Phe 100, Tyr 51, Phe 21, Leu 2, Trp 18, Trp 19, Ala 22, Asp 23, Gly 31, Asp 48, Tyr 27, Tyr 63, Arg 30, Cys 28, Gly 29, His 47 and Cys 44 reacted with the ligand, 17-Hydroxyazadiradione. Phe 5, Phe 100, Tyr 51, Phe 21, Leu 2, Trp 18, Trp 19, Ala 22, Asp 23, Gly 31, Asp 48 and Tyr 27 interacted with the ligand, 17-Hydroxyazadiradione through van der Waals interactions. Tyr 63 Arg 30 interacted with the ligand, 17-Hydroxyazadiradione through conventional hydrogen bond. Cys 28 interacted with the ligand, 17-Hydroxyazadiradione through Carbon-Hydrogen bond. Gly 29 interacted with the ligand, 17-Hydroxyazadiradione through pi-donor hydrogen bond. His 47 interacted with the ligand, 17-Hydroxyazadiradione through pi-pi stacked bond.

Cys 44 interacted with the ligand, 17-Hydroxyazadiradione through pi-alkyl bond.

A potent drug molecule must be able to get to its target site at a concentration that is enough to illicit its therapeutic effect before it can be accepted as a drug. It must also be bioavailable at the target site for an expected period of time. In this in silico analysis, the SwissADME aided in the prediction of physicochemical properties, pharmacokinetics and drug-likeness, which explained the absorption, distribution, metabolism and excretion of the potent molecule or drug (Daina et al., 2017). Under pharmacokinetics, for a drug to be orally absorbed in the gastrointestinal (GI) tract, it must be able to withstand certain physiological conditions such as low pH and the degrading activities of some metabolizing enzymes present in the gastrointestinal tract **(**Jennifer Le, 2022). The ligands that showed binding affinities ranging from -7.0 to -9.0 in the molecular docking showed high absorption in the gastrointestinal tract with the exception of 7-desacetyl-7-benzoylazadiradione and isoquecitrin (Table 2). This implies that about 83% of the 12 ligands will be absorbed in the gastrointestinal tract when the drug (Azadirachta indica) is administered orally. Analysis of the capacity of the ligands to cross the blood-brain barrier was also done. Considering the blood-brain barrier (BBB), it is a cellular phospholipid protein bilayer barrier of the brain, which is highly permeable and selective. It separates the circulating blood from the extracellular fluid of the brain in the Central Nervous system (Fong, 2016). From the results of the SwissADME, 6, 7-dehydroferruginol, ferruginol and nimbiol were permeable to the blood-brain barrier. These exceptions may

have the possibilities of interfering with the way neurons send, receive and process signals through neurotransmitters, therefore resulting in brain toxicity. The ability of the ligands to act as substrate for P-glycoprotein was also evaluated. The P-glycoprotein is widely distributed in intestinal epithelium which transports xenobiotic back to intestinal lumen and from capillary endothelial cells of the brain back into the capillaries (Ranjith and Ravikumar, 2019). Gedunin, meliantriol, 17-hydroxyazadiradione and nimbolide are very good substrates (Table 2), hence will not inhibit the p-glycoprotein. They can be bound and transported by this protein. The biotransformation of the ligands by Ctyochrome P450 was also assessed. CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 (Table 3) are isoenzymes of Cytochrome P450 that metabolize more than 50-90% of xenobiotics in the liver. Inhibition of these metabolizing enzymes is an indication that a particular drug or ligand will remain in the liver for a longer period of time and cause toxicity. Gedunin, isoquercitrin, meliantriol, nimbolide and nimbin are not inhibitors of these cytochrome P450 isoenzymes. However, 7-desacetyl-7-benzoylazadiradione which showed the highest binding affinity of -9.0 inhibited CYP2C9 and CYP3A4. 17-hydroxyazadiradione which also showed the highest binding affinity of -9.0 inhibited CYP3A4. Also, ellagic acid inhibited CYP1A2. The rest of the ligands inhibited either two or three of the isoenzymes.

Drug-likeness examines the probability for a molecule to become an oral drug in terms of bioavailability (Ranjith and Ravikumar, 2019). 17-Hydroxyazadiradione, Ellagic acid, gedunin, meliantriol, nimbiol, nimbolide and quercetin showed drug likeness. However, these ligands; 6,7-dehydroferruginol,

7-desacetyl-7-benzoylazadiradione, Ferruginol, Isoquercitrin, Nimbin did not show drug likeness because they had either 1 or 2 violations.

To further this research, analysis was done based on the results from SwissADME to identify the ligands that would effectively inhibit the Phospholipase A2. 7-desacetyl-7-benzoylazadiradione,17-hydroxyazadiradione, elagic acid, nimbin, and quercitin were considered based on the pharmacokinetics, physicochemical and druglikeness properties of theses ligands from the SwissADME results. 7-desacetyl-7-benzoylazadiradione had low GI absorption rate, inhibited p-glycoprotein, CYP2C9, CYP3A4 and had two violations as a drug. 17-Hydroxyazadiradione had high GI absorption rate, a good substrate to p-glycoprotein, inhibited CYP 3A4 and had no violations as a drug. Elagic acid inhibited CYP1A2 and had no violations as a drug. Nimbin had one violation as a drug. Quercetin inhibited CYP1A2, CYP2D6, CYP3A4, and had no violation.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATION

6.1 CONCLUSION

Twenty bioactive compounds of *Azadirachta indica* were used for the molecular docking. Out of the 20 bioactive compounds used, 12 were potent inhibitors of the Phospholipase protein of the snake venom. It be concluded that compounds present in *Azadirachta indica* (neem) have the potential of inhibiting Phospholipase A2 of the snake venom.

To narrow the research, analysis was done using SwissADME to determine the absorption, distribution, metabolism and excretion of the ligands. 17-Hydroxyazadiradione having the highest binding affinity of -9.0 showed drug-likeness, however, it was a substrate of P-glycoprotein and inhibited CYP3A4. Ellagic acid having a binding affinity of -8.7 also showed drug-likeness but would only inhibit CYP1A2 of the Cytochrome P450 isoenzymes. It can be concluded that ellagic acid is a potential ligand that may have inhibitory effects on the Phospholipase protein in the snake venom.

6.2 RECOMMENDATION

It is important to clarify that the findings of this work cannot conclude on the inhibitory of the bioactive compounds on Phospholipase A2 of snake venom since the work was computational and only gave a prediction. It is therefore, suggested that experimental studies, both in vitro and in-vivo be conducted to validate the findings made.

REFERENCES

Adjorlolo, L. K., Timpong-Jones, E. C., Boadu, S., & Adogla-Bessa, T. (2016). Potential contribution of neem (Azadirachta indica) leaves to dry season feeding of ruminants in West Africa. *Development*, *28*(5).

Alzohairy, M. A. (2016). Therapeutics role of Azadirachta indica (Neem) and their active constituents in diseases prevention and treatment. *Evidence-Based Complementary and Alternative Medicine*, *2016*.

Asif, M. (2012). Antimicrobial potential of Azadirachta indica against pathogenic bacteria and fungi. *Journal of Pharmacognosy and phytochemistry*, *1*(4), 78-83.

Bala, E., Hazarika, R., Singh, P., Yasir, M., & Shrivastava, R. (2018). A biological overview of Hyaluronidase: A venom enzyme and its inhibition with plants materials. *Materials today: proceedings*, *5*(2), 6406-6412.

Blaney, J. (2012). A very short history of structure-based design: how did we get here and where do we need to go?. *Journal of computer-aided molecular design*, *26*(1), 13-14.

Carvalho, B. M. A., Santos, J. D. L., Xavier, B. M., Almeida, J. R., Resende, L. M., Martins, W., ... & Marchi-Salvador, D. P. (2013). Snake venom PLA2s inhibitors isolated from Brazilian plants: synthetic and natural molecules. *BioMed Research International*, *2013*.

Chaube, S. K., Shrivastav, T. G., Tiwari, M., Prasad, S., Tripathi, A., & Pandey, A. K. (2014). Neem (Azadirachta indica L.) leaf extract deteriorates

oocyte quality by inducing ROS-mediated apoptosis in mammals. *SpringerPlus*, *3*(1), 1-5.

Casasola, A., Ramos-Cerrillo, B., de Roodt, A. R., Saucedo, A. C., Chippaux, J. P., Alagón, A., & Stock, R. P. (2009). Paraspecific neutralization of the venom of African species of cobra by an equine antiserum against Naja melanoleuca: a comparative study. *Toxicon*, *53*(6), 602-608.

Chippaux, J. P., Diouf, A., Stock, R. P., Parra, H. J., & Massougbodji, A. (2011). Report of the 4th international conference on envenomations by snakebites and scorpion stings in Africa, Dakar, April 25–29, 2011. *Toxicon*, *58*(5), 426-429.

Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific reports*, *7*(1), 1-13.

Drabu, S., Khatri, S., & Babu, S. (2012). Neem: Healer of all ailments. *Res. J. Pharm. Biol. Chem. Sci*, *3*(1), 120-126.

Eldeen, I. M. S., Mohamad, H., Tan, W., Siong, J. Y. F., Andriani, Y., & Tengku Muhammad, T. S. (2016). Cyclooxygenase, 5-lipoxygenase and acetylcholinesterase inhibitory effects of fractions containing, α-guaiene and oil isolated from the root of xylocarpus moluccensis. Research Journal of Medicinal Plants, 10(4), 286–295

Fong, C. W. (2015). Permeability of the blood–brain barrier: molecular mechanism of transport of drugs and physiologically important compounds. *The Journal of membrane biology*, *248*(4), 651-669.

Ferreira, L. G., Dos Santos, R. N., Oliva, G., & Andricopulo, A. D. (2015). Molecular docking and structure-based drug design strategies. *Molecules*, *20*(7), 13384-13421.

Girish, K., & Shankara, B. S. (2008). Neem–a green treasure. *Electronic journal of Biology*, *4*(3), 102-111.

Tomar, A., Singh, K. K., Phogat, S., & Dhillon, R. S. (2008). Neem: An Introduction. In *Neem: A Treatise* (p. 546). IK International Publishing House Pvt. Ltd.

Gilles, H., & Warrel, D. (1999). Bruce Chwatts Essential malariology. Terceraedición

Gowda, S. K., & Sastry, V. R. B. (2000). Neem (Azadirachta indica) seed cake in animal feeding-Scope and Limitations-Review. *Asian-Australasian Journal of Animal Sciences*, *13*(5), 720-728.

Gutiérrez, J. M., Calvete, J. J., Habib, A. G., Harrison, R. A., Williams, D. J., & Warrell, D. A. (2017). Snakebite envenoming. *Nature reviews Disease primers*, *3*(1), 1-21.

Habib, A. G., Kuznik, A., Hamza, M., Abdullahi, M. I., Chedi, B. A., Chippaux, J. P., & Warrell, D. A. (2015). Snakebite is under appreciated: appraisal of burden from West Africa. *PLoS neglected tropical diseases*, *9*(9), e0004088.

Kumar, V. S., & Navaratnam, V. (2013). Neem (Azadirachta indica): prehistory to contemporary medicinal uses to humankind. *Asian Pacific journal of tropical biomedicine*, *3*(7), 505-514.

Kumari, P., Geat, N., Maurya, S., & Meena, S. (2020). Neem: Role in leaf spot disease management: A review. *Journal of Pharmacognosy and Phytochemistry*, *9*(1), 1995-2000.

Lauridsen, L. P., Laustsen, A. H., Lomonte, B., & Gutiérrez, J. M. (2017). Exploring the venom of the forest cobra snake: Toxicovenomics and antivenom profiling of Naja melanoleuca. *Journal of proteomics*, *150*, 98-108.

Luiselli, L., & Angelici, F. M. (2000). Ecological relationships in two Afrotropical cobra species (Naja melanoleuca and Naja nigricollis). *Canadian Journal of Zoology*, *78*(2), 191-198.

Mirtschin, P. J., Dunstan, N., Hough, B., Hamilton, E., Klein, S., Lucas, J., ... & Nias, T. (2006). Venom yields from Australian and some other species of snakes. *Ecotoxicology*, *15*(6), 531-538.

Nunes,P.X. Silva, S.F. Guedes,R.J. and Almeida, S. (2012). Biological oxidations and antioxidant activity of natural products. in Phytochemicals as Nutraceuticals—Global Approaches to Their Role in Nutrition and Health, In Tech.

Orwa C, Mutua A, Kindt R, Jamnadass R and Simons A 2009: Agroforestree Database: a tree reference and selection guide. Version 4.0. (http://www.worldagroforestry.org/af/treedb/).

Patel, S. M., Venkata, K. C. N., Bhattacharyya, P., Sethi, G., & Bishayee, A. (2016, October). Potential of neem (Azadirachta indica L.) for prevention

and treatment of oncologic diseases. In *Seminars in cancer biology* (Vol. 40, pp. 100-115). Academic Press.

Paul, R., Prasad, M., & Sah, N. K. (2011). Anticancer biology of Azadirachta indica L (neem): a mini review. *Cancer biology & therapy*, *12*(6), 467-476.

Pramely, R., & Raj, L. S. (2012). Prediction of biological activity spectra of a few phytoconstituents of Azadirachta indicia A. Juss. *Journal of Biochemical Technology*, *3*(4), 375-379.

Ranjith, D., & Ravikumar, C. (2019). SwissADME predictions of pharmacokinetics and drug-likeness properties of small molecules present in Ipomoea mauritiana Jacq. *Journal of Pharmacognosy and Phytochemistry*, *8*(5), 2063-2073.

Shabbir, A., Shahzad, M., Masci, P., & Gobe, G. C. (2014). Protective activity of medicinal plants and their isolated compounds against the toxic effects from the venom of Naja (cobra) species. *Journal of ethnopharmacology*, *157*, 222-227

Shine, R., Branch, W. R., Webb, J. K., Harlow, P. S., Shine, T., & Keogh, J. S. (2007). Ecology of cobras from southern Africa. *Journal of Zoology*, *272*(2), 183-193.

Singh, J. (2010). *Antivenom activity of albizia lebbeck bark extracts against naja kaouthia snake venom* (Doctoral dissertation, RGUHS).

Sithisarn, P., Supabphol, R., & Gritsanapan, W. (2005). Antioxidant activity of Siamese neem tree (VP1209). *Journal of Ethnopharmacology*, *99*(1), 109-112.

Soares, A. M., Ticli, F. K., Marcussi, S., Lourenco, M. V., Januario, A. H., Sampaio, S. V., & Pereira, P. S. (2005). Medicinal plants with inhibitory properties against snake venoms. *Current Medicinal Chemistry*, *12*(22), 2625-2641.

Streets, R. J. (1962). Exotic forest trees in the British Common- wealth. Clarendon Press, Oxford. 765

Subapriya, R., & Nagini, S. (2005). Medicinal properties of neem leaves: a review. *Current Medicinal Chemistry-Anti-Cancer Agents*, *5*(2), 149-156.

Tan, C. H., Wong, K. Y., Tan, N. H., Ng, T. S., & Tan, K. Y. (2019). Distinctive distribution of secretory phospholipases A2 in the venoms of Afro-Asian cobras (Subgenus: Naja, Afronaja, Boulengerina and Uraeus). *Toxins*, *11*(2), 116.

Timpong-Jones E C 2011: Estimation and management of forage production in the Coastal Savannah Plains of Ghana. PhD. Thesis. Department of Animal Science, School of Agriculture, University of Ghana, Legon

Trento, M. V. C., Sales, T. A., de Abreu, T. S., Braga, M. A., Cesar, P. H. S., Marques, T. R., & Marcussi, S. (2019). Exploring the structural and functional aspects of the phospholipase A2 from Naja spp. *International journal of biological macromolecules*, *140*, 49-58.

Udeinya, J. I., Shu, E. N., Quakyi, I., & Ajayi, F. O. (2008). An antimalarial neem leaf extract has both schizonticidal and gametocytocidal activities. *American journal of therapeutics*, *15*(2), 108-110.

Vashist, H., & Jindal, A. (2012). Antimicrobial activities of medicinal plants–Review. *Int J Res Pharm Biomed Sci*, *3*(1), 222-30.

Wilkins, D., Burns, D. S., Wilson, D., Warrell, D. A., & Lamb, L. E. M. (2018). Snakebites in Africa and Europe: a military perspective and update for contemporary operations. *BMJ Military Health*, *164*(5), 370-379.

Wüster, W., Chirio, L., Trape, J. F., Ineich, I., Jackson, K., Greenbaum, E., … & Hall, C. (2018). Integration of nuclear and mitochondrial gene sequences and morphology reveals unexpected diversity in the forest cobra (Naja melanoleuca) species complex in Central and West Africa (Serpentes: Elapidae). *Zootaxa*, *4455*(1), 68-98