UNIVERSITY OF NAIROBI

MODELING AND SYNTHESIS OF ANTIPLASMODIAL NAPHTHOQUINONES FROM NATURAL PRODUCTS OF KENYA

BY
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I56/79108/2012

A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science (Environmental Chemistry) of University of Nairobi.

2016

DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

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DEDICATION

I dedicate this work to my beloved and caring parents, my siblings especially my brother Arthur Oyim, my fiancée Joy Otuya and all who have given me ample time to successfully complete my project work.

ACKNOWLEDGEMENT

I would like to appreciate the guidance of my supervisors Dr Albert Ndakala and Dr Solomon Derese. I would also like to acknowledge the Grand Challenges Canada for the research grant (S4 0260-01) which facilitated the purchase of equipment and chemicals used in the project. Many thanks to the Novartis Institute for Biomedical Research for their donation of work stations and other equipment used in this research.

I would like acknowledge Dr. Hoseah M. Akala of United States Army Medical Research Unit at Kenya Medical Research Institute (KEMRI) for running the antiplasmodial assays and Dr. Mathias Heydenrich of the University of Postdam, Germany for NMR spectroscopic analysis. Not forgetting the Open Eye Scientific software for their academic license which we used extensively in docking studies. Lastly, I would like to acknowledge the University of Nairobi and the Department of Chemistry, in particular, for the opportunity and support throughout my study.

ABSTRACT

While there have been many approved drugs for treatment and prevention of malaria, drug resistance has compromised the efficacy of some of them necessitating the development of new antimalarial drugs. Using computer aided drug discovery (CADD), this study exploited a newly validated enzyme target; *Plasmodium falciparum* dihydroorotate dehydrogenase (*Pf*DHODH), for antimalarial drug discovery. Natural products of Kenya containing 1,4-naphthoquinone scaffold were targeted for investigation.

To facilitate the CADD studies, a searchable web based *in silico* database; the Mitishamaba database (http://*Mitishamba*.uonbi.ac.ke/) consisting of 1102 bioactive natural products of Kenya was developed. An assessment on the relevance of the database in drug discovery proved that 55.4% of the compounds in the *Mitishamba* database fell within the lead space and therefore ideal for drug discovery.

In search for new *Pf*DHODH inhibitors, the 1,4-napthoquinones in the *Mitishamba* database were subjected to binding studies. Psychorubin (**5**) which has previously been established to be active against *Plasmodium falciparum* emerged as the best structure, which was modelled to generate a number of analogs, out of which 2-acetylamino-1,4 naphthoquinone (**38**) and 2-amino-1,4-naphthoquinone (**39**) were synthesized and biologically evaluated against *Plasmodium falciparum.* 2,4-Dinitro-1-naphthol (**35**), which was one of the intermediates in the synthesis of compound **39** was also tested and found to exhibit activity of 1.67 ± 0.20 µg/ml against the chloroquine resistant K1 isolate and 4.22 ± 2.99 µg/ml against the chloroquine sensitive 3D7 isolate. Compound **38** had an activity of 8.23 ± 1.67 µg/ml against the chloroquine resistant K1 isolate and 3.86 ± 1.21 µg/ml against the chloroquine sensitive 3D7 isolate, while compound **39** had an activity of 24.74 ± 3.56 µg/ml against the chloroquine resistant K1 isolate and 12.51 ± 1.19 µg/ml against the chloroquine sensitive 3D7 isolate.

The promising antiplasmodial activities of the computational models demonstrate that the *Mitishamba* database can be used in lead design and a source of lead compounds for drug discovery.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACTs: Artemisinin-Based Combination Therapies

3D7: Chloroquine Sensitive Strain of *P. falciparum*

K1: Multi-drug Resistant strain of *P. falciparum*

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

FMN: Flavin mononucleotide

HTS: High Throughput Screening

IC_{50}: Half Maximal Inhibitory Concentration

*Pf*DHODH: *Plasmodium falciparum* dihydroorotate dehydrogenase

QSAR: Qualitative Structure Activity Relationship

SDF: Structure Data File

SQL: Structure Query Language

VS: Virtual Screening

W2: Chloroquine Resistant Strain of *Plasmodium falciparum*

HSQC: Heteronuclear Single Quantum Coherence

HMBC: Heteronuclear Multiple Bond Correla

CHAPTER ONE

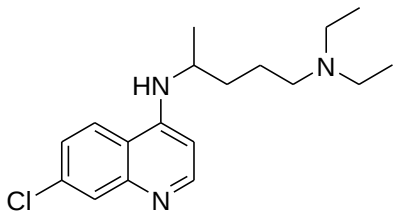
INTRODUCTION

1.1 Background Information

According to the World Health Organization, over 200 million cases of malaria are diagnosed each year (88% of which occur within the WHO African Region) with over 430 000 deaths per year (WHO, 2015). Malaria is caused by the *Plasmodium* parasite. Among the *Plasmodium* species, *Plasmodium falciparum* is the most lethal (WHO, 2015).

Malaria is both preventable and treatable and various cost-effective interventions have been put in place to help curb malaria. These include vector control interventions to help reduce parasite transmission (WHO, 2015) using indoor residual spraying (IRS) and insecticide-treated mosquito nets (ITNs), chemoprevention interventions and chemotherapy (WHO, 2006).

In Africa, more than half of the suspected cases always turn out to be positive (WHO, 2015) therefore malaria treatment becomes the center of focus. Of the various available antimalarial drugs for treatment of malaria, chloroquine (**1**) was for decades the drug of choice, because of its safety, effectiveness and cost (Batista *et al.*, 2009). The parasite developed resistance to chloroquine (**1**) rendering it ineffective (Dondorp *et al.*, 2009). As a result in April 2001, the WHO recommended the use of artemisinin-based combination therapies (ACTs) as first-line treatment of malaria (WHO, 2006).



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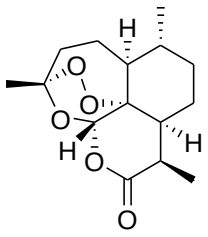
Artemisinin (**2**), which is used in ACTs is a natural product that was isolated from *Artemisia annua* L. (Asteracea) (Abdi, 1995). Artemisinin (**2**) is used in combination therapy to ensure high cure rates and reduce the potential of development of drug resistance (Dondorp *et al.*, 2009). Increasing the access to artemisinin-based combination therapies (ACTs) in malaria-prone countries in Africa has been integral to the remarkable recent success in reduction of the global malaria cases (WHO, 2015). Despite these efforts, resistance has already been observed in some parts of the world such as Thailand and Cambodia borders (Dondorp *et al.*, 2009). With the resistance to artemisinin already an emerging issue, there is an urgent need to discover alternative drugs.



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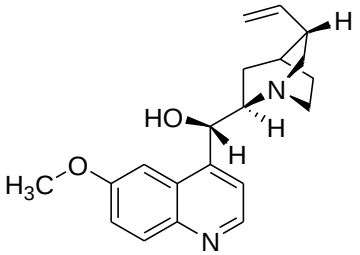
Natural products has for years been a dependable source of drugs (Harvey, 2000). For example, the two major anti-malarial drugs; artemisinin (**2**) and quinine (**3**) were derived from traditional medicinal plants that were used to treat malaria. Africa has a rich flora which has been used in folklore medicine to treat various ailments. In East Africa for instance, about 1200 plant species have been used by different communities for medicinal purposes (Kokwaro, 2009).



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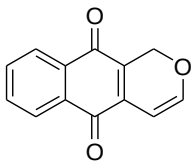
Despite this extensive use in traditional medicine, their use in drug discovery has not been exploited. However, there are extensive research that has been undertaken on the flora of Kenya resulting in the isolation of thousands of natural products with diverse skeletons. Some of the compounds have very good activities and these compounds or their derivatives could yield the next generation of drugs against malaria and other ailments.

Among the natural products that have been isolated from plants of Kenya, quinones have shown promising antiplasmodial activities. Pentalogin (**4**) and psychorubin (**5**) which were isolated from *Pentas longiflora* plant are examples of the quinones from Kenyan plants that have shown good antiplasmodial activity (Endale *et al.*, 2012). Other studies have also shown that quinones show promising antiplasmodial activities (Kayembe *et al.*, 2010; Philippe, 2009).Among the quinones

there is a growing interest in naphthoquinones owing to the fact that lapachol (**6**) and atovaquone (**7**) are currently used as antimalarial drugs (Basco, 2003; Wells, 2011).Therefore there is need to investigate this class of compounds as leads in antimalarial drug discovery.



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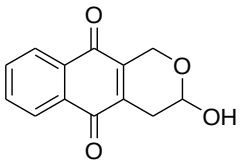

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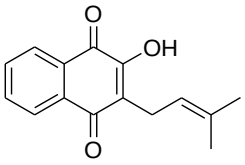

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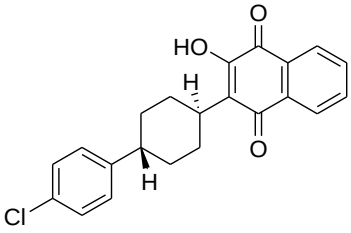

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Developing new potent drugs is one of the most complex processes in the pharmaceutical industry because conventional drug design strategies are slow and expensive, taking up to 20 years and up to $800 million (DiMasi *et al.,* 2003; Jakobsen *et al.,* 2011). Over the past few years, computer-aided drug design (CADD) has emerged as an important tool for identifying compounds with desired properties because it is faster and less costly (Cramer, 2004).

CADD mainly uses computational chemistry to study and discover drugs and other related biological active molecules by use of sophisticated computer programs. Most fundamental goal of CADD is to predict whether a given molecule will bind to a target and if so how strong, and usually by incorporating qualitative structure activity relationship studies (Ooms, 2000; Rapaka and Hawks, 1993). In this study, the CADD approach is used to model napthoquinones derived from natural products of Kenya for their antiplasmodial potential.

1.2 Statement of the Problem

Resistance of *Plasmodium falciparum* parasite to antimalarial drugs has undermined malaria control and eradication efforts. The parasite has already developed resistance to chloroquine (**1**) and there are already signs of resistance to artemisinin (**2**) jeopardizing the success already achieved in the fight against malaria using ACTs. Therefore, there is need to develop new antimalarial agents.

Natural product research in Kenya has been a major source of diverse chemical structures with promising biological activities such as antiplasmodial activity. Despite this extensive research, the natural products of Kenya have not been utilized in CADD studies because a database of natural products of Kenya that would allow easy access to all these structures does not exist. Therefore there was need to develop such a database.

1.3 Objectives

1.3.1 General Objective

The general objective of this study was to develop a web-based *in silico* database of natural products of Kenya for design and synthesis of antiplasmodial compounds based on the napthoquinone scaffold.

1.3.2 Specific Objectives

The specific objectives of this study were:

i. To develop a searchable web-based *in silico* database of natural products of Kenya
ii. To evaluate the antiplasmodial potential of the naphthoquinones in the database by virtual screening against *Plasmodium falciparum* dihydroorate dehydrogenase enzyme (*Pf*DHODH)
iii. To synthesize the synthetically accessible naphthoquinones with high virtual *Pf*DHODH inhibition and evaluate their antiplasmodial activity by *in vitro* assay.

1.3 Justification and Significance

Natural products have provided diverse classes of bioactive compounds. Among these compounds, naphthoquinones such as lapachol (**6**) and atavaquone (**7)** have been shown to exhibit antimalarial activity (Basco, 2003; Wells, 2011).Therefore focusing on this class of compounds was most appropriate.

Conventional drug design methods are expensive and time consuming but with current advanced computers, CADD has emerged as powerful drug design tool, a remedy for time and cost. To apply CADD to this study, a searchable database of natural products of Kenya was needed for virtual screening to identify promising napthoquinones for antimalarial drug discovery. Despite the extensive body of knowledge on natural products of Kenya, the lack of a searchable database was

drawback for CADD studies. There was therefore the urgent need to develop such a database for this study, which would also be useful to other researchers in industries and academia.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Disease and Management

Malaria is a common disease (in the tropical and sub-tropical regions) caused by protozoan parasites of the *Plasmodium* species and is transmitted by the female Anopheles mosquito (Guerra *et al.,* 2008). Among the *Plasmodium* species: *falciparum, vivax, ovale* and *malariae* , *Plasmodium falciparum* causes the deadliest form of malaria (WHO, 2006). Globally malaria leads to about 430 000 deaths per year (WHO, 2015). In Africa, it is most prevalent in Sub-Saharan Africa predominantly affecting expectant mothers and children under the age of five (WHO, 2015).

Malaria is both preventable and treatable. The cost-effective interventions recommended by WHO ("Malaria Policy Advisory Committee to the WHO," 2013) to help curb malaria are summarized in Figure 2.1.

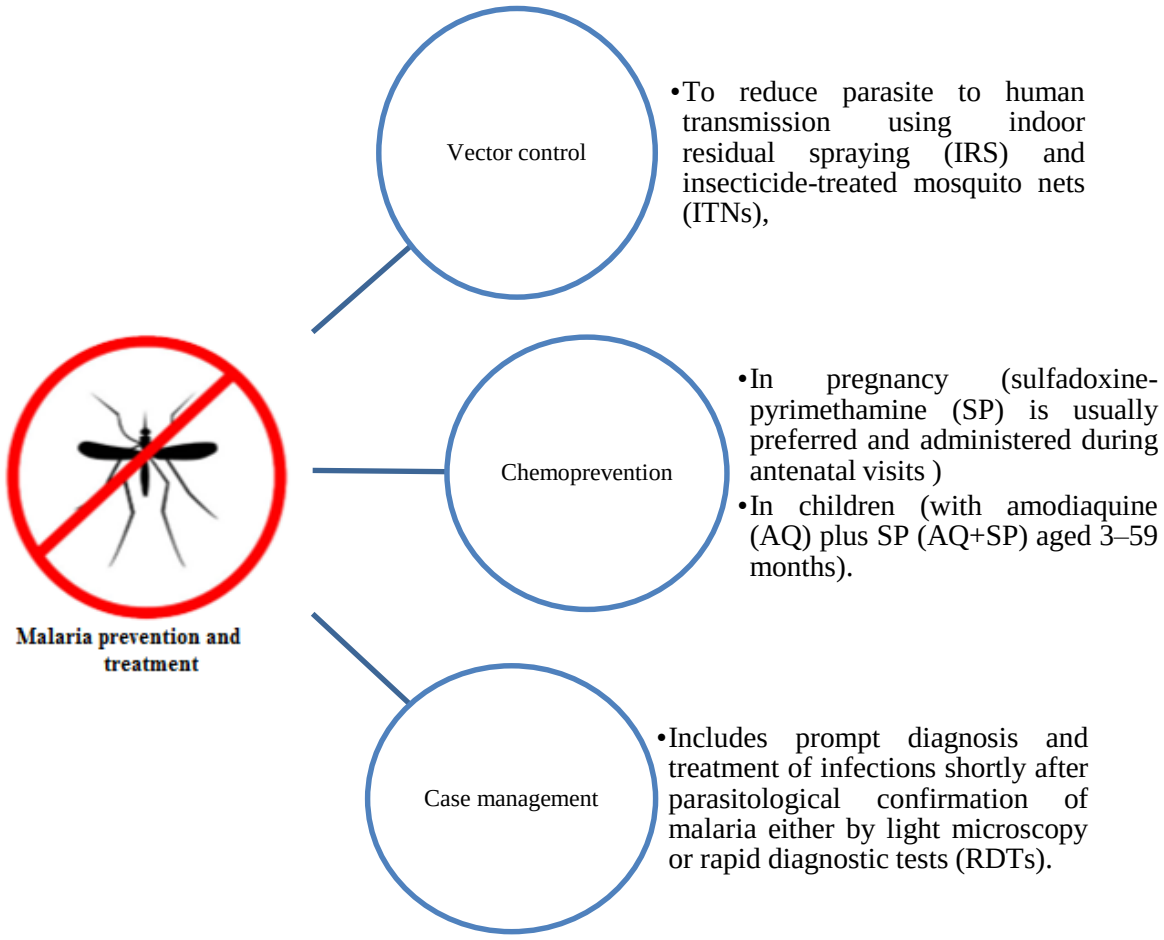


Figure 2:1: Malaria treatment and prevention strategies

Of all these strategies, chemotherapy remains the most common approach to curb malaria. Currently, artemisinin-based combination therapy (ACT) is the most preferred treatment option and has helped in malaria mortality reduction especially in children aged 1–23 months by 99% (range: 94–100%), and in children aged 24–59 months by 97% (range: 86–99%) ("Malaria Policy Advisory Committee to the WHO," 2013). The introduction of Artemisinin-based combination

therapies (ACT) coupled with vector control measures has led to better progress in reducing the malaria burden (Rosenthal, 2008; WHO, 2015).

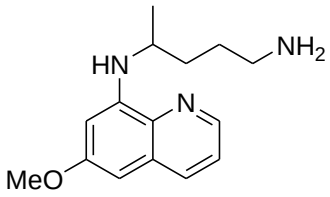
Despite the progress that has been achieved in curbing malaria using ACTs, the reports of incidences of artemisinin resistance especially in western Cambodia (WHO, 2015), have the potential of reversing the gains made in controlling malaria. Drug resistance to majority of known anti-malarials has been reported, giving evidence to the ease by which parasite populations can adapt and survive by developing resistance (WHO, 2006). Therefore there is an urgent need to discover new antimalarial drugs. It is noteworthy that most of the current antimalarial drugs are either natural products or inspired by natural products.

2.2 Natural Products in Malaria Chemotherapy

The two most effective antimalarial drugs, quinine (**3**) and artemisinin(**2**), are obtained from *Cinchona succirubra* (Jones *et.,* 2015) and *Artemisia annua* (Haynes, 2006), respectively, traditional medicinal plants used for the treatment of malaria. In addition, lapachol (**6**), a natural naphthoquinone obtained from the bark of *Tabebuia avellanedae* (Bignoniaceae) (Said *et al.*, 2003) is also an antimalarial drug (Renou *et al.*, 2003). These have inspired the development of synthetic aminoquinolines, endoperoxide sesquiterpene lactones and naphthoquinone derived antimalarial drugs.

2.2.1 Quinine and its Derivatives in Malaria Treatment

Quinine (**1**), a quinoline natural product isolated from the bark of the cinchona tree in 1820,was for many years the only remedy for malaria treatment (Meshnick and Dobson, 2001). With the revolution of synthetic organic chemistry in the late 19th century, many chemists tried synthesizing quinine including William Henry Perkins, an English chemist who tried to synthesize quinine but failed in 1856 (Meshnick and Dobson, 2001). With continued search of antimalarial drugs, quinine was later synthesized in 1944, and guided by in-depth studies on quinine, it was discovered that the quinoline scaffold was the one responsible for its antimalarial property (Jones, 2015). Based on this scaffold, the aminoquinoline antimalarial drugs chloroquine (**1**) and primaquine (**8**) were developed (Foley and Tilley, 1998).



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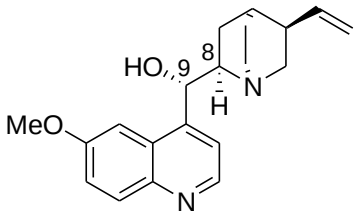
Modification on the stereochemistry was also found to be essential in determining the efficacy. Among the four stereoisomers, quinine (**3**) and quinidine (**9**) with 8*S*, 9*R* and 8*R*, 9*S* configurations are active, (chloroquine sensitive strain D-6 of *P. falciparum*; IC_{50} =29.3±9.5nM and 13.4±4.6 nM, respectively). The other two stereoisomers, 9-epiquinidine (**10**) and 9-epiquinine (**11**) with 8*R*,9*R*

and 8*S*,9*S* configurations, respectively, are inactive (chloroquine sensitive strain D-6 of *P. falciparum;* IC_{50} =2700±704 nM and 3471±797 nM, respectively) (Gorka *et al.*, 2013).



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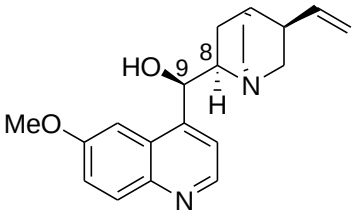

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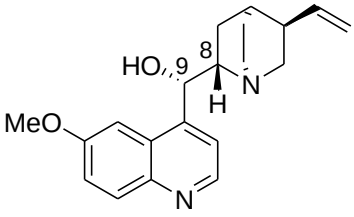

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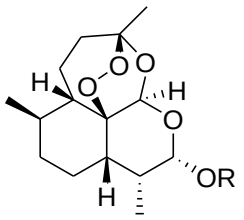
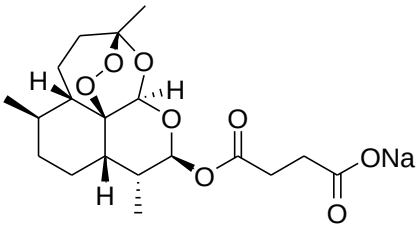
2.2.2 Artemisinin and Artemisinin-based Combination Therapies (ACTs)

Artemisinin (**2**) is a natural product that was isolated from *Artemisia annua* L. (Asteracea) (Abdi, 1995), which is currently being used as an antimalarial drug because of its high potency and low toxicity (Davis *et al*, 2005). However, as a drug, artemisinin (**2**) has a low half-life ($t_{1/2}$ -2 hours) and low solubility in water and oil leading to low bioavailability in the body (Davise *et al.*, 2005). Due to its short half-life, artemisinin is administered frequently over a short period of time and patients compliance became a major drawback (WHO, 2006). These shortcomings necessitated structure modification to enhance its bioavailability and half-life, leading to the design of various semi-synthetic analogues such as dihydroartemisinin (**12**), artemether (**13**), arteether (**14**) and sodium artesunate (**15**) (Rachel *et al*, 2015).



R =H **12**
R =Me **13**
R =Et **14**



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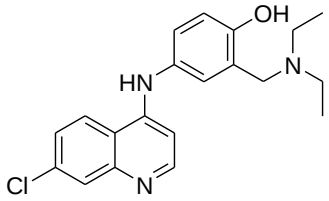
The short half-life of artemisinin (**2**) may potentially lead to the fast development of drug resistance. To address this problem, artemisinin-based combination therapies (ACTs) was recommended by WHO; where artemisinin is administered with one or more longer-acting and slowly-eliminated antimalarial drugs with independent modes of action such as amodiaquine (**16**), piperaquine(**17**), lumefantrine (**18**), mefloquine (**19**), chlorproguanil (**20**) /dapsone (**21**), and sulfadoxine (**22**) /pyrimethamine (SP) (**23**) (Aweeka and German, 2012; "Malaria Policy Advisory Committee to the WHO," 2013).



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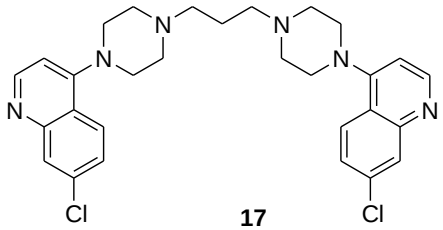

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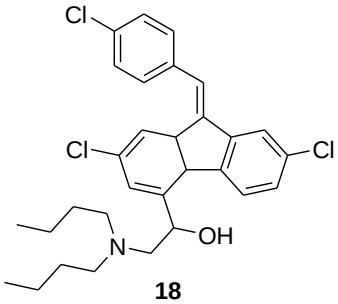

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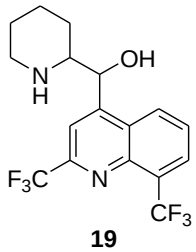

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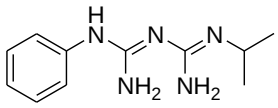

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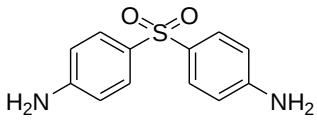

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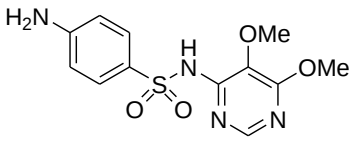

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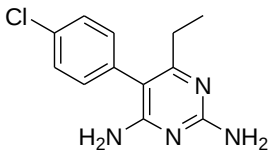

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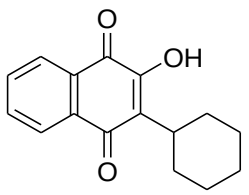
Besides quinolines and sesquiterpenes, other classes of natural products such as quinones, particularly naphthoquinones have antiplasmodial potential (Riffel *et al.*, 2002).

2.2.3 The Napthoquinone Scaffold in Antimalarial Drug Design

Among the natural quinones, naphthoquinones, specifically 1,4-naphthoquinones are widely reported to show antimalarial activity (Ball *et al.*, 1947; Induli *et al.*, 2012; Pérez-Sacau *et al.*, 2005; Riffel *et al.*, 2002; Said *et al.*, 2003). Lapachol (**6**), a natural 1,4-naphthoquinone from the bark of Tabebuia avellanedae (Bignoniaceae) (Said *et al.*, 2003) exhibits antimalarial activity (IC_{50} = 1.8 - 12.2 μg/ml) (Mooberry and Anderson, 2014; Renou *et al.*, 2003). It occurs in various plant

families, including Bignoniaceae, Leguminosae, Sapotaceae, Scrophulariaceae, Verbenaceae, Malvaceae, and Proteaceae, and exhibits an impressive list of biological activities (Hussain *et al.*, 2007).

Lapachol's (**6**) antiplasmodial activity has been linked to several mechanisms such as the competitive inhibition of the cytochrome bc1 complex, generation of reactive oxygen species and enzymatic inhibition (e.g., glutathione reductase, dihydroorotate dehydrogenase and glycerol) (Ball *et al.*, 1947). Despite this, lapachol (**6**) was not successful as a drug because it had poor oral bioavailability which necessitated structure optimization to various analogues such as atovaquone (**7**), a synthetic 1,4-naphthoquinone currently used as a drug (Malarone) for prophylaxis and treatment of uncomplicated tropical malaria (Maier *et al.*, 2009). Parvaquone (**24**) and buparvaquone (**25**) are additional important 1,4-naphthoquinones used as drugs for the treatment of malaria (Sharma *et al.*, 2013), and this highlights the importance of this class of compounds as a scaffold in the development of novel antimalarial drugs.



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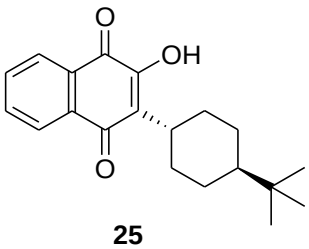

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Phytochemical investigation of *Pentas longiflora*, an important medicinal plant traditionally used in Kenya for treatment of malaria (Kokwaro, 2009), led to the isolation of the 1,4-naphthoquinones; pentalongin (**4**) and psychorubrin (**5**) with IC_{50} values of 0.27 ± 0.09 and 0.91

± 0.15μg/ml against W2 (CQ-R) and 0.23 ± 0.08 and 0.82 ± 0.24μg/ml against D6 (CQ-S) strains of *Plasmodium falciparum*, respectively (Endale *et al.,* 2012). All these support the need to investigate compounds bearing the 1,4-naphthoquinone scaffold in antimalarial drug discovery.

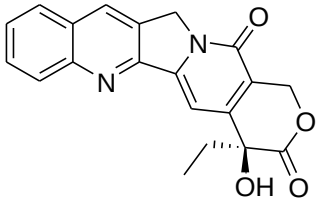
2.3 Drug Discovery and Development

The drug discovery and development is a process that brings potential drugs from the laboratory to the market. The drug development process consists of four stages: drug discovery, development, regulatory review and marketing, which may take several years (an average of 10 years). Research and development (R and D) costs of each successful drug is expensive and has been estimated to range from $1.3 billion to 3.7 billion (DiMasi *et al.,* 2003; Paul *et al.,* 2010). The drug discovery process has evolved overtime from the traditional random drug discovery to modern strategies of drug discovery that use bioinformatics and chemoinformatics for the design of drugs (Rao and Srinivas, 2011).

2.3.1 Traditional Drug Discovery

The traditional drug discovery is a process of chance based on serendipitous observations of natural systems (e.g. plant or fungal extracts or exudates) that exhibited interesting biological effects. The natural tendency of such an observation would be to determine the active components through isolation, characterization and biological evaluation. An example of such a serendipitous discovery is that of penicillin in 1928 by Alexander Fleming (Ban, 2006). While working on influenza, Fleming noticed that one of his *Staphylococcus* cultures was contaminated by a mould

(possibly from the dusty old building he was working in) and a bacteria-free circle developed. Fascinated by the bacteria–free circle, he went ahead and isolated the mold in pure culture which he discovered that it produced a substance that destroyed many of the common bacteria that affect humans, and called it "penicillin", after the fungus *Penicillium notatum* (Ban, 2006). Another approach is random screening, where selected classes of compounds or randomly selected plants are screened for the purpose of discovering a new drug. Drugs such as camptothecin (**26**) and paclitaxel (**27**) were discovered through this random approach (Harvey, 2000).



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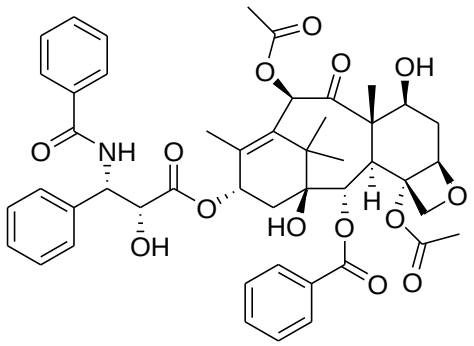

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Serendipitous and random drug discovery heavily relies on chance and because of this the probability of hitting a jackpot, discovering a drug, is usually very low and therefore it is not a very dependable strategy for drug discovery. The evolution of better technologies ushered in modern methods, which although expensive, do not rely on serendipity anymore but utilize a highly planned process.

2.3.2 Modern Drug Discovery

The modern drug discovery, unlike the traditional approach, is more systematic and involves target identification, target validation, assay development, screening to find hits, lead identification and lead optimization to increase potency, bioavailability and selectivity. When these requirements have been met, the lead compounds go through clinical development processes before being delivered to the market, Figure 2.2. (Steinhagen, 2011).

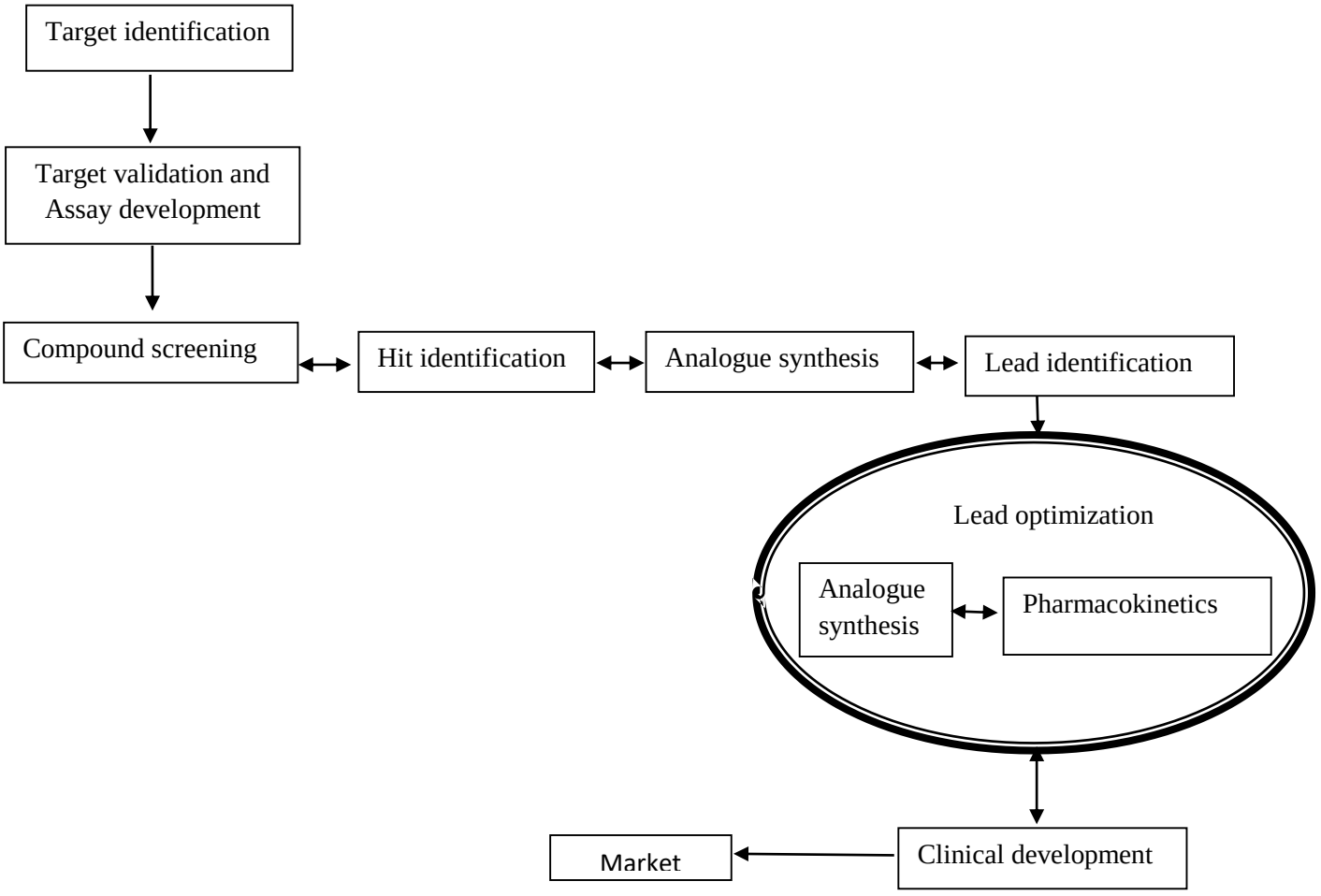


Figure 2:2: The processes of modern drug discovery

A target is a region (e.g. in a protein, nucleic acid, ion channel, receptor, DNA/RNA, ribosome etc.) in a living organism that allows ligands/drugs to bind and consequently leads to a change in its function or behavior (Lindsay, 2003). Drug-target interactions cannot be observed directly but the subsequent ripple effects are noticeable. Because of this target discovery is largely dependent on x-ray crystallographic structures of ligand-bound macromolecules, where drug targets are treated as fixed objects (Imming *et al.,* 2006). Some proteins are not easy to crystallize and other techniques including biochemical data have been used instead to discover targets, for example in the discovery of G-protein-coupled receptors (GPCRs), the proof of the re-organization of the ligand bound receptor was obtained from a biochemical data (Saunders, 2005). Biochemistry and pharmacology have now provided a better understanding of enzymes and cellular receptors. These utilize techniques such as proteomics to examine mRNA or protein levels so as to establish whether they are expressed in disease progression, bioinformatics and genomics to study the genetic associations for purposes of drug target discovery (Lindsay, 2003). Bioinformatics approach has not only been used in target identification but also in prioritizing and selecting potential drug targets (Hughes *et al.*, 2011).

After the targets have been identified, they are then validated by demonstrating their role in the disease cycle. Different tools have been used in drug target validation, including whole animal models, for example in the knockout animal model, the lack of any observable effect by a drug administered to an animal (e.g. mice) that lacks a certain target provides a strong support that the target is directly linked to that expected effect (Imming *et al.*, 2006; Zambrowicz and Sands, 2003). For enzyme targets ,molecular interaction of the enzyme inhibitors with other identical but

structurally unrelated enzymes are studied and the target validated based on the different observed binding conformations if any, while for receptor targets, the antagonists, agonists and the inverse agonist (if available) are retested to provide the proof of their effect. Gene expression or protein/enzyme functions have also been used to support target selection before they are used in drug discovery (Lindsay, 2003).

Target identification and validation is done in first stages in modern drug discovery and is critical to the success of the drug discovery process (Cao and Wang, 2015). After a target has been identified and validated, an assay is then developed. The developed assay is used in screening molecules to identify hits (compounds that have the desired level of activity). Varieties of screening methods exist which include high throughput screening (HTS) (involves screening a very large library of compound against a target),focused screening (previously identified hits for specific class of targets or having similar structures), fragment screen (where small compounds are soaked into crystals so as to get low millimolar activity compounds which are then used for building larger molecules) and NMR screen (where small compounds are screened by soaking into a protein target of known NMR or crystal structure to find hits having low millimolar activity) (Hughes *et al.*, 2011). Although, the compounds screened usually come from natural products or synthesis, recent advances in combinatorial chemistry have enabled medicinal chemists to access thousands of compounds (Olliaro and Yuthavong, 1999).

The hits are studied to identify promising lead compounds (compounds that have good pharmacological activity with drug-like properties but whose structure might still require

optimization to improve their potency). The identified leads are then ushered into lead optimization stage, where they are formulated to ease target delivery by studying their pharmacological properties and preliminary toxicology in order to improve their bioavailability, efficacy and safety (Shivaputra *et al.*, 2012). These processes take a lot of time and resources.

Although the quick adoption of HTS and combinatorial chemistry helped to increase the rate of lead identification, a proportionate increase in introduction of new drugs into the market was not observed. This is simply because the chemical space is still too wide for random screening as most compounds are generally not drug like and any hits identified are not amenable to further development because of issues such as poor ADMET (absorption, distribution metabolism, elimination and toxicity) properties (Rao and Srinivas, 2011). Furthermore, the cost of setting up HTS and combinatorial chemistry programs are also very high. These limitations have led to the emergence of computer aided drug design (CADD) method that provides compounds with high probability of being a drug (Kore *et al.*, 2012).

2.4 Computer-Aided Drug Design (CADD)

Computer-aided drug design (CADD) is a specialized strategy that utilizes computers to virtually simulate drug-target interactions without the need to synthesize and bioassay the compounds (Kore et al., 2012). Computational methods have accelerated drug discovery by reducing the chemical space for actual synthesis and assay, which has helped greatly in providing more potent molecules. CADD heavily relies on chemoinformatics, bioinformatics, structural databases, protein databanks, high performance computers and software. CADD is employed in various stages of

drug development, starting from target identification to preclinical stage (Figure 2.3) (Cramer, 2004).

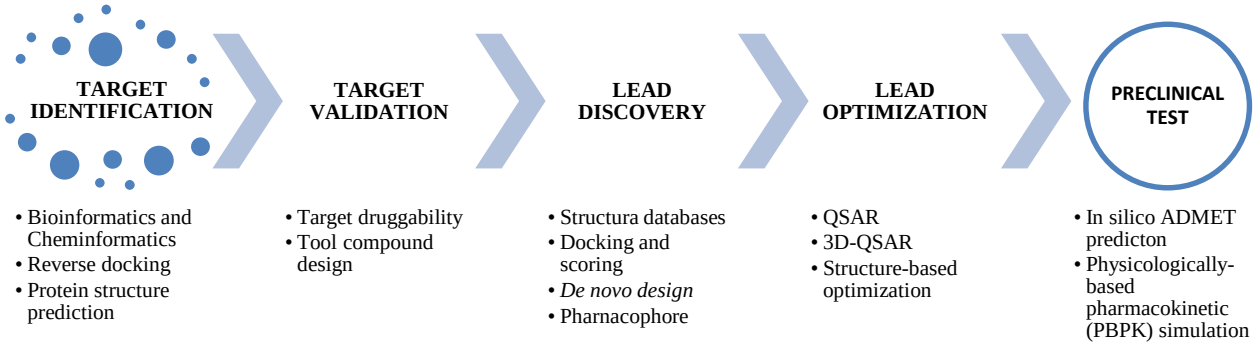


Figure 2:3: Application of CADD to various drug development stages (Cramer, 2004)

Identifying targets among thousands of candidate macromolecules is tedious and CADD has been employed to address this through genomic and proteomic approaches (Wang *et al.*, 2004). Protein ligand interaction fingerprint (PLIF) and Ligand-based interaction fingerprints (LIft) method have been used to identify GPR17 human neurodegenerative diseases and p38α kinase target for anticancer drug development respectively (Cao and Wang, 2015; Eberini *et al.*, 2011).

Once the target is identified and validated, CADD is employed in the lead discovery stage to virtually screen libraries of compounds against target. The libraries of compounds are obtained from natural products as well as synthetic databases. Virtual screening uses software to dock libraries against the target followed by scoring in order to identify possible active compounds (Miller, 2002). Virtual screening has been successful in the recent years due to development of

high performance computers, robust computational algorithms and better scoring functions. These have made it even more practical as it provides a much higher hit rate of more lead-like or drug-like molecules as compared to high throughput screening (Rognan, 2006).

The two virtual screening approaches that are used in lead discovery are ligand based drug design and structure based drug design, LBDD and SBDD, respectively. In LBDD, prior knowledge about other molecules that bind to the biological target of interest is required. This is used to derive the basic pharmacophore model that defines the minimum structural features required in order to bind to a target and this information is then used to design more effective inhibitors. In the absence of any structural information, quantitative structure activity relationship (QSAR) is usually the most preferred ligand based approach. A number of diverse 5-Lox inhibitors were designed through this process by quantitatively investigating their similar chemical characteristics (Aparoy *et al.,* 2012). The SBDD, on the other hand, requires knowledge of the three-dimensional (3D) structures of the target protein enzyme/receptor, which is usually obtained from protein databases such as the "Protein Databank (PDB)" (Rognan, 2006). The libraries of compounds are then docked against the 3D structure of the target to identify molecules that bind to the target. Examples where SBDD approach has been used for drug development was the discovery of the antiretroviral nelfinavir and amprenavir by the GlaxoSmithKline (GSK) company in which the known structures of the HIV protease enzyme assisted in designing of the inhibitors (Simmons *et al*, 2010).

The 3D protein structures in the PDB database contain in addition to the 3D structure of the protein several experimentally determined 3D coordinates and protein–ligand complexes that can be

searched and retrieved from a user defined query (Berman *et al.*, 2000). The results are available for download in various file formats for structure based virtual screening (Berman *et al.,* 2000) against structure databases using docking programs.

After structural studies of a novel antimalarial compound from natural compound possibly from SAR data, identification of the basic pharmacophoric unit (s) is made possible, which then allows for a systematic database or libraries search of known compounds for structural analogs (Golbraikh *et al.,* 2012)

In addition to the 3D structures of the receptor, the PDB contains both 2D and 3D structures of co-crystallized ligands/inhibitors, which can also be used in LBDD to generate a QSAR model and identify basic pharmacophore unit(s) that is Pharmacophore modeling. This then allows for further systematic database or library search of compounds for potent analogue (Golbraikh *et al.,* 2012). Pharmacophore modeling is widely applied in LBDD to identify potent inhibitors and an example of such approach was the discovery of c-Myc oncoprotein inhibitors (Mustata *et al.,* 2009) where a pharmacophore model was generated using known inhibitors and successfully used to identify other nine potent inhibitors, four of which were active *in vivo* and inhibited the growth of the c-Myc-overexpressing cells (Mustata *et al.,* 2009).

2.4.1 Potential Application of CADD in Antimalarial Drug Discovery

In order for CADD to be successfully employed in drug discovery, there is need for validated targets and appropriate sources of structure databases. The completion of the *Plasmodium*

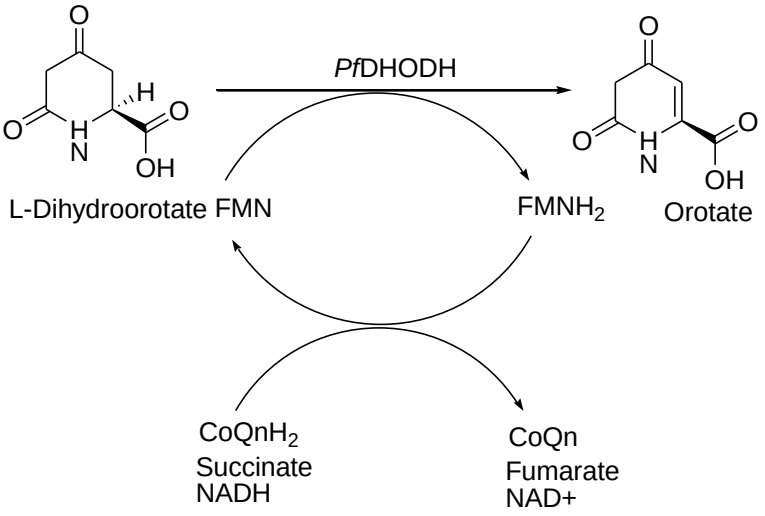
falciparum genome (Gardner *et al.*, 2002) has helped in identifying various novel validated drug targets such as *Plasmodium falciparum* dihydroorate dehydrogenase (*Pf*DHODH) for the application of CADD in antimalarial drug discovery (Phillips and Rathod, 2010). In addition to validated targets, there are diverse sources of chemical databases (including natural products databases) that can facilitate the use of CADD in the discovery of antimalarials.

2.4.1.1 *Plasmodium falciparum* Dihydroorate Dehydrogenase as a Promising Malaria Target

Major drug targets in *P. falciparum* are located in the digestive food vacuole, parasite channels and transporters, nucleus, cytosol, mitochondrion and apicoplast. They may also be identified as enzymes in processes associated with nucleic acid biosynthesis and membrane phospholipid biosynthesis (Agüero *et al.*, 2008; Athar , 2009).

Generally, the biosynthesis of pyrimidine bases in DNA and RNA in biological systems can be through the *de novo* pathway and/or the salvage pathway (Phillips and Rathod, 2010). The human host for *Plasmodium* parasite synthesizes pyrimidine bases through both pathways unlike the parasite which only uses the *de novo* pathway (Olliaro and Yuthavong, 1999). This difference can be exploited in designing antiplasmodial drugs that inhibit the *de novo* pathway. The fourth step, which is the rate determining step, in the *de novo* pyrimidine synthesis pathway that oxidizes dihydroorotate (DHO) to orotate is catalyzed by *Plasmodium falciparum* dihydroorate dehydrogenase (*Pf*DHODH), Scheme 2.1 (Gardner et al., 2002). Inhibition of *Pf*DHODH will block pyrimidine synthesis leading to the eventual death of the parasite. Additional selective

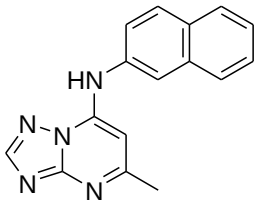
inhibition is made possible by the difference in amino acid sequence between *Pf*DHODH and human DHODH, which also explains the specie-selectivity in inhibitor binding modes (Deng *et al.*, 2009) and therefore, making *Pf*DHODH an attractive target for antimalarial drug discovery.



Scheme 2.1: The fourth step in the *de novo* pyrimidine biosynthesis in *Plasmodium falciparum*

Plasmodium falciparum DHODH has been validated as a drug target through selective inhibition of the enzyme using antimalarial triazolopyrimidine-based inhibitors (**28,29, 30**) (Phillips *et al.*, 2008). These inhibitors including selective human DHODH inhibitors; A77 1726 (**31**), and brequinar (**32**), have been observed to bind in different modes which demonstrated the enzyme's conformational flexibility and that it can also house different scaffolds (Deng *et al.*, 2009; Phillips and Rathod, 2010).These inhibitors have been shown to inhibit *Pf*DHODH by competitive inhibition of CoQ-dependent FMN oxidation, leaving out FMN-dependent DHO oxidation (Phillips *et al.*, 2008). The possibilities of this has been proposed where inhibitor binding sites are

not overlapping but blocking the transfer of electrons from FMN to CoQ and vice versa , or by stabilizing the *Pf*DHODH conformation, excluding CoQ binding (Deng *et al.,* 2009).



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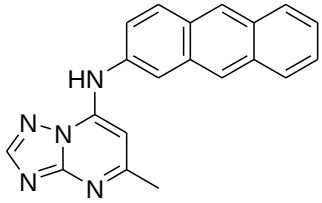

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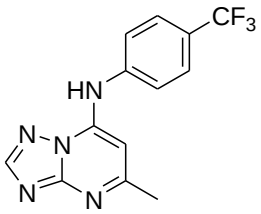

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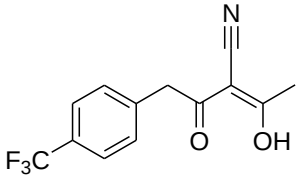

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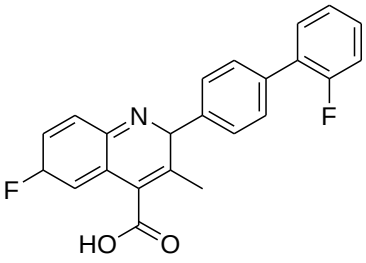

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In a malaria mouse model, the metabolically stable triazolopyrimidine analogue containing phenyl-trifluoromethyl (**30**), was able to suppress infection caused by *Plasmodium bergheii*, which provides the basic proof that *Pf*DHODH inhibitors can exhibit *in vivo* anti-malarial activity (Gujjar *et al.,* 2009).

A recent research targeting the enzyme led to the discovery of potent *Pf*DHODH inhibitors where a combination of 3D pharmacophore modelling and structure based virtual screening, that utilized the National Cancer Institute database, helped in the identification of NSC336047 (Diethyl 2-[[3-

(dimethylamino)anilino]methylidene]propanedioate) as the most potent inhibitor with an IC_{50} value of 26 μM among other nine compounds with inhibition values of >25% at a concentration of 10μM (Pavadai et al., 2016).

2.4.1.2 Natural Products Databases for Drug Development

Natural products are promising source of potential antimalarial drugs recognizing the fact that most of the current antimalarial drugs are either natural products or inspired by natural products. Nature provides chemical compounds with diverse 3D structures and functionality. Natural products, secondary metabolites, are synthesized by organisms for diverse ecological functions such as protection from various diseases and survival in the environment making them suitable source of bioactive molecules. Natural products are an attractive source of bioactive molecules as compared to synthetic compounds because through evolutionary processes they have developed structural features that help them bind to biological targets (Harvey, 2015). In addition, natural products being natural metabolites, cover a better chemical space of the biologically relevant compounds which gives them an advantage over synthetic compounds (Harvey, 2015). This is very essential for virtual screening and gives the basis of focus on natural product database development for drug discovery.

Recognizing the importance of natural products as source of library for drug discovery, a number of natural products databases have been developed. Some of the most important natural products databases that have been developed include TCM Database@Taiwan (***tcm.cmu.edu.tw***) (Chen, 2011) which is a database of compounds from Chinese traditional medicine, NuBBE database from

the rich Brazilian Amazon flora (Valli *et al.*, 2013) and CamMedNP a database of Cameroonian natural products representing the rich African tropical flora (Ntie-Kang *et al.*, 2013). Kenya also has a rich biodiversity and an extensive folklore in the use of plants for treatment of various ailments (Dharani and Yenesew, 2010; Kokwaro, 2009). There have also been extensive phytochemical studies on Kenya's biodiversity leading to the identification of structurally diverse natural products with unique scaffolds and promising biological activities with a potential to yield the next generation of drugs. However, this information is scattered in various forms of publications and therefore not structured for CADD studies. Therefore, there is need to organize these in a form of database.

CHAPTER THREE

MATERIALS AND METHODS

In this section, the procedures, materials and methods used to realize the objectives of this study are described.

3.1 Development of a Searchable Web-based *In Silico* Database of Natural Products of Kenya

The steps involved in development of a searchable web-based *in silico* database of natural products of Kenya were:

i. Data mining of natural products of Kenya from different literature sources
ii. Tabulating information on the natural products which included structure, name, class of compound, botanical source, plant part, place of collection, bioassay data and literature references
iii. Calculation of the physicochemical properties of the natural products.
iv. Organization of the data as a web-based database

3.1.1 Data Mining

The first step in the development of the database involved sourcing compounds isolated from plants of Kenya from literature such as review papers, journal articles, conference proceedings, book of abstracts, dictionary of natural products and students' research reports.

3.1.2 Tabulating Data

The next step after data mining involved capturing the data of each natural product in an Excel spread sheet tabulating its structure, name (trivial and IUPAC), class, botanical source (plant species, family and place of collection).The structures of the compounds were drawn using Accelrys draw 4.1 software. This software was also used to generate their IUPAC names and 1D structures (SMILES).

3.1.3 Calculation of Physicochemical Properties

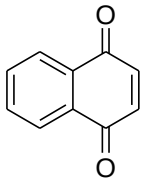
The third step in the development of the database was the calculation of the physicochemical properties of the compounds. The properties that were calculated were molecular weights (MW), Merck molecular force field (MMFF) energy, rotatable bonds, heavy atoms, hydrogen bond acceptor (HBA), hydrogen bond donors (HBD), log P, polar surface area (PSA). These physicochemical properties were calculated using Open Eye's scientific software application tool kit called "MolProp TK" (Blum and Reymond, 2009).

3.1.4 Creation of the Web-Based *In Silico* Database

The last step in the development of the database was the organization of the data into a searchable web-based database. Discovery studio software (BIOVIA, San Diego, USA) was used to organize the data into a single chemical table file (SDF) and then uploaded to a relational database; MySQL (Oracle Corporation, USA). The searchable database was implemented on a Linux server using HTML5 (W3C, 2014), MySQL5.0 (Oracle corporation, USA) and PHP (The PHP Group, 2016). While the search engine was developed based on the Norbert Haider's MolDB5R package, which uses *checkmol* and *matchmol* programs for structure searching and matching (Haider, 2010).

3.2 Virtual Screening of Natural Naphthoquinones of Kenya

The database of the natural products developed was queried for the1,4-naphthoquinone scaffold (**33**) to create a library of 1,4-naphthoquinones for virtual screening against the target.



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3.2.1 Preparation of Library

The library generated was prepared for virtual screening against *Pf*DHODH receptor using the different applications in the Open Eye software suite (Santa Fe, NM, USA). The *molcharge* program in the *quacpac* application was used to assign appropriate atomic partial charges to the molecules and save as a mol file. The *omega* program was then used to convert the mol file into 3D structures and saved as zipped Open Eye binary (oeb.gz) file.

3.2.2 Preparation of *Pf*DHODH Receptor

The preparation of the receptor involved downloading the 3D protein-ligand complex structure of *Pf*DHODH (PDB ID = 1TV5.pdb) with a bound ligand (2Z)-2-cyano-3-hydroxy-*N*-[4-(trifluoromethyl) phenyl] but-2-enamide) from the protein data bank (PDB-http://www.rcsb.org/pdb/explore/explore.do?structureId=1tv5) and preparing using graphical user interface program called *make_receptor* in *oedocking***.**

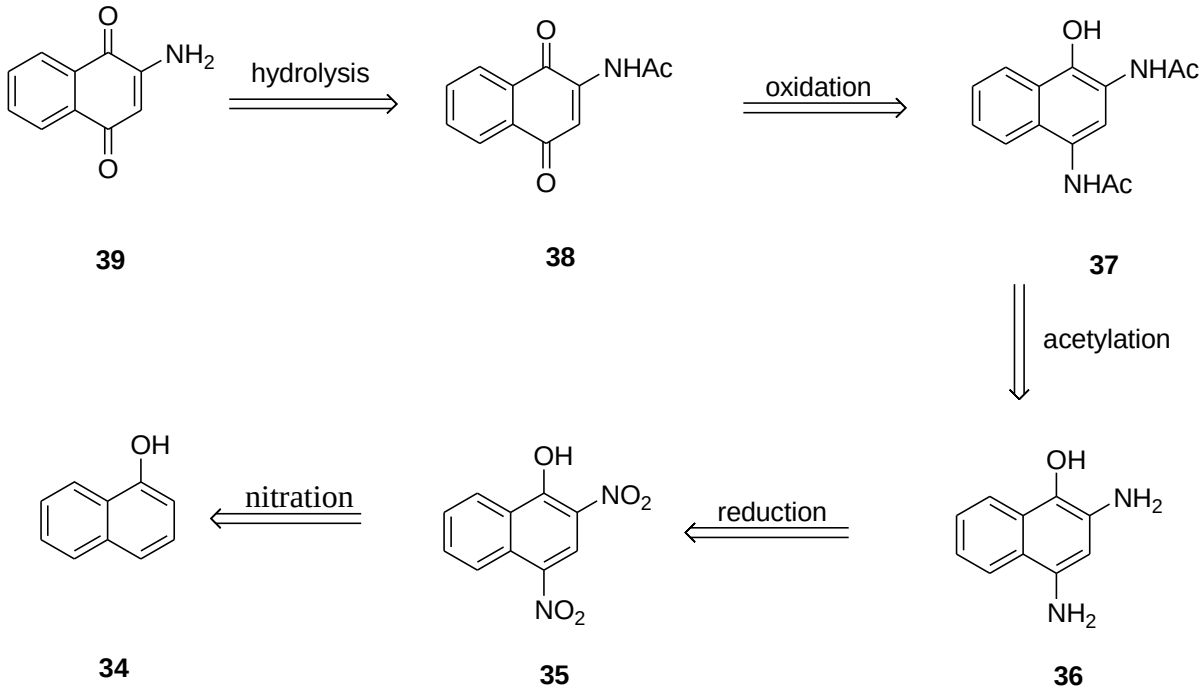
3.2.3 Virtual Screening of Library against *Pf*DHODH

The prepared library was subjected to virtual screening against the prepared *Pf*DHODH receptor using the *Hybrid* docking program found in the *oedocking* application. The docking reports of the docked molecules together with their binding scores were generated in pdf format using the *docking_report* program that gave extra details including residue fingerprints, shape interactions, hydrogen bonding, ligand and protein desolvation scores. *Brood* program was then used to generate analogs, which were then docked using *hybrid*. The binding scores and synthetic

accessibility of the compounds guided the selection of the 1,4-naphthoquinones that were targeted for synthesis

3.3 Synthesis of Target Naphthoquinones

The synthetic approach employed in the synthesis of the target naphthoquinones was based on literature procedures in Louis and Kenneth (1979), and is as highlighted in the retrosynthetic pathway in Scheme 3.1.



Scheme 3.1: Retrosynthetic pathway for synthesis of the target naphthoquinones

3.3.1 General Procedure

The chemical used in the synthesis were of synthetic grade, purchased from Sigma-Aldrich. The ^{1}H NMR spectra were obtained at 600 and 500 MHz with TMS as an internal standard and deuterated dichloromethane and acetone as solvents. Reactions were monitored on analytical TLC silica gel plates using fluorescent indicator 254 nm. The specific procedures used in the synthesis of the target naphthoquinones were as follows:

3.3.2 Synthesis of 2,4-Dinitro-1-naphthol (35)

Concentrated H_2SO_4 (5mL, 93 mmol) was added to 1-naphthol (**34**) (2.5 g, 17 mmol) in a 50-mL Erlenmeyer flask. The mixture was heated on a hot plate for 5 min and then cooled in an ice bath. Concentrated HNO_3 (3mL, 71 mmol) was then added slowly to the chilled solution with swirling and the solution was left to sit for 5 minutes then warmed to 50 ºC. The product separated as a yellow paste which was precipitated with water and filtered under vacuum. The product was then transferred to a mixture of hot water (75 mL) and concentrated ammonium hydroxide (2.5 mL) and heated to boiling while stirring to dissolve the solid. Ammonium chloride (5g, 93 mmol) was added to salt-out the ammonium salt and then the mixture was filtered. The filtrate was then allowed to cooling an ice bath, filtered and washed with water containing 1 % ammonium chloride to obtain 2,4-dinitro-1-naphthol (**35**) as orange crystals (3.47 g, 85 % yield).

Physicochemical properties: mp 137-139 °C: ^{1}H NMR (500 MHz, Acetone-d6) δ 8.97 (*s*, 1H), 8.73 – 8.62 (*m*, 2H), 8.10 (*ddd*, *J* = 8.6, 7.0, 1.4 Hz, 1H), 7.92 (*ddd*, *J* = 8.2, 6.9, 1.1 Hz, 1H). ^{13}C NMR (126 MHz, Acetone) δ, 157.7, 138.4, 133.9, 128.6, 128.6, 126.6, 125.9, 125.4, 123.8, 120.1.

3.3.3 Synthesis of 2-Acetylamino-1,4-naphthoquinone (38)

Sodium hydrosulfite (2.0 g, 11 mmol) was added to a beaker containing 2,4-dinitro-1-naphthol (**35**) (3.0 g, 12.6 mmol) in 100 mL water. The mixture was stirred until the orange color disappeared and a tan precipitate was formed. The mixture was cooled in ice and then washed in a solution of sodium hydrosulfite (1 g, 5.7 mmol) in water (50mL) to yield 2,4-diamino-1-naphthol (**36**) as a tan product. This product was converted to a dihydrochloride salt using 1 M HCl (15 mL), filtered under vacuum and washed with dilute HCl to give 2, 4-diamino-1-naphthol dihydrochloride as a stable salt. The vacuum filtration was done through activated charcoal to remove the oxidation product of **36**

The crude 2,4-diamino-1-naphthol dihydrochloride (10 mL, 56 mmol) was immediately converted to 2,4-diacetylamino-1-naphthol (**37**) by reacting it with acetic anhydride (3 mL, 31.7 mmol) in the presence of sodium acetate (3 g, 36.6 mmol). Sodium hydrosulfite (0.1g, 0.6 mmol) in water (30 mL) was then added to the mixture to obtain a white solid, which was collected by suction filtration and then dissolved in 0.27 M sodium hydroxide solution (27.5 mL) at room temperature to hydrolyze any triacetate present. The solution was acidified by gradual addition of 21 mL of 0.14 M hydrochloric acid. The solution was allowed to crystallize, filtered and washed with water

to provide 2,4-diacetamino-1-naphthol (**37**) (1.2 g, 76.6% yield) which was used without further purification.

The crude 2,4-diacetamino-1-naphthol (**37**) (0.92 g, 3.5 mmol) dissolved in hot acetic acid (5 mL) and diluted with hot water (10 mL) was reacted with 0.13 M iron (III) chloride solution (5 mL, 0.65 mmol). The resultant solution was cooled, filtered and the residue recrystalized from ethanol/water to obtain 2-acetylyamino-1,4- naphthoquinone (**38**) as yellow crystals (0.56 g, 74% yield).

3.3.4 Synthesis of 2-Amino-1,4-naphthoquinone (39)

A mixture of concentrated sulfuric acid (1 mL) and compound **38** (0.25 g, 1.16 mmol) was heated with swirling for five minutes to provide a deep red solution. This was then cooled, filtered and the residue washed with cold water to obtain a deep red solid which was recrystallized from ethanol/water to obtain red needle like crystals of 2-amino-1,4-naphthoquinone **(39)** (0.14 g, 70% yield).

Physicochemical properties; mp 205-2017 °C, M/z 174 (M+1), ^{1}H NMR (400 MHz, Acetonitrile-d3) δ 8.02 (*ddd*, *J*= 7.63, 1.42, 0.56 Hz, 1H), 7.98 (*ddd*, *J* = 7.66, 1.38, 0.55 Hz, 1H), 7.77 (*td*, *J* = 7.5, 7.54, 1.4 Hz, 1H), 7.69 (*td*, *J* = 7.5, 7.46, 1.4 Hz, 1H), 5.92 (*s*, 1H), 5.80 (*s*, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 188.2, 187.3, 139.8, 138.7, 137.4, 136.1, 134.7, 131.1, 130.7, 108.8.

3.4 *In Vitro* Antiplasmodial Assay

The antiplasmodial assay procedure of the target naphthoquinone was adopted from (Juma et al., 2011) using a modified none-radioactive assay method that utilized a flourochrome DNA dye called “SYBR Green I” (Johnson *et al.,* 2007; Smilkstein *et al.,* 2004) against chloroquine resistant K1 and chloroquine sensitive 3D7 isolate strains of *Plasmodium falciparum*

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Study outline

In this study, a searchable *in silico* database of natural products of Kenya (The *Mitishamba* Database, http://*Mitishamba*.uonbi.ac.ke/) was created. This was then queried for naphthoquinones, which were virtually screened against *Pf*DHODH to model antiplasmodial naphthoquinones. The synthetically accessible modeled naphthoquinones were synthesized and assayed against chloroquine sensitive and resistant strains of *Plasmodium falciparum*.

4.2 The *Mitishamba* Database of Natural Products of Kenya

The data mining of natural products of Kenya from various literature sources resulted in the collation of 1112 compounds. The name of the database was coined from a Swahili word '*Mitishamba*' which refers to traditional herbal medicine. Besides chemical structures, which can be searched and downloaded in different file formats, the database also provides information on the trivial name, IUPAC name, botanical source and physicochemical properties of each structure. The *Mitishamba* database is hosted at the University of Nairobi and is accessible through http://*Mitishamba*.uonbi.ac.ke, Figure 4.1.

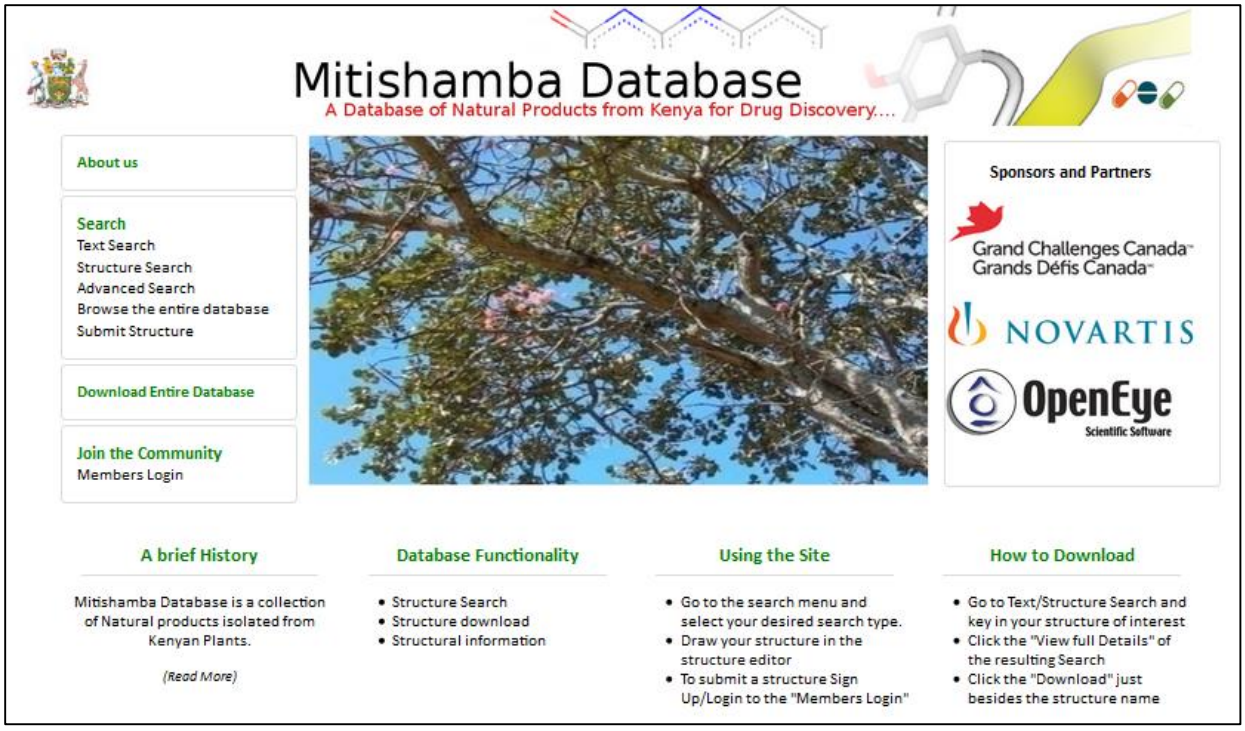


Figure 4:1: The *Mitishamba* database web page

The search engine for the database was developed based on the Norbert Haider's MolDB5R package (Haider, 2010) where the background structure searching and matching is performed by checkmol and matchmol programs. Checkmol reads the query structure to generate descriptors for a preliminary database search while matchmol does the full structure comparison of the input structure from checkmol (Figure 4.2) (Haider, 2010). A JavaScript molecular applet (JME applet) is incorporated in the structure search on the client side, where a client specified structure (query structure) or a substructure can be used to search the database. The internet acts as an interface between the client using a web-browser and the *Mitishamba* database (MySQL relational database) located in a server. The web server receives queries in form of PHP scripts from the browser,

interprets the scripts and creates SQL (structured query language) scripts to query the database. The results are sent back to the web server in the form of SQL, which are interpreted and then sent to the browser as PHP or HTML (hypertext markup language) scripts for further interpretation for the user, Figure 4.2.

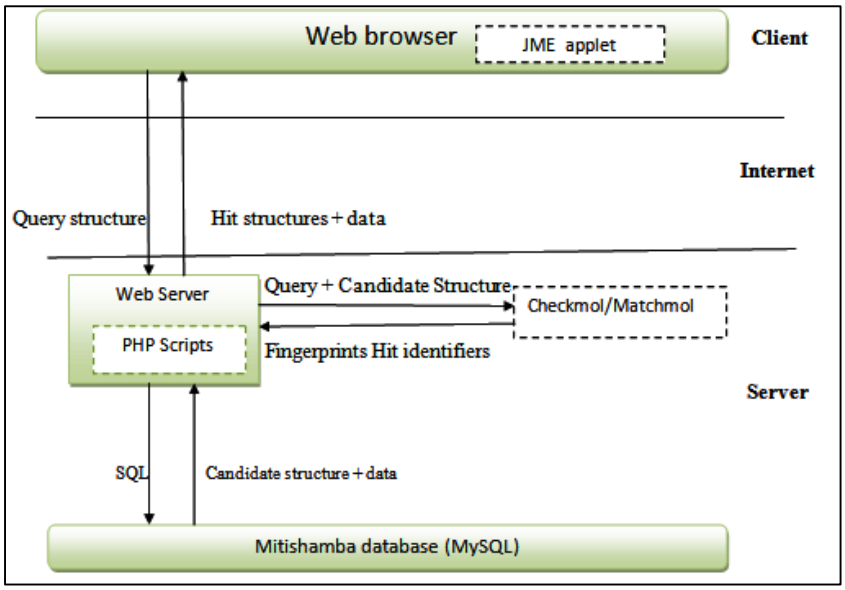


Figure 4:2: Framework for development of the *Mitishamba* database showing the clients/internet/server interaction (Adapted from Haider (2010))

4.3 Features of the *Mitishamba* Database

The *Mitishamba* database allows one to search for structures, browse and download the entire database or contribute to the database by submitting structures, Figure 4.3.

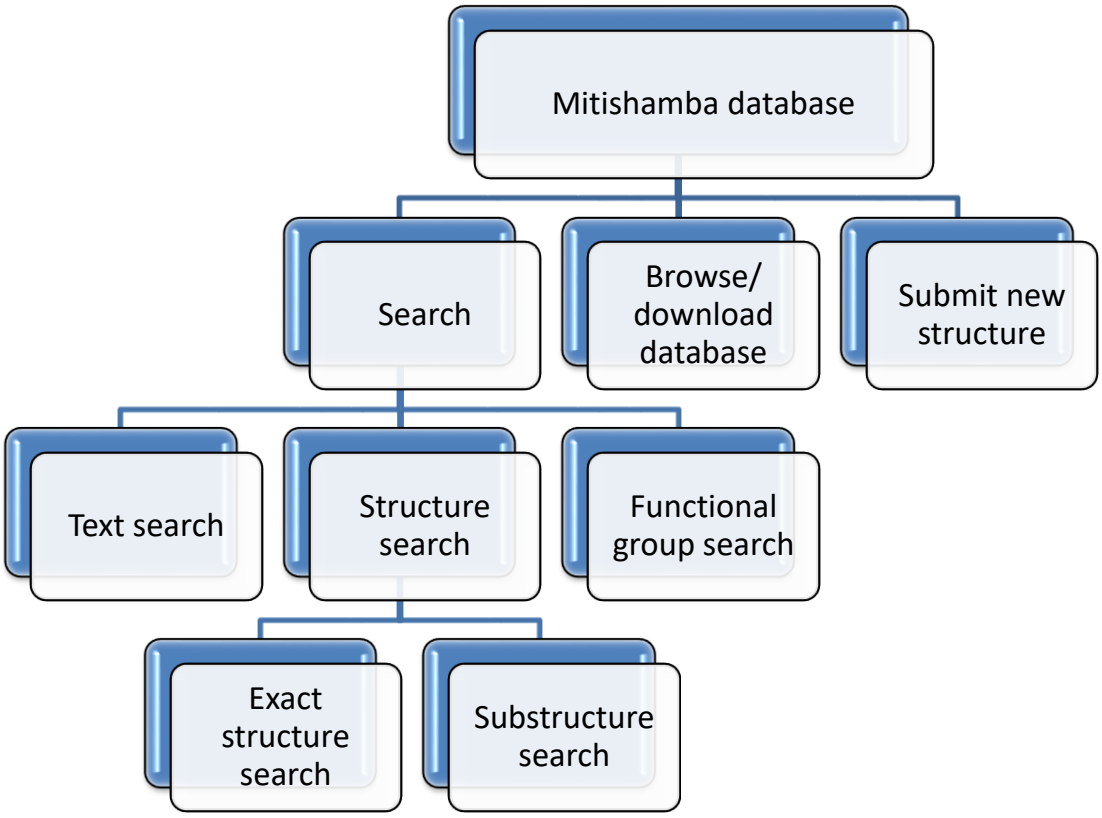


Figure 4:3: Features of the *Mitishamba* database

4.3.1 Search Options

The *Mitishamba* database allows for three structure search options: one can search using text, structure of functional group search. In the text search, users can do search based on IUPAC (Figure 4.4) or trivial name (Figure 4.5) of a compound.

Figure 4:4 : Structure text search using IUPAC name

Figure 4:5: Structure text search using trivial name

In structure search, a structure is drawn for an exact search (Figure 4.6) or substructure search (Figure 4.7). When conducting a substructure search, the output will be compounds that have the substructure in the database.

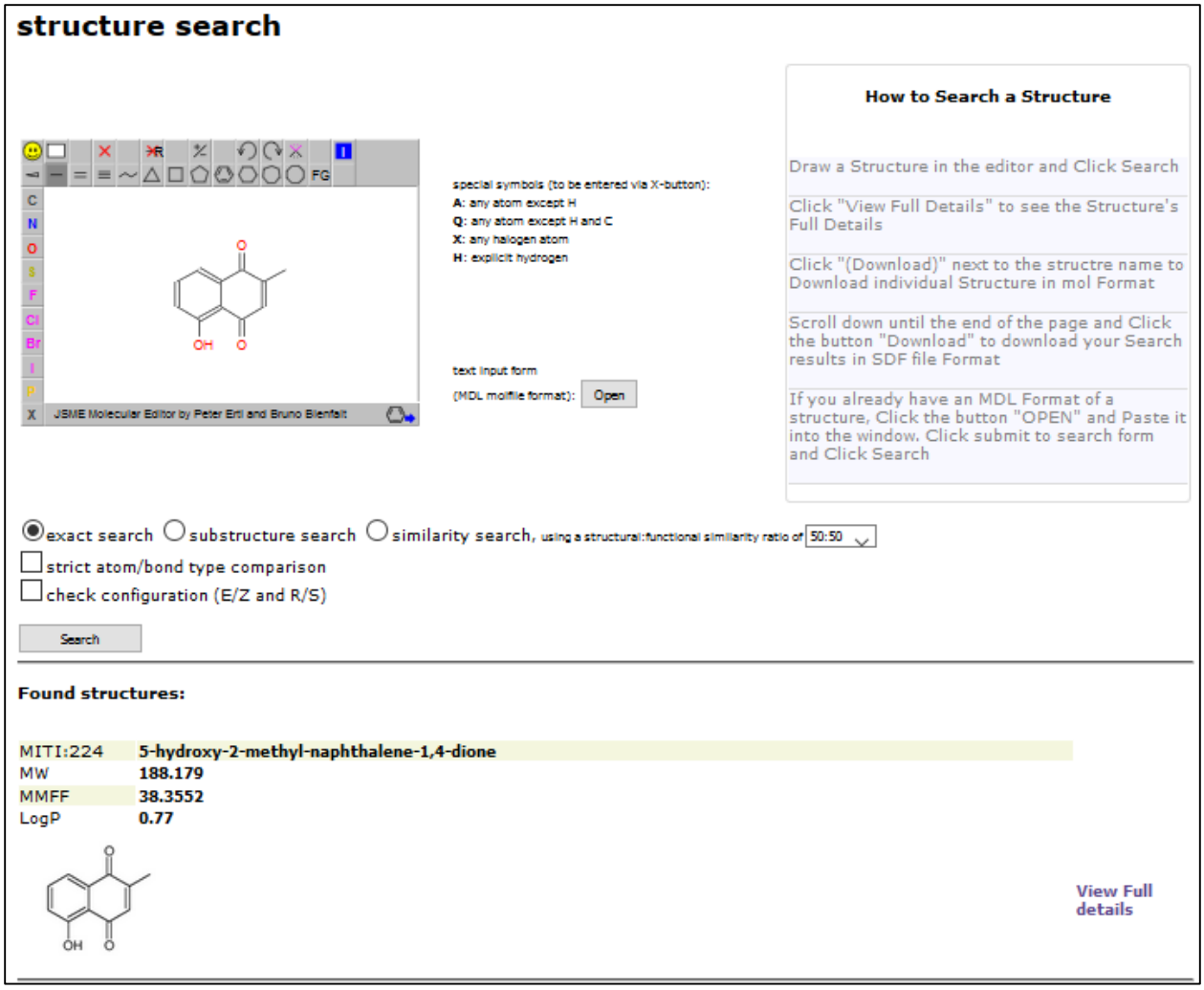


Figure 4:6: Structure search by exact structure search option

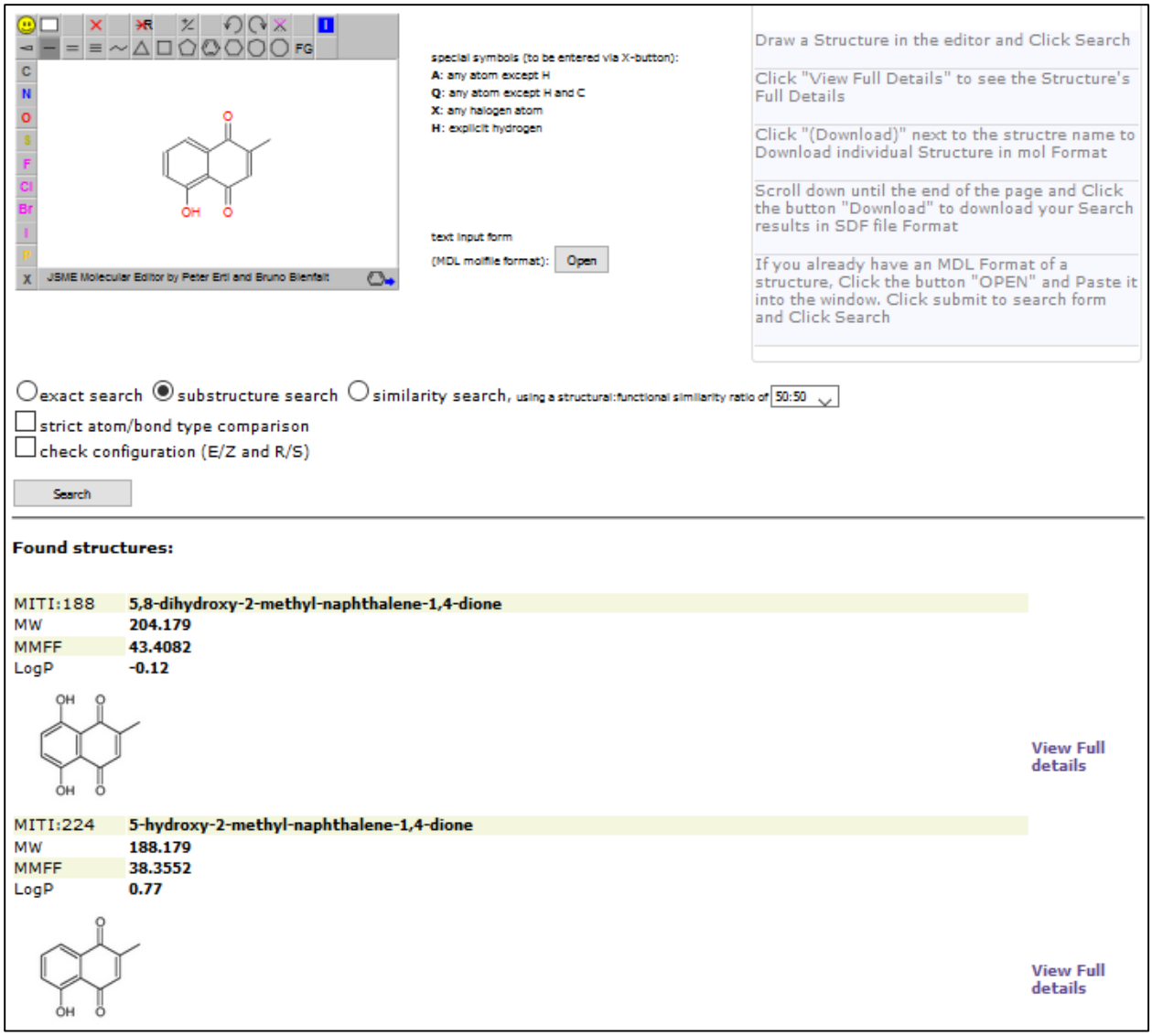


Figure 4:7: Structure search by Substructure search option

The database also allows for searching for compounds based on functional groups and the output will be that structure with the specified functional group, (Figure 4.8).

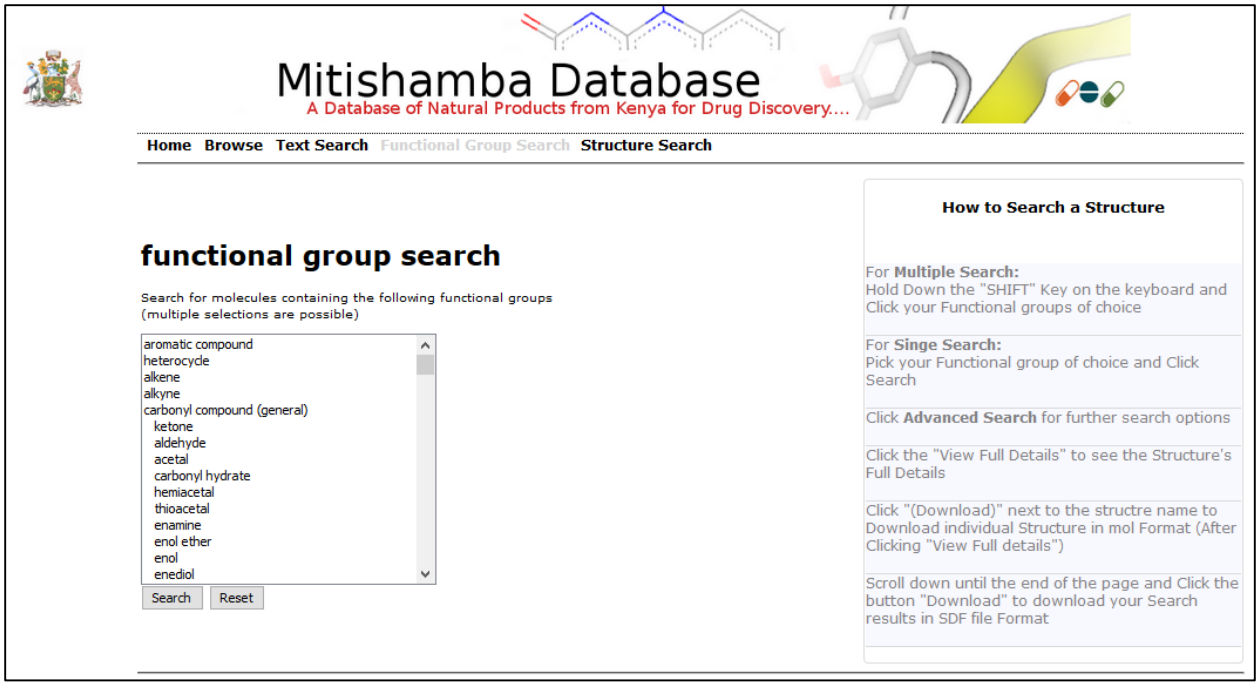


Figure 4:8: Structure search by functional group

For each output, full information on the compound can be retrieved by clicking on the “view full details” on the search output page. The details include physicochemical properties, botanical source (family, species and place of collection) and the references as illustrated in Figure 4.9.

You are viewing details for:

2-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione (Download)

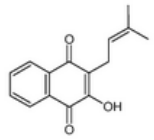


image not found

mw	242.27
mmff	42.5833
logp	1.7
psa	54
smiles	CC(=CCC1=C(C(=O)c2ccccc2C1=O)O)C
rotatable_bonds	2
hydrogen_acceptors	2
hydrogen_donors	1
heavy_atoms	18
plant_family	Bignoniaceae
plant_species	Kigelia africana
plant_part	Root bark
compound_type	Napthoquinone
common_name	Lapachol
authors	Dharani,N., and Abiy Y., (2010)Medicinal Plants of East Africa:An Illustrated guide.Nairobi.Kenya:NajmaDharani.
name	2-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione

Figure 4:9: Full details structure search output

4.3.2 Browsing and Downloading the Database

The database allows users to browse and download the entire database. Downloads are available in mol, sdf, oeb and smiles files formats.

4.3.3 Submitting Structure

Since the database is not comprehensive, there is need to upload more structures. It also requires continuous updating as new natural products are reported from plants of Kenya. Researchers working on the phytochemistry of plants of Kenya are encouraged to contribute by submitting structures of their compounds as a PDF file that contains the full details of the natural products

(name, botanical source, place, and biological activity if any). The database administrators will verify and update the database.

4.4 The Status of the *Mitishamba* Database within the Lead Space

Since the *Mitishamba* database is designed for use for drug discovery, the compounds in the database were assessed to determine their drug likeness and their position within the lead space. This was done by assessing how the compounds obey the Lipinski's rule of five; a rule that was developed after the analysis of compounds from the world drugs index database, with an aim of identifying physicochemical properties that were important for any orally active drug. These properties and values are molecular weight ≤ 500, hydrogen donors groups ≤ 5, hydrogen acceptors group ≤ 10 and calculated LogP value ≤+5 (Lipinski *et al.,* 2012). Table 4.1 gives the percentage of compounds in the *Mitishamba* database that satisfy the Lipinski's rule of five.

Table 4.1 : The percentage of compounds in the *Mitishamba* database that satisfy the Lipinski's rule of five

Physicochemical property	**% of Compounds that satisfy Lipinski's rule**
Molecular weight (MW)	80.3
Calculated Log P (cLog P)	84.2

Physicochemical property	**% of Compounds that satisfy Lipinski's rule**
Hydrogen bond acceptors (HBA)	90.1
Hydrogen bond donors (HBD)	87.1
Overall (MW, log P, HBA, HBD, RB)	55.5

As can be inferred from Table 4.1, 80.3% of the compounds have MW ≤ 500, 84.2 % of the compounds have cLogP ≤ +5, 90.1 % of the compounds have HBA ≤ 10 and 87.1 % of the compounds have HBD ≤ 5. Overall, when all the physicochemical properties are considered, 55.5 % of the compounds satisfy the Lipinski's rule of five and are therefore drug like. Extending the rule of five to rule of three for lead like structures, 55.4 % of the compounds were found to be lead like (MW ≤ 300, cLogP ≤ 3, HBA ≤ 3, HBD ≤ 3 and rotatable bonds (RB) ≤ 3). This is much better than synthetic databases used for CADD such as Zinc database which only has 29.4 % of the compounds being lead like (Harvey, 2015).

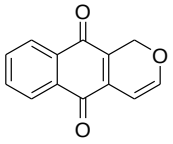
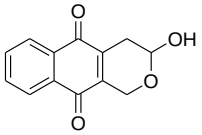
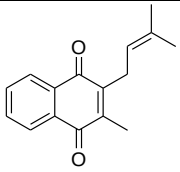
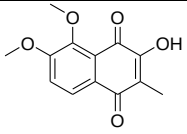
Furthermore, the physicochemical properties, polar surface area (PSA) and the numbers of rotatable bonds (RB) have been found to play an important role in oral bioavailability of drugs. In order for a drug to be orally bioavailable, the acceptable values for PSA and RB are ≤ 140 Å and ≤ 10, respectively (Veber *et al.*, 2002). It is worth noting that 82.7 % and 89.8 % of the compounds in the

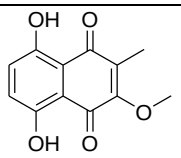
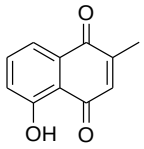
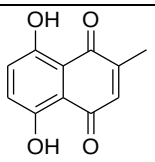
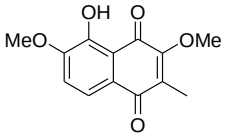
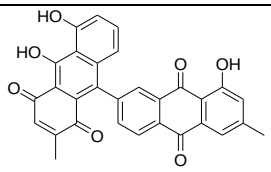
Mitishamba database have PSA ≤ 140 Å and RB ≤ 10. Therefore, the *Mitishamba* database can be considered as an attractive source for orally bioavailable lead like compounds for drug discovery.

4.5 The Library of 1,4-Naphthoquinones in the *Mitishamba* Database

A substructure search for the 1,4-naphthoquinone scaffold **(33)** was done on the *Mitishamba* database which yielded nine structures (Table 4.2).

Table 4.2: 1,4-Naphthoquinones in the *Mitishamba* database

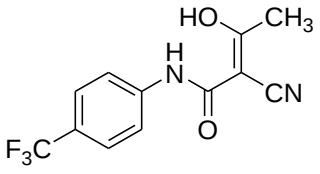
Name	**Structure**
Pentalongin **(4)**	
Psychorubin**(5)**	
Lapachol **(6)**	
3-Hydroxy-5,6-dimethoxy-2 methylnaphthalene-1,4-dione **(40)**	

Name	Structure
5,8-Dihydroxy-2-methoxy-3-methylnaphthalene-1,4-dione **(41)**	
Plumbagin **(42)**	
5,8-Dihydroxy-2-methyl-1,4-naphthoquinone **(43)**	
5-Hydroxy-3,6-dimethoxy-2-methylnaphthalene-1,4-dione **(44)**	
Abyquinone A **(45)**	

The 1,4-naphthoquinone library was prepared using *omega* to give energy minimized 3D structures. These were then used for virtual screening against a prepared *Pf*DHODH receptor.

4.6 Virtual Screening of 1,4-Naphthoquinones of the *Mitishamba* Database against the *Pf*DHODH Receptor

The prepared structures were evaluated for their drug-likeness and only those with favorable drug like properties were docked. The *hybrid* program was used in the docking process because it utilizes the bound ligand, (2Z)-2-cyano-3-hydroxy-*N*-[4-(trifluoromethyl) phenyl] but-2-enamide (**A77 1726**) (**46**), information and performs a systematic and exhaustive examination of all possible poses within the *Pf*DHODH receptor's active sites (Figure 4.10). It filters and ranks poses based on their shape and chemical complementarity to a known bound ligand and scores using the Chemgauss4 scoring function.



46

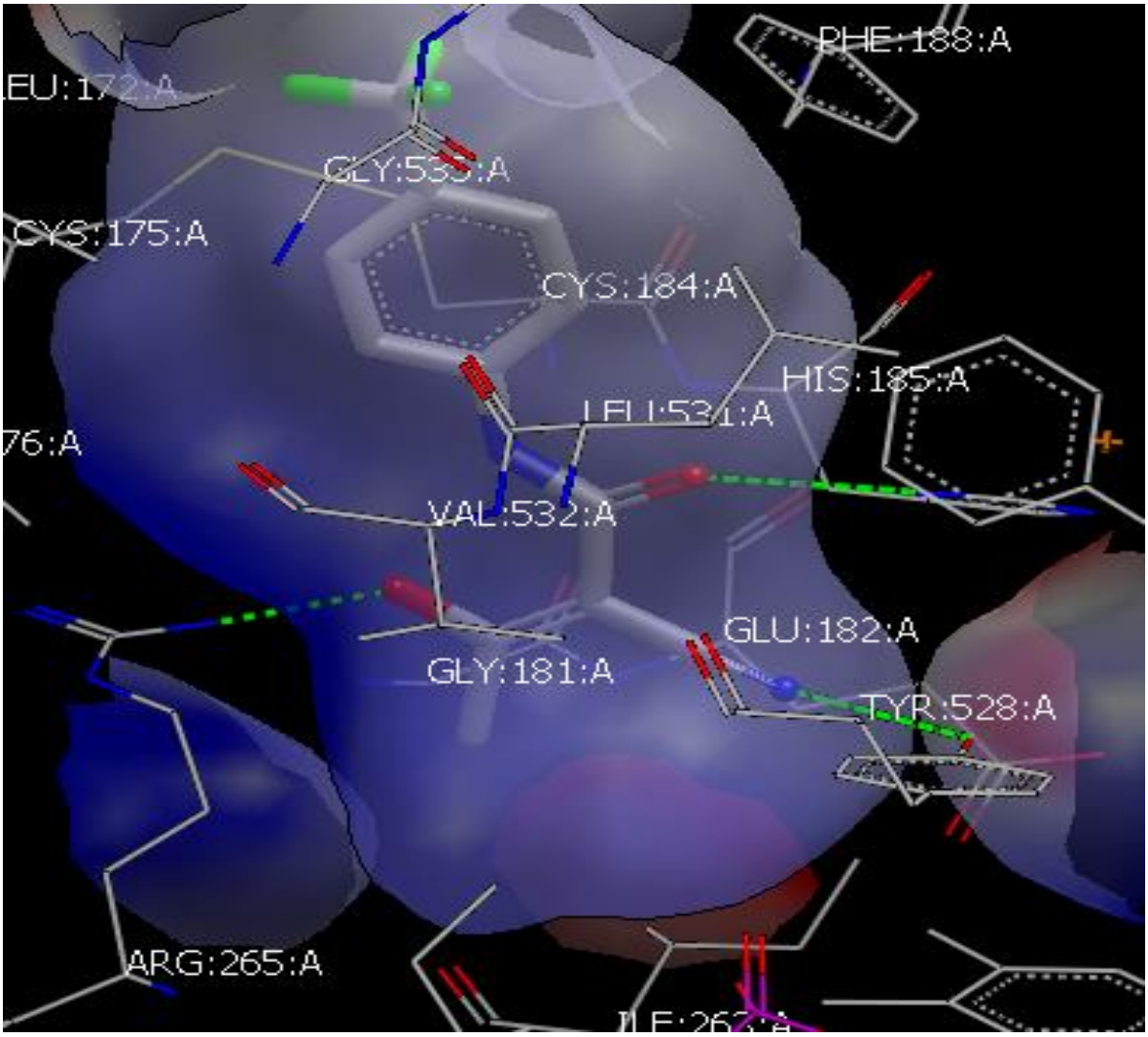
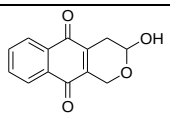
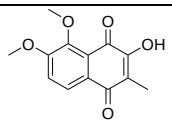
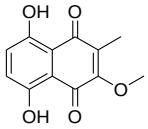
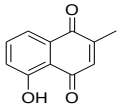
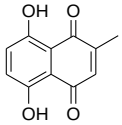
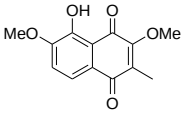
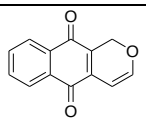
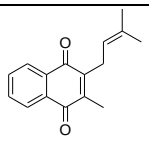
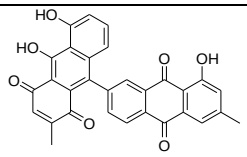


Figure 4:10: The active site of *Pf*DHODH

The ranked results of the virtual screening of the 1,4-naphthoquinones in the *Mitishamba* database against *Pf*DHODH are tabulated in Table 4.3.

Table 4.3: Ranking of the hybrid docking results of *Mitishamba* database of 1,4-naphthoquinones

Ranking	**Structure**	**Name**	**Hybrid Score**
1		Psychorubin**(5)**	-11.03
2		3-Hydroxy-5,6-dimethoxy-2 methylnaphthalene-1,4-dione**(40)**	-10.76
3		5,8-Dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione**(41)**	-10.52
4		Plumbagin **(42)**	-10.51
5		5,8-Dihydroxy-2-methyl-1,4-naphthoquinone **(43)**	-10.40
6		5-Hydroxy-3,6-dimethoxy-2-methylnaphthalene-1,4-dione **(44)**	-10.25
7		Pentalongin **(4)**	-9.97

Ranking	Structure	Name	Hybrid Score
8		Lapachol **(6)**	-8.92
9		Abyquinone A **(45)**	Rejected

Among the 1,4-napthoquinones of the database, psychorubin **(5)** exhibited the best pose. This compound was isolated from the root extract of *Pentas longiflora* and has been shown to exhibit high *in vitro* antiplasmodial activity; IC_{50} of 0.91 ± 0.15 and 0.82 ± 0.24 μg/mL against chloroquine resistant (W2) and sensitive (D6) strains of *Plasmodium falciparum,* respectively (Endale *et al.*, 2012).

4.7 Comparison between the Binding Interactions of Psychorubin (5) and A77 1726 (46) with the *Pf*DHODH Receptor Binding site

Noting the importance of shape, size and intermolecular interactions in the binding of a ligand and a receptor, a comparison was made between the interaction between the best performing 1,4-naphthoquinone (psychorubin (**5**)) and the bound inhibitor A77 1726 (**46**). It is apparent that the inhibitor's interaction with the *Pf*DHODH receptor is mainly hydrophobic and through hydrogen bonds with the three polar amino acids Arg265, Tyr528A and His185, Figure 4.11. The interaction

of psychorubin (**5**) with the binding site is also mainly hydrophobic but forms hydrogen bonds with four amino acid residues (Ala225A, Lys429A, Ser477A, Lys229A) as illustrated in the docking report, Figure 4.12. Considering that the 1,4-naphthoquinone psychorubin (**5**) had more interactions with the receptor than the inhibitor, it is worth modeling 1,4-naphthoquinones to enhance these binding interactions.

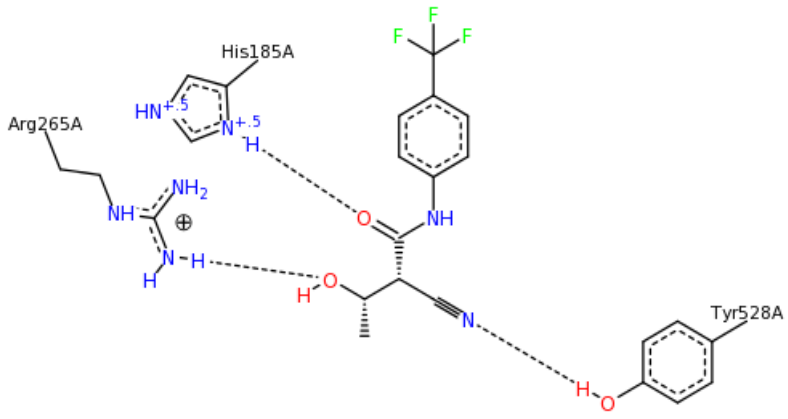


Figure 4:11: Binding interactions of A77 1726 (**46**) with the *Pf*DHODH receptor

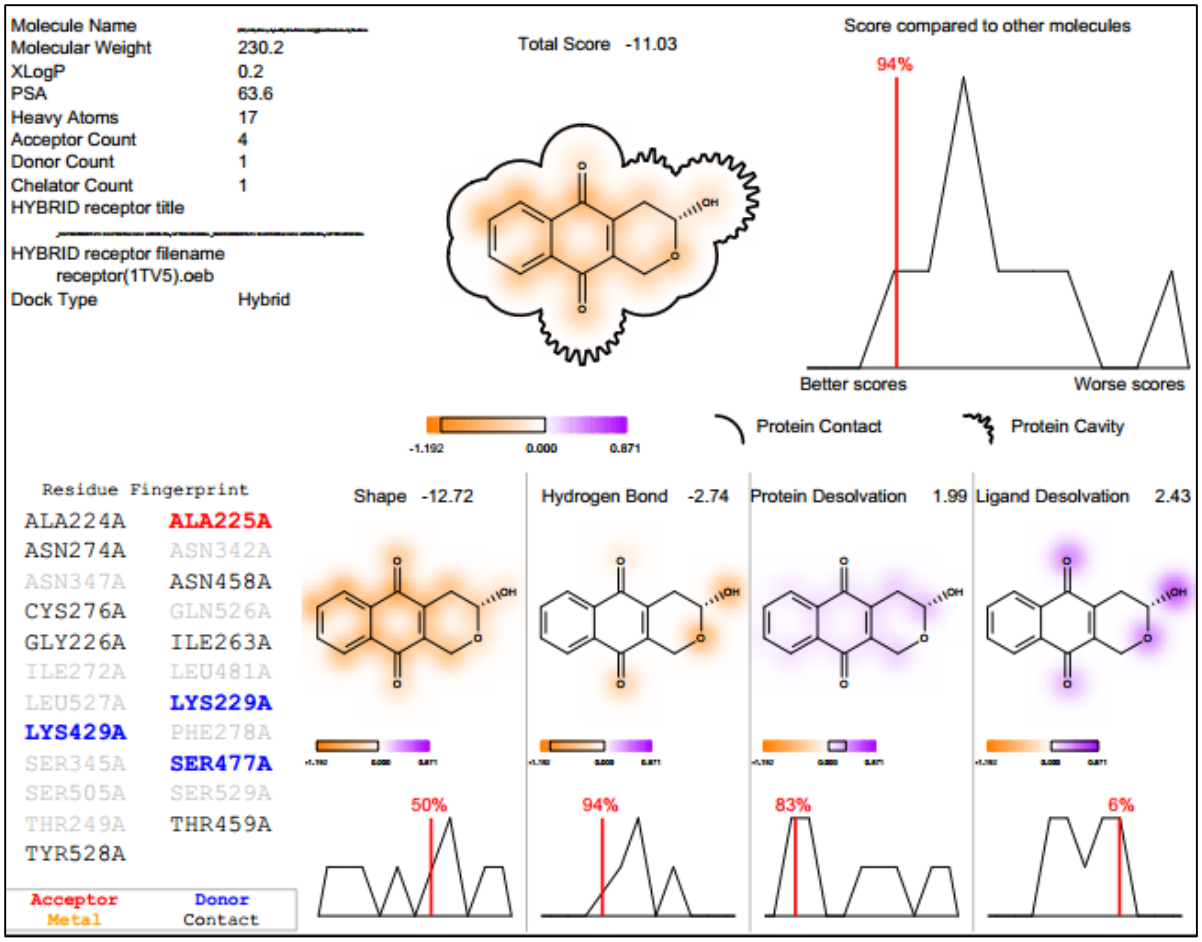


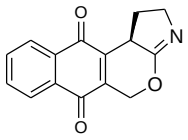
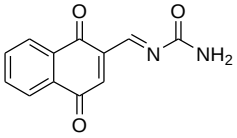
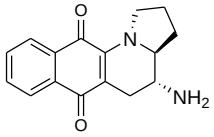
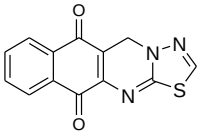
Figure 4:12: Docking report for psychorubin against *Pf*DHODH

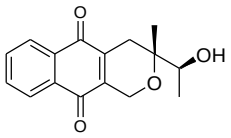
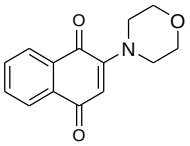
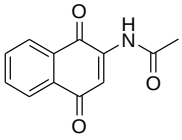
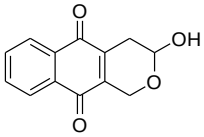
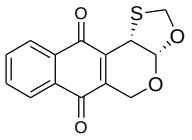
4.8 Modelling of Potent and Synthetically Accessible 1,4-Naphthoquinones

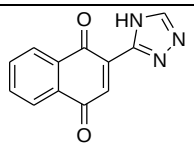
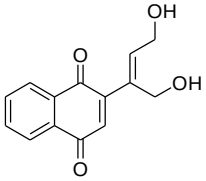
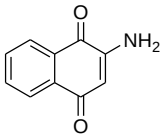
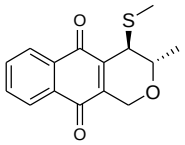
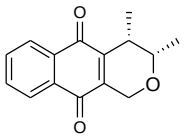
Guided by the docking score, psychorubin was modeled with the aim of improving potency (hybrid score) and the identification of synthetically accessible analogues. The *Brood* program was used to explore the chemical space around the 1,4-napthoqinone scaffold by replacing different fragments on the scaffold with those that have similar shape and electrostatics in order to generate

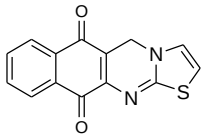
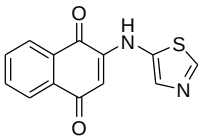
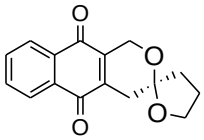
different drug-like analogues (compounds **38, 39, 47-60**), which were docked and their hybrid scores tabulated, Table 4.4.

Table 4.4: Hybrid docking scores for the 1,4-naphthoquinone model structures

Structure	**Hybrid score**
 **47**	-12.88
 **48**	-12.44
 **49**	-12.20
 **50**	-11.62

Structure	Hybrid score
 **51**	-11.56
 **52**	-11.15
 **38**	-11.13
 **5**	-11.03
 **53**	-10.84

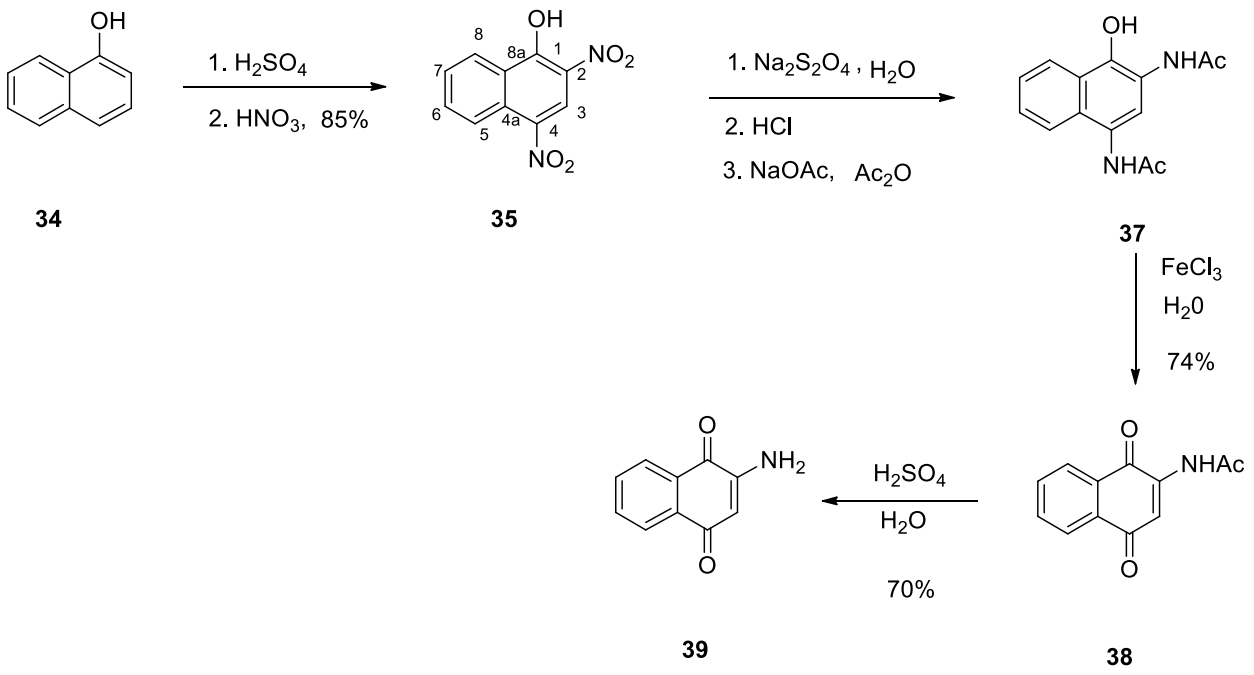
Structure	Hybrid score
 **54**	-9.89
 **55**	-9.85
 **39**	-9.82
 **56**	-9.76
 **57**	-9.68

Structure	Hybrid score
 **58**	-9.59
 **59**	-9.45
 **60**	Rejected

Among the above compounds, the synthetically accessible compounds **38** and **39** were synthesized and evaluated for their *in vitro* antiplasmodial activities.

4.9 Synthesis of the Target 1,4-Naphthoquinones

The synthetically accessible 1,4-naphthoquinones that were identified from the modelling study were synthesized based on a literature protocol (Fieser, 2003) as outlined in Scheme 4.1. Each of the steps of the synthesis are discussed in Sections 4.9.1 – 4.9.3.



34 **35** **37** **38** **39**

Scheme 4.1: Synthesis of the target naphthoquinones

4.9.1 Synthesis of 2,4-Dinitro-1-naphthol (35)

2,4-Dinitro-1-naphthol (**35**) was obtained by sulfonating 1-naphthol (**34**) and treating the sulfonated product (disulfonic acid) with nitric acid. This two-step indirect method of introducing the nitro groups was chosen to avoid side products arising from the oxidation of compound **34**. The kinetic product (disulfonic acid) is less liable to oxidation compared to 1-naphthol. 2,4-Dinitro-1-naphthol (**35**) was obtained in high yield (85%) as orange crystals.

The structure of this compound was determined using NMR. The ^{13}C NMR showed peaks for resonating ten carbons (Table 4.5). Using HSQC the ten carbons were categorized as quaternary (C-1, C-2, C-4, 4a and 8a) and methine (C-5, C-6, C-7 and C-8). The peak at δ_C 157.7 was assigned

to a phenolic carbon at C-1. The other carbons were assigned using a combination of HSQC and HMBC. In the ^{13}C NMR (126 MHz, Acetone) at δ_C 157.7, 128.6, 120.1, 138.4, 123.8 134.0, 128.6, 125.4, 126.6, 125.9. The signal at δ_C 157.7 was assigned to an oxygenated carbon while the peaks at δ_C 128.6 and 138.4 to the nitrated carbon.

The assignment of the ^{1}H NMR chemical shifts was as follows: δ8.97 (*s*, 1H), 8.67 (*dt J*=8.72, 0.82 Hz, 1H), 8.10 (*ddd, J* = 8.54, 7.00, 1.36 Hz, 1H), 7.92 (*ddd, J* = 8.24, 7.01, 1.07 Hz, 1H) and 8.70 (*ddd, J*= 8.46, 1.32, 0.64 Hz, 1H)

Table 4.5: NMR chemical shifts for 2,4-dinitro-1-naphthol (**35**)

Position	**δ_C (ppm)**	**δ_H (ppm), *m*, (*J* in Hz)**	**HMBC correlations**
1	157.7		
2	128.6		
3	120.1	8.97 *s*	C-1, C-4a, C-4, C-2
4	138.4		
5	123.8	8.67 *dt* (8.72, 0.82, 0.82)	C-4a, C-4
6	134.0	8.10 *ddd* (8.54, 7.00, 1.36)	C-4a

Position	δ_C (ppm)	δ_H (ppm), *m*, (*J* in Hz)	HMBC correlations
7	128.6	7.92 *ddd* (8.24, 7.01, 1.07)	C-8a
8	125.4	8.70 *ddd* (8.46, 1.32, 0.64)	C-1
4a	126.6		
8a	125.9		

4.9.2 Synthesis of 2-Acetylamino-1,4-Naphthoquinone (38)

2,4-Dinitro-1-naphthol (**35**) was reduced to 2,4-diamino-1-naphthol (**36**) using sodium hydrosulfite which was them immediately converted into its more stable salt 2,4-diamino-1-naphthol dihydrochloride. The salt was acetylated using acetic anhydride in the presence of sodium acetate to yield 2,4-diacetylamino-1-naphthol (**37**), which was then reacted with ferric chloride to obtain 2-acetylamino-1,4-naphthoquinone (**38**).

4.9.3 Synthesis of 2-Amino-1,4-Naphthoquinone (39)

2-Amino-1,4-naphthoquinone (**39)** was obtained thorough hydrolysis of 2-acetylamino-1,4-naphthoquinone (**38**) using concentrated sulfuric acid. The structure of this compound was determined using NMR. The ^{13}C NMR showed peaks for ten carbons resonating at δ_C 188.2, 187.3,

139.8, 138.7, 137.4, 136.1, 134.7, 131.1, 130.7 and 108.8. The signals at δ_C 187.3 and 188.2 were assigned to the two carbonyl carbons (C-1 and C-4, respectively) while the signal at δ_C 138.7, 136.1 and 134.7 was assigned to C-2, C-8a and C-4a, respectively. The peaks at δ_C 108.8, 139.8, 137.4, 131.1 and 130.7 were assigned to C-3, C-6, C-7, C-8, and C-5, respectively.

The ^{1}H NMR peaks were assigned as follows: δ 8.02 (*ddd, J*= 7.63, 1.42, 0.56 Hz, 1H), 7.98 (*ddd, J* = 7.66, 1.38, 0.55 Hz, 1H), 7.77 (*td, J* = 7.5, 1.4 Hz, 1H), 7.69 (*td, J* = 7.5, 1.4 Hz, 1H), 5.92 (*s*, 1H), 5.80 (*br,s*, , NH2), Table 4.6.

Table 4.6: NMR chemical shifts for 2-amino-1,4-naphthoquinone (39)

Position	**δ_C (ppm)**	**δ_H (ppm), *m*, (*J* in Hz)**
1	187.3	
2	138.7	
3	108.8	5.92 (*s*, 1H)
4	188.2	
5	130.7	8.02 *ddd* (7.63, 1.42, 0.56)
6	139.8	7.77 *td*, (7.57, 7.54, 1.4)
7	137.4	7.69 *td*, (7.52, 7.46, 1.37)

8	131.1	7.98 *ddd* (7.66, 1.38, 0.55)
4a	134.7	
8a	136.1	
2-NH_2		5.80 *br,s*

4.10 *In vitro* Antiplasmodial Activity

The modeling of 1,4-napthoquinones of the *Mitishamba* database identified potential *Pf*DHODH inhibitors which were synthesized and their antiplasmodial activities determined against Chloroquine resistant K1 isolate and Chloroquine sensitive 3D7. The results are tabulated in **Table 4.7**

Table 4.7: *In vitro* antiplasmodial activity of the synthesized compounds

Sample	**IC_{50} (µg/mL)** **CQ KI**	**IC_{50} (µg/mL) CQ 3D7**
2,4-Dinitro-1-naphthol (**35**)	1.67 ± 0.20	4.22 ±2.99

Sample	**IC_{50} (µg/mL)** **CQ KI**	**IC_{50} (µg/mL) CQ 3D7**
2-Acetylamino-1,4 naphthoquinone (**38**)	8.23 ± 1.67	3.86 ± 1.21
2-Amino-1,4-naphthoquinone (**39**)	24.74 ±3.56	12.51 ± 1.19
Chloroquine*	0.46±0.04	0.0063±0.0022
Mefloquine*	1.15±1.97	0.00084±0.0004

*Standards

Among the compounds assayed, compound **35,** one of the intermediates in the synthesis of **38**, was found to be the most active against the K1 isolate while compound **38** was most active against the 3D7 isolate, with values falling within the WHO recommended range for highly active compounds (less than 10 µg/mL). The rest of the values were found to be within WHO moderately active region that is IC_{50} values between 10 and 50 µg/mL.

Structure **35** exhibited activity of 1.67 ± 0.20 µg/ml against the chloroquine resistant K1 isolate and 4.22 µg/ml against the chloroquine sensitive 3D7 isolate. 2-Acetylamino-1,4 naphthoquinone (**38**) had an activity of 8.23 ± 1.67 µg/mL against the chloroquine resistant K1 isolate and 3.86 ± 1.21 µg/ml against the chloroquine sensitive 3D7 isolate. It is noteworthy, from the docking report

(Figure 4.13) that compound **35** interacts favorably with the receptor and forms hydrogen bonds with more amino acid residues (Asn342A, Lys429A, Ser505A, Thr249A, Ser477A) compared to **38** and **39**.

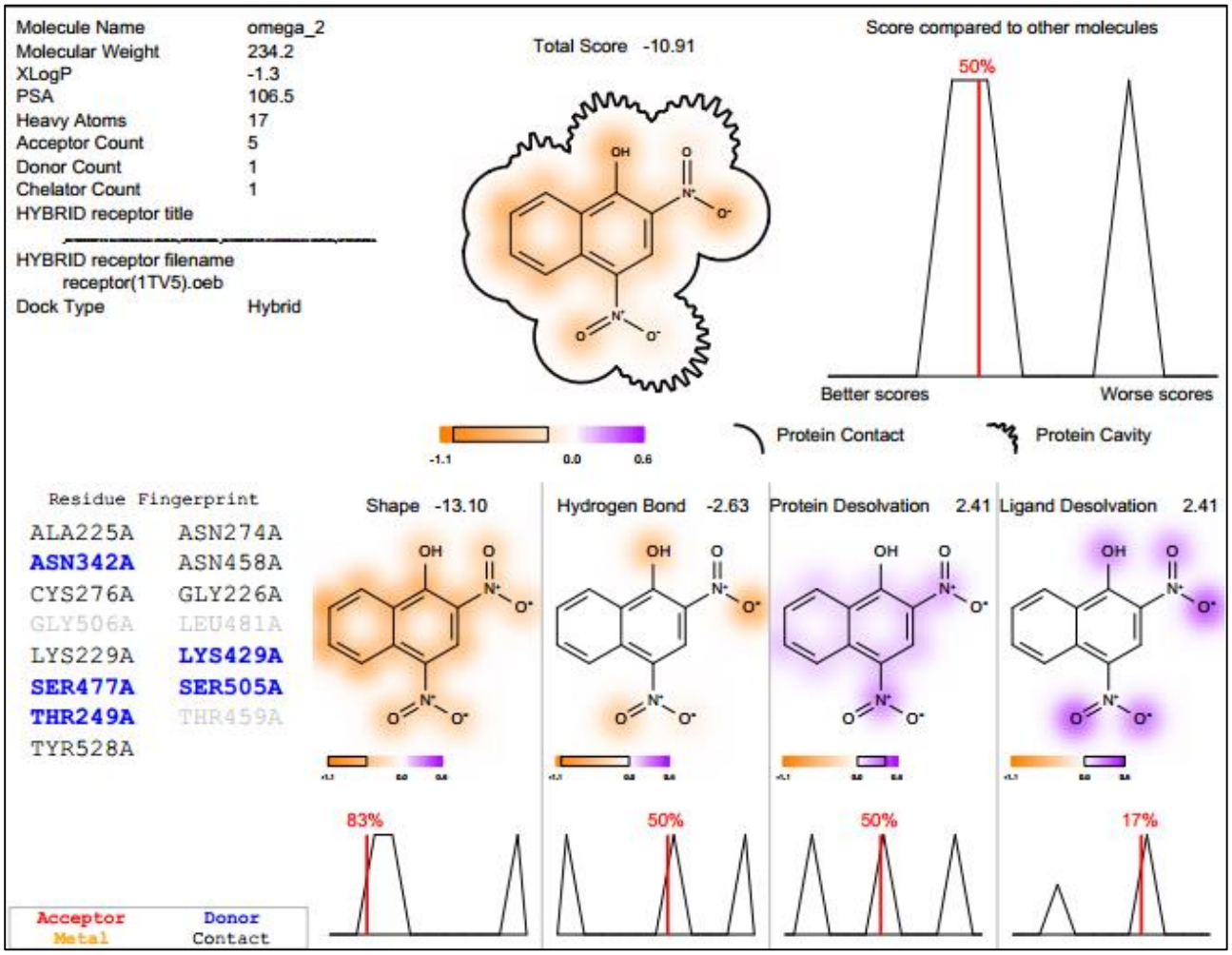


Figure 4:13: Docking report for 2,4-dinitro-1-naphthol (**35**)

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

The main objective of this study was to develop a web-based *in silico* database of natural products of Kenya for design and synthesis of antiplasmodial compounds based on the 1,4-napthoquinone scaffold with the following specific objectives: Develop a searchable web-based *in silico* database of natural products of Kenya, evaluate the antiplasmodial potential of the 1,4-naphthoquinones in the database by virtual screening against *Plasmodium falciparum* dihydroorate dehydrogenase enzyme (*Pf*DHODH), synthesize the synthetically accessible 1,4-naphthoquinones with high virtual *Pf*DHODH inhibition and evaluate their antiplasmodial activity by *in vitro* assay. Summarized below are the conclusions and recommendations of the study.

5.1 Conclusions

In this study:

i. A web-based database of natural products of Kenya called the *Mitishamba* database, (http://*Mitishamba*.uonbi.ac.ke/) was generated.
ii. Structure based virtual screening of the 1,4-napthoquinones in the database identified psychorubin **(5)** as a promising inhibitor of the *Pf*DHODH enzyme. This was used to model 1,4-naphthoquinone analogues to improve virtual activity and synthetic accessibility leading to the selection of 2-acetylamino-1,4 naphthoquinone (**38**) and 2-amino-1,4-naphthoquinone (**39**) for synthesis and bioassay.

iii. Among the I,4-naphthoquinones synthesized, 2-acetylamino-1,4 naphthoquinone (**38**) was found to be highly active after evaluation in an *in vitro* antiplasmodial assay.

5.2 Recommendations

The *Mitishamba* database is the first searchable database of natural products of Kenya and is an essential tool than can be used in natural products and medicinal chemistry research for drug discovery. Since the database contains over a thousand diverse lead-like molecules, I recommend that:

i. The database be periodically updated so as to capture any new natural products of Kenya that are isolated.
ii. Other scaffolds be identified in database, modeled and investigated for anti-infective activity against other diseases.
iii. The antiplasmodial assay should be focused towards the targeted enzyme (*Pf*DHODH) to assess its correlation with virtual screening results.

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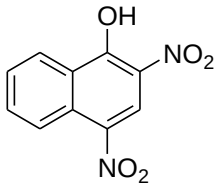
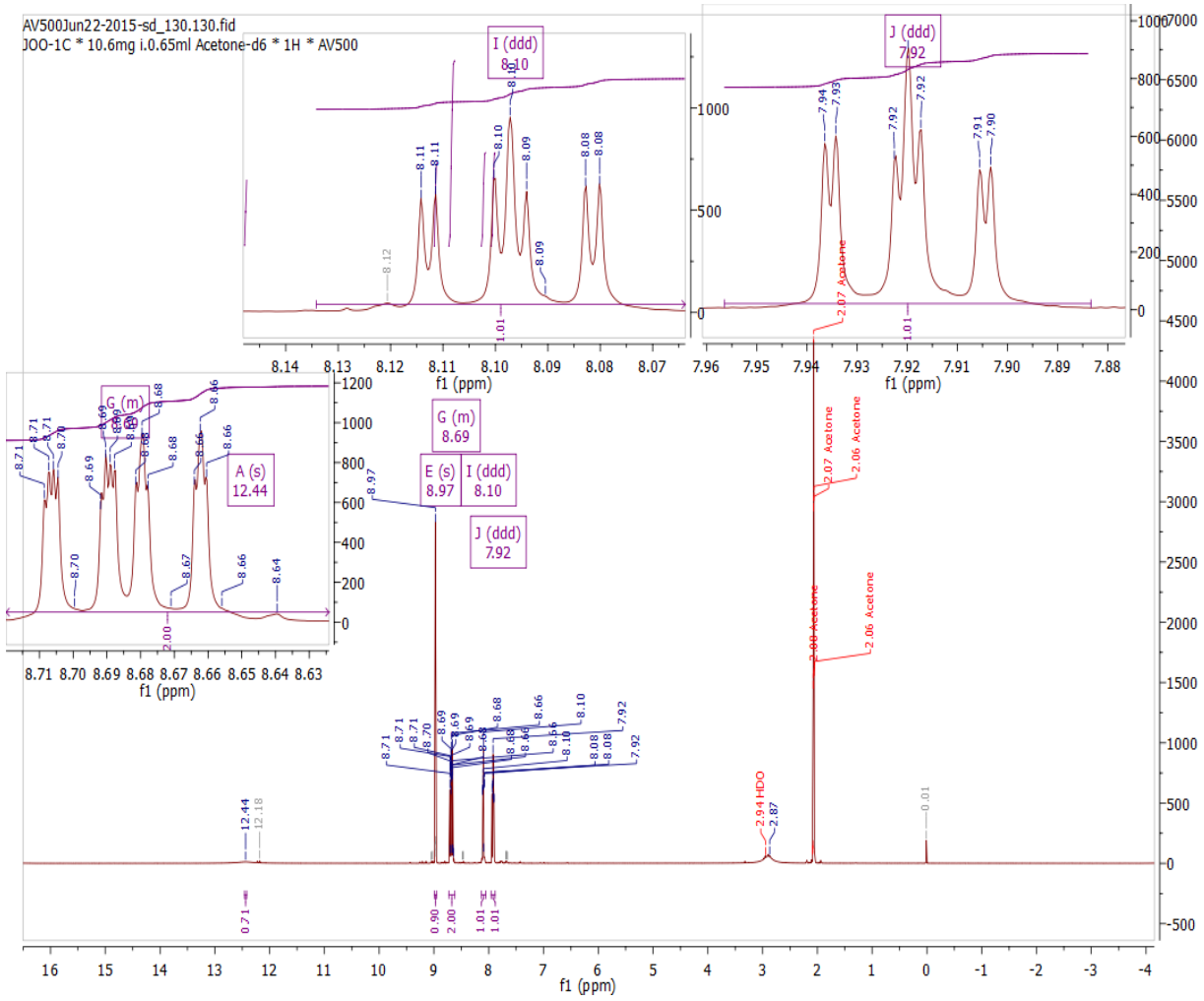
APPENDICES

Appendix A: NMR Spectra for 2,4-Dinitro-1-naphthol (35)

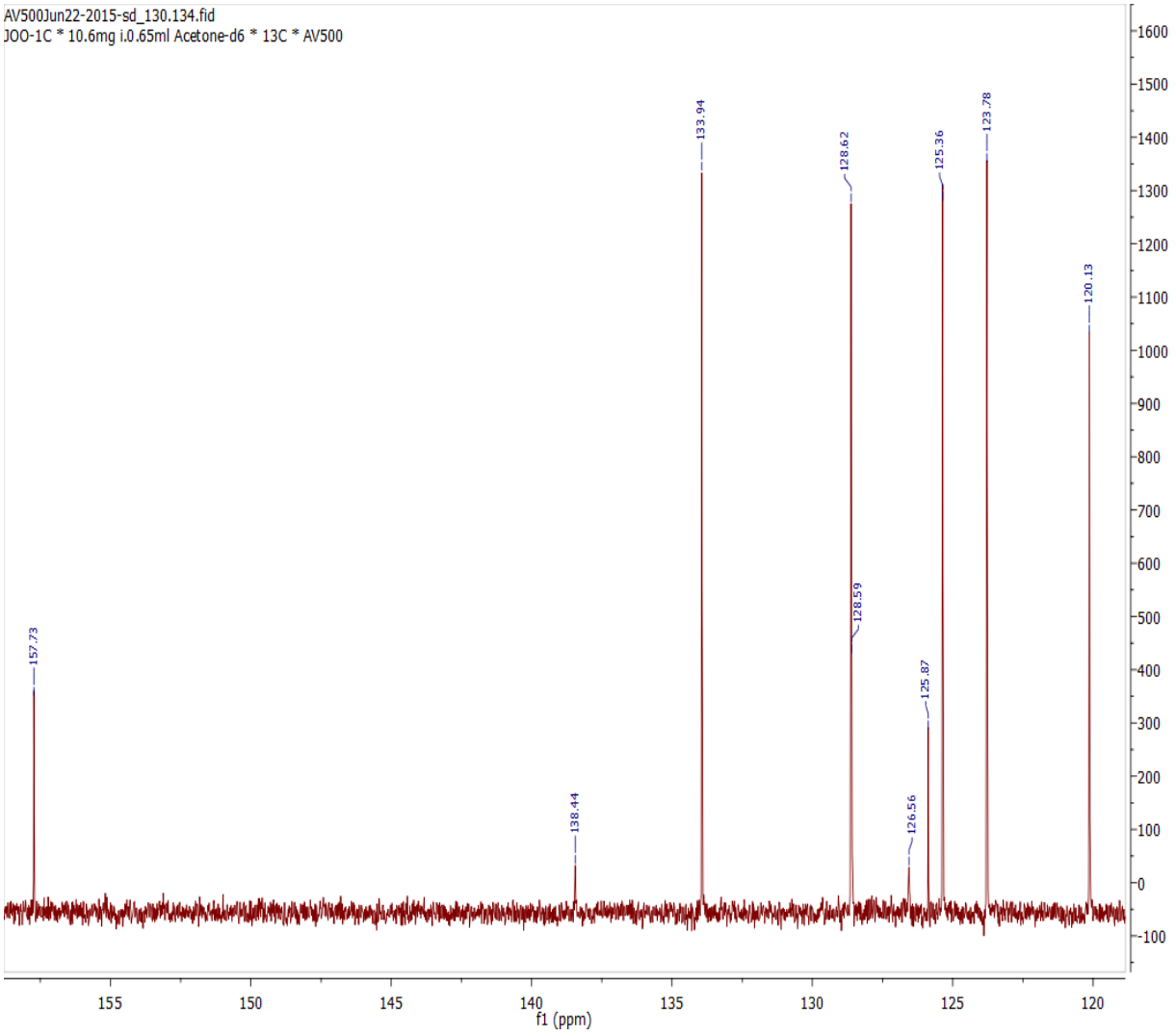


35

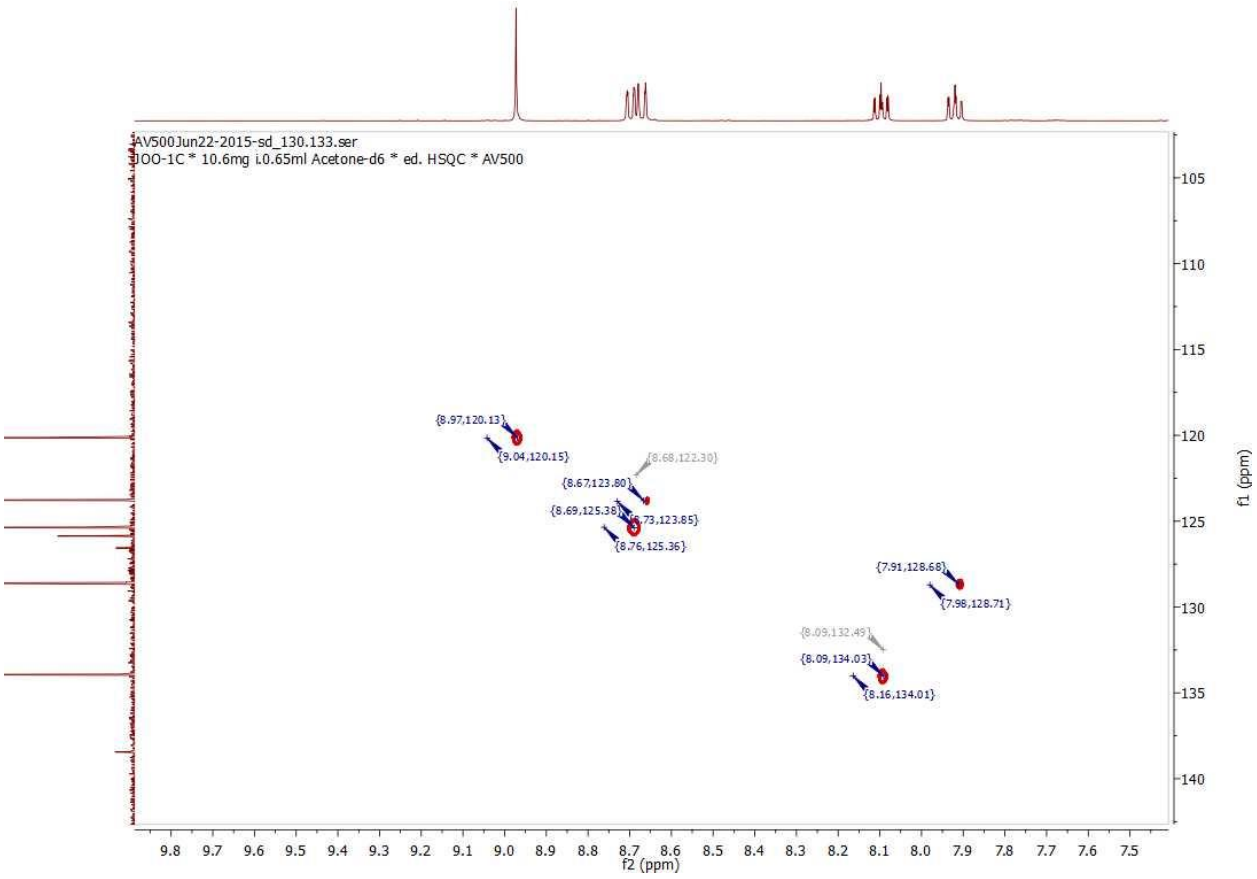
[1]HNMR Spectrum for **35**



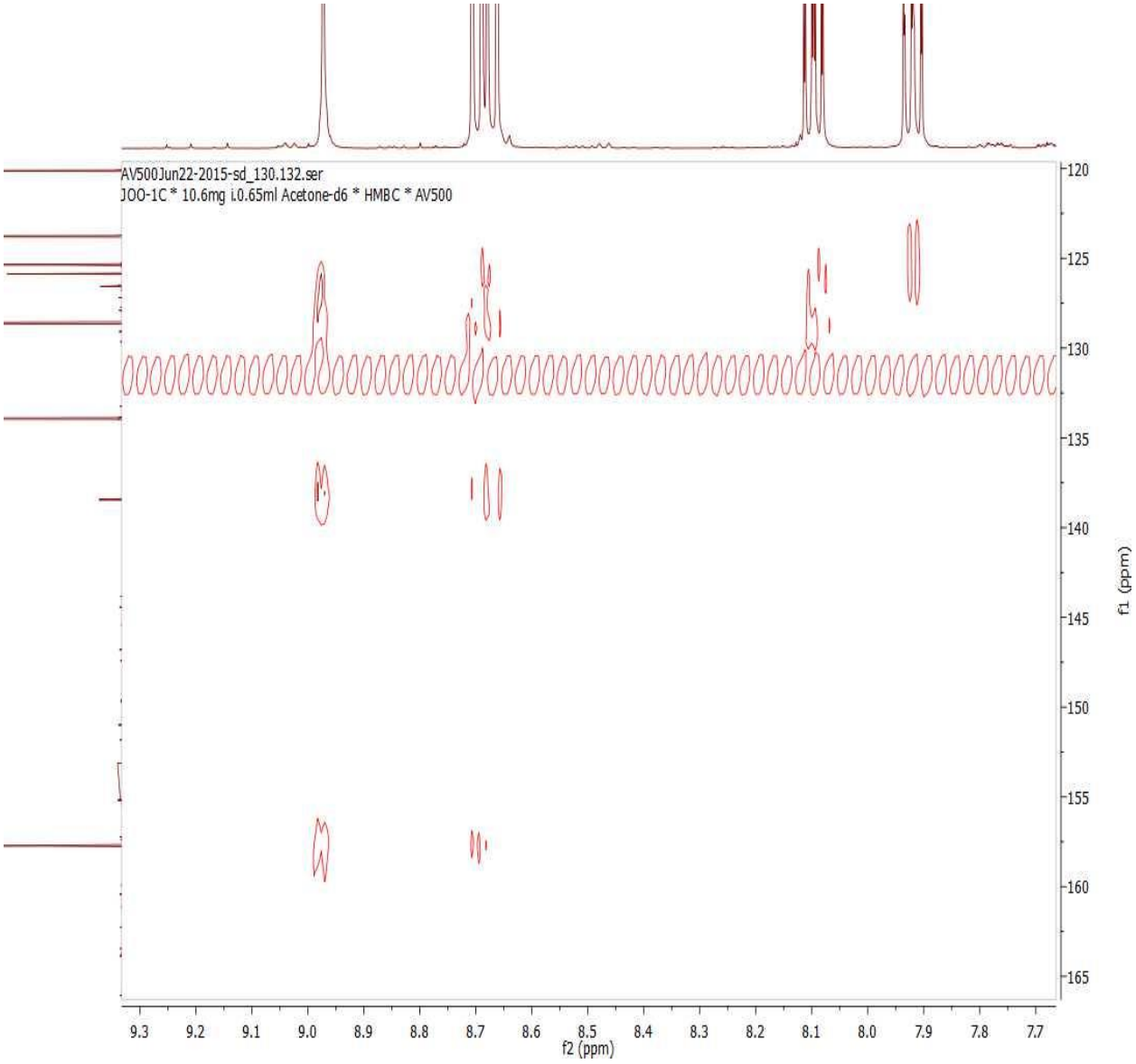
^{13}C NMR Spectrum for **35**



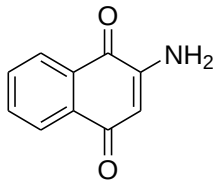
HSQC Spectrum for **35**



HMBC Spectrum for **35**

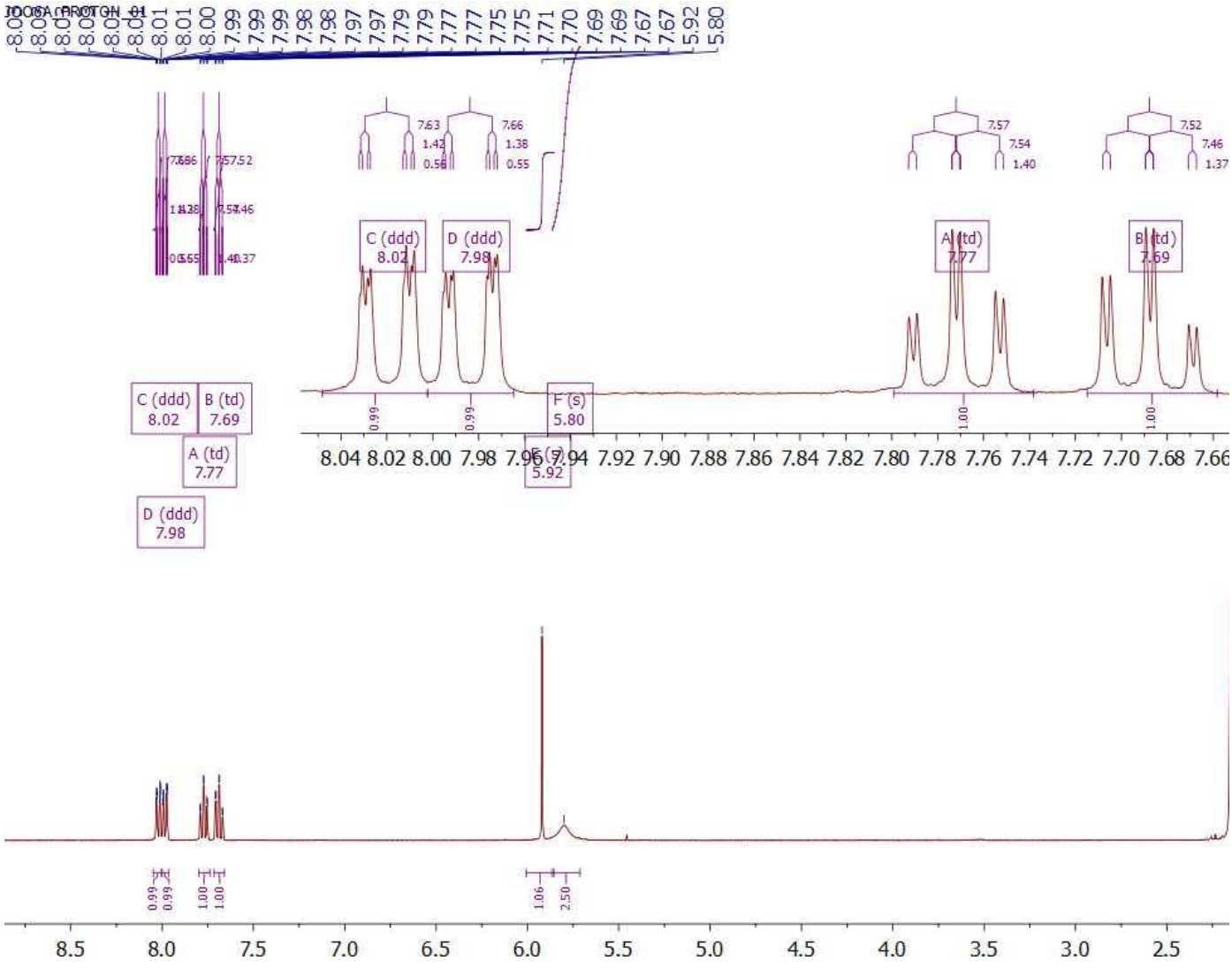


Appendix B: NMR Spectra for 2-amino-1,4-naphthoquinone (39)

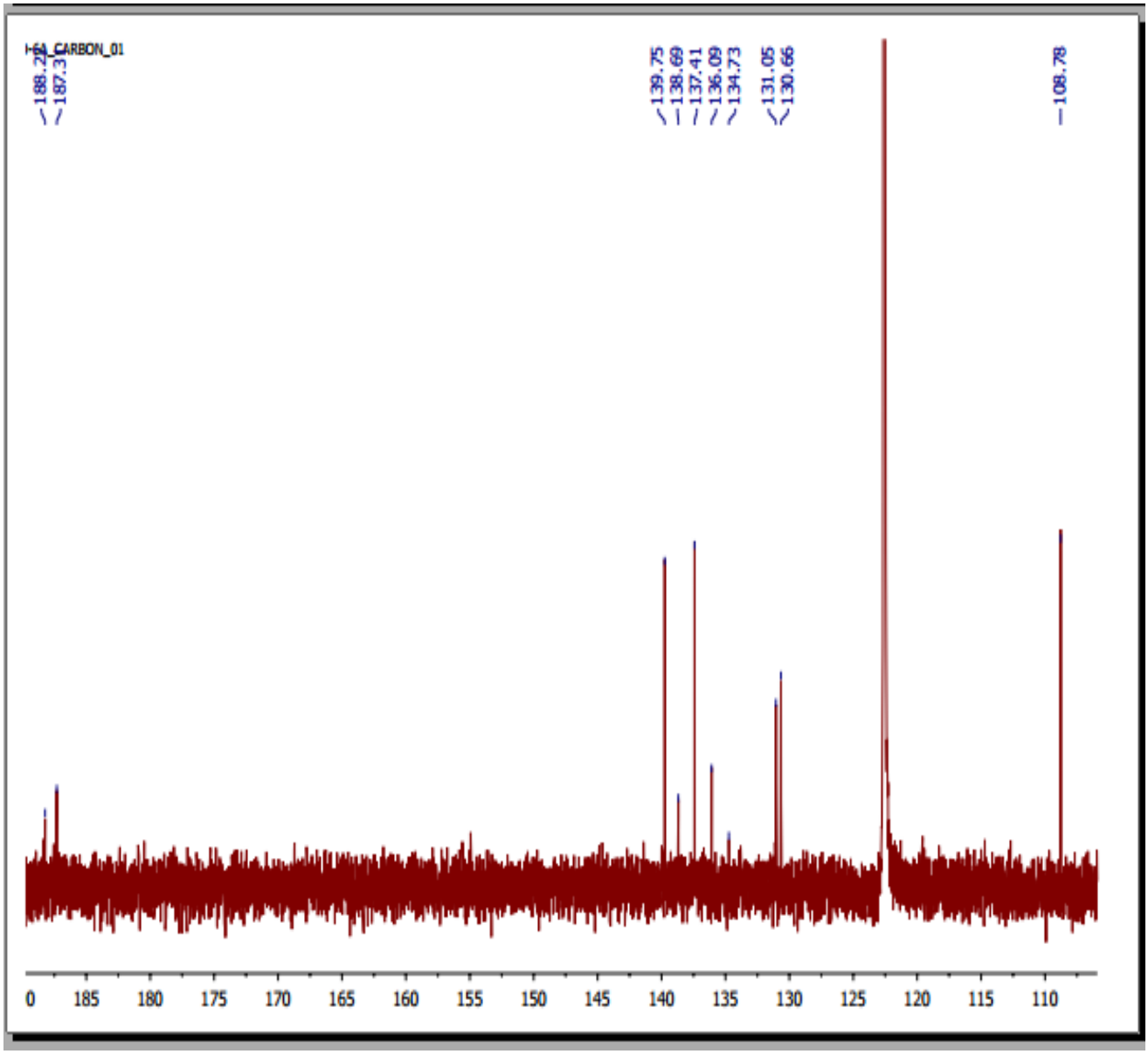


39

^{1}HNMR Spectrum for **39**


C (ddd)
8.02
D (ddd)
7.98
A (td)
7.77
B (td)
7.69
F (s)
5.80
8.04 8.02 8.00 7.98 7.96 7.94 7.92 7.90 7.88 7.86 7.84 7.82 7.80 7.78 7.76 7.74 7.72 7.70 7.68 7.66
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5

^{13}CNMR Spectrum for **39**



MS Spectrum for **39**