BIOCHEMICAL AND PHARMACOINFORMATIC APPROACH FOR SCREENING POTENTIAL ANTIMALARIAL PHYTOCONSTITUENTS IN DEVELOPMENT OF HERBAL FORMULATION FROM THE SELECTED MEDICINAL PLANTS

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Botany

By

Ibrahim Isyaku Muhammad

Registration Number: (12112720)

Supervised By

Dr. Devendra Kumar Pandey (15673)

Department of Botany

Lovely Professional University

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB
2025

DEDICATION

This thesis is dedicated to

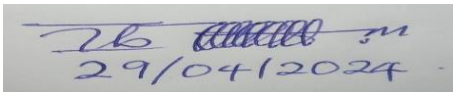
Yahaya Said (my sponsor),

My beloved parent

& entire family

DECLARATION

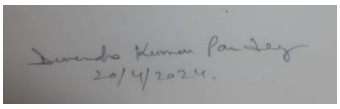
I, hereby declared that the presented work in the thesis entitled "**Biochemical and pharmacoinformatic approach for screening potential antimalarial phytoconstituents in development of herbal formulation from the selected medicinal plants"** in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of **Dr. Devendra Kumar Pandey**, working as **Professor**, in the Department of Botany, School of Bioengineering and Biosciences, Lovely Professional University, Punjab, India. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



Ibrahim Isyaku Muhammad
Registration No.: 12112720
Department of Botany
School of Bioengineering and Biosciences
Lovely Professional University, Punjab, India
Date: 23/01/2025

CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Biochemical and pharmacoinformatic approach for screening potential antimalarial phytoconstituents in development of herbal formulation from the selected medicinal plants"** submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in Botany, School of Bioengineering and Biosciences, is a research work carried out by Ibrahim Isyaku Muhammad, reg. no.: 12112720. It is bonafide record of his original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



Dr. Devendra Kumar Pandey
Associate Professor
Department of Botany
School of Bioengineering and Biosciences
Lovely professional University, India
Date: 23/01/2025

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ABSTRACT

The increased burden of antimalarial drug resistance becomes the major threat to African and Asian countries. This led to an increase in global mortality, which accounts for 627,000 as of 2021 according to the WHO report. *P. falciparum* demonstrates drug resistance to some of the available antimalarial medicines. This results in the quick search of new drugs with diverse modes of action. Moreover, most synthetically drugs were shown to contain more carcinogens than the herbal drugs. Therefore, herbal remedies could serves as alternative treatments for not only malaria but other ailments with less cancer risk. The current work employs High Performance Thin Layer Chromatography and Gas Chromatography Mass Spectrophotometry to developed antimalarial herbal formulation from the four selected medicinal plants.

In silico study of bioactive substances from the four selected medicinal plants were carried out against the three protease enzymes of *P. falciparum.* The derivatives of quercetin, artemisinin, quinine and Flavone, 5-hydroxy-7,8-dimethoxy- from the selected medicinal plants revealed the excellent binding affinity, convenient molecular interactions and ADMET properties.

Many phytochemicals, including alkaloids, flavonoids, terpenoids, saponin, cardiac glycoside, tannins, quinones, and phenolic compounds, were found in both extracts of the selected medicinal plants when the extracts were screened qualitatively for phytochemicals. On the other hand, alkaloids were absent from *S. thomsonii* extracts and quinones were absent from *A. annua* extracts. Moreover, saponin, and phenol were found absence from *C. occidentalis* extracts.

HPTLC result revealed that the petroleum ether extract of *A. annua* (***PEARA)*** had the highest artemisinin content with **(0.71% ± 0.02 w/w**), followed by methanol extract with (0.25% ± 0.02 w/w), chloroform extract with (0.14% ± 0.01 w/w), and water extract with (0.06% ± 0.01 w/w). While in *C. officinalis,* methanol extract yield higher content of quinine compound with **(2.60% ± 0.01 w/w**), followed by aqueous extract with (1.44 % ±

0.01 w/w), chloroform extract with (1.13% ± 0.01 w/w) and Petroleum ether extract with (0.76% ± 0.01 w/w). However, in *C. occidentalis* the chloroform extract yield the highest quercetin compound content with (**4.10% ± 0.01w/w),** followed by the methanol extract at (2.37% ± 0.01 w/w), the petroleum ether extract with (2.09% ± 0.01w/w), and the water extract with (1.79% ± 0.01 w/w). The methanol extract of *Swertia thomsonii* ***(MESWT)*** was characterized using GC-MS. The GC-MS profile of ***(MESWT)*** showed the existence of various important phytochemicals including the flavone, 5-hydroxy-7,8-dimethoxy- which exhibited excellent interactions with all the three target receptors while *in silico* study.

The extracts with highest yield of the target analyte were selected (***CFCAO, MECIO, PEARA*** *and* ***MESWT*****)** and were used for the *in vitro* assessment of antiplasmodial activity against *3D7* and *Dd2* clones of *P. falciparum*.

From the *in vitro* study while evaluating the antimalarial efficacy of the selected extracts, ***PEARA*** was found to be very active when tested on Dd2 strain with average mean of inhibition and standard error (SEM) on the mean of (0.920 ± 0.083 µg/mL) than in 3D7 strain with mean inhibition of (1.046 ± 0.101 µg/mL). Methanol extract of *C. officinalis* ***(MECIO)*** were found to be very active against both the strains with mean inhibition value of (0.741 ± 0.122 µg/mL when tested against Dd2) and (0.882 ± 0.071µg/mL when tested against 3D7). Chloroform extract of *Cassia occidentalis* ***(CFCAO)*** shows good activity against the both strains with mean inhibition (10.67 ± 0.147 µg/mL in 3D7) and (11.22 ± 1.32µg/mL in Dd2). However, in methanol extract of *Swertia thomsonii* ***(MESWT)*** Moderate activity were found in 3D7 with mean inhibition value of (36.98 ± 11.57 µg/mL) and less activity in Dd2 strain with mean inhibition value of (86.19 ± 20.11 µg/mL). All the fractions of the selected extracts from the selected medicinal plant were largely comparable between the two isolates (3D7 and Dd2) with around 2-fold activity difference at a maximum, which is not unusual for crude preparations given that they are a mixture of many individual compounds and thus resistance is not expected. Therefore, both the isolates show no resistance to the tested fractions of the crude extracts of the

selected medicinal plants. Fractions showed varying levels of activity from highly active with IC_{50} values less than 1μg/mL (*A. annua* and *C. officinalis*) to moderate active with IC50 value 10x higher in *C. occidentalis* and 30x higher in *S. thomsonii.* The control compounds; Chloroquine phosphate and Artesunate are extremely active and showed IC_{50} values of 9 nM (0.009μg/mL) and 4 nM (0.004μg/mL) against the sensitive isolate (3D7) respectively and were also extremely active with IC50 value of 0.232μg/mL and 0.005μg/mL against the resistant isolate (Dd2) respectively. As expected for the CQ-resistant strain of *P. falciparum* (Dd2), the artesunate IC50 is around the same range at approximately 5nM (0.005μg/mL) in both the two strains (3D7 and Dd2), while the Chloroquine (CQ) IC50 is significantly higher in 3D7 (0.009μg/mL) than in Dd2 (0.232μg/mL). Therefore, the mean of inhibition of the three tested extracts ***(PEARA, MECIO & CFCAO)*** on the two isolates of *P. falciparum* were found comparable with the control. Although the IC50 value of ***MESWT*** was not comparable to the standard drug (**CQ)** (IC50 values 0.009 ± 0.002μg/ml for 3D7 strain and 0.232μg/mL for Dd2 strain), but the outcomes of the results were deemed satisfactory and warrant further investigation. The extracts displayed a degree of inhibition in parasite growth, suggesting that the herbal formulation derived from these plant components could offer advantages. The cytotoxicity analysis showed that all active extracts tested on the neonatal mouse dermal fibroblast cell line had lethal concentrations (LC50) more than 100μg/ml, indicating their safety. Finally, after assessing the antimalarial activity of the individual extracts of the selected medicinal plants, the herbal formulation was created using the effective doses of all of the selected active extracts of the medicinal plants. The herbal remedy derived from these extracts can serve as a potential solution to the prevailing issue of resistance in *Plasmodium falciparum.*

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CHAPTER ONE

1.0 Background of the study

Alphonse Laveran is a distinguished military physician in France. He achieved a landmark discovery in 1880 by identifying the *plasmodium* parasite. In 1878, he was assigned to a military hospital in Algeria, a French territory, where he diligently served as a military doctor. At that time, malaria posed a significant threat to the army, prompting Dr. Laveran to embark on a thorough investigation of the disease's clinical manifestations and anatomical pathology. His meticulous efforts resulted in an exemplary histologic portrayal of cerebral malaria, contributing substantially to the understanding of this debilitating ailment (CDC, 2012). Since then, malaria has continued to be battled with worldwide. Malaria is a severe illness that poses a threat to human life and is caused by the *Plasmodium* parasite. Transmission of the disease occurs through the bite of female *Anopheles* mosquitoes (Muhseen *et al.,* 2021). Antimalarial drug resistance referred to the ability of *plasmodium* parasite to survived and replicate after being exposed of drug at doses equivalent to or above standard prescriptions (Shibeshi *et al.*, 2020). This phenomenon is evidenced by the decline in effectiveness of previously recommended medications. For instance, chloroquine (CQ) has historically served as the predominant treatment and preventative measure for malaria for more than fifty years. However, its efficacy diminished over time due to the emergence of parasite resistance, despite its initial high effectiveness, affordability, and widespread accessibility. Consequently, a combined treatment of sulfadoxine/pyrimethamine (SP) was endorsed as the primary therapeutic option. Nevertheless, this regimen proved effective for only a brief period as resistance to the medication swiftly emerged. In 2006, the WHO authorized artemisinin combination treatments as the preferred treatment for mild to severe malaria (Ogbole et al., 2014). Subsequently, Artemisinin Combination Therapies (ACTs) have demonstrated notable efficacy. Nonetheless, the evolution of artemisinin resistance has been documented in Western Cambodia (Kigen, 2019). The increased burden of antimalarial drug resistance becomes the major threat to African and Asian countries. This lead to

increase in global mortality associated with malaria which accounts for 627,000 deaths as of 2021 according to WHO report. This high mortality rate was primarily attributed to the prevalence of *Plasmodium falciparum*, the most perilous human malaria parasite (Pillay *et al.,* 2008). Over the last fifty years, *Plasmodium falciparum* demonstrates drug resistance to some of the available antimalarial medicines mefloquine, and piperaquine an so on (Shibeshi *et al.,* 2020). These results the urgent creation of novel drugs with diverse modes of action (Adewole, 2020).

Medicinal plants have were historically been utilized as a source of medicine in almost every civilization on the planet. A significant proportion, exceeding 80%, of individuals in low-income nations depend on herbal medicine as their primary form of healthcare. Moreover, more than 1,200 plant species are utilized in the treatment of malaria. Comprehensive information regarding the utilization of these medicinal plants to cure malaria is accessible; but, there is inadequate scientific research elucidating the precise mechanisms of action for the majority of these botanical remedies (Kigen, 2019). Phytochemicals including flavonoids, alkaloids, terpenes, and peptides serve as bioactive constituents found in medicinal plants, contributing to the plant's defense mechanisms. From a medical perspective, these compounds exhibit physiological activity by interfering with pathogenic pathways in human pathogens, thus offering therapeutic potential for various illnesses. Artemisinin and quinine, key chemotherapy medications for malaria, are derived from medicinal herbs. Additionally, other antimalarial drugs have been developed based on the molecular structures of these phytochemicals (Muhseen *et al.,* 2021).

The majority of antimalarial drugs with therapeutic significance focus on combating the parasite during its blood stage, prompting extensive research efforts to develop additional treatments targeting this stage. This includes interventions that hinder the degradation of host-cell hemoglobin (Patrick, 2020). Recently, high-throughput approaches for *in-silico* drug screening against molecular targets have gained popularity. Various softwares and online tools are been utilized for screening lead candidates from huge libraries (Verma *et al.,* 2020). This methodical approach enhances the efficiency of drug discovery, aligns

with specific objectives, and proves cost-effective. The present study used the protease enzymes of *P. falciparum* which appear to be among interesting targets for the development of new classes of malaria treatment drugs. These enzymes include; Plasmepsin-II with (pdb id: 1lee), Falcipain-II with (pdb id: 7ei0) and Aminopeptidase with (pdb id: 3q44) which all functions during blood stage of the parasite's life cycle to degrade hemoglobin of the human host and hence essential enzyme for *P. falciparum* survival.

Plasmepsin II is an aspartic protease that degrades hemoglobin. This protein marks the initial instance of structural identification among *Plasmodium* protozoans. Among the catalytically active enzymes within the feeding vacuole *of Plasmodium falciparum*, it is one of the four known plasmepsins. These enzymes initiate the degradation of hemoglobin through cleavage of the alpha-chain at the Phe33-Leu34 site (Asojo *et al.,* 2003). Falcipain-II, a cysteine protease generated from *Plasmodium falciparum*, is an excellent target for the development of antimalarial drugs. Falcipain-II enzyme is very critical in the life cycle of *P. falciparum* because it facilitates the cleavage of ankyrin and other proteins, as well as the degradation of hemoglobin during the early trophozoite stage (Brown, 2008). Aminopeptidase is another important enzyme of interest that may be used as a therapeutic target. Our research aims to offer important informations for the logical development of more potent inhibitors of this enzyme. These inhibitors hold potential for the creation of novel anti-malarial medications (Velmourougane *et al*., 2011). These enzymes are very vital for the survival of *Plasmodium* parasite through a variety of mechanisms.

In this study, virtual screening through molecular docking, molecular interaction studies and ADMET analysis were utilized aiming to screen the phytocompounds from the selected medicinal plants with the inhibitory activity against the target receptors were carried out following the established method of (Feng *et al.,* 2021). Cold maceration technique (CMT) was also developed which is safe, effective, low-cost, and rapid for extracting the screened compounds from the selected medicinal plant. A validated HPTLC and GC-MS method was also established for the characterization of the screened

compounds. Therefore, an expedited sample preparation and a validated method for the quantification of these compounds utilizing the HPTLC and GC-MS method were established.

Additionally, an *in vitro* approach was used to assess the antimalarial efficacy of the chosen extracts on the strains of *P. falciparum*, namely 3D7 and Dd2. Furthermore, a toxicity study of the tested extracts was conducted using a neonatal dermal fibroblast cell line to ascertain their safety profiles. Finally, after the assessment of the antimalarial efficacy of the individual extracts of the selected medicinal plants, the herbal formulation was developed from the effective doses of all the selected active extracts of the medicinal plants. The formulation created from these extracts could be employed as one of the solutions to the current issue of resistance in *Plasmodium falciparum.*

1.1 Hypothesis

1. The phytoconstituents of the selected medicinal plants do not have antiplasmodial activity against the 3d7 and Dd2 strains of *P. falciparum*
2. The herbal formulation made from the selected medicinal plants does not have antiplasmodial activity against the 3d7 and Dd2 strains of *P. falciparum*.

1.2 Research Aim

This research focuses on screening of phytoconstituents with antiplasmodial activity from the selected medicinal plants using *in silico* and *in vitro* approach for the development of herbal formulation with antiplasmodial activity.

1.3 Objectives of the research

1. Virtual screening and analysis of potential phytocompounds with antimalarial activity using *in silico* approach from medicinal plants.
2. Collection and screening of medicinal plants
3. Assessment of antiplasmodial activity of the extracts against *Plasmodium falciparum*
4. Preparation of herbal formulation having potential antimalarial activity

1.4 Identification of research gap

The rise of drug resistance in malaria presents a significant challenge as it reduces the effectiveness of current antimalarial medications, particularly against the most lethal *Plasmodium* parasite species known as the *P. falciparum* (Muhseen *et al.,* 2021). This resistance poses a substantial threat to African and Asian nations and contributes to a heightened global mortality rate associated with malaria, which reached 627,000 deaths in 2021 according to the World Health Organization (Pillay et al., 2008). Over the past five decades, *Plasmodium falciparum* has shown resistance to several available antimalarial drugs (Shibeshi *et al.,* 2020), necessitating the urgent development of new drugs with diverse mechanisms of action (Adewole, 2020). The economic and social impact of malaria is substantial, with patients experiencing hospitalization, lost work days, and increased financial burdens associated with seeking medical care and burial expenses. In Nigeria alone, malaria is estimated to cost approximately N132 billion annually due to sickness-related productivity losses, treatment expenses, and other associated costs (Satish *et al.,* 2017). Furthermore, malaria contributes to absenteeism from work and school, exacerbates poverty, and adversely affects pregnancy outcomes. To address the challenges posed by drug resistance in *P. falciparum*, this study utilized virtual screening techniques, molecular docking, molecular interaction studies, and ADMET analysis to identify herbal derivatives with inhibitory activity against target receptors. *In vitro* assays were conducted to assess the antimalarial efficacy of these extracts against different clones of *Plasmodium falciparum* (3D7 and Dd2). Subsequently, a herbal formulation was developed from the most effective doses of the active extracts, with the aim of providing an alternative remedy to combat drug resistance in *Plasmodium falciparum.* This research highlights the potential of herbal remedies as a promising avenue for the development of novel malaria treatments.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Antimalarial drug resistance

Antimalarial drug resistance refers to the potential of a *Plasmodium* parasite (the causative agent of malaria) to survive and replicate after being exposed to of antimalarial drugs in doses equivalent to or higher than the prescribed ones (Nsanzabana *et al.,* 2018). The global efforts to control malaria face a significant challenge due to the rise of resistance to previously effective first-line drugs. Notably, chloroquine (CQ) which served as a cornerstone in malaria treatment and control for over five decades. However, its efficacy dwindled over time as parasite resistance developed, despite its initial effectiveness, affordability, and widespread availability. Subsequently, the combination therapy of sulfadoxine/pyrimethamine (SP) was introduced as the primary treatment option. However, its tenure as the first-line therapy was short-lived, as resistance swiftly proliferated. In response, WHO endorsed artemisinin combination therapies in 2006 as the principal regimen for managing both uncomplicated and severe malaria cases (WHO, 2010). Subsequent evaluations have affirmed the considerable efficacy of ACTs. Nonetheless, the emergence of Artemisinin resistance has been documented in Western Cambodia and the Greater Mekong subregion (Kigen, 2019; Cui et al., 2012).

P. falciparum is the parasite that causes the most severe type of malaria in humans, presents a significant challenge in world efforts to manage and eradicate the disease. This resistance phenomenon arises primarily due to the parasite's ability to adapt and evolve in response to various antimalarial interventions, including drugs and insecticides.

2.1.1 Chloroquine Resistance

Historically, chloroquine was the cornerstone of malaria treatment. However, widespread resistance to chloroquine emerged in *P. falciparum* during the latter half of the 20th century, rendering it ineffective in many regions (Ursos & Roepe, 2002). Chloroquine-resistant *P. falciparum* first emerged independently in Southeast Asia, Oceania, and South America in the late 1950s and early 1960s (Mita & Tanabe, 2012). Over time, resistance spread to nearly all *falciparum* malaria transmission areas worldwide. Despite apparent elimination of parasites, treatment failures occur due to submicroscopic levels of resistant *P. falciparum* surviving. Parasites can become dormant when exposed to multiple antimalarials. *P. falciparum* has also developed resistance to not only chloroquine but to other antimalarials like sulfadoxine/pyrimethamine, mefloquine, halofantrine, and quinine. The principal mechanism behind chloroquine resistance is mutations in the PfCRT gene. Mutated PfCRT reduces chloroquine accumulation in the parasite's vacuole (Sidhu *et al.*, 2002).

2.1.2 Sulfadoxine-Pyrimethamine (SP) Resistance

Resistance to sulfadoxine-pyrimethamine (SP), another widely used antimalarial drug, has become prevalent *in Plasmodium falciparum* populations, particularly in sub-Saharan Africa. SP, a potent antimalarial agent, is formed by combining sulphadoxine and pyrimethamine (Barnes & White, 2005). Children infected with parasites that possess various single to quintuple DHFR/DHPS mutations exhibit inconsistent responses to SP. Quintuple mutant genotypes are predominantly linked to treatment failures. SP resistance has now spread globally. Concurrent studies on factors contributing to resistance development are essential (Mishra et al., 2006).

2.1.3 Artemisinin Resistance

ACTs are currently the frontline treatment for malaria. However, resistance to artemisinins has been reported in Southeast Asia, threatening the efficacy of this vital treatment strategy. In certain regions of Southeast Asia, resistance to artemisinins has

emerged, prompting ongoing research into resistance mechanisms and the exploration of new treatment options (Pousibet *et al.,* 2016). The WHO recommend ACTs as the primary treatment for both uncomplicated *P. falciparum* malaria and chloroquine-resistant *P. vivax* malaria. ACTs combine an artemisinin derivative with another drug. Resistance to artemisinins primarily stems from mutations in the PfK13 propeller domain, which confer parasites with enhanced power to enter a dormant state. Particularly in Cambodia, artemisinin resistance has been documented, leading to concerns over delayed parasite clearance and treatment failures. Although widespread artemisinin resistance hasn't been observed in Africa, recent studies have hinted at partial resistance in Rwanda (Dambach *et al.,* 2018; Paloque *et al.,* 2016). Researchers persist in investigating resistance mechanisms, emphasizing the need for urgent containment measures to curb further spread. Understanding artemisinin resistance is pivotal for effective malaria control, necessitating collaborative efforts to confront this formidable challenge.

2.2 Herbal drugs as alternative for malaria treatment

Herbal medicines have been explored as potential alternatives for malaria treatment**,** especially in regions where conventional antimalarial drugs face challenges due to resistance. Malaria, which is caused by *Plasmodium falciparum,* continues to be one of the huge public health concerned worldwide. The advent of resistance to traditional antimalarial medications has spurred experts to look into herbal therapies as viable alternatives (Oghenesuvwe et al., 2021). Previous research has studied the interaction between herbal antimalarial medications and conventional antimalarial drugs (CAMDs), and several herbal antimalarial treatments revealed synergistic benefits when coupled with CAMDs. While most interactions were synergistic, some studies reported antagonistic effects. Herbal remedies showing synergistic effects with CAMDs hold promise for developing standardized antimalarial-herbal combination therapies. Such approaches could help combat malaria resistance to conventional antimalarial treatments (Erhirhie *et al.,* 2021). However**,** It’s important to note that while herbal medicines are

commonly used in communities as alternatives to modern antimalarial agents, their efficacy and safety need further investigation (Ocan *et al.*, 2023). Garlic, ginger, and onion are common culinary components and have been proven to have antimalarial properties when taken together (Etewa & Abaza, 2011). Indigenous medicinal plants have been utilized for millennia to treat malaria. These plants are still being studied for potential antimalarial properties (Evbuomwan *et al.,* 2023). Creating standardized herbal remedies could supplement current treatments and manage resistance. Herbal medications show promise as complementary treatments for malaria. Therefore, ongoing research explores the potential of herbal drugs to enhance malaria treatment and address resistance issues.

2.3 Epidemiology of malaria

Malaria is a major vector-borne disease in sub-Saharan Africa and India. It is very prevalent to these regions, putting fourty percent of the global population at risk (Bharati & Saha, 2017). The epidemiology of malaria in India and Africa exhibits considerable variability owing to expansive geographic regions characterized by diverse topography and climatic conditions. This variability is further influenced by factors such as high genetic diversity among parasite strains, evolving drug resistance, differential distribution of vector species, emergence of insecticide resistance, inherent diversity in human genetics, and historical evolutionary trajectories. Moreover, the shifting patterns of climate have likely exerted a notable influence on malaria epidemiology. Consequently, there has been a notable shift in recent years, with *Plasmodium falciparum* malaria surpassing *P. vivax* in prevalence. This shift has prompted adjustments in medication and insecticide application policies in India (Singh *et al.,* 2009).

2.4 *Plasmodium* parasite culture

The cultivation of human malaria parasites was a daunting problem until 1976, when Trager and Jensen discovered the vital function of the low-oxygen environment within coagulated red blood cells (RBCs) within capillaries in the growth of the *P. falciparum*

life cycle (Cox, 2010). A year later, this technique evolved into a system similar to current methodologies, in which cryopreserved parasitized RBCs could be resuscitated and cultured in washed human RBCs in RPMI medium supplemented with human serum, all in controlled culture flasks maintained in a hypoxic environment (Barnwell *et al.*, 1989). Initially, the reduced oxygen atmosphere was created by lighting a candle at the flask's entry to lower oxygen concentration. Subsequent methodology refinements have improved reproducibility; candles have been replaced by low-oxygen gas with a defined composition, and a standardized culture medium containing RPMI supplemented with AlbuMAX has largely replaced the variable human serum composition (Adams et al., 1990). Prior to the 1970s, *P. falciparum* proteins were isolated predominantly through direct extraction from malaria-infected patients, which had several disadvantages. Trager and Jensen's key work in 1976, which established continuous parasite culture, represented a transformative advance by providing a fresh laboratory reservoir of parasite proteins. Traditional purification methods encountered significant hurdles, mostly due to the presence of erythrocyte protein contaminants and insufficient yield of isolated parasite proteins**.**

2.5 Types of malaria parasite infecting human

There are five *plasmodium* parasite species which infect humans and cause malaria. These species of *Plasmodium* parasites are as follow;

i. P. vivax

They can be found throughout the world, but the most common are in India, Central and South America. In the human body, the disease's symptoms appear after about 8-13 days of incubation. Infection with this parasite can sometimes result in a life-threatening splenic rupture. They hide in the liver and can return once a person has been infected. Although this species does not produce difficult malaria, recent investigations have found complications in vivax malaria (Singh et al., 2009; Sharma, 2007).

ii. P. ovale

They are found mostly throughout Africa. This kind of malaria has an 8-17 day period to developed in infected individuals and can hide in the liver of partially cured patients before returning later. This species is extremely rare, with only a few sightings documented from India's eastern states.

iii. P. malariae

They are found in most parts of the world, but are less common than other malaria parasites. This parasite incubates for 2-4 weeks in the affected person. If left untreated, the condition might persist for many years. This species is also less frequent than *vivax* and *falciparum*, which account for more than 95% of malaria positives.

iv. P. falciparum

They caused most of the deadly type of malaria, causing the majority of deaths worldwide, and can be found anywhere. This parasite has an incubation period of 5–12 days. They are resistant to the majority of malaria treatment and prevention medications. Over the last 15 years, there has been a shift in the replacement of P. *vivax* with *P. falciparum*, most likely due to *P. falciparum's* rising chloroquine resistance. According to WHO projections, 70% of the hundred million total cases in Southeast Asia occur in India, with *P. vivax* accounting for more than 65%.

v. P. knowlesi

There is also a fifth, rare species called *Plasmodium knowlesi*, which can infect people and monkeys. However, it is mostly located in Southeast Asia (Wu, 2023).

2.6 Life cycle of *plasmodium* parasite

The life cycle of *Plasmodium* is complex, involving both sexual (gametogony) and asexual (sporogony) stages, which occur in two hosts: the mosquito, where sexual reproduction takes place exclusively, and the human, serving as the intermediate host for the three stages of asexual reproduction (Gautam *et al.*, 2020). The journey of the P. falciparum parasite begins when sporozoites, transmitted by an infected Anopheles mosquito during a blood meal, enter the human bloodstream as the secondary host. These sporozoites invade liver cells via the bloodstream, where they transform into schizonts, each containing over a thousand haploid merozoites. About a week later, the schizonts rupture, causing hepatocyte death and releasing millions of merozoites into the bloodstream, initiating a cycle of asexual exoerythrocytic schizogony. This stage sees a greater number of red blood cells becoming infected as schizonts and erythrocytes rupture every 2-3 days, releasing merozoites that further invade uninfected erythrocytes. Some merozoites differentiate into gametocytes during this stage and remained dormant until taken by a mosquito vector during a blood meal. Upon ingestion, female mosquitoes become infected with gametocytes, which travel to the mosquito's midgut and differentiate into male microgametes and a female macrogamete. Fusion of these gametes results in the formation of a diploid zygote, which undergoes meiotic division to produce an ookinete. The ookinete migrates and penetrates the mosquito gut wall, facilitated by the peritrophic matrix, eventually transforming into an oocyte. Upon rupture, sporozoites are released into the mosquito's hemocoel cavity and migrate to the salivary glands, poised to infect a host during subsequent blood feeding. The parasite's transformation through various developmental stages is crucial for its propagation and continuation of the disease cycle. Combatting malaria requires diverse strategies targeting specific stages of this intricate life cycle in both the primary and secondary host systems (Kushwaha et al., 2020).

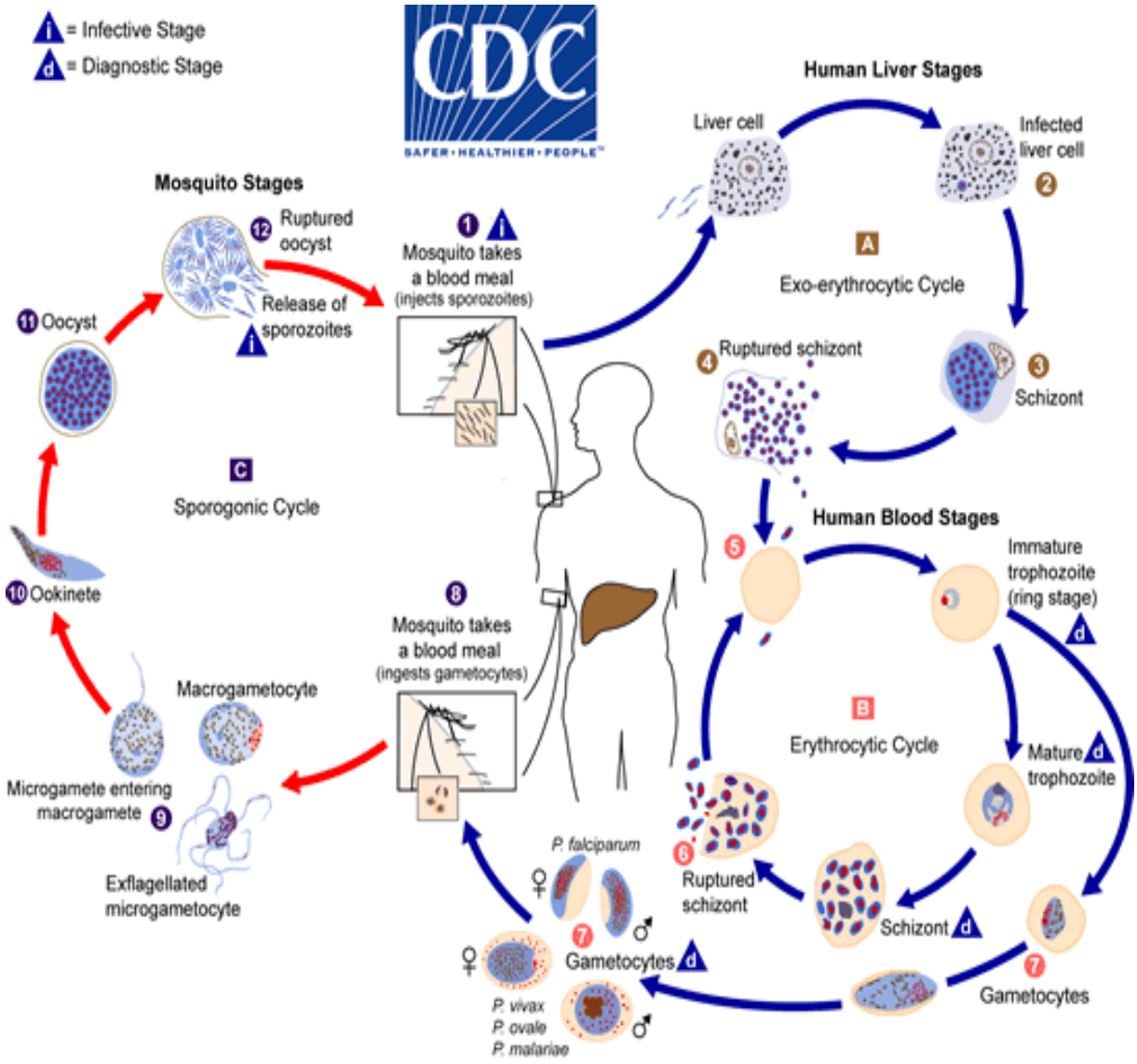

Figure 2.1: Malaria life cycle

2.7 Burden of complicated malaria during pregnancy

Available data in India show that fatality rates for difficult cases of P. falciparum malaria vary by area. Mortality rates were reported at 7.9% in Vellore, Tamil Nadu, whereas higher rates of 25.6% and 30% were observed in Jabalpur, Madhya Pradesh, and Rourkela, Odisha, respectively as reported by (Kumar et al., 2007; Kumar et al., 2012). Pregnant women are classified as a high-risk population for malaria infection, particularly in endemic areas (Mutabingwa, 1994; Okoko et al., 2003). Such infections might result in consequences like cerebral malaria and severe anemia.
In countries with low malaria transmission rates, maternal mortality rates are believed to be about 1%, but in Africa, this figure might range from 84 to 2,000 per 100,000 live births reported by (Egwunyenga *et al.,* 1997; Melba, 2002).

2.8 Malaria control and elimination strategies

The National Malaria Eradication Programme (NMEP), later rebranded as the National Anti-Malaria Programme (NAMP), implemented in the 1950s, was successful in controlling malaria in roughly three-quarters of India by using DDT and other insecticidal measures. This initiative shown promising progress towards malaria elimination. By 1965-66, malaria cases had been reduced with reliable surveillance, and deaths had been nearly eradicated. Malaria was a disease that primarily affected rural areas, with fewer cases occurring in urban. The escalation of malaria cases in both urban and rural areas in India was attributed to the concerted efforts of local municipalities and governmental bodies in town and metropolitan districts. The spread of malaria, even in urban settings, was facilitated by a breakdown in municipal regulations, rapid industrialization, urban expansion, limited healthcare access, and a lack of health services extension. The discovery of chloroquine-resistant strains in Assam in 1973 coincided with a surge in *falciparum* malaria cases. The prevalence of chloroquine resistance has been reported in several Indian states, including Assam, Bihar, Tamil Nadu, Maharashtra, and Karnataka. Recent findings also indicate the rise of chloroquine-resistant strain in Rameshwaram, Tamil Nadu.

The implementation of the Five-Year Plans in India during the 1950s and 1960s contributed significantly to the successful control of malaria. The advancements made during these plans also led to improvements in the overall health of the Indian population. Noteworthy achievements included the eradication of smallpox, plague, and cholera, alongside a significant reduction in the prevalence of malaria and associated mortality rates.

Malaria's return in India has been ascribed to a number of obstacles, including pesticide resistance, strong exophilic vector behavior, treatment resistance in malarial parasites, and financial constraints that prevent enough resources from being allocated to disease management activities. This resurgence has rendered malaria control strategies ineffective, resulting in the reemergence of malaria-related mortality and epidemics that were previously reduced during eradication efforts.

The malaria control approach in India was mostly based on indoor residual spraying activities. However, discontinuing these efforts frequently resulted in a return of malaria with greater severity. Notably, in 2000, India reported 2.02 million malaria cases, with *P. falciparum* accounting for 50% of the illness burden. Malaria in India follows a cyclical pattern, with outbreaks affecting all age groups and having an immediate negative impact on local economy. These problems highlight the need for long-term and comprehensive approaches to malaria management, including initiatives to address increasing medication and insecticide resistance as well as increased financial and infrastructure assistance.

2.9 Selected medicinal plants

2.9.1 Cassia occidentalis Linn.

Cassia occidentalis is also known as *Negro coffee*, belongs to the *leguminose* family. This plant has a rich history of traditional use and is known for its diverse biological activities. It is frequently used in Indian traditional medicine (*Ayurveda*) to treat various diseases including malaria. It is an erect, perennial plant and have traditional practice, as well as wide phytochemicals and having diverse pharmacological effects *(*Sharma *et al.*, 2014). It's widely distributed and commonly found in wastelands and plains up to an altitude of 900 meters. It has a pantropical distribution, likely originating in tropical America (Mahanthesh *et al.,* 2019). In India, it grows wild throughout the plains, including coastal areas, and is found up to an altitude of 1,000 meters in the Himalayas and also thrives in the deltaic regions of western, eastern, and southern India (Das and Duarah, 2013; Manikandaselvi *et al.,* 2016). *C. occidentalis* contains various chemical groups, including alkaloids, anthocyanosides, phenolics, proteins, phlobatannins, steroids, tannins, flavonoids, anthraquinones, saponins, terpenes which contributed to its numerous pharmacological effects such as antimicrobial, anthelmintic, larvicidal activity, insecticidal, antioxidant, antianxiety, antidepressant, antimutagenic, antidiabetic, vasoconstrictor, anticancerous, antihemorrhagic, digestive stimulant, wound healing, hepatoprotective, renoprotective, sun protective, smooth muscle relaxation, immune-modulating, anti-inflammatory, analgesic and antipyretic (Khan and Mali, 2012).

The phytochemicals isolation from *Cassia occidentalis* yielded different compounds, most of which are reported for the first time and belongs to the class of glycosides (Chukwujekwu *et al.,* 2016). According to (Mahanthesh et al., 2019), a high concentration of alkaloids was discovered in the stem, leaves, and fruits of Ethiopian plants. Malaria remains a life-threatening infectious disease in tropical and subtropical places around the world. Quercetin, a natural flavonoid found in various plants, has garnered attention for its potential therapeutic properties. Studies have shown that quercetin possesses antimalarial activity against *Plasmodium falciparum*, the causative

agent of malaria. In vitro experiments have revealed significant antimalarial effects of quercetin against both K1 strain and 3D7 strain of *P. falciparum*, with IC50 values of 28.47 μM and 50.99 μM, respectively. Quercetin functions by inhibiting glycogen synthase kinase-3β (GSK3β), a key regulator of pro- and anti-inflammatory responses. Through modulation of cytokine production, quercetin contributes to maintaining immune balance during malarial infection, supporting its traditional use in malaria treatment. Furthermore, quercetin's interactions with Plasmodium falciparum lactate dehydrogenase (Pf LDH) suggest its potential as a candidate for future antimalarial drug development. Thus, quercetin's dual role as an antimalarial agent and a cytokine modulator underscores its promising potential in the management of malaria as reported by (Chaniad *et al.,* 2021).

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2.9.3 *Artemesia annua Linn.*

Artemisia annua L. is also known as sweet wormwood which belongs to the *Asteraceae* family. It's an aromatic annual herb which is the source of artemisinin and essential oils. It is well documented in old pharmacopoeias from several Asian and European countries. The World Health Organization classifies it as a possible pharmacological agent and antimalarial medication (Ahamd *et al.*, 2023). *Artemisia annua* brew has long been used in Africa to cure malaria. The essential element responsible for its antimalarial efficacy is artemisinin, which serves as the chemical foundation for the world's antimalarial programs and combinatorial curative therapy (Czechowski *et al.*, 2019). Artemisinin demonstrates efficacy against all species of the malaria-causing protozoan genus Plasmodium. It is especially valuable in treating infections caused by chloroquine-resistant parasites and multidrug-resistant strains of *P. falciparum*, which is considered the most lethal malaria protozoan. Artemisinin exert its effects across the asexual blood stages of the Plasmodium life cycle, including targeting sexual gametocyte stages, potentially limiting disease transmission in regions with low malaria prevalence (O'Neill *et al.*, 2010). Studies have determined that the principal antimalarial action of artemisinin is attributed to its unique internal structures known as endoperoxide linkages (peroxide bridges) (Xiong et al., 2021). Consequently, the multifaceted pharmacological properties of artemisinin extend beyond malaria treatment, rendering it a noteworthy compound in combating this lethal disease. According to current studies, artemisinin is not only efficient against malaria, but it also kills human breast cancer cells. The characterisation and extraction of artemisinin from *Artemisia annua* has sparked worldwide interest. The secondary plant product artemisinin, known in Chinese traditional medicine as qinghasu, is an antimalarial with less adverse effects than quinine, chloroquine, and other antimalarials. Quinolines and sulphadozine-pyrimethamine have lost clinical efficacy in Africa due to biocidal resistance by malaria parasites. Artemisinin is effective in cases of biocide resistance. However, monotherapy with artemisinin leads to high rates of recrudescence, raising worries about the emergence of biocidal resistance. However, monotherapy with artemisinin leads to high rates of recrudescence, raising concerns

about the development of biocidal resistance. As a result, the WHO recommend artemisinin to be used with other effective antimalarials. Artemisinin-based combination medicines are becoming increasingly popular in Africa. According to the World Health Organization (1981), the herb is harmful for use during pregnancy due to its uterine and menstrual stimulating effects; however, there is insufficient or accurate information in this regard, thus it should not be taken during breastfeeding. In light of the aforementioned, the current study performed the phytochemical screening and evaluation of the possible histopathological effects of the herb on the testes and ovaries, the primary reproductive organs of albino Wistar rats, and therefore the fertility status (Ajah & Eteng, 2010). Therefore, *Artemisia annua* stands as a potent medicinal herb with diverse properties, and its antimalarial potential continues to be a subject of research and global interest.

2.9.5 Swertia thomsonii Linn.

Swertia thomsonii is commonly known as Thomson's Swertia. It's a herbaceous perennial plant native to the Himalayan regions of Pakistan and northwest India (Wani *et al.,* 2023). Bitter compounds such as xanthones and iridoids contribute immensely to its medicinal properties. The antimalarial activity of *Swertia thomsonii* has been studied, particularly in the context of traditional medicine. The plant's roots and leaves are used to create decoctions or powders that are believed to be effective against malarial fever. While more research is needed to fully understand its mechanisms, this Himalayan herb holds promise as a natural remedy for combating malaria. Moreover, the extracts of *Swertia spp.* and a few other herbs are sold commercially as an antimalarial. In addition to its antimalarial activity, it has been demonstrated that *Swertia spp.* contains antihepatotoxic, antiulcerogenic, and blood sugar-lowering properties as reported by (Bhat & Surolia, 2001). **5-hydroxy-7,8-dimethoxyflavone** is also known as **flavone**. It is also found in *S. thomsonii.* This compound has garnered attention due to its potential pharmacological properties, including antimalarial effects. Flavones are a subclass of flavonoids, which are natural compounds found in various plants. Flavones exhibit

diverse biological activities, including antioxidative, anti-inflammatory, antimicrobial, and antiviral effects (Anh *et al.*, 2024). Research has revealed that flavones possess antimalarial properties. Specifically, **5-hydroxy-7,8-dimethoxyflavone** has shown promise in combating malaria. The precise mechanism by which flavones exert their antimalarial effects is not fully understood. However, they likely interfere with essential processes in the malaria parasite, such as nucleic acid synthesis or protein function. Flavones are typically evaluated in vitro against *Plasmodium falciparum*, the deadliest malaria parasite. Researchers assess their activity using IC50 values, which represent the concentration required to inhibit parasite growth by 50%. Flavones with IC50 values ≤ 11 μM are considered promising (Bero *et al.*, 2009). Flavones could serve as templates for designing novel antimalarial drugs. Their natural origin makes them attractive candidates for further exploration. Therefore, while more research is needed, flavones, including **5-hydroxy-7,8-dimethoxyflavone**, hold potential in the fight against malaria.

2.9.7 Cinchona officinalis Linn.

Cinchona officinalis is a plant native to South America and has been known in Europe since the 1640s. It has been used for treating malaria since the 1820s (Hariyanti *et al.*, 2022). It primarily grows in the wet tropical biome of the Andes region in South Ecuador. However, apart from its native habitat, *Cinchona officinalis* has been introduced into several other regions: Colombia, Guinea, Jamaica, Peru, Puerto Rico, St. Helena etc (POWO, 2024). In India, *Cinchona officinalis* is found in the Western Ghats region and it's widely cultivated (Vattakaven *et al.*, 2016). The main phytochemical content of *Cinchona* bark is quinoline alkaloids, including quinine, cinchonidine, cinchonine, and cinchonidine. Quinoline alkaloids from *Cinchona* are well-known for their antimalarial properties. Besides antimalarial effects, Cinchona alkaloids are being explored for their various properties including anticancer (Raza *et al.*, 2021). Quinine, derived from the bark of the cinchona tree, holds a historical significance in malaria treatment and is considered one of the most fortuitous discoveries in medical history. It marked the pioneering use of a chemical compound to combat an infectious disease, with its usage dating back to the 1600s when Jesuit missionaries in South America employed it for malaria treatment, known by various names including the "Jesuits' bark," "cardinal's bark," or "sacred bark" (Achan et al., 2011). Quinine primarily targets the blood stage of the malaria parasite's life cycle, functioning as a rapid-acting erythrocytic schizonticide against the endoerythrocytic forms of all *Plasmodium* malaria causing species (Quadros *et al.*, 2021). Quinine exhibits gametocytocidal effects against *Plasmodium vivax* and *Plasmodium malariae* but not against *Plasmodium falciparum*. While noteworthy for its analgesic properties, quinine does not possess antipyretic effects. Its historical significance and continued relevance in malaria management emphasize its importance. Despite its longstanding efficacy, the precise mechanism underlying quinine's antimalarial activity remains partially elucidated. Nearly 400 years since its inception, quinine remains relevant in malaria treatment, albeit encountering challenges in its usage such as;

1. **Tolerability**: Quinine can cause adverse effects, impacting patient compliance.
2. **Complex Dosing Regimens**: Administering quinine requires adherence to intricate schedules.
3. **Emergence of Efficacious Alternatives**: More effective antimalarial drugs are now available.

Table 2.0: Taxonomy classification of the selected medicinal plants

Classfication	**Selected medicinal plants**			
Hierarchy	***A. annua L.***	***C. officinalis L.***	***C. occidentalis L***	***S. thomsonii L***
Kingdom	Plantae	Plantae	Plantae	Plantae
Division	Tracheophyta	Streptophyta	Tracheophyta	Tracheophyta
Class	Magnoliopsida	Equisetopsida	Magnoliopsida	Magnoliopsida
Sub-class	---	Magnoliidae	Rosidae	---
Order	Asterales	Gentianales	Fabales	Gentianales
Family	*Asteraceae*	*Magnoliidaes*	*Leguminoseae*	Gentianaceae
Genus	*Artemesia*	*Cinchona*	*Cassia*	Swertia
Specie	*Artemesia annua*	*Cinchona officinalis*	*Cassia occidentalis*	*Swertia thomsonii*
Scientific name	*Artemesia annua* Linn.	*Cinchona officinalis* Linn.	*Cassia occidentalis* Linn.	*Swertia thomsonii* Linn.

2.10 Computational technique in drug development

Bioinformatics offers methodologies for pharmaceutical development, including virtual screening of compounds to initiate lead generation, subsequent optimization of the lead to enhance its properties, and visualization of potential drug-target interactions. Furthermore, candidate treatments undergo evaluation for drug-likeness based on Lipinski's rule of five (Verma and Pathak, 2022). From conception to market, the drug development pipeline includes seven important phases: disease and drug target identification, lead molecule discovery, lead optimization, preclinical and clinical trial review, and pharmacogenomics refinement (Kiriiri et al., 2020). Chemical entities for testing can be derived naturally from plants, animals, and microbes, or they can be chemically created. Compounds exhibiting low activity or unfavorable pharmacokinetic profiles may be rejected during the screening process (Kigen, 2019).

Given the strict regulatory landscape governing drug testing implementation, achieving reductions in time and cost during later stages of drug development appears improbable. Therefore, efforts aimed at improving drug development efficiency primarily concentrate on ligand identification and optimization stages (Hopkins *et al.,* 2014). Overcoming the substantial difficulties of discovery of antimalarial drug is made feasible through the application of *in silico* screening tools. The adoption of high-throughput technology for *in silico* screening of significant compounds against the target proteins has gained significant attraction in recent years. Within this framework, compounds sourced from extensive libraries undergo screening using various computational methodologies, notably docking and ligand-based similarity searches, alongside computer-aided prediction of properties. These endeavors result in the identification of a modest yet noteworthy number of novel candidates for subsequent biological evaluation (Verma *et al.,* 2020). Consequently, this systematic and rational approach substantially enhances the efficiency, target orientation, and overall value of the drug development endeavor.

2.11 Pharmacophore modelling in antimalarial drug discovery

Pharmacophore is a molecular structure that holds (phoros) the critical features necessary for a drug's (pharmacon) biological activity (Güner *et al.*, 2014). While the essential ideas of pharmacophore have not changed over the last century, its conceptual scope and practical applicability have expanded significantly. Pharmacophore model is "an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response." In addition to this official classification, several similar descriptions and analyses have been provided in scientific discourse. Pharmacophore models can be developed using either a ligand-based approach, which involves aligning active molecules to extract common chemical features critical to their bioactivity, or a structure-based method, which involves exploring potential interaction sites between macromolecular targets and ligands. Pharmacophore techniques have proven extremely useful in virtual screening, de novo design, and other aspects of lead identification and optimization (Langer et al., 2001).

Numerous examples demonstrate the effectiveness of pharmacophore approaches in drug discovery, notably in the areas of optimization and multi-target drug design (Abdolmaleki et al., 2017). Notable among these accomplishments is the work of (Brunner et al., 2013) who identified a pharmacophore conducive to high antimalarial action in pharmaceutical substances. Their findings revealed substances capable of reducing the growth of both drug-sensitive and drug-resistant strains of erythrocytic *P. falciparum* at nanomolar doses, comparable to recognized antimalarials like chloroquine and artesunate

2.12 Target receptors

2.12.1 Plasmepsin-II

Plasmepsins are a group of aspartic proteases found in the malaria parasite *Plasmodium falciparum*. These enzymes play a crucial role in the degradation of **human hemoglobin** within the parasite. During its lifecycle, *P. falciparum* resides within human red blood cells. To sustain its growth and development, the parasite needs amino acids derived from hemoglobin. Plasmepsins are responsible for breaking down hemoglobin into these essential building blocks (Asojo *et al.,* 2003). Given their central role in hemoglobin catabolism, plasmepsins are attractive targets for antimalarial drug development. If we can inhibit these enzymes, we might disrupt the parasite's ability to obtain nutrients, ultimately thwarting its survival (Nezami *et al.,* 2002). Plasmepsin II (PMII) has two catalytic aspartases: D34 and D214. These residues are critical for its proteolytic activity. PMII specifically cleaves hemoglobin, releasing amino acids that the parasite utilizes. This makes it a prime candidate for drug intervention (Gulati *et al.,* 2015)

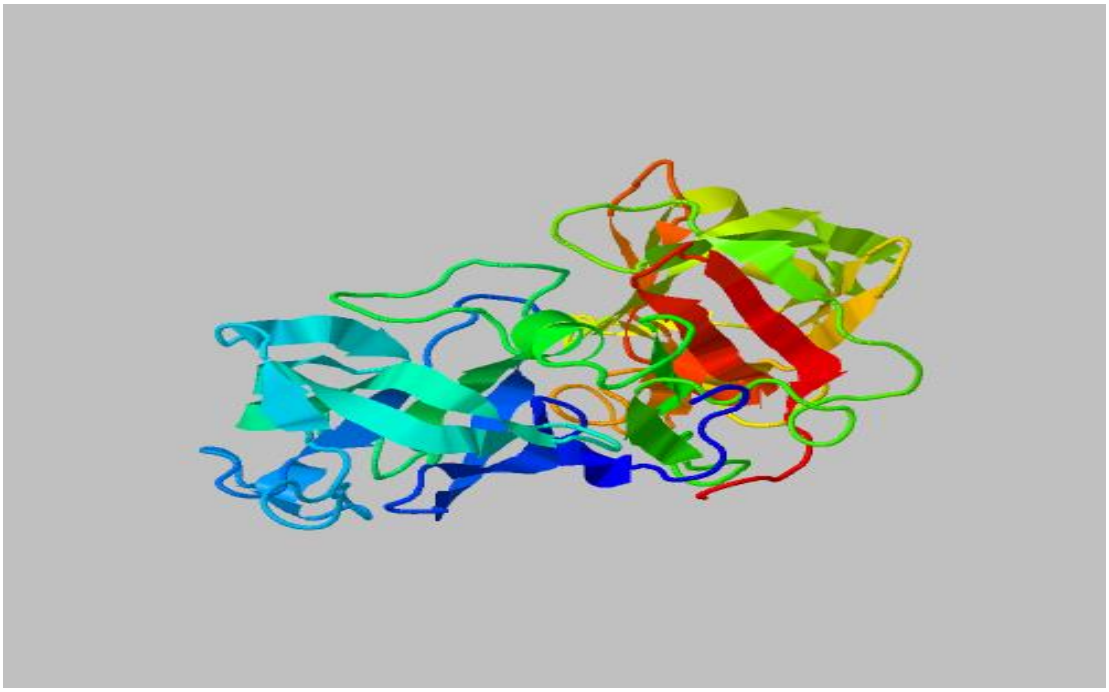

Figure 2.2: 3D Crystal structure of Plasmepsin-II (PDB ID: 1LEE)

2.12.2 Falcipain-II

Falcipain-2 is a cysteine protease found in *P. falciparum*, the deadliest malaria parasite species. Its primary function is to degrade host red cell hemoglobin within the food vacuole. By breaking down hemoglobin, falcipain -2 provides essential amino acids for parasite protein synthesis and metabolic needs. It is vital for the survival of the malaria parasite *Plasmodium falciparum*. Inhibition of Falcipain-2 disrupts this process and impairs parasite development. Falcipain-2 also plays a role in cleaving ankyrin and band 4.1 protein. These proteins are essential for the stability of the red cell membrane. Disruption of cytoskeletal elements affects the parasite's survival (Brown, 2008). Researchers have screened alkaloids as potential falcipain-2 inhibitors. Alkaloids hold promise as anti-malarial agents due to their diverse chemical structures. A total of 340 alkaloids were considered for study using computational pipelines.

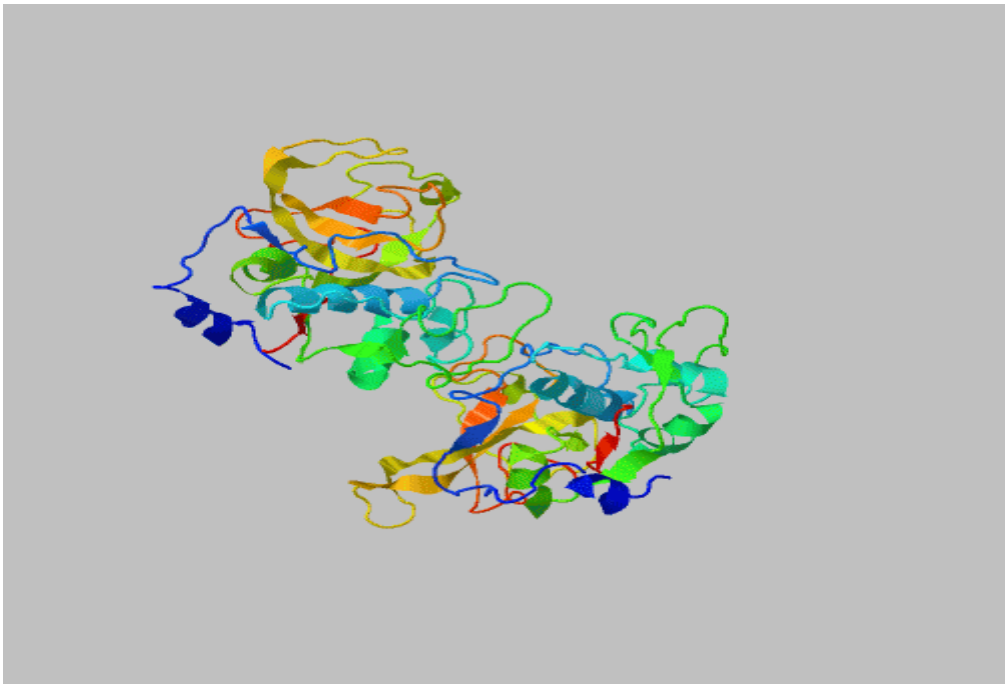

Figure 2.3: 3D Crystal structure of Falcipain-II (PDB ID: 7ei0)

2.12.3 Aminopeptidase

Aminopeptidases are enzymes involved in peptide degradation. They play a crucial role in the parasite's survival by breaking down hemoglobin. Hemoglobin degradation provides essential amino acids for the parasite's growth and development. PfAM is an aminopeptidase discovered in Plasmodium falciparum, the most lethal malaria parasite species. It belongs to the M1 family of metalloproteases. PfAM1 is essential for the parasite's intra-erythrocytic stages (Edgar et al., 2023). PfAM1 metallo-aminopeptidase in malaria is thought to be a potential therapeutic target. Bestatin, a natural product dipeptide mimic, is a strong PfA-M1 inhibitor (Velmourougane *et al.*, 2011).

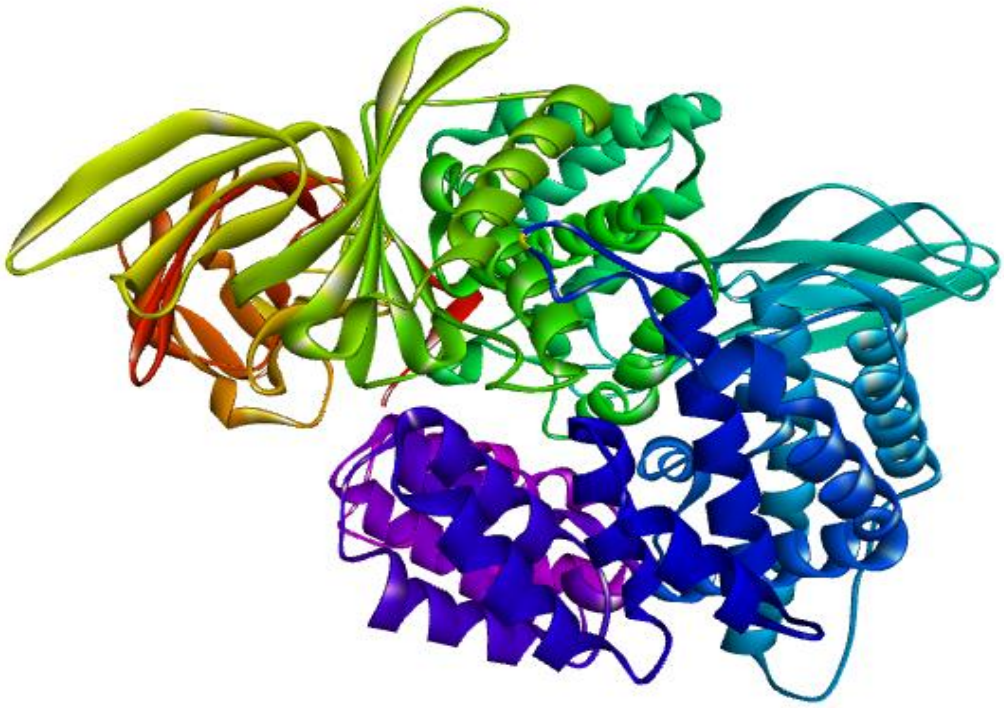

Figure 2.4: 3D Crystal structure of aminopeptidase (PDB ID: 3Q44)

CHAPTER THREE

3.0 Materials and Methods

3.1 In silico Study

3.1.0 Sources of data

The ligands and target proteins used in the in silico analysis were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) and the Protein Data Bank (https://www.rcsb.org/).

3.1.1 Ligands/phytochemicals search

A literature survey was firstly conducted to identify the phytochemicals found in the selected medicinal plants. In *C. occidentalis* plant, a total of 13-ligand compounds (n=13) were identified and retrieved, in *A. annua* plant, a total of 123-ligand compounds (n=123) were identified and retrieved, in *C. officinalis*, a total of 7-ligand compounds (n=7) were identified and retrieved and *S. thomsonii* plant, a total of 11-ligands (n=11) were revealed using GC-MS and their respective 3D structures were retrieved from pubchem. The chemical structures of the identified phytochemicals were obtained and stored in SDF format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov). Additionally, the structures of licensed malaria medication (chloroquine) were acquired and saved in SDF format for empirical comparison with potential ligands during computational analysis.

3.1.2 Ligand Preparation

All the chemical structures of the identified phytochemicals/ligands of the selected medicinal plants was downloaded from the Pubchem database and saved in SDF format. The ligands were also all converted into PDBQT format prior to docking using PyRx software.

3.1.3 Protein preparation

The protein data bank https://www.rcsb.org/ was used to retrieve the 3D crystal structure of *Plasmodium falciparum's* proteins (PDB entry code: 1lee, 7ei0 and 3q44). However, before docking, the co-crystallized ligands, water molecules and other heteroatoms present in the co-crystallized protein molecules were removed using BIOVIA Discovery Studio 2021. All the target proteins were converted into PDBQT format prior to docking using PyRx software. The BIOVIA Discovery Studio 2021 was used to prepare the PDB file as per standard protocol described in (Verma *et al.,* 2020).

3.1.4. Virtual screening and molecular docking

Molecular docking was carried out using the PyRx tool to evaluate the binding affinity and different sorts of interactions between the ligand molecules and target proteins. PyRx is a virtual screening tool based on AutoDock 4.2 and Vina. Target proteins and ligand molecules were loaded into PyRx as macromolecules and ligands respectively together with the control ligand (chloroquine). For each receptor, a grid box was placed with particular dimensions for aminopeptidase, falcipain II, and plasmepsin II. All ligands were docked, and the maximal exhaustiveness was calculated for every one of them. All other program settings were kept to their default values. Analysis and comparison of the docking results with the control group were conducted according to the method described by (Memvanga *et al.,* 2015). Biovia Discovery was used to create 2D and 3D diagrams showing the interactions between ligand atoms and amino acid residues of the target proteins.

3.1.5 ADME Analysis

The ADME properties of the screened ligands were forecasted utilizing the SWISSADME website (http://www.swissadme.ch/index.php), an online resource offering curated information on chemicals with established Absorption, Distribution, Metabolism, and Excretion (ADME) profiles (Daina *et al.,* 2017).

3.1.6 Toxicity Analysis

Before the drug molecule utilized as a medicine, the toxicity analysis of the molecule has to be done to ensure its safety. pkCSM (http://biosig.unimelb.edu.au/pkcsm/) is simply an online pharmacokinetics prediction database which predict Small-molecules for ADMET properties. The ligands toxicity was analyzed by entering the ligand SMILE format in to the database (Pires *et al.*, 2015).

3.2 Experimental/wet lab study

3.2.1 Collection and identification of plant materials

3.2.2 Artemisia annua L.

Artemisia annua L. plant with voucher number 8620-KASH, were collected from Kawoosa Budgam, Kashmir region of India in August, 2022. The collection site is located at coordinates (74.3051, 34.0614) and has an altitude of 1585 meters. The plant's authentication was conducted by a curator at Kashmir University, Department of Botany, Jammu and Kashmir, India. The authenticated plant specimen were deposited in the KASH herbarium. After collection, the plants were washed, subjected to shade drying for a period of 2-3 weeks until they were completely dry, and then powdered.

3.2.3 Cassia occidentalis L.

Cassia occidentalis L. with voucher number 9134-KASH plant was collected from Rama mandi, Jalandhar, Punjab region of India. The collection site is located at coordinates (31^{o} 30’86, 75^{o} 62’97”). The plant's authentication was conducted by a curator at Kashmir University, Department of Botany, Jammu and Kashmir, India. After collection, the plants were washed, subjected to shade drying for a period of 2-3 weeks until they were completely dry, and then powdered.

3.2.4 Swertia thomsonii L.

Swertia thomsonii L. plant with voucher number 9085-KASH, were collected from Kashmir region of India in November, 2023. The plant's authentication was conducted by a curator at Kashmir University, Department of Botany, Jammu and Kashmir, India. The authenticated plant was deposited in the KASH herbarium. After collection, the plants were washed, subjected to shade drying for a period of 2-3 weeks until they were completely dry, and then powdered.

3.2.5 Cinchona officinalis (Bark)

The bark of *Cinchona officinalis* L. was obtained from a local market in Jalandhar district of Punjab, India. The bark was mashed into a fine powder using a blender. This fine powder was used for experimental purpose.

3.3 Plant processing

The selected medicinal plants were washed with water and air dried in a shade for 7-days. The dried samples of the selected medicinal plants were pounded to coarse powder using electric blender. The mass of the powder was weighed using analytical weighing balance. The powder was packed in a sealed bag, label and stored in a dry cool place for further experiment.

3.4 Plant extraction protocol

The powdered plant samples from the selected medicinal plants were extracted using four different solvents namely: (Petroleum ether → Chloroform → Methanol→ Distilled water (ddH_2O). About 50g of each plant sample from the selected medicinal plants were extracted in 200ml of each solvents by cold maceration method started from less polar to high polar solvents in this series: (Petroleum ether → Chloroform → Methanol→ Distilled water (ddH_2O) successively. The powdered plant materials were kept in a conical flask in contact with the solvent in a stoppered container for a week with frequent agitation until soluble matter become dissolved. The mixture of plant samples was allowed to soaked for 7-days so that plant molecules could move through the solvent according to their polarity. After a week, filtration was performed with Whatmann filter

paper no.1. Subsequently, the extracts underwent concentration utilizing a rotary evaporator under vacuum conditions at 600 mmHg. For chloroform and petroleum ether extracts, heating water to 50°C was employed, while the methanol extract required a slightly higher temperature of 60°C. Evaporation of solvents in aqueous extracts was facilitated by subjecting the extract to a hot air oven set at 50°C under a pressure of 1 mmHg (Bashir *et al.,* 2021). The extracts were weighed and the percentage yield of the crude extracts were calculated and stored in sterile vials at 4^{o}C for further assays.

$$\text{Percentage yield of the crude extracts} = \frac{\text{Weight of concentrated extract}}{\text{Weight of sample used}} \times 100$$

3.5 Preliminary phytochemical analysis

The extracts generated from the selected medicinal plants were subjected to qualitative phytochemical analysis in order to establish distinct phytoconstituent profiles, as stated by (Riaz *et al.*, 2018; Usman *et al.*, 2020). Each extract was accurately weighed and subsequently dissolved in its corresponding parent solvents to attain a concentration of 10 mg/ml, equivalent to a stock solution containing 1% extract (w/v). This standardized preparation facilitated the execution of various tests, each conducted independently on the respective extracts.

3.5.0 Test for Alkaloids using Meyer's test

1 ml of 1 percent hydrochloric acid solution was placed in a test tube before adding 3 ml of plant extract. Following that, a small volume of potassium mercuric iodide solution was put into the mixture. The presence of alkaloids is indicated by the formation of a faint yellowish turbidity or precipitate (Usman *et al.,* 2020).

3.5.1 Test for Terpenoids using Salkowski reagent.

In separate test tubes of 5 ml of each plant extract, 2 ml of chloroform and 3 mL concentrated sulfuric acid were added to form distinct layer. Evidence of terpenoid was observed by the red brownish color developed (Usman *et al.*, 2020).

3.5.2 Test for Saponin

To generate a stable and enduring froth, 0.5 ml of each plant extract was vigorously shaken in a test tube containing 2 ml of distilled water. The presence of saponins is indicated by the persistence of a foamy lather lasting beyond ten minutes (Usman *et al.,* 2020).

3.5.3 Test for Flavonoids

In each extracts, a small amount of sodium hydroxide (NaOH) solution was introduced into a test tube. Flavonoids were confirmed by the yellow color development that dissipated in the presence of a weak acid.

3.5.4 Test for Tannins

Following the addition of a small quantity of 5% ferric chloride solution to 2-3 ml of the extract, the ensuing change in coloration was documented. Condensed tannins yield a dark green hue, whereas hydrolysable tannins yield a blue-black coloration (Riaz *et al.,* 2018; Usman *et al.,* 2020).

3.5.5 Gelatin Test

In the presence of the test solution, 1 ml of 1% gelatin solution and 1 ml of 10% NaCl were added. The emergence of a white precipitate of gelatin signified the presence of tannins (Riaz *et al.,* 2018; Usman *et al.,* 2020).

3.5.6 Test for Cardiac Glycosides (Keller-Killani Test).

In separate test tubes, 5mL of each plant extract was mixed with 2mL of glacial acetic acid, followed by a few drops of ferric chloride solution. Next, 2mL of concentrated H2SO4 was added to the mixture. A dark-brown ring appeared at the contact, confirming the presence of glycoside (Riaz *et al.,* 2018; Usman *et al.,* 2020).

3.5.7 Test for Phenols (Ferric Chloride Test)

The crude extract of the selected medicinal plant was carefully combined with 3-4 drops of ferric chloride solution. The presence of phenols was indicated by the appearance of a bluish-black coloring (Riaz *et al.,* 2018; Usman *et al.,* 2020).

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3.5.8 Test for Quinones

Concentrated H_2SO_4 was introduced to 2 ml of the test substances and vigorously agitated for a duration of 5 minutes. The appearance of a red hue signifies the presence of quinone (Riaz *et al.,* 2018; Usman *et al.,* 2020).

3.6 Characterization of the compounds

The qualitative assessment and characterization of the methanol extract of Swertia thomsonii were conducted utilizing gas chromatography mass spectrophotometry (GC-MS). This analytical technique was used to verify the presence of various phytochemicals in the extracts, employing established methodologies.

3.6.1 High-performance Thin Layer Chromatography (HPTLC)

The extracts derived from the chosen medicinal plants underwent analysis via High-Performance Thin-Layer Chromatography (HPTLC), adhering to the standard protocol outlined in (Nazir *et al.,* 2021), with slight modifications made as necessary.

3.6.1 Reference compounds for HPTLC analysis

Prior to HPTLC analysis, we performed a virtual screening, molecular docking, and ADMET analysis of the ligands molecules in all the selected medicinal plants with three target proteins of *P. falciparum* which lead to screening of some few compounds hypothesized to be the bioactive molecules possessing inhibitory activity. The derivatives of quercetin in (*C. occidentalis L.),* artemisinin in (*A. annua* L.) and quinine in (*C. officinalis L.*) were considered as reference/standard compounds for HPTLC analysis. However, GC-MS analysis of *Swertia thomsonii* L. revealed a total of 11 bioactive

compounds which were virtually evaluated for antiplasmodial activity and Flavone, 5-hydroxy-7,8-dimethoxy- emerged as the excellent candidate. All reference/standards compounds were procured from Sigma–Aldrich (USA) and stored at -20°C. All solvents used in our experiments were of HPLC grade that were procured from HIMEDIA.

3.6.2. Preparation of standards (Artemisinin, quinine and quercetin)

To create a stock solution of artemisinin, quinine and quercetin at a concentration of 1 mg/mL, 6 mg of all the three standards were dissolved in 6 mL of methanol. Subsequently, final concentrations of 0.1 mg/mL of all the standards were achieved by dissolving 1 mL of the stock solution in 10 mL of methanol.

3.6.3 Preparation of test samples (Extracts)

To prepare concentration of 1 mg/mL, 10 mg of free extracts were each dissolved in 10 mL of their respective solvents. All sample solutions were filtered using a 0.22 µm polypropylene membrane filter before being subjected to HPTLC analysis.

3.6.4 Separation of bioactive compounds using HPTLC

High-Performance Thin-Layer Chromatography was employed for the separation of bioactive compounds. HPTLC analysis was performed by selecting four extracts from each selected medicinal plants. The HPTLC system consists of a Linomat-5 applicator with a 100 µl syringe and a TLC scanner3 from CAMAG Medtech AG, Switzerland. All processes were carried out with the Win CATS program version 1.4.6.2002. The HPTLC run was performed on a silica gel 60F254 pre-coated plate of 20 × 10 cm with a mobile phase of toluene, chloroform, and acetone (2:8:2). Merck Millipore catalog number: 100390. Samples were applied using a Linomat-5 CAMAG applicator with a 100µl syringe. The parameters included a 6 mm band length, a 10 sec/mL application rate, and an 8 mm gap between applications. The distance from the plate's side edge was maintained at 1.5 cm. The unchangeable parameters included a 75.0 mm developing distance and a 12-15 minute development timen and detection with anisaldehyde reagent. Derivatization was performed after chromatography for 5 minutes in a hot air oven set to 100 °C. Camag TLC scanner 3 and Win Cats software were used to scan densitometry at 550 nm for artemisinin, 254 nm for quercetin, and 226 nm for quinine reflectance. Before drying, the plates were sprayed with anisaldehyde reagent, which consisted of 1 mL anisaldehyde, 20 mL glacial acetic acid, 170 mL methanol, and 10 mL concentrated

sulfuric acid. Before scanning, the generated plates were dried in a hot air oven at 100°C for 5 minutes. The scanning The densitometric parameters were a 4.00 × 0.30 mm slit, a scanning speed of 20 mm/s, and data resolution of 100 mm per step. The peak of separated substances in each extract was detected by comparing it to that of the standards, and the retention factor (Rf.) Value was calculated. The concentrations of each target compound were determined using the calibration curves.

3.7 HPTLC Validation

The HPTLC method was validated in accordance with the ICH guidelines (ICH guidelines, 2005) as follow;

3.7.0 Linearity

Calibration curves for artemisinin and quercetin were established by applying varying volumes of standard solutions (5, 10, 15, and 20µl), resulting in a linearity range of 5-20 µg/spot. Similarly, calibration curve for quinine were established by applying various quantities of quinine standard solution (4, 8, 12, and 16µl) to achieve a linearity range of 4-16 µg/spot. Peak area versus concentration graphs were constructed at their respective resolved wavelengths. Utilizing the regression equation and consistent peak areas, the yields of artemisinin, quercetin, and quinine were estimated.

3.7.1 Precision

To determine the precision and accuracy of the method, 500ng/spot of all the standards with (n = 4) was spotted in four different concentrations in triplicates. Reproducibility and repeatability of the results were determined by computing the coefficient of variation for Intra-day and inter-day precision with different concentrations of all the standards three times on the same day and three days later respectively. These observations were repeated three times and the results were presented as mean ± %RSD.

3.7.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were computed on the bases of standard error (SE) of the response and the slope of the calibration curve of the standards using the relations below:

$$\textbf{LOD} = \frac{(3.3 \times SE)}{Slope}$$

$$\textbf{LOQ} = \frac{(10 \times SE)}{Slope}$$

Where SE = Standard error of the response

3.7.3. Densitometry and evaluation of peak spectra

To ascertain the purity and specificity of artemisinin, quinine and quercetin within the extracts, densitometry scanning was conducted using a CAMAG TLC scanner 3 operating in absorption mode. The scanner was linked to a personal computer equipped with the user-friendly winCATS software (version 1.4.2). For artemisinin, quercetin, and quinine, respective wavelengths of 550 nm, 254 nm, and 226 nm were selected based on preliminary experiments, aligning with the near absorption maxima of the marker compounds. The scanning parameters included a slit dimension of 5 mm × 0.4 mm, a scanning speed of 20 mm/s, and a data resolution of 100μm/step. Analysis involved assessing artemisinin, quercetin, and quinine based on their retention factor, UV spectrum, and peak area measurement. Chromatographic peak wavelengths were monitored within the range of 400 to 550 nm for artemisinin, 200 to 254 nm for quercetin, and 200 to 226 nm for quinine. This methodology enabled accurate characterization and quantification of artemisinin, quercetin, and quinine, furnishing valuable insights into their presence, purity, and concentration within the examined extracts.

3.8 *In vitro* antimalarial screening of the extracts from the selected medicinal plants

The test samples were evaluated in triplicate over a 72-hour period on two separate occasions, targeting the 3D7 (chloroquine-sensitive clone) and Dd2 (chloroquine-resistant clone) of *P. falciparum*, the parasite organism that causes human malaria. Cultures of asexual erythrocyte stages of *P. falciparum* were continuously maintained using the approach reported by (Trager and Jensen, 1976) with minor adjustments made as needed. The quantitative evaluation of antiplasmodial activity *in vitro* was conducted through the parasite lactate dehydrogenase assay as outlined by (Makler *et al.,* 1993). This assay relies on the colorimetric assessment of parasite viability, in which the breakdown of a dye by glycolytic enzymes in living parasites serves as a marker for survival.

3.8.0 Preparation test samples (extracts)

All the test samples (*extracts*) from the selected medicinal plants were prepared to a 200 mg/mL stock solution in 100% solvent (DMSO). Samples were tested as a suspension if not completely dissolved. Further dilutions to the desired starting concentration were freshly prepared in growth media on each occasion of the experiment.

3.8.1 Preparation of standards (Chloroquine phosphate and Artesunate)

Chloroquine phosphate and artesunate were utilized as standard medicines in this work, and a 10mmol/L stock was generated using the same process as the extracts for future use.

3.8.2 In vitro culture of malaria parasite

The malaria parasite *P. falciparum*, which included the chloroquine-resistant strain (Dd2) and sensitive strain (3D7) was procured from the H3D laboratory at the University of Cape Town. Fresh A+ erythrocytes suspended in RPMI-1640 medium supplemented with HEPES, D-glucose, sodium bicarbonate, antibiotics, and 10% heat-inactivated AB+ serum were used for the culturing of two *P. falciparum* strains. The parasite cultures were

maintained at 37°C with 5% CO_2 and the culture medium was refreshed every 24 hours with a new batch containing 10% heat-inactivated AB+ serum to ensure that parasitemia levels remained less than 2 %.

3.8.3 Antimalarial activity screening

In order to establish the efficacy of the investigated samples as antimalarial agents, chloroquine (CQ) and artesunate (Arts) which are widely recognized standard malaria drugs, were employed as reference/control drugs throughout the study. A comprehensive dose-response analysis was conducted utilizing a 96-well plate format, aimed at determining the concentration at which 50% inhibition of parasite growth (IC50-value) occurred. Initial evaluations of the materials were conducted at a concentration of 100 µg/mL, followed by serial 2-fold dilutions in growth medium to establish a range of tested concentrations. A consistent dilution protocol was adhered to for all samples. Standard drugs were initially evaluated at a concentration of 1 µg/mL to ensure comparability in assessment parameters.

The parasites were also subjected to a maximum solvent concentration of approximately 1%, which did not demonstrate any discernible impact on parasite viability. Subsequently, the assay plate was positioned within a hermetically sealed gas chamber and maintained at 37°C for duration of 72 hours under controlled atmospheric conditions comprising 3% O_2, 4% CO_2 and the remaining nitrogen. After incubation, gently agitate each well in the assay plate and transfer 15 µL to a duplicate plate with 100 µL of Malstat reagent and 25 µL of nitroblue tetrazolium solution. The plates were then incubated in darkness at room temperature for 20 minutes before being measured in a spectrophotometer at 620 nm.

3.8.4 Determination of IC50 values

To determine the surviving parasite population at each concentration of the test samples, the absorbance of each well was compared to that of a control well containing no test sample. To calculate IC50 values, a non-linear dose-response curve fitting analysis was carried out with the Dotmatics software platform and GraphPad Prism. Survival versus concentration was plotted. The extracts were classed as active (<20 μg/ml), intermediate (20-40 μg/ml), or inert (>40 μg/ml) based on their IC50 values.

3.8.5 *In vitro* cytotoxicity study

To assess the safety of the selected extracts for human consumption, toxicity tests were conducted utilizing the MTT assay, which employs 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

3.8.6 MTT cell viability assay

Toxicity assessments employing the MTT assay were carried out following established protocols as described in the literature (Geetha et al., 2014; Kavitha, 2018; Teh et al., 2013). The tests were conducted utilizing a neonatal mouse dermal fibroblast (NMDF) cell line to evaluate the safety profile of the extracts for human consumption across different dosage levels.

3.8.7 Cell line preparation

An assay was conducted using a neonatal mouse dermal fibroblast cell line (NMDF), which was first cultivated in DMEM medium (Dulbecco's Modified Eagle’s medium) supplemented with antibiotics (10 μg/ml streptomycin and 100 μg/ml penicillin). Until the cells reached 80% confluency, they were kept at 37°C in a humidified environment with 5% CO2.

3.8.8 Extract concentration preparation

The extracts were produced as stock solutions in 0.1% DMSO at a concentration of 1000 µg/ml. Next, serum-free DMEM media was serially diluted to yield working solutions at five distinct concentrations (500, 300, 200, 100, and 50 µg/ml). The extracts were filtered through a 0.22 µm nylon syringe filter to sterilize them before application.

3.8.9 Assay procedure

To achieve a cell concentration of 1.0×10^{-5} cells/ml, the initial cell stock was diluted with DMEM supplemented with 10% FBS. Following this, 100 µl of the diluted cell suspension was dispensed into each well of a 96-well microtiter plate. The plates were then incubated for 24 hours at 37°C ± 1°C in an atmosphere with 5% CO_2 to allow for the adherence of the NMDF cell monolayer to the well surfaces. After incubation, the supernatant was removed gently and the cell monolayer was washed with 200 µl of 1XPBS (pH 7.4). The cells were then subjected to 200 µl of test extracts at different doses (500, 300, 200, 100, and 50 µg/ml) in microtiter plate. To produce positive controls, 200 µl of fresh DMEM without extract was added to separate wells containing the NMDF cell monolayer. Negative controls received 200 µl of fresh media. The plates were then put in a humidified environment with 5% CO_2 at 37°C ± 1°C for 72 hours. After incubation, the sample solution was aspirated, leaving a cell monolayer which was then washed with 200 µl of 1X PBS.

200 µl of 0.5 mg/ml MTT solution was added in 1X PBS to each well and mixed slowly. The plates were incubated for 4 hours at 37°C ± 1°C in a humid atmosphere with 5% CO_2. After removing the supernatants and washing the cells with 200 µl of 1X PBS, 100 µl of DMSO was added to each well to dissolve the purple formazan crystals. After being placed on a microplate reader, the absorbance at 570 nm was measured. The study was carried out in triplicate, and each extract replicate's average absorbance was calculated. Following that, the percentage of cell cytotoxicity for each replicate was computed using the relation below:

$$\text{Percent (\%) cytotoxicity} = 100 - \frac{(Abs_{\text{treated cells}})}{(Abs_{\text{Pos. control (average)}})} \times 100$$

.

"$Abs_{\text{treated cells}}$" = Absorbance of extract-treated cells, whereas

"$Abs_{\text{Pos. control}}$" = Average absorbance of the positive control (untreated cells).

The average percentage of cell cytotoxicity versus the plant extract concentrations was plotted.

3.9 Preparation of herbal formulations

Finally, after assessment of antimalarial efficacy of the individual extracts of the selected medicinal plants, the herbal formulation was developed from the effective doses of all the selected active extracts of the medicinal plants. The formulation derived from these extracts can thus be employed as one of the solutions to the present resistance problem in *Plasmodium falciparum.*

3.10 Data and statistical analysis

To reduce experimental error, experiments were reproduced three times. Values were expressed as Mean ± SD and SEM in triplicates. Statistical analyses were conducted using the graph pad prism and Microsoft Excel program. The *in silico* results were examined using standard bioinformatics methods, taking into account binding affinity, appropriate chemical interactions between the ligands and the receptor molecule, and ADMET analysis, as indicated in the methodology section.

CHAPTER FOUR

4.0 Results and discussions

4.1 *In-silico* analysis results

Through virtual screening, a total of 3 phytochemicals from the three selected medicinal plants were screened based on docking score, ADMET properties and convenient molecular interactions between the ligands and amino acid residues of the three target proteins of *P. falciparum*. The three screened phytochemicals includes; Artemisinin from *A. annua*, quinine from *C. officinalis* and quercetin from *C. occidentalis*. Drug likeness as well as the ADMET properties of these lead compounds was evaluated and found suitable to be used as drugs. However, with regard to *S. thomsonii* plant, the GC-MS profile of its methanol extract revealed the presence of total 11-compounds. These 11-compounds were subjected to virtual screening through molecular docking experiment with the three target proteins of *P. falciparum* and identify 5-hydroxy-7,8-dimethoxy flavone as the most excellent compound to inhibit the activity of the target proteins (i.e *Plasmepsin-II, Falcipain-II and Aminopeptidase*) as shown in **Table 4.1.**

4.1.0 Molecular docking analysis

Binding affinity quantifies the strength of the interaction between a ligand (such as a drug or compound) and a target biomolecule (in this study, the Plasmepsin-II, Falcipain-II and Aminopeptidase enzymes). A more negative binding affinity indicates a stronger interaction, where the ligand is more likely to bind with enzyme. For quercetin, the calculated binding affinity was found to be -7.5, -8.2, and 9.0kcal/mol with 1lee, 7ei0 and 3q44 respectively. For artemisinin, the binding affinity was found to be -7.0, -7.5 and -8.8kcal/mol with 1lee, 7ei0 and 3q44 respectively. For quinine, the binding affinity was found to be -7.0, -6.5 and -7.9kcal/mol with 1lee, 7ei0 and 3q44 respectively. And finally, for 5-hydroxy-7,8-dimethoxy flavone, the calculated binding affinity was found to be -7.2, -7.1 and -8.78kcal/mol with 1lee, 7ei0 and 3q44 respectively. This suggests robust and favorable interactions with the target enzymes. This high negative value

indicates a strong binding strength, signifying that quercetin, artemisinin and quinine has the potential to effectively interact with and influence the enzyme's activity. However, In the case of the control compound, the binding affinity was found to be -6.0, 5.6 and 7.2kcal/mol with 1lee, 7ei0 and 3q44 respectively. This indicates a slightly weaker interaction compared to the selected compounds. Nonetheless, these negative values still indicates a favorable binding interaction between the control compound and the target enzymes. The results indicate that both the selected compounds and the control compound exhibit negative binding affinities, suggesting strong and favorable interactions with the target receptors. The details of molecular docking results were presented in **table 4.1**. Molecular interactions between the ligand molecules and the target enzymes illustrated in **figure 4.1-4.15** likely play a significant role in these binding affinities. However, further analysis and understanding of these interactions could provide insights into the potential inhibitory of these compounds on the selected enzymes activity.

Table 4.1: Binding affinity and protein–ligand interactions profile of the target proteins with the selected compounds

Targeted receptors with pdb ID	Compounds	Binding affinity Kcal/mol	Conventional H-bond interactions	Carbon Hydrogen interactions	π–σ interactions	π-Alkyl & Alkyl interactions
Plasmepsin-II (1LEE)	Chloroquine	-6.0	---	TYR A: 272(5.26)	---	Val A: 160(4.75 & 6.06), Arg A: 307 (5.70)
	Artemisinin	-7.0	---	---	---	Ile A:277 (23.06)
	Quinine	-7.0	Asn A: 13(5.19), Tyr A: 272(4.98)	Glu A:271 (5.54)	---	Ile A: 277(5.02 & 6.46), Val A: 160(5.91), Arg A: 307 (5.30)
	5-hydroxy-7,8-dimethoxy flavone	-7.2	Arg A:307(4.87)	Gln A:275(3.19)	---	Val A: 160(5.30), Ile A:(5.05)
	Quercetin	-7.5	Tyr A: 272(4.98), Arg A: 307(6.22), Ala A: 325(3.54)	Lys A: 163(5.13)	---	Val A: 160 (5.51 & 5.38), Lys A: 327 (6.30)
Falcipain-II (7EI0)	Chloroquine	-5.6	Val B:71(3.96)	Asp B:109(4.99), Ala B:110(5.32),	---	Tyr B:78(4.40)
	Artemisinin	-7.6	Trp B:206(20.20)	Gly B:40(27.28)	---	---
	Quinine	-6.5	---	Asp B: 35	---	His B:174(4.91), Ala B:157(4.87), Trp B:206(5.04 & 6.79)
	5-hydroxy-7,8-dimethoxy flavone	-7.1	Ala A:110(4.29), Ser A:108(5.48), Gln A:68(5.76), Arg B:213(7.28)	---	---	Cys A:80(4.64 & 5.95), Pro B:32(5.27)

	Quercetin	-8.2	Asp A:109(4.36), Asp A:72(2.65), Asn A:112(4.94), Cys A:114(3.65), Val B:30(4.30)	---	Pro A:111(5.11)	Pro B:32(6.24)
Aminopeptidase (3Q44)	Chloroquine	-7.2	---	Tyr A:575(5.92)	Tyr A:580(4.09)	Tyr A:575(3.98), Val A: 459(5.86 & 4.87), Met A: 462(5.82)
	Artemisinin	-8.8	Asn A:638(22.35)	---	---	Lys A:632(17.32), Ala A:637(21.47)
	Quinine	-8.1	Glu A:319(3.94), Met A:462(4.41)	---	---	Val A:493 (3.78), Val A:459 (5.07 & 4.63), Tyr A: 580(6.46), His A:496(4.03), Tyr A: 575(4.32 & 5.36)
	5-hydroxy-7,8-dimethoxy flavone	-8.7	Glu A:497(4.81)	---	Tyr A:575 (4.23 & 5.0)	Val A:459(5.00), Tyr A:580(5.45)
	Quercetin	-9.0	Glu A:497(4.96), Glu A:463(4.68), His A:496(4.92), Met A:462(4.16), Glu A:319(3.68 & 5.06), Gln A:317(5.64)	---	Val A:459(4.97)	Val A:459 (4.97 & 5.26), Tyr A:580(6.78), Tyr A:575(4.78& 4.68)

4.1.1 Molecular interactions study of the selected compounds with the three target proteins

The interactions between ligands and target biomolecules are vital for understanding the mechanism of action. These interactions can involve various forces, such as hydrogen bonding, hydrophobic interactions, and electrostatic interactions. The docking score alone provides false positive results for protein-ligand binding strength and stability (Cheng *et al.,* 2023). Supporting the docking result by taking into account the key amino acid residues involved in the docking process can make the docking result more reliable and convincing, as well as make it easier to verify inhibitory activity of the ligands against the target protein.

The detail of molecular interactions between the selected ligands and the target receptors were vividly presented in **Table 4.1** above. The results suggest that both the selected ligand compounds and the control compound exhibit favorable interactions with key amino acid residues of the three target receptors in their active binding site.

The presence of hydrogen bonds, carbon hydrogen interactions, π–σ bond and π-alkyl/alkyl interactions indicates that these ligands are effectively interacting with the enzyme's binding site. These interactions pattern could potentially lead to inhibition of the enzyme's activity, which is desirable in drug design targeting enzyme inhibition. The details of these interactions between the selected compounds with the target proteins were summarized in T**able 4.1.** The interaction observed in the selected compounds and that of control contributes in stabilizing them in the binding pocket of the receptors as illustrated in (**Figures 4.1-4.15**).

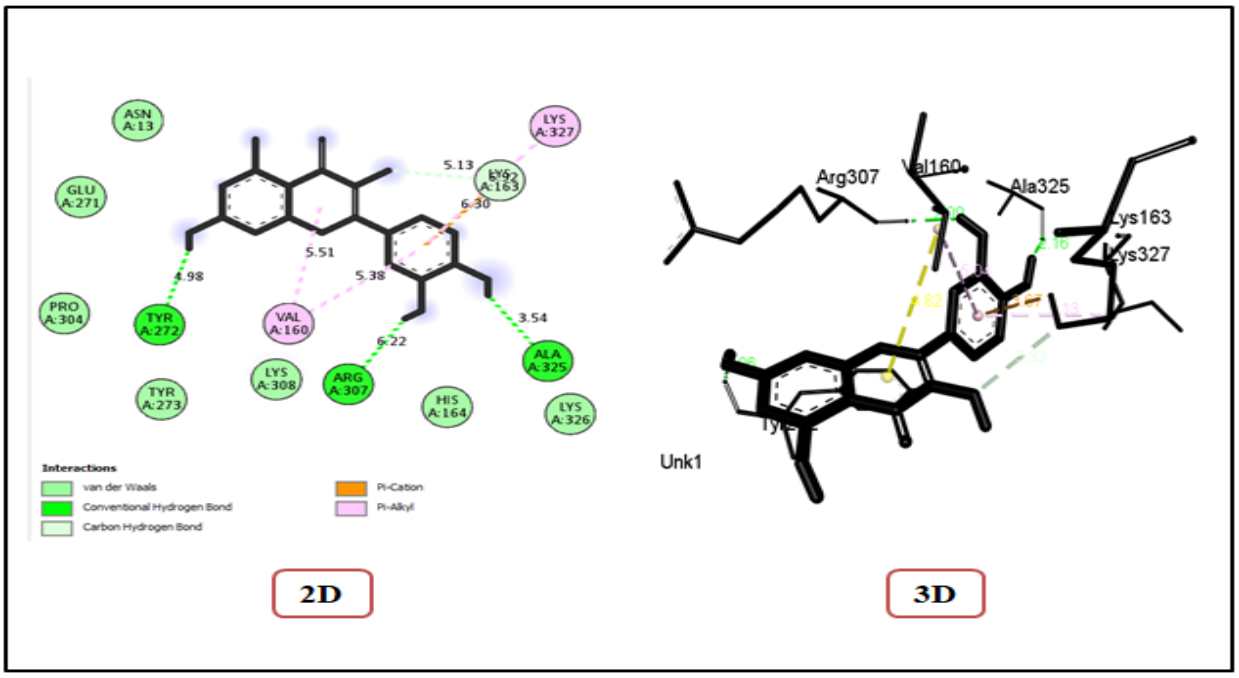


Figure 4.1: Complex of Quercetin-1lee in 2D and 3D format

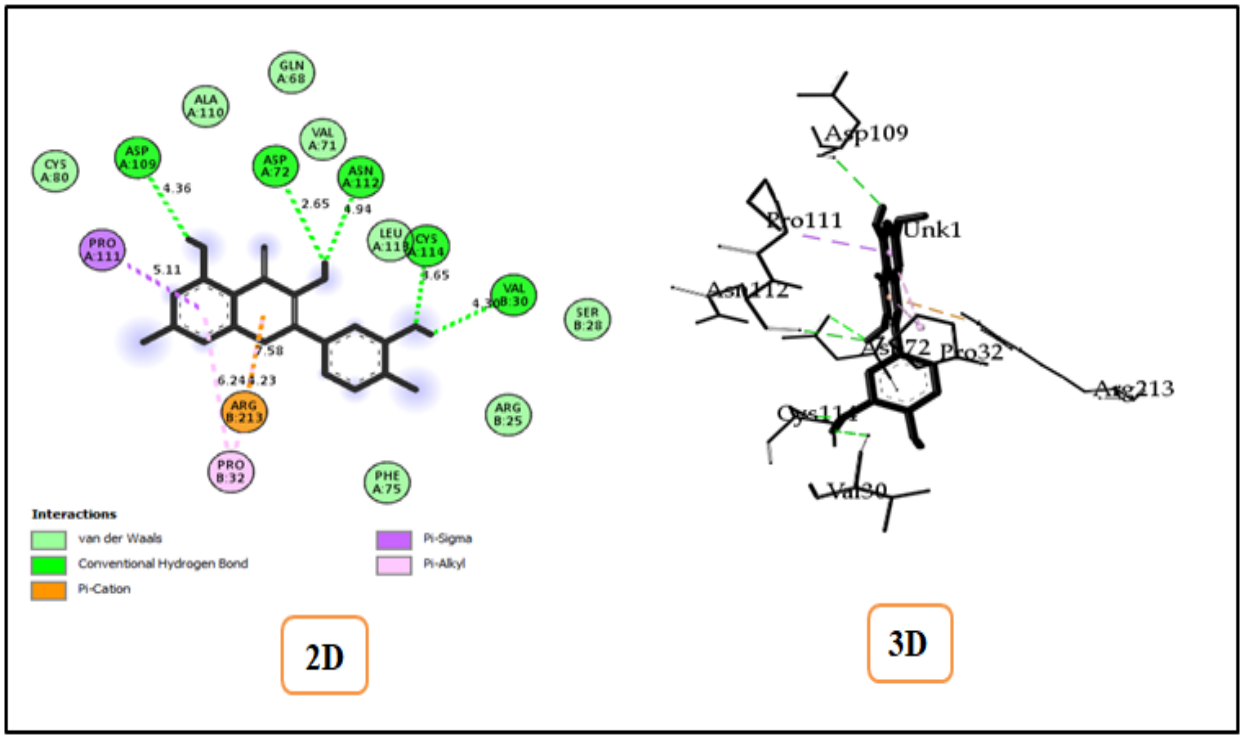


Figure 4.2: Complex of Quercetin-7ei0 in 2D and 3D format

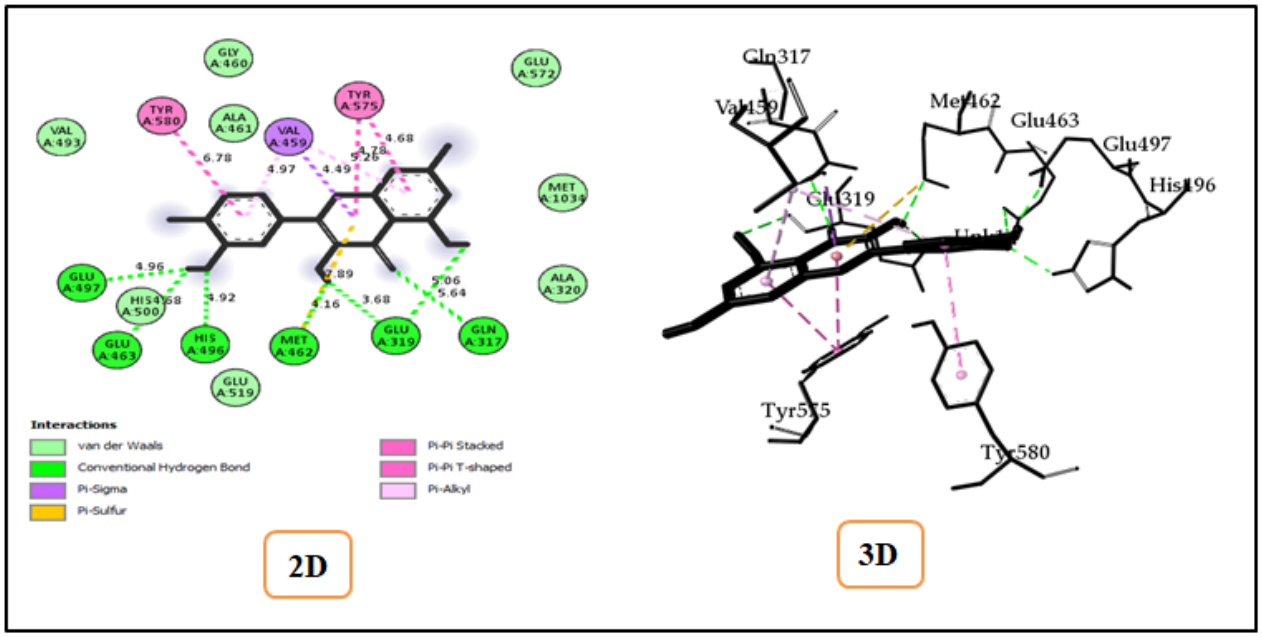


Figure 4.3: Complex of Quercetin-3Q44 in 2D and 3D format

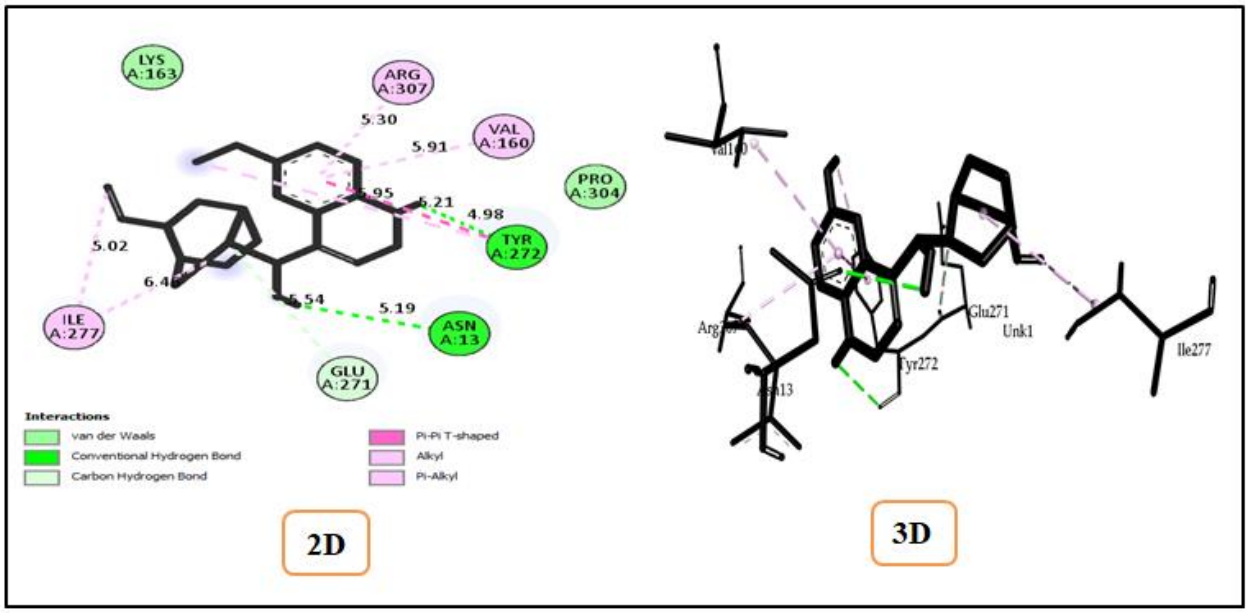


Figure 4.4: Complex of Quinine-1lee in 2D and 3D format

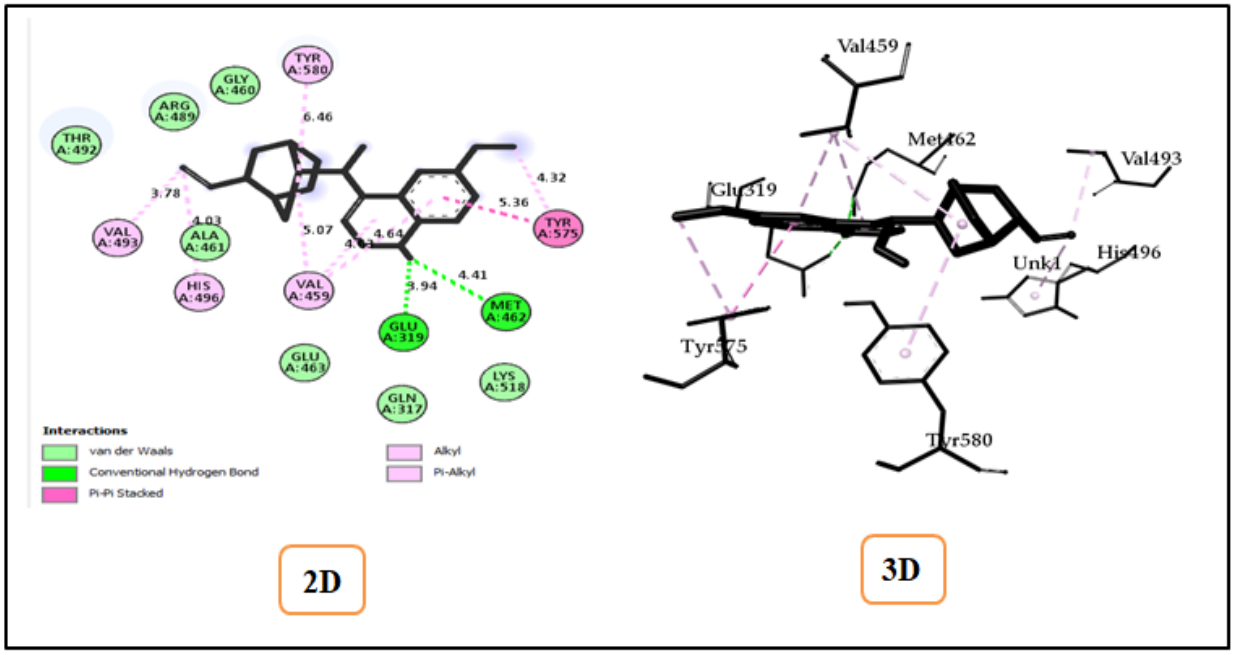


Figure 4.5: Complex of Quinine-3q44 in 2D and 3D format

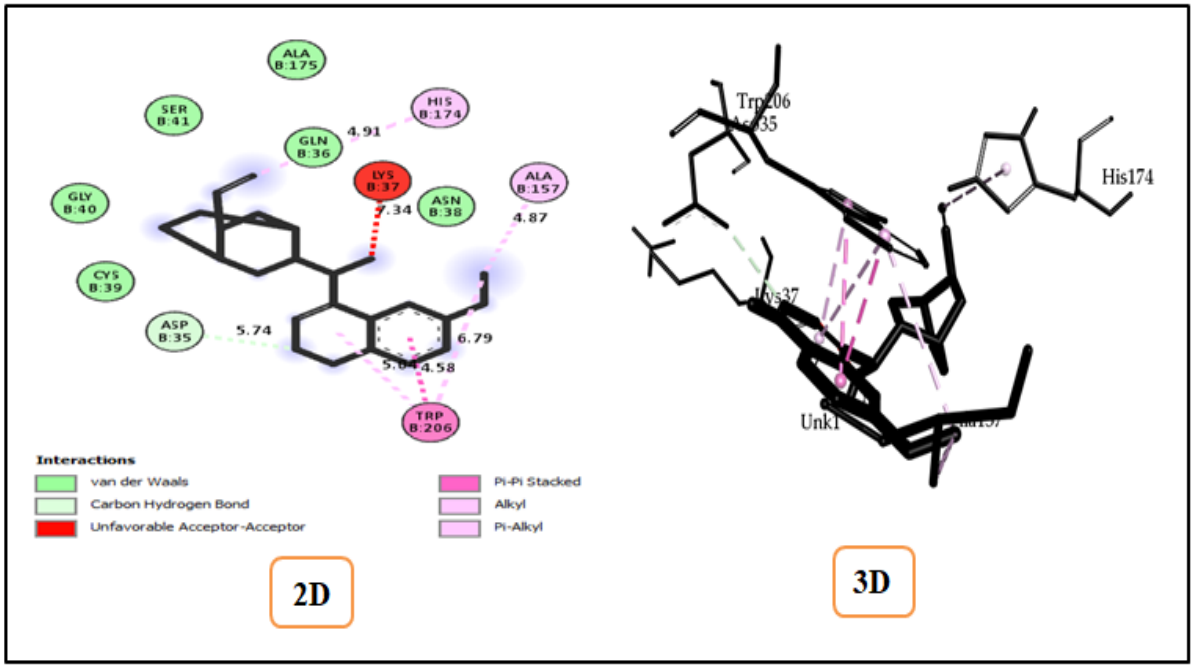


Figure 4.6: Complex of Quinine-7ei0 in 2D and 3D format

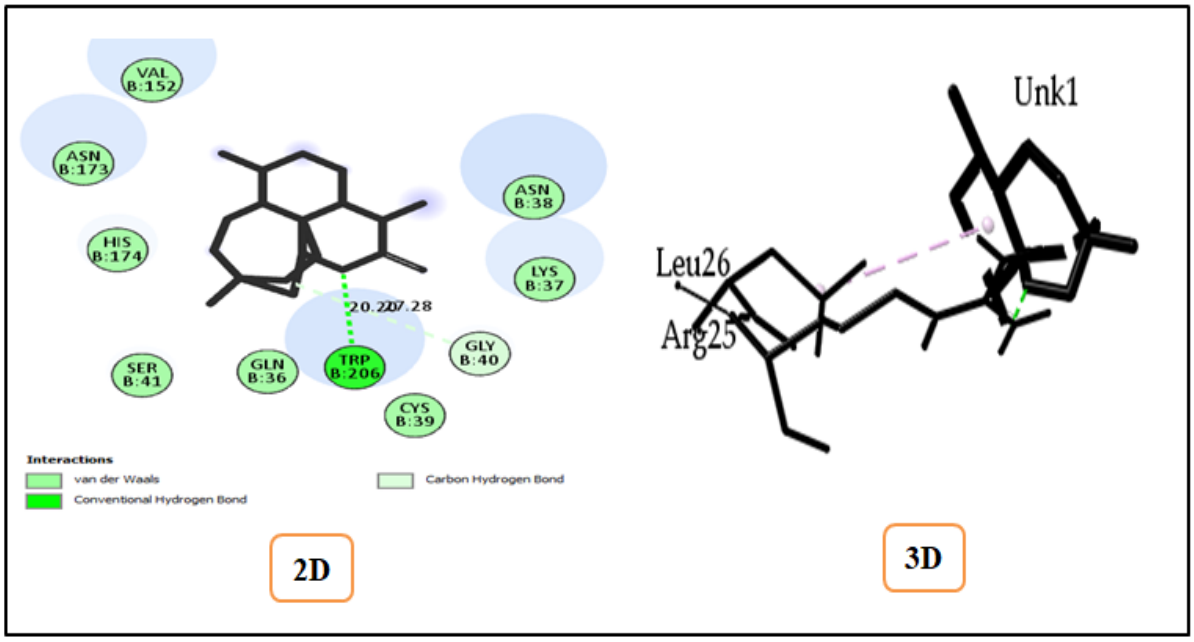


Figure 4.7: Complex of Artemisinin-7ei0 in 2D and 3D format

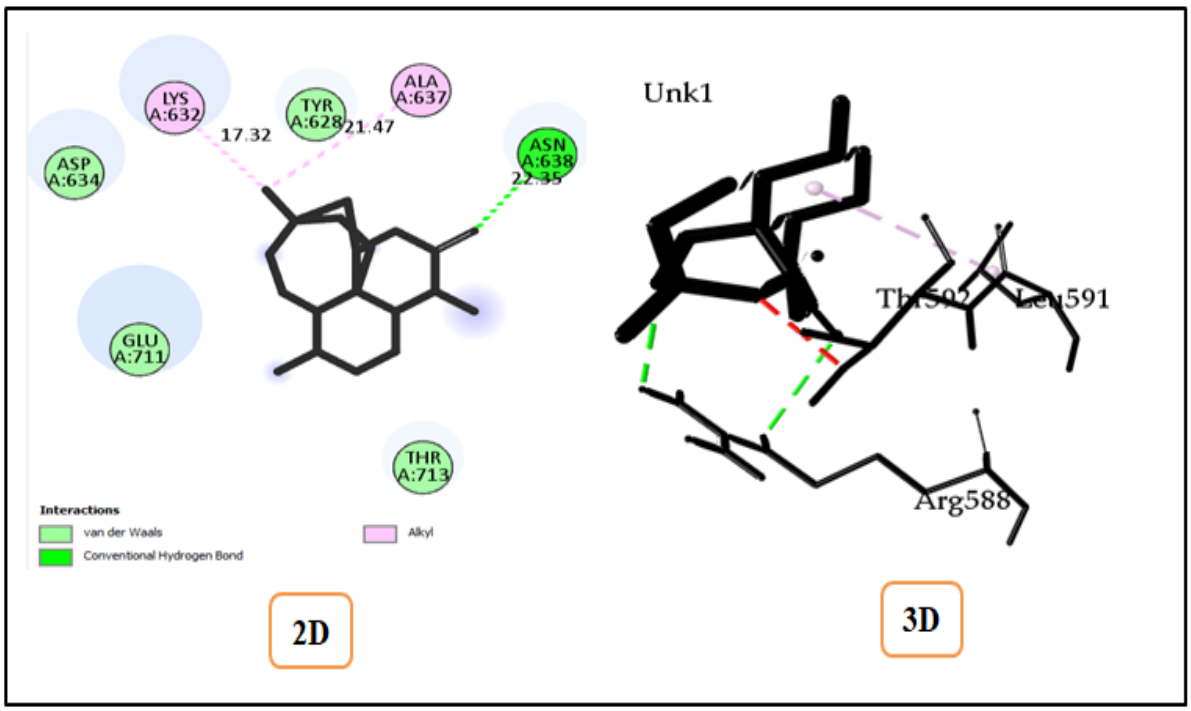


Figure 4.8: Complex of Artemisinin-3q44 in 2D and 3D format

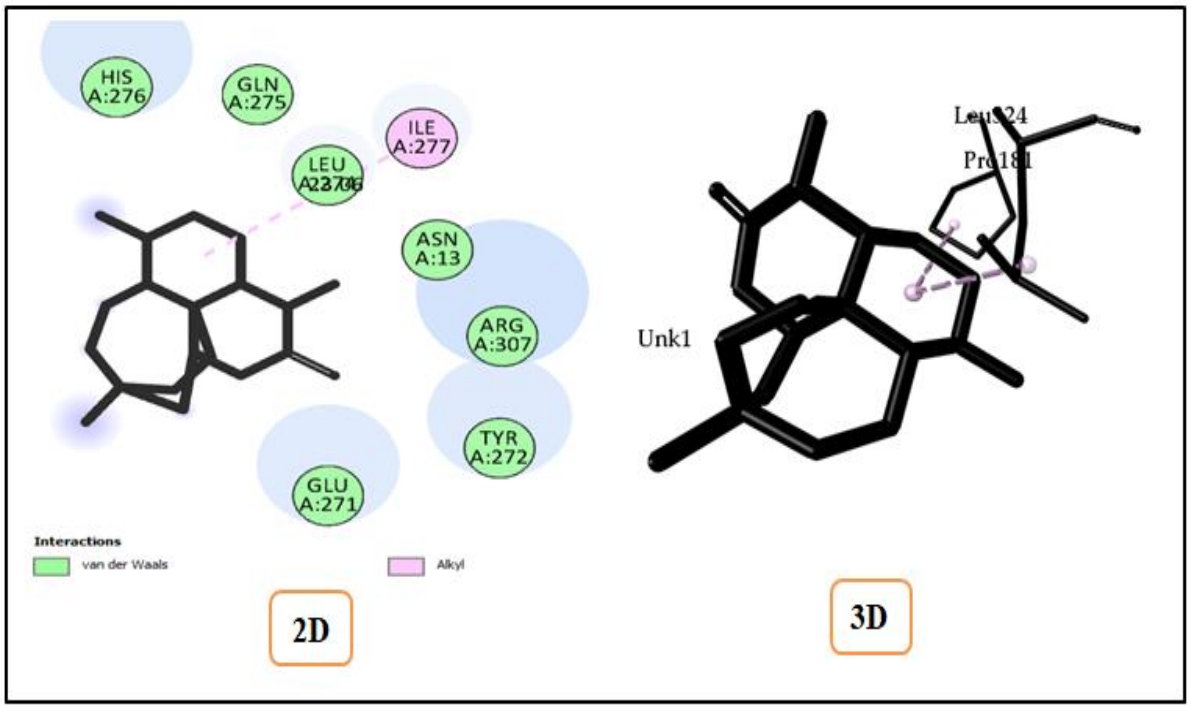


Figure 4.9: Complex of Artemisinin-1lee in 2D and 3D format

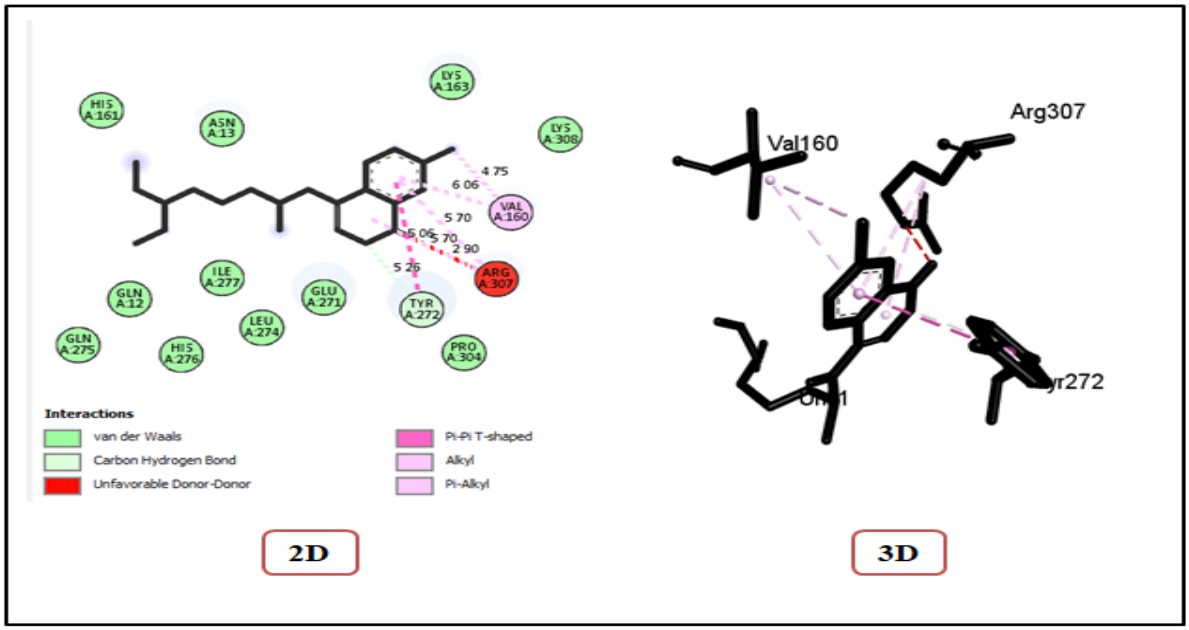


Figure 4.10: Complex of Chloroquine-1lee in 2D and 3D format

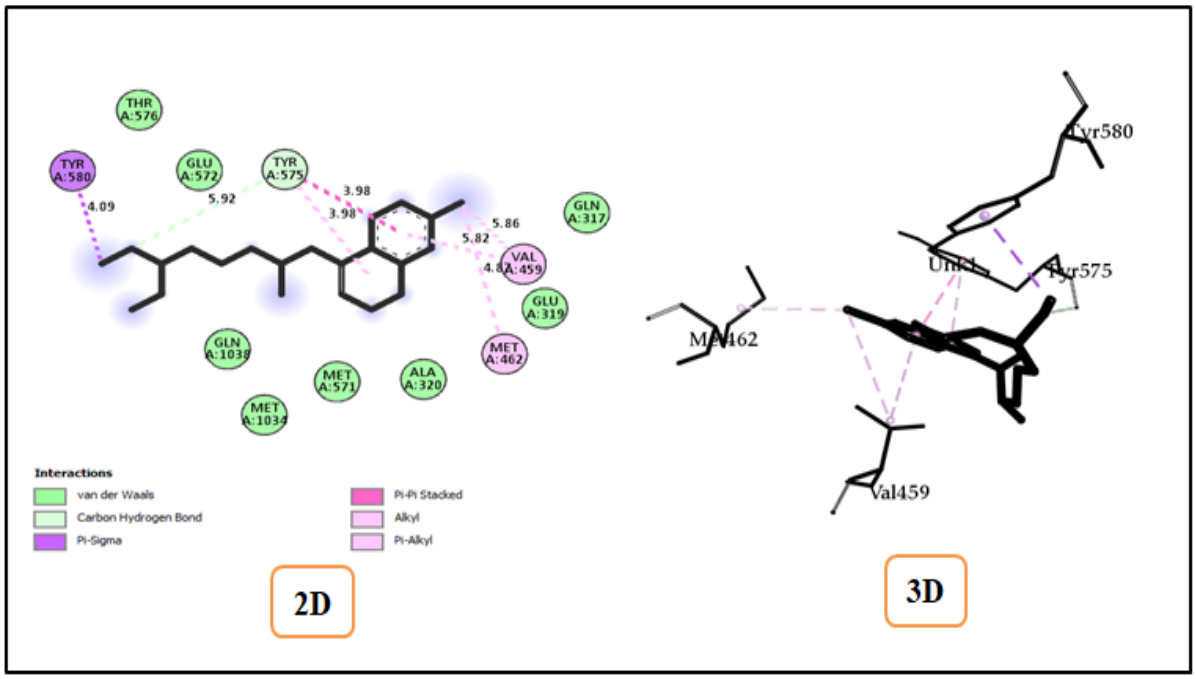


Figure 4.11: Complex of Chloroquine-3q44 in 2D and 3D format

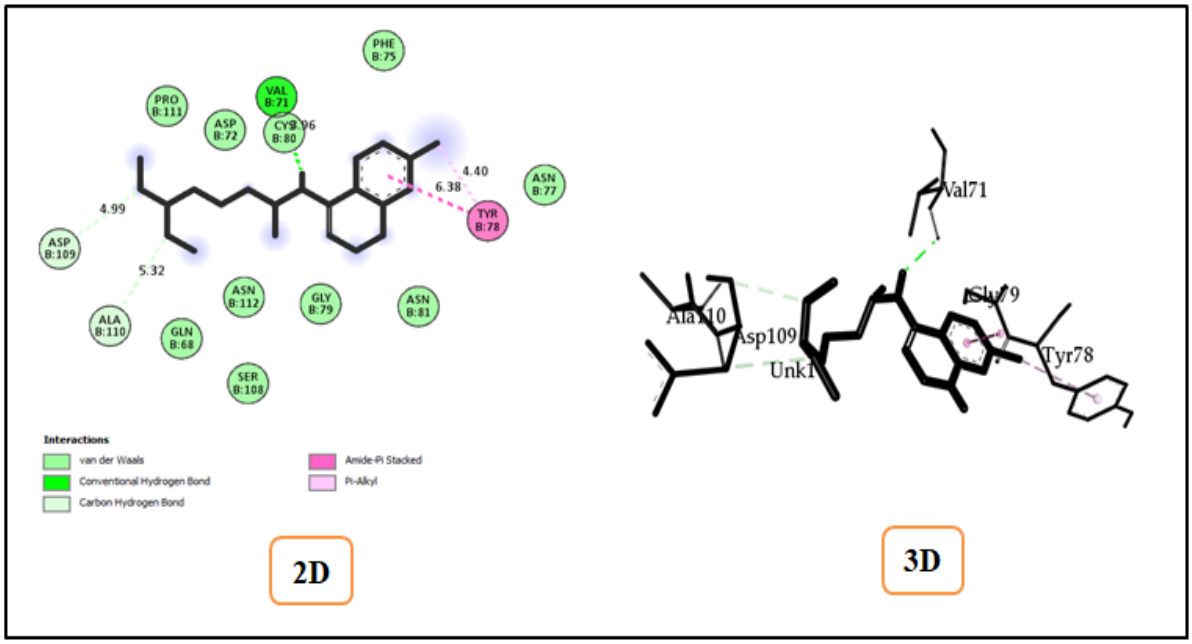


Figure 4.12: Complex of Chloroquine-7ei0 in 2D and 3D format

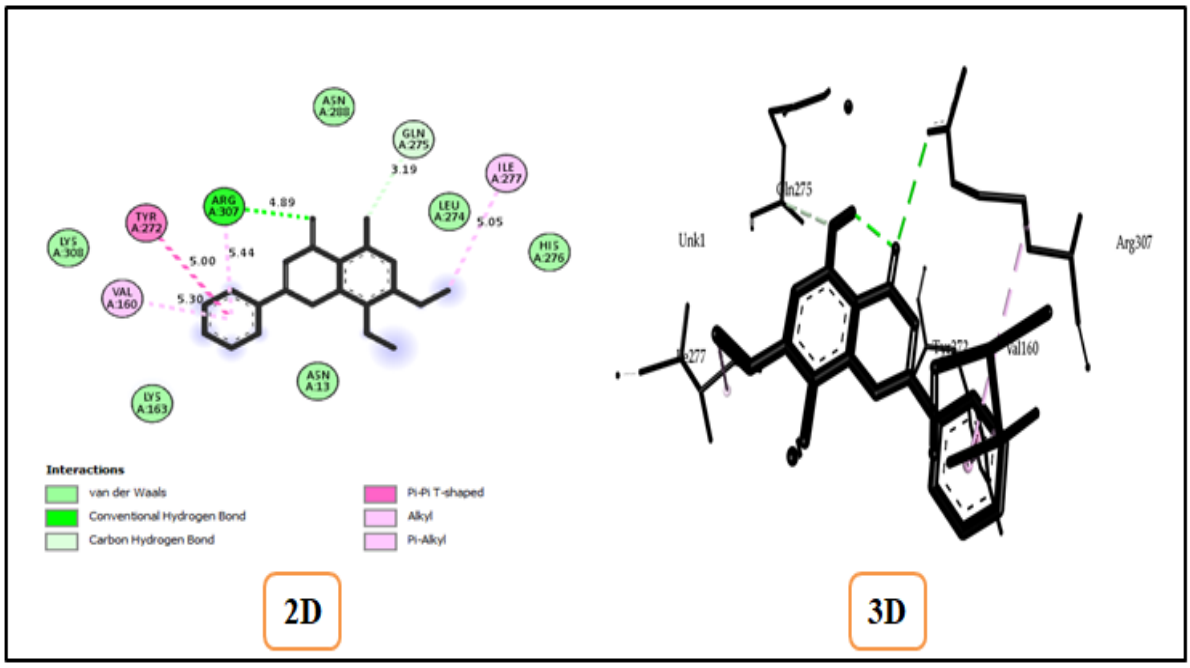


Figure 4.13: Complex of 5-hydroxy-7,8-dimethoxy flavone-1lee in 2D and 3D format

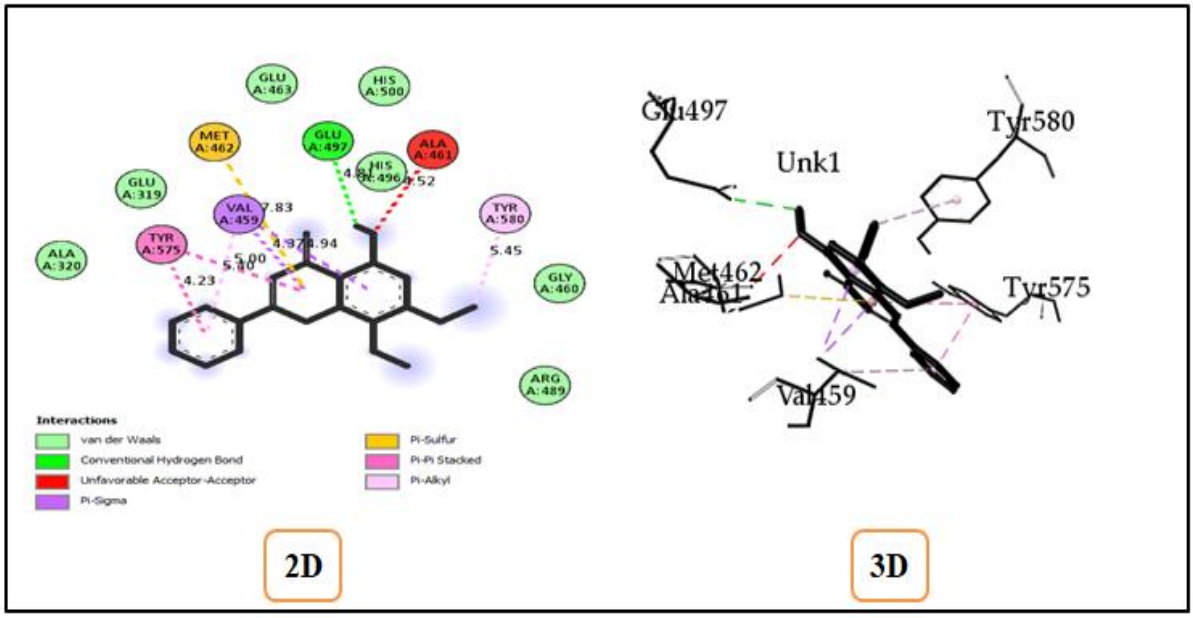


Figure 4.14: Complex of 5-hydroxy-7,8-dimethoxy flavone-3q44 in 2D and 3D format

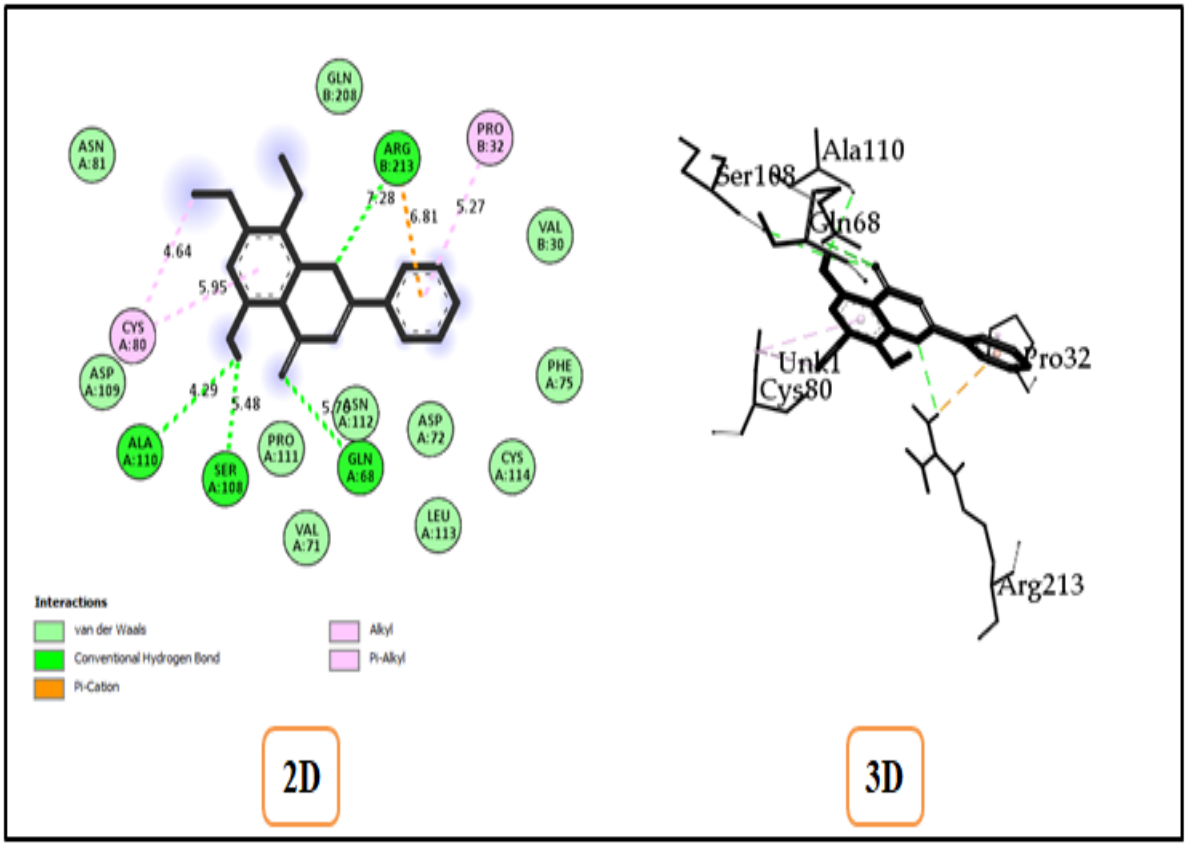


Figure 4.15: Complex of 5-hydroxy-7,8-dimethoxy flavone-7ei0 in 2D and 3D format

4.1.2 Major ADME Properties of the Selected Compounds and Control

The major ADME properties of artemisinin, quinine, quercetin and 5-hydroxy-7,8-dimethoxy flavone were evaluated and analyzed along with control compound using SWISSADME online tool (http://www.swissadme.ch/index.php) and the results are presented in **Table 4.2.** The result indicated that, all the compounds exhibited substantial drug-like properties as per the major parameters evaluated. Generally, orally active medicines delivered via the trans-cellular route should not have a TPSA greater than 120Å^2, while poorly absorbed compounds have a TPSA greater than 140 Å^2 (Zagórska and Jaromin, 2020; de la Nuez and Rodríguez, 2008). In this study, all the ligands had TPSA range between (28.16 to 131.36Å^2) and hence good at permeating cell membrane**.** The number of rotatable bonds of the all the ligands have nrotb within the recommended range (0 to 8). A drug compound with a number of rotatable bonds (nrotb) of ≤ 10 generally possessed good bioavailability. All the molecules shows good liphophilicity (XLogP3) value using the LogS (ESOL) not higher than 6 as a criteria. The pharmacokinetic properties of all the ligand molecules were predicted and presented in table 4: All the compounds obey the lipinski's rule of five without violation and have a good bioavailability score of [F>10%] as shown in **Table 4.2**.

Table 4.2: Major ADME parameters of the Selected Compounds and Control

Molecules	Water Solubility Log S (ESOL)	Lipophilicity Xlogp3	Polarity TPSA ($Å^2$)	Flexibility rotatable (0<_<9)	GI absorptions	BBB Permeants	P-gp substrate	Lipinsk violatio	Bioavailabity score
Molecule 1	-4.55	4.63	28.16	8	High	Yes	No	0	0.55
Molecule 2	-3.47	2.90	53.99	0	High	Yes	No	0	0.55
Molecule 3	-3.71	2.88	45.59	4	High	Yes	No	0	0.55
Molecule 4	-3.16	1.54	131.36	1	High	No	No	0	0.55
Molecule 5	-4.12	3.32	68.90	3	High	Yes	No	0	0.55

Key: Molecule 1: Chloroquine, Molecule 2: Artemisinin, Molecule 3: quinine, Molecule 4: quercetin and Molecule 5: 5-hydroxy-7,8-dimethoxy flavone

4.1.3 Toxicity Analysis of the Selected Compounds and Control

The toxicity analysis was carried out for artemisinin, quinine, quercetin, 5-hydroxy-7,8-dimethoxy flavone and control. The findings were presented in **Table 4.3.** All the compounds with exceptions of artemisinin and quinine have no AMES toxicity. Moreover, acute oral rat toxicity (LD50), the Maximum recommended tolerated dose for a human, hERG I inhibitor, hERG II inhibitor, hepatotoxicity, Skin sensitization and *T. Pyriformis* were evaluated and the all the compounds was found to be safe for used. The details of the toxicity analysis were presented in (**Table 4.3)**.

Table 4.3: Toxicity analysis of the Selected Compounds and Control

Selected Compounds	AMES toxicity	Max. tolerated human dose (log mg/kg/day	hERG I inhibitor	hERG II inhibitor	LD50 (mol/kg)	LOAEL (log mg/kg	Hepato toxicity bw/day)	Skin sensitization	T.Pyriformi
Molecule 1	Yes	-0.167	No	Yes	2.85	1.026	Yes	No	1.558
Molecule 2	Yes	0.065	No	No	2.459	1.00	No	No	0.322
Molecule 3	Yes	-0.416	No	Yes	2.728	0.635	Yes	No	0.488
Molecule 4	No	0.499	No	No	2.471	2.612	No	No	0.288
Molecule 5	No	-0.061	No	No	2.537	1.238	No	No	0.476

Key: Molecule 1: Chloroquine, Molecule 2: Artemisinin, Molecule 3: quinine, Molecule 4: quercetin and Molecule 5: 5-hydroxy-7,8-dimethoxy flavone

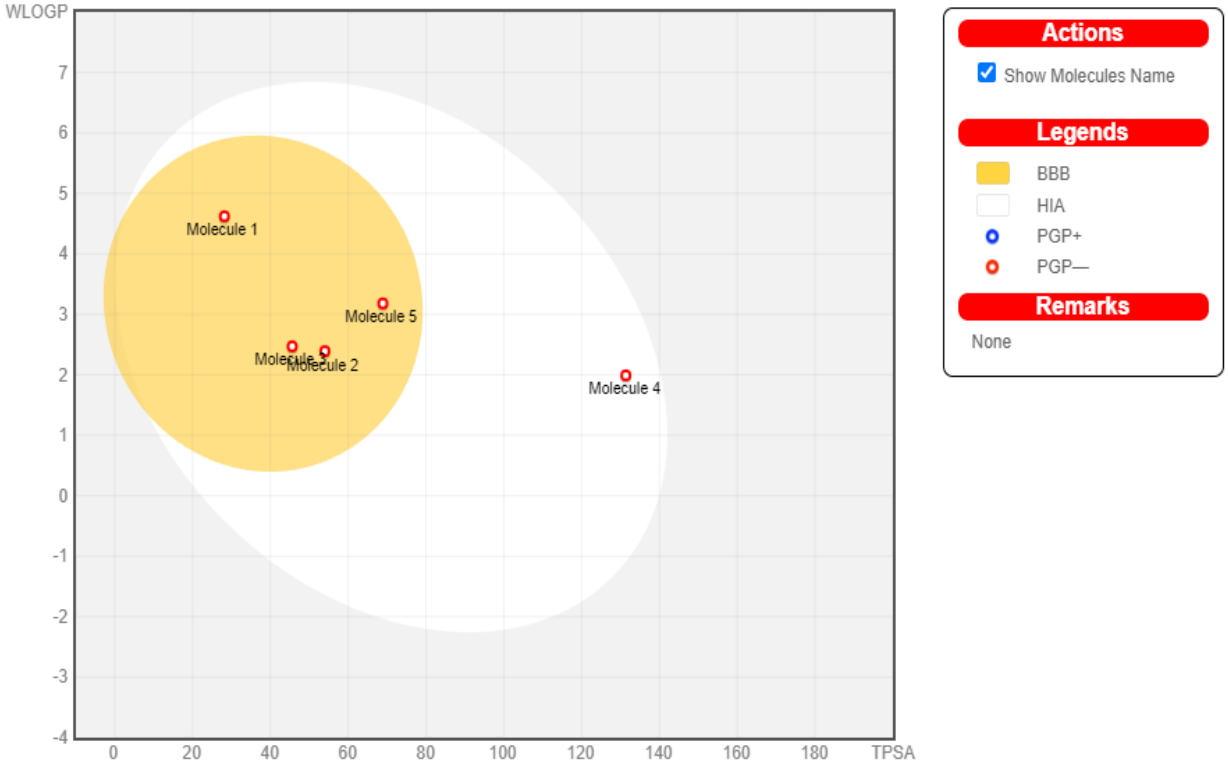


Figure 4.16: The Boiled-egg depicted molecule 1 as a Control (Chloroquine) and Molecule 2 to 5 as the investigative compounds.

4.2 Procurement and identification of plant materials

In the present study, the four medicinal plants namely; *Cassia occidentalis, Artemesia annua, Swertia thomsonii and Cinchona officinalis* were collected from various parts of India as follow;

4.2.1 Artemisia annua L.

Artemisia annua L. plant with voucher number 8620-KASH, were collected from Kawoosa Budgam, Kashmir region of India in August, 2022. The collection site is located at coordinates (74.3051, 34.0614) and has an altitude of 1585 meters. The plant's authentication was conducted by a curator at Kashmir University, Department of Botany, Jammu and Kashmir, India. The authenticated plant specimen was deposited in the KASH herbarium. After collection, the plants were washed, subjected to shade drying for a period of 2-3 weeks until they were completely dry, and then powdered.

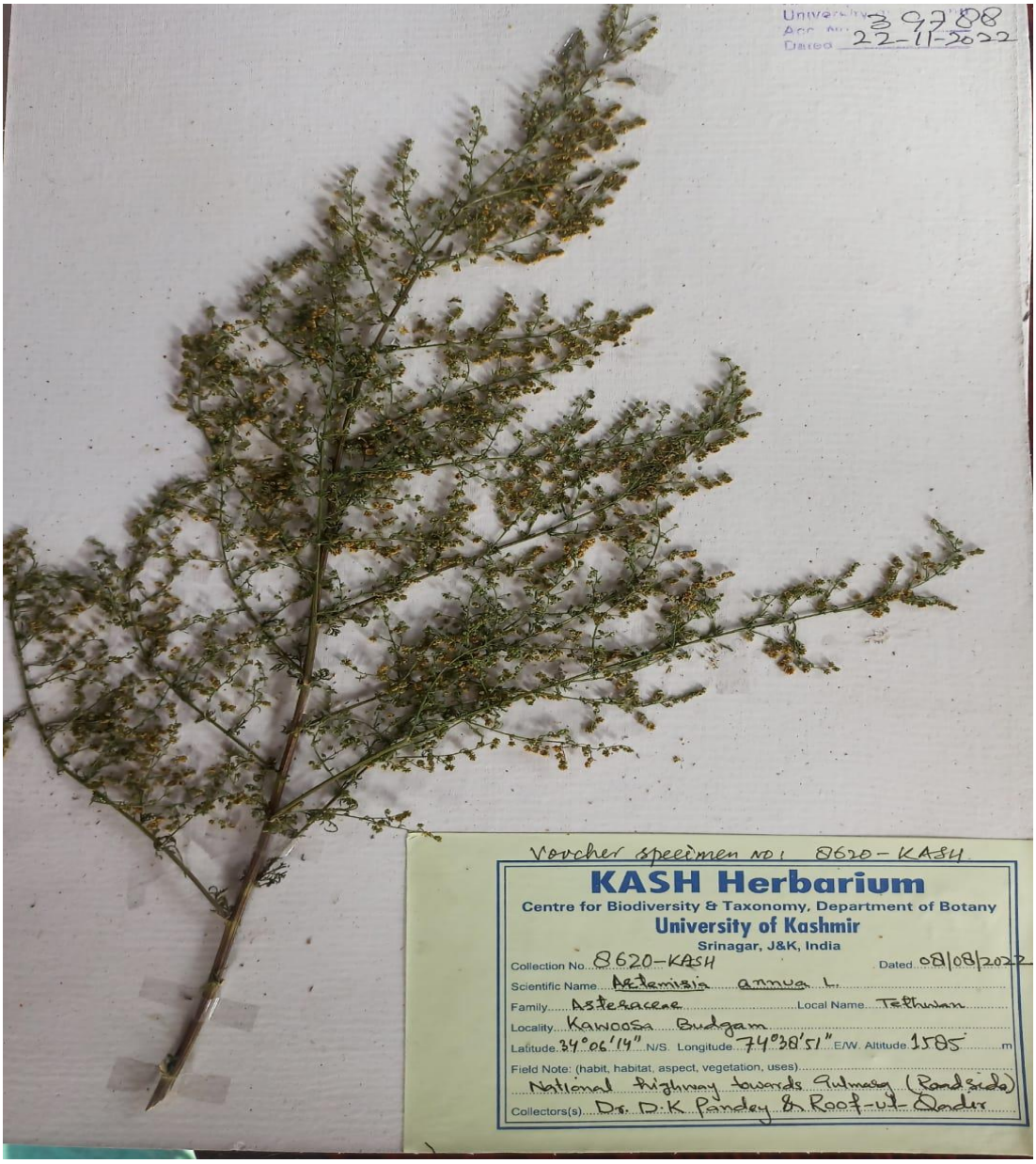


Figure 4.17: Herbarium of *A. annua* L.

4.2.2 Cassia occidentalis L.

Cassia occidentalis L. plant with voucher number 9134-KASH were collected from Rama mandi, Jalandhar, Punjab region of India. The collection site is located at coordinates (31^{o} 30’86, 75^{o} 62’97”). The plant's authentication was also conducted by a curator at Kashmir University, Department of Botany, Jammu and Kashmir, India. The authenticated plant specimens were deposited in the KASH herbarium. After collection, the plants were washed, subjected to shade drying for a period of 2-3 weeks until they were completely dry, and then powdered.

Figure 4.18a: *Cassia occidentalis* L. showing (A) = leaves, (B) = Flower, (C) = Pods

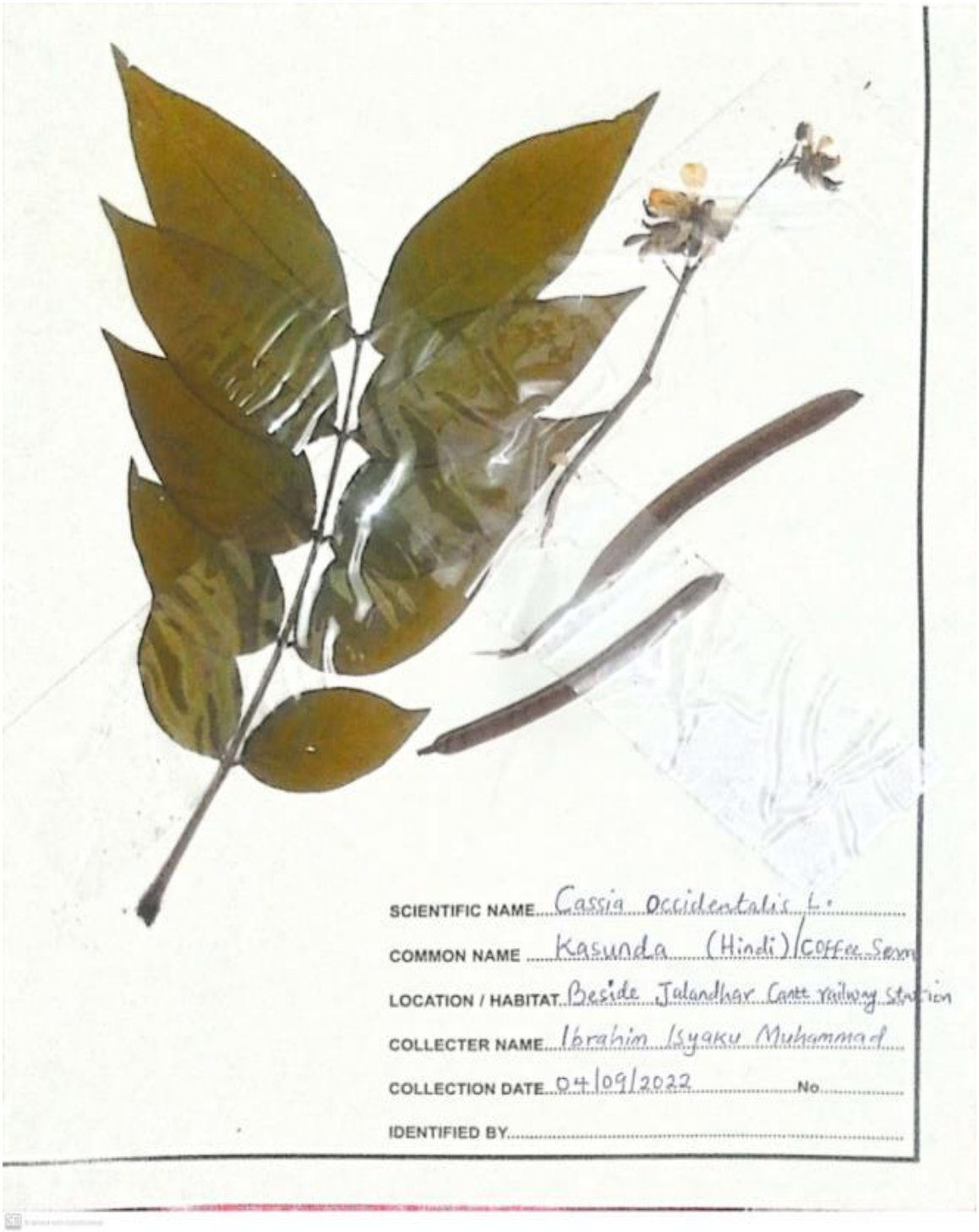


Figure 4.18b: Herbarium of *C. occidentalis L.*

4.2.3 Swertia thomsonii L.

Swertia thomsonii with voucher number 9085-KASH was collected from Kashmir region of India. The plant's authentication was also conducted by a curator at Kashmir University, Department of Botany, Jammu and Kashmir, India. The authenticated plant specimens were deposited in the KASH herbarium. After collection, the plants were washed, subjected to shade drying for a period of 2-3 weeks until they were completely dry, and then powdered.

Figure 4.19: ***Swertia thomsonii L.***

4.2.4 Cinchona officinalis L

The bark of *Cinchona officinalis* was obtained from the local market in Jalandhar district of Punjab, India. The bark was mashed into a fine powder using a blender. This fine powder was used for experimental purposes.

Figure 4.20: *Cinchona officinalis L.* (Bark powder)

4.3 Characterization of compounds

4.3.1 Preliminary phytochemical screening

4.3.2 Percentage of extract yields recovered

Because of solvents various degrees of polarity and ability to dissolve various compounds found in the samples, solvents are important factors in determining the yield of extract obtained from plant samples.

In the extraction of biomolecules from plants, a variety of solvents are employed, chosen based on the polarity of the desired solute. The solute will dissolve correctly in a solvent that has a similar polarity to it. Several solvents may be used one after the other to lower the amount of comparable compounds in the intended yield (Sultana *et al.,* 2009). The polarity, from least polar to most polar, of a solvents used in this experiment is as follows: Petroleum ether< Chloroform < Methanol < Water.

After extraction, diverse quantities of extracts were obtained, which varied according to the plant species and the solvent system employed. The quantities of each extract recovered (expressed in grams) alongside their corresponding percentage yields are provided below.

Table 4.4: Total amount of extract recovered per solvent used

	Solvents used for the extractions			
Medicinal Plants	**Petroleum ether**	**Chloroform**	**Methanol**	**Water**
C. occidentalis	2.12(4.24%)	3.40(7.10%)	7.14(16.1%)	13.17(35.3%)
A. annua	4.97(16.56%)	4.46(17.8%)	1.0(4.86%)	0.86(4.6%)
S. thomsonii	3.66(7.32%)	0.28(0.6%)	7.05(15.3%)	1.27(3.26%)
C. officinalis	3.09(6.18%)	2.63(5.6%)	4.88(11.0%)	7.12(18.07%)

The findings presented in **Table 4.4** indicate that the majority of plants yield higher quantities of extract when water and methanol are employed as solvents, followed by petroleum ether and chloroform. This observation can be attributed to the significant influence of solvent polarity on the extraction of phytochemical compounds. Specifically, the moderate polarity of methanol positions it between the polar nature of water and the non-polar characteristics of petroleum ether, thereby enabling it to effectively elute a broad spectrum of compounds with polarities spanning the middle range of solvents. This rationale is further supported by previous studies conducted on various plant materials (Nawaz et al., 2020).

The effectiveness of solvents utilized in this investigation for extracting diverse plant parts is contingent upon the specific plant species and the phytochemical composition inherent to each species. As delineated in the preceding **Table 4.4,** the highest yield of extract was achieved from the distilled water extraction of *C. occidentalis*, yielding 13.17 g (with a percentage yield of 35.3%), followed by *C. officinalis* with 7.12 g (with a percentage yield of 18.07%), both employing double distilled water as the solvent. Methanolic extraction exhibited the second-highest extract yield, with C. occidentalis yielding 7.14 g (16.1%) in aqueous extract and *C. officinalis* yielding 4.88 g (11.0%). However, in *Artemesia annua* plant, the highest extract yield was obtained in petroleum ether extract with 4.97g (16.56%) followed by chloroform and methanol extracts with 4.46g (17.8%) and 1.0g (4.86%) respectively. The lowest extract yield in *Artemesia annua* were obtain from aqueous extract with 0.86g (4.6%) as presented in the table above. In *S. thomsonii* the highest extract yield was obtained in methanol extract with 7.05g (15.30%) followed by petroleum ether and water extracts with 3.66g (7.32%) and 1.27g (3.26%) respectively. The chloroform solvent was found to yield the least amount of extract of about 0.28g (0.6%) in *S. thomsonii* (**Table 4.4**).

The quantity of extract recovered using petroleum ether and chloroform solvents exhibits variability among different plant species. Interestingly, certain extracts yield more when extracted with petroleum ether despite chloroform possessing a higher polarity index.

This observation underscores the complex interplay of factors influencing extraction yield, wherein the chemical composition of the plant, extraction duration, temperature, solvent concentration, and other variables play pivotal roles alongside solvent polarity. A similar phenomenon is observed between aqueous and methanolic extracts, suggesting that multiple factors beyond solvent polarity alone contribute to the extraction process (Iloki-Assanga et al., 2015).

The sequential extraction method is considered exhaustive since it is one of the key procedures used to ensure that the phytocompounds are extracted to their maximum potential. Given the diverse polarity indices of various compounds present in plants, a sequential extraction approach was employed. Recognizing that a single solvent may not ensure the comprehensive extraction of all plant compounds, the utilization of multiple solvents within a single extraction procedure emerges as a thorough method for recovering a wide array of compounds from the same plant or plant parts. This strategy allows for the extraction of compounds with differing polarities, thereby enhancing the comprehensiveness of the extraction process and facilitating the recovery of a broader spectrum of phytochemicals (Bimakr *et al.*, 2011).

4.3.3 Qualitative phytochemical analysis of the extracts from the selected medicinal plants

Many phytochemicals, including alkaloids, flavonoids, terpenoids, saponin, cardiac glycoside, tannins, quinones, and phenolic compounds, were found in both extracts when the extract samples of the selected medicinal plants were screened qualitatively for phytochemicals. On the other hand, alkaloids were absent from *Swertia thomsonii* extracts and quinones were absent from *A. annua* extracts. Moreover, flavonoids, saponin, and phenol were found absence from *C. occidentalis* extracts **(Table 4.5).** Plant extract is a mixture of numerous kinds of phytocompounds, each with unique biological features that make plants a major source of innovative medication (Farnsworth, 1966). The presence of flavonoids, alkaloids, and quinones in plant extracts has been shown to have a direct association with antiplasmodial action, hence adding to overall plant activity. Flavonoids have emerged as an important bio-therapeutic class of natural

compounds with promising antimalarial properties. They may also play a role in reversing antimalarial drug resistance, providing dual therapeutic benefits (Chaturvedi *et al.*, 2021). Some flavonoids, including those from *A. annua* L. have been proposed to act synergistically with artemisinin (sesquitarpene lactone) or independently as antimalarials (Czechowski *et al.*, 2019). Previous research has demonstrated that *Swertia spp*. contains a variety of phytochemicals, including xanthones (a type of flavonoid), mangiferin, and phenolic compounds, all of which are known for their significant role in antioxidant activities (Negi et al., 2010). Alkaloids, a class of natural compounds found in numerous plants, have been investigated for their potential as antimalarial agents. Quinine, derived from the *Cinchona* tree, was the first effective antimalarial medication and is still used today to combat multidrug-resistant malaria (Uzor, 2020). The details of the initial phytochemical screening results are presented in **Table 4.5.**

Table 4.5: Phytochemical compositions of the selected medicinal plants

Phytochemical tests	*C. occidentalis*				*A. annua*				*S. thomsonii*				*C. officinalis*			
	PE	**CF**	**ME**	**WT**	**PE**	**CF**	**ME**	**WT**	**PE**	**CF**	**ME**	**WT**	**PE**	**CF**	**ME**	**WT**
Test for Alkaloids (Meyer's Test)	+	+	-	-	-	-	+	-	-	-	-	-	+	+	-	+
Test for flavanoids	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
Test for Terpenoids (Salkowski test)	-	-	-	+	-	-	+	-	-	-	+	-	-	-	+	-
Test for Saponins (Foam Test).	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+
Test for Tannins	-	-	+	+	-	-	+	-	-	-	-	+	-	-	+	+
Test for Cardiac Glycosides (Keller-Killani Test).	-	-	+	+	-	-	+	-	-	-	+	+	-	+	+	-
Test for Phenols (Ferric Chloride Test)	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+	+
Test for Quinones	-	-	-	+	-	-	-	-	-	-	+	+	-	-	+	+

Key: present = (+), absent = (-)

4.4 High performance thin layer chromatography

4.4.1 Qualitative and Quantitative Estimation of Artemisinin using HPTLC-densitometry method

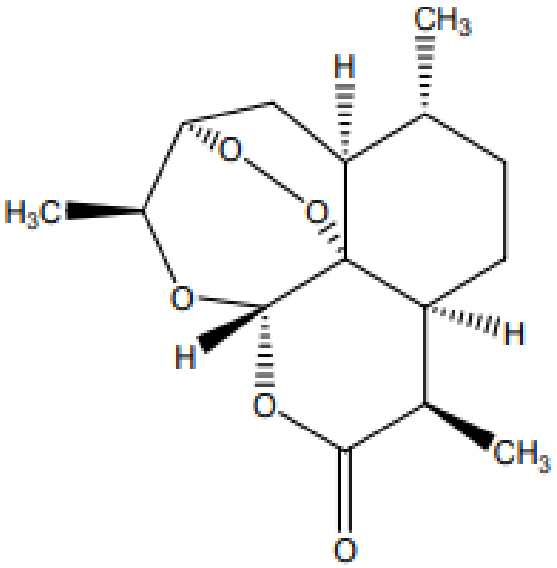


Figure 4.21: Chemical structure of artemisinin

4.4.2 Development of optimum mobile phase ratio for artemisinin separation

The TLC mobile phase ratio was optimized to achieve proper separation of artemisinin. The poor separation of artemisinin was observed towards the end of the TLC plate while using solvent system of petroleum ether : ethyl acetate in the ratio 8.5 : 1.5 (75 : 25, v/v) as shown in (**Figure 4.22)**. However, while adjusting the mobile ratio to ethyl acetate : petroleum ether in the ratio (75 : 25, v/v), an excellent separation of artemisinin was observed towards the middle of the plate with retention factor (0.56) as shown in (**Figure 4.23).** Therefore, the ethyl acetate: petroleum ether in the ratio 8.5 : 1.5 (75 : 25, v/v) is the suitable solvent system for the separation of artemisinin.

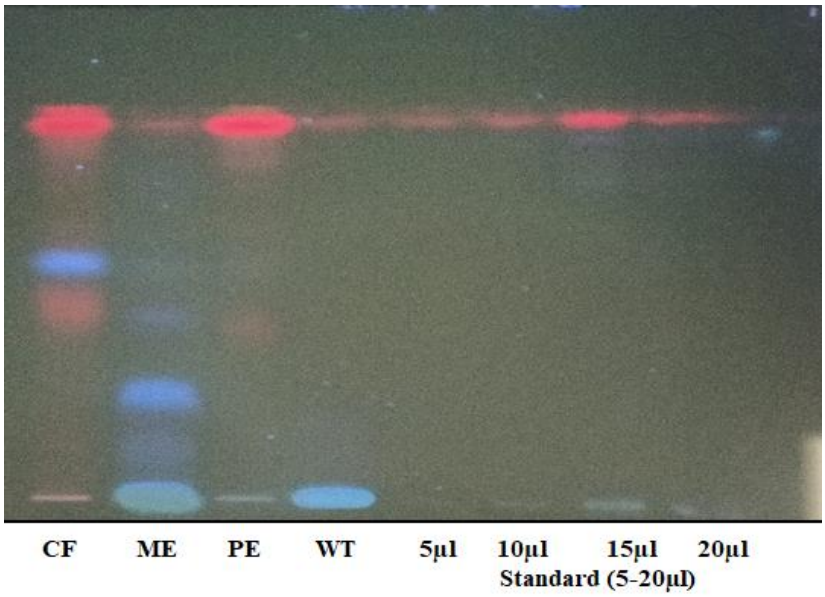


Figure 4.22: HPTLC fingerprinting of four different extracts of *Artemesia annua*

Key: **CF** = Chloroform extract; **ME** = Methanol extract; **PE** = Petroleum ether extract; **WT** = Water extract; Artemisinin Standard = (5-20µl)

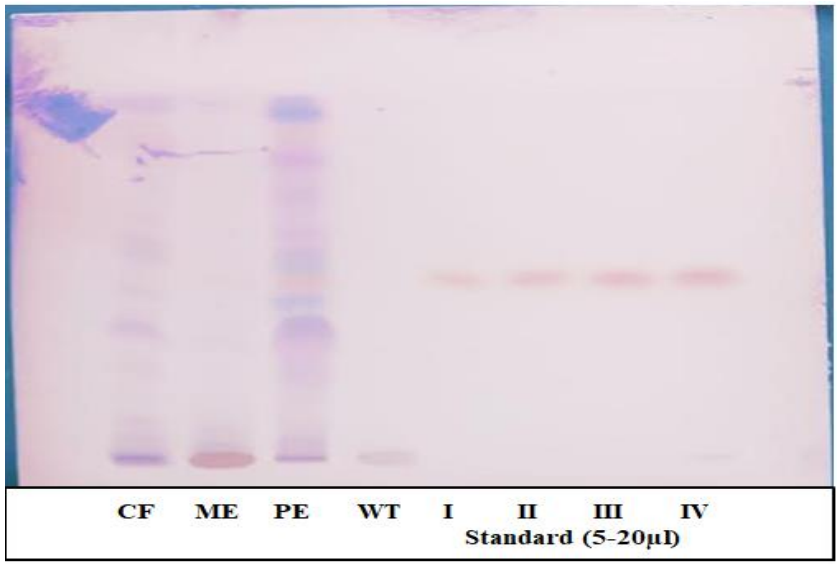


Figure 4.23: HPTLC fingerprinting of four different extracts of *Artemesia annua*

Key: **CF** = Chloroform extract; **ME** = Methanol extract; **PE** = Petroleum ether extract; **WT** = Water extract; Accessions (I-1V) = Artemisinin Standard (5-20µl)

4.4.3 Influence of solvents on artemisinin yield

The selection of an appropriate solvent system is a crucial factor that significantly influences the separation of components on HPTLC plate. The artemisinin compounds were quantified from four different extracts of *Artemisia annua* L. using high-performance thin-layer chromatography. To achieve this, four solvents with varying polarities was employed. The artemisinin content was determined using the peak area parameter and percentage mean on dry weight basis. The results obtained were presented in **table 4.6.** Petroleum ether extract yield the maximum artemisinin content followed by methanol and chloroform extract. However, very little amount of artemisinin was obtained in aqueous extract. Therefore, among all the studied extracts of *A. annua,* petroleum ether extract (PE) displayed higher content of artemisinin compound with **(0.71% ± 0.02**), followed by methanol extract with (0.25% ± 0.02), chloroform extract with (0.14% ± 0.01) and water extract with (0.06% ± 0.01) as presented in **Table 4.6.**

Wide range of solvents has been used for artemisinin extraction, including hexane, ethanol, methanol, chloroform, petroleum ether and ethyl acetate. Based on the previous study, ethanol was found to be very efficient for the artemisinin extraction. A study by (Babacan *et al.,* 2022) found ethanol as the most effective solvent for extracting artemisinin from *Artemisia annua.* However, the present result shows the maximum yield of artemisinin in petroleum ether. Another study by (Cao *et al.,* 2017; Kundu *et al.,* 2016) found petroleum ether as the efficient solvent for extracting high quality artemisinin from *Artemisia annua,* but the yield was lower compared to other solvents like designed hydrophobic and hydrophilic solvents. The study also found that the purity of artemisinin extracted using petroleum ether was higher than that obtained using other solvents. This is in agreement with the present finding as per densitometry graphs obtained **(Figure 4.25).** Therefore, based on the previous study, petroleum ether has been found to be less efficient in extracting artemisinin compared to other solvents like methanol, ethanol, and ethyl acetate. However, it has been shown to selectively extract lipophilic compounds like artemisinic acid and casticin, which may have potential for anti-malarial activity. But

the present study, show the efficiency of petroleum ether for extracting the maximum content as well as high quality artemisinin with little or no impurities from *A. annua L.*

Table 4.6: Influence of solvents on artemisinin yield

S/no.	Extraction solvents	Mean % of Artemisinin ± SD (dry wt. basis)	Rf value
1.	Chloroform (CF)	0.14 ± 0.01	0.59
2.	Methanol (ME)	0.25 ± 0.02	0.56
3.	Petroleum ether	**0.71 ± 0.02**	0.51
4.	Water (WT)	0.06 ± 0.01	0.56

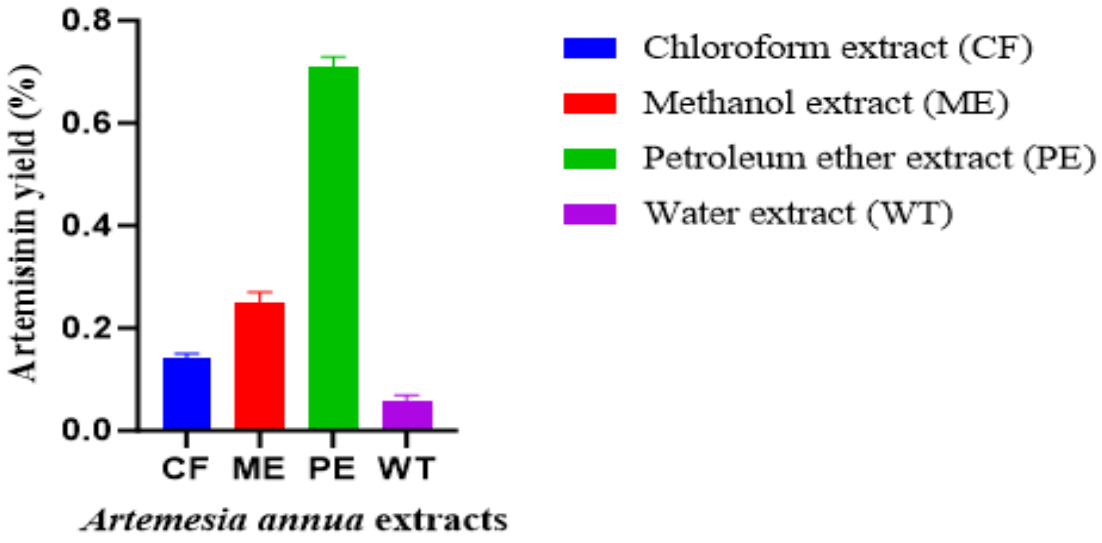
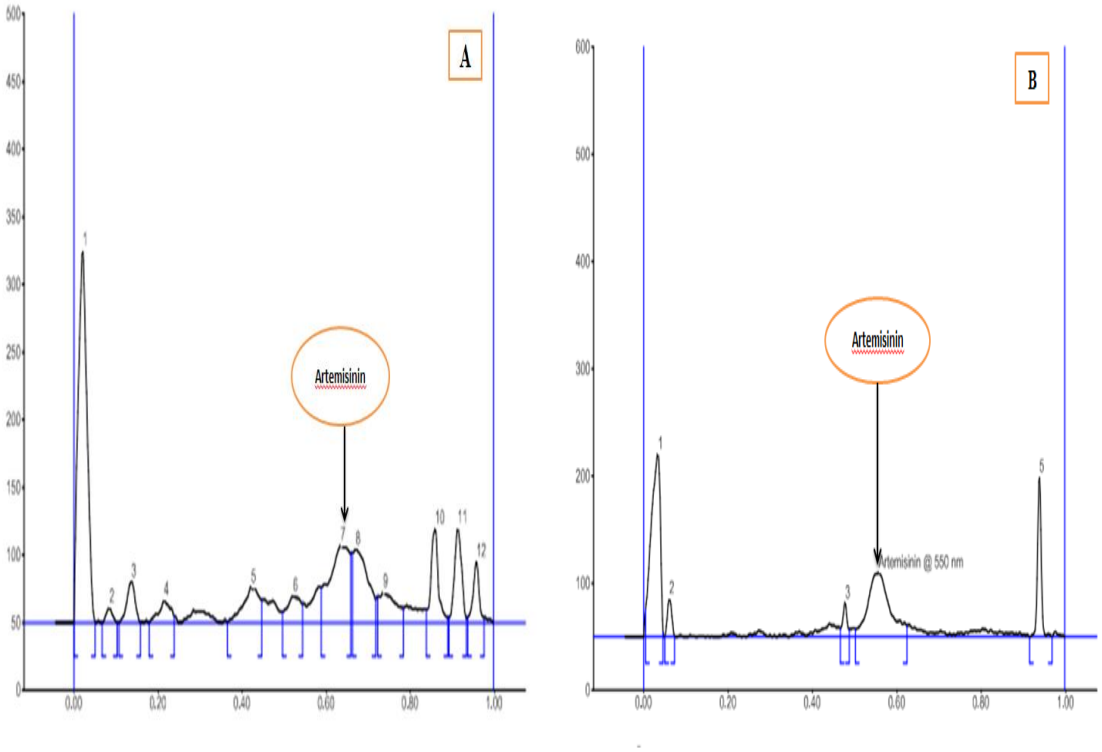
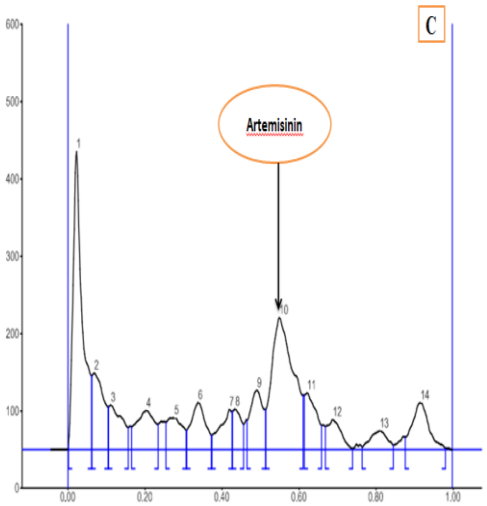


Figure 4.24: Artemisinin content in different extracts of *Artemisia annua* plant

4.4.4 Specificity (Artemisinin)

To confirm the specificity of the artemisinin compound in the extract, a comparison was made between the Rf (retention factor) values of the extract and that of the standard bands. These comparisons were depicted in **figures 4.25**. The results indicated that the method employed was specific, as it facilitated the clear separation of artemisinin, compounds in *A. annua L.* extracts.




C
Artemisinin
1
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3
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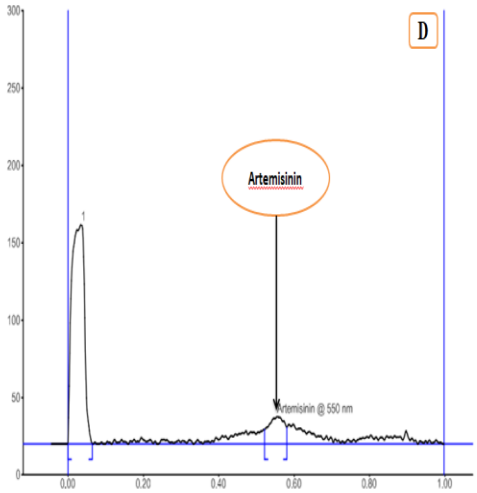
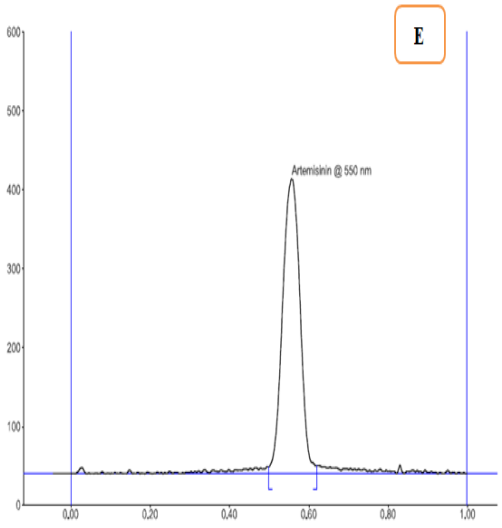
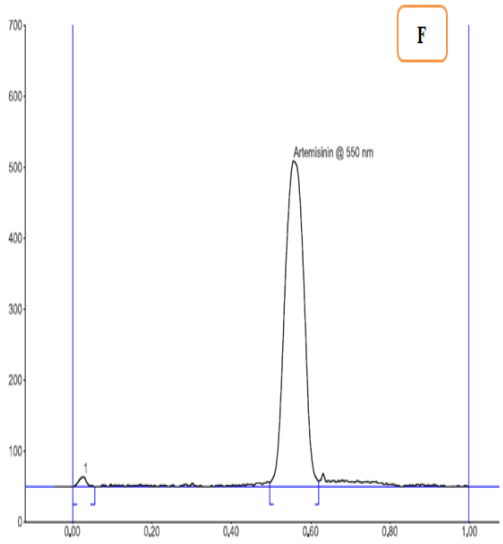

D
Artemisinin
1
Artemisinin @ 550 nm


E
Artemisinin @ 550 nm


F
Artemisinin @ 550 nm
1

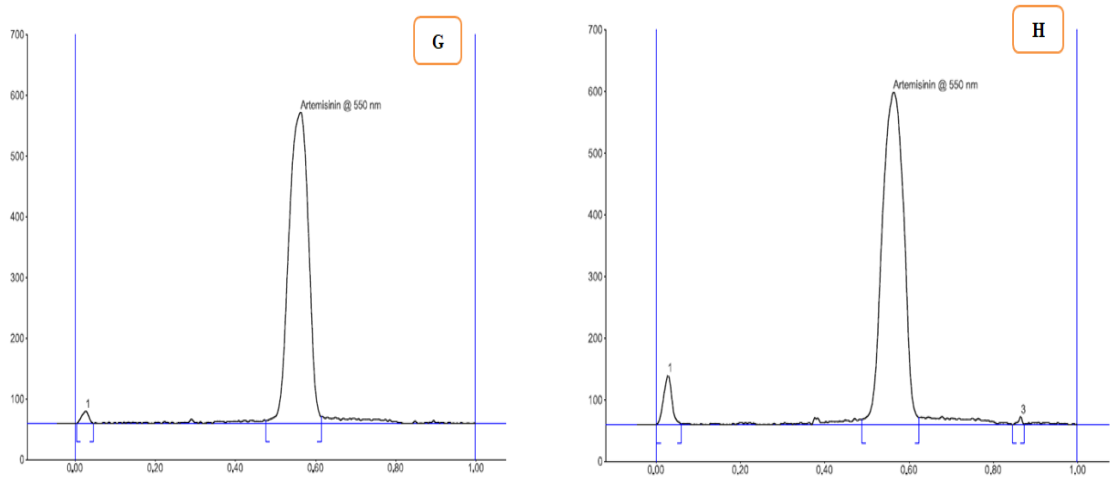


Figure 4.25: Densitometry graph showing isolation of Artemisinin compound from **(A**) = Chloroform extract, (**B)** = Methanol extract, **(C)** = Petroleum ether extract, (**D)** = Water extract of *A. annua*, (**E-H) =** Artemisinin standard (5-20µl) at 550nm.

4.4.5 Linearity (Artemisinin)

To assess the linearity, various concentrations of working standard solutions of artemisinin were applied. The correlation coefficient (r^2) was ascertained by performing a linear least square regression analysis on the resultant data. The correlation coefficient (r^2) of 0.9609 was obtained for artemisinin standard indicating a robust linear association between the concentration of the standard and those of the corresponding responses. The linearity range of the calibration curve for artemisinin was established as 5-20 µg/spot. **Figure 4.26** illustrates the calibration curve graph for artemisinin showing their linearity range.

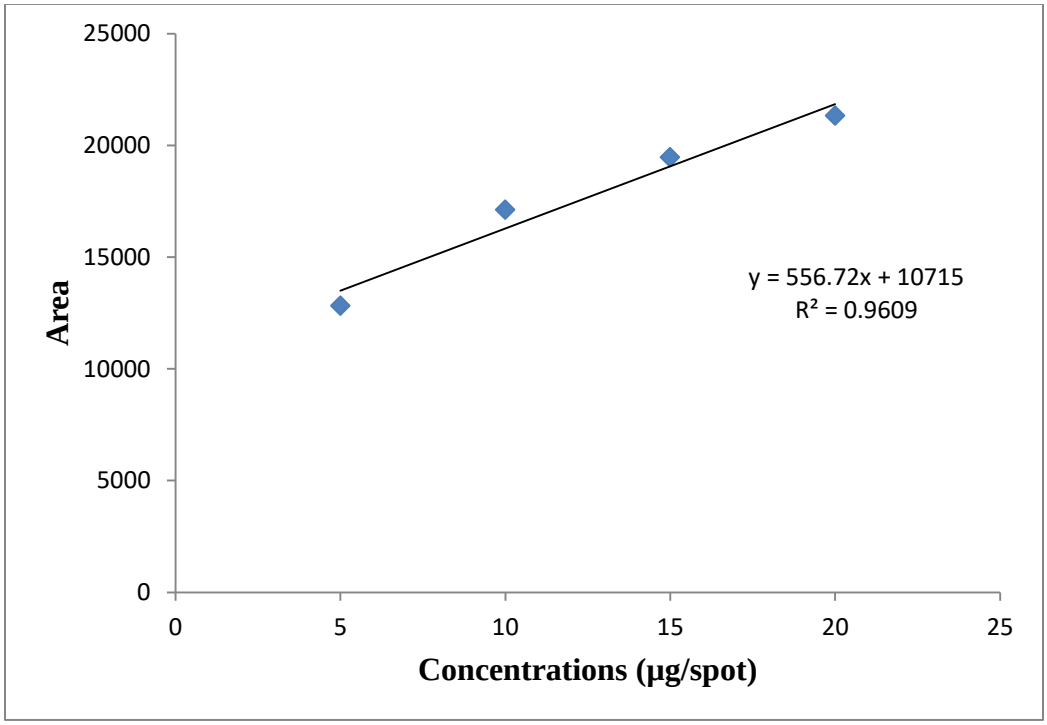


Figure 4.26: Calibration curve of artemisinin standard

4.4.6 Intra-day and inter-day precision (artemisinin)

An intra-day and inter-day precision analysis method were carefully evaluated in four separate determinations of artemisinin (n = 4) on distinct tracks. Each track underwent scanning, and the resultant chromatograms were recorded. The consistency of these measurements was quantified using the percent coefficient of variation (RSD%) for the peak area of the standard compounds integrated into the extracts. Intra-day precision was found to be 1.48% ± 0.001, and inter-day precision was found to be 1.50% ± 0.021 for artemisinin. **Figures 4.27** represent the 3D chromatogram data of both standard and extracts for artemisinin while **Figure 4.28** represents the spectral comparison of artemisinin standards and that of the extracts. The results obtained affirmed that the method showcases a remarkable degree of precision for the analysis of artemisinin in *A. annua* plant. The presence of low RSD% values implies negligible variation in peak areas, underscoring the method's exceptional measurement repeatability.

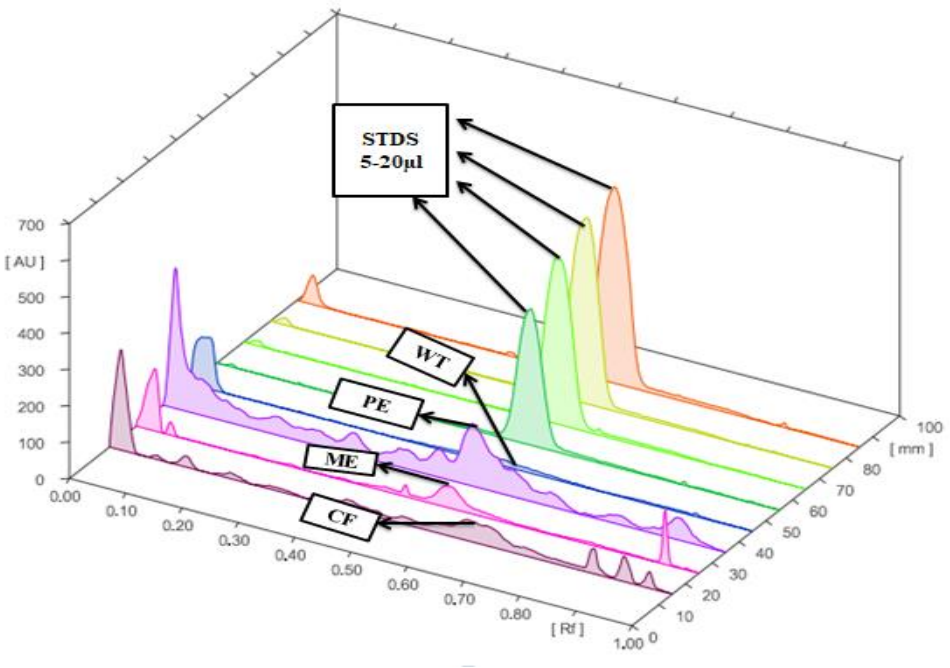


Figure 4.27: 3D Chromatogram of artemisinin from four different extracts of *A. annua* and standard (STDS)

Key: **CF** = Chloroform extract; **ME** = Methanol extract; **PE** = Petroleum ether extract; **WT** = Water extract;

STDS = Artemisinin Standard (5-20µl)

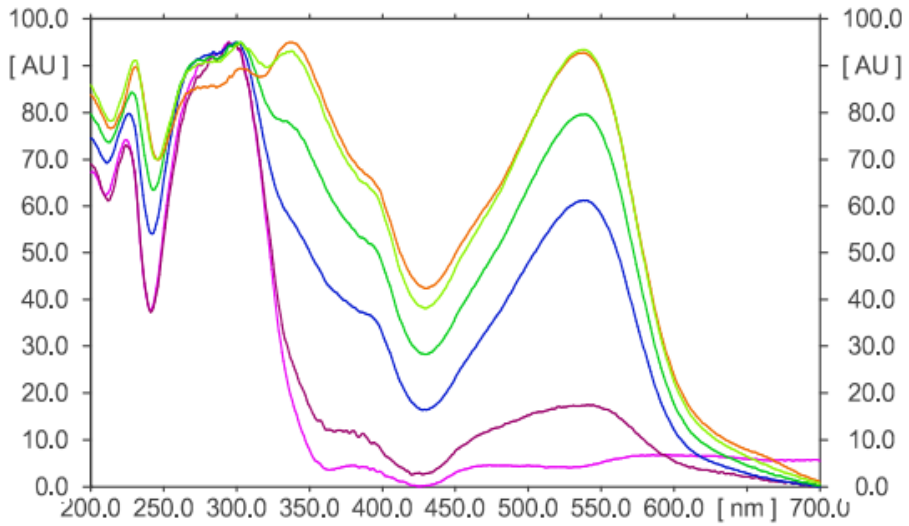


Figure 4.28: Overlay spectral comparisons of artemisinin standard and extracts

4.4.7 Limit of Detection and Limit of Quantitation of artemisinin

As shown in **Table 4.7,** the limit of quantitation (LOQ) for artemisinin was found to be 15.93 µg/spot, while the limit of detection (LOD) was found to be 5.23 µg/spot. The response's standard error (SE) and slope were used to get these values. Consequently, this outcome underscores the sensitivity of the method and its ability to accurately detect and quantify artemisinin, quinine, and quercetin compounds even at low concentrations, demonstrating a high level of reliability and precision.

Table 4.7: HPTLC validated parameters for artemisinin estimation

Parameters	Artemisinin
Specificity	Specific
Regression equation	Y = 556.72X + 10715
Correlation coefficient (r^2)	0.9609
Correlation coefficient (r)	0.9803
Slope	556.72
Standard error of the slope	882
Linearity range (µg/spot)	5-20
Limit of detection (LOD) µg/spot	5.23
Limit of quantitation (LOQ) µg/spot	15.93

4.5 Qualitative and quantitative estimation of Quercetin by HPTLC-densitometry method

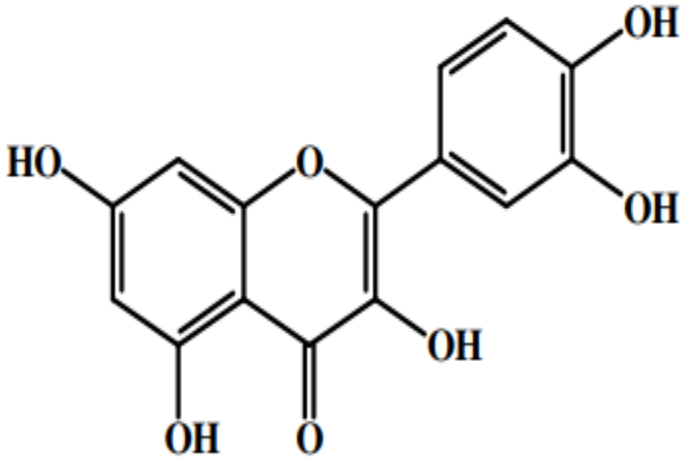


Figure 4.29: Chemical structure of quercetin

4.5.1 Development of Optimum Mobile Phase ratio for Quercetin separation

Using the solvent system of toluene: chloroform: ethyl acetate: and formic acid: in the ratio (3:2:4.9:0.1%), as seen in **Figure 4.30**, we were able to successfully separate quercetin.

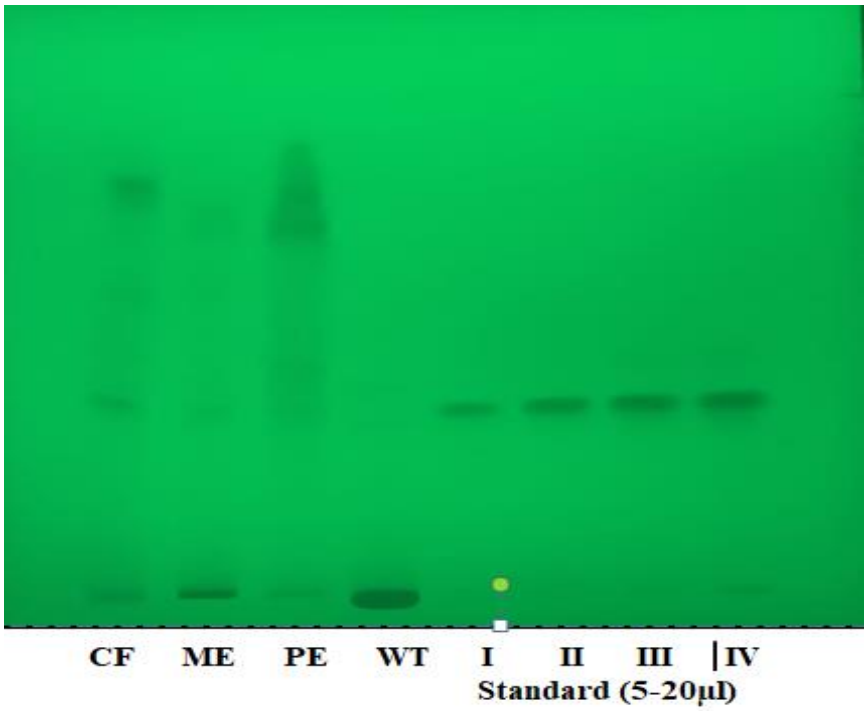
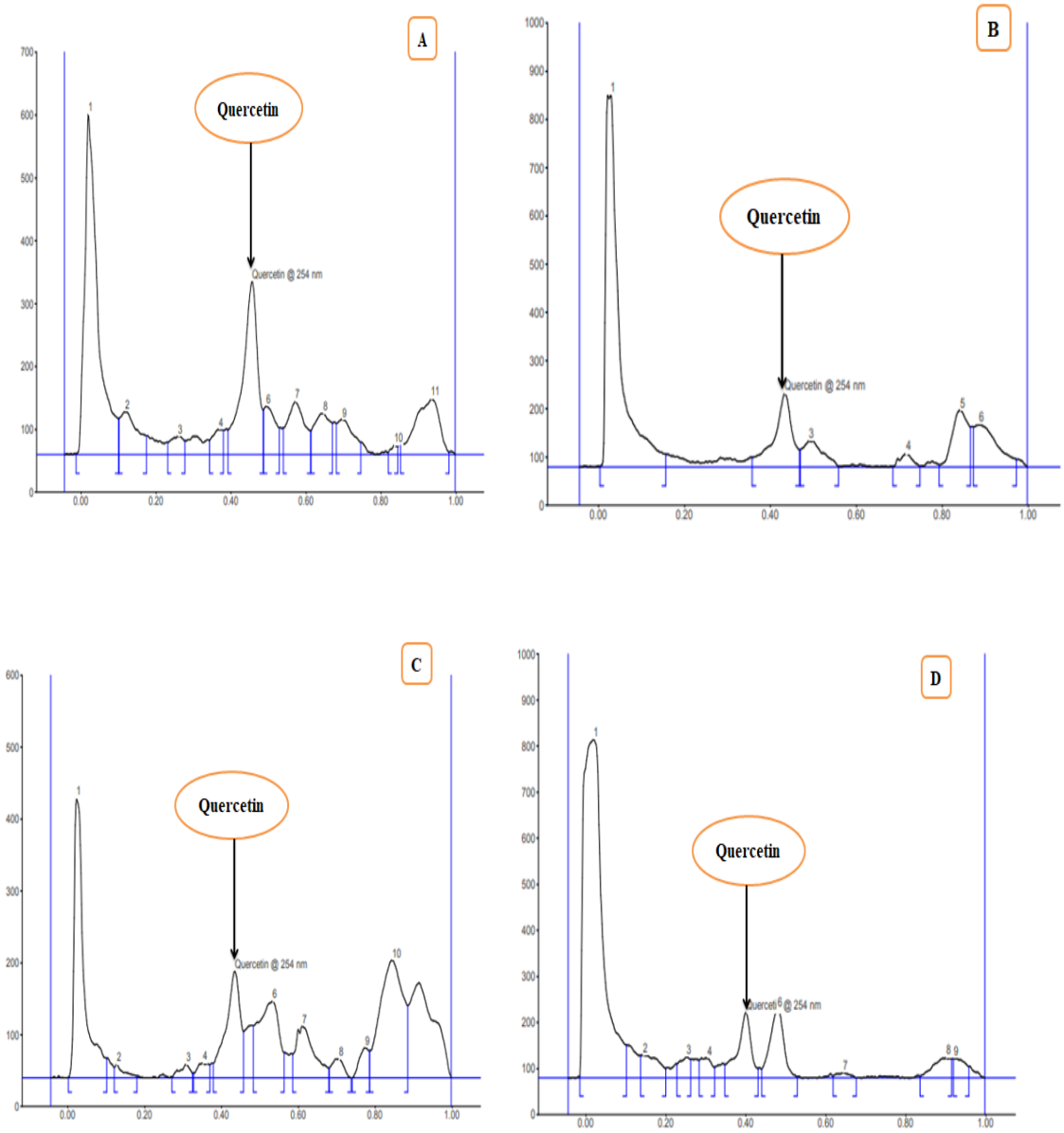


Figure 4.30: HPTLC fingerprinting of four different extracts of *C. occidentalis*
Key: **CF** = Chloroform extract; **ME** = Methanol extract; **PE** = Petroleum ether extract; **WT** = Water extract; Accessions (I-1V) = Quercetin Standard (5-20µl)

4.5.2 Specificity (quercetin)

To confirm the specificity of the quercetin compound in the extracts, a comparison was made between the Rf (retention factor) values of the extract and that of the standard spots. This comparison was depicted in **Figure 4.30**. The results indicated that the method employed was specific, as it facilitated the clear separation of quercetin compound in all four extracts. However, a well-defined, symmetrical peak were obtained in chloroform extract (**Figure 4.31**) indicating the good separation and high purity of quercetin compound better than the other three solvents which appears to have impurities or co-elution of other compounds.


A
Quercetin
Quercetin @ 254 nm
B
Quercetin
Quercetin @ 254 nm
C
Quercetin
Quercetin @ 254 nm
D
Quercetin

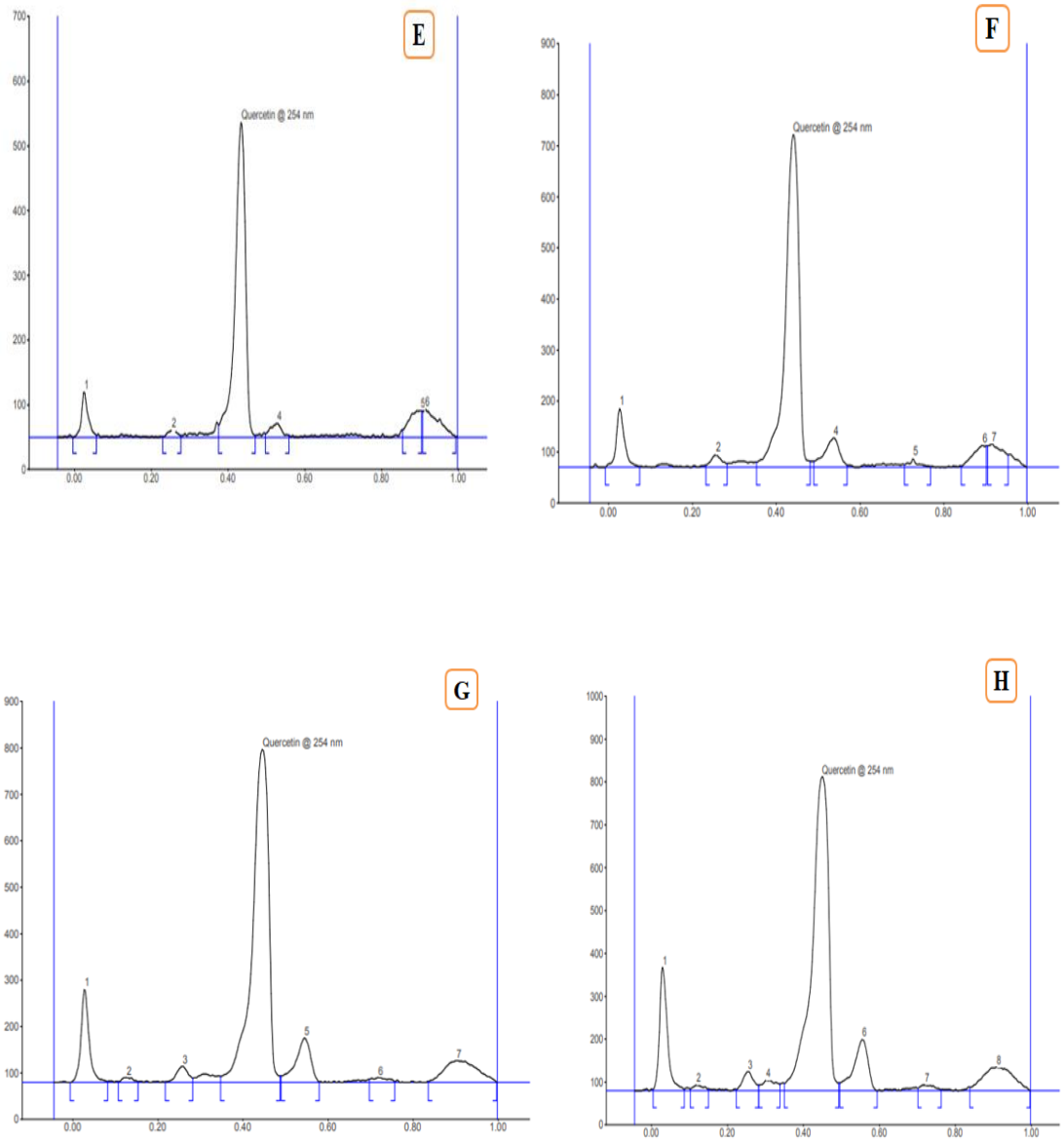


Figure 4.31: Densitometry graph showing isolation of Quercetin compound from (**A)** = Chloroform extract; **(B)** = Methanol extract; **(C) =** Petroleum ether extract; **(D)** = Aqueous extract of *Cassia occidentalis*; (**E-H) =** Quercetin standard (5-20µl) at 254nm.

4.5.3 Influence of solvents on quercetin yield

The choice of an appropriate solvent system stands as a pivotal determinant significantly influencing the separation of components on an HPTLC plate. In this study, the primary objective was to isolate and quantify the high-quality quercetin compound from four distinct extracts of *C. occidentalis L*. (leaf) using high-performance thin-layer chromatography. To accomplish this, four solvents with varying polarities; petroleum ether, chloroform, methanol, and water were employed. The amount of quercetin compounds present was determined through the utilization of peak area parameters and mean percentages based on dry weight, as detailed in (**Table 4.8).** Among the four extracts, the chloroform extract exhibited the highest quercetin content, succeeded by methanol, petroleum ether, and aqueous extracts, in that order. Consequently, within the scope of all the examined *C. occidentalis* extracts, the chloroform extract (CF) showcased the highest quercetin compound content at **4.10% ± 0.01** w/w, followed by the methanol extract at 2.37% ± 0.01 w/w, the petroleum ether extract at 2.09% ± 0.01w/w, and the water extract at 1.79% ± 0.01 w/w. This outcome diverges from the findings presented by (Gaikwad *et al.* 2018), who reported distinct quercetin content yields in different parts of *C. occidentalis*, specifically, 0.033% in the stem part and 0.037% in the flower part. As such, a comparison with the current results underscores that quercetin is more abundantly concentrated in the leaf part of *C. occidentalis* compared to its stem and flower parts. This variation is likely attributed to the specific plant parts utilized for quantification.

Table 4.8: Influence of solvents on quercetin yield

S/no.	Extraction solvents	Mean % of Quercetin ± SD (dry wt. basis)	Rf value
1.	Chloroform (CF)	**4.10 ± 0.01**	0.46
2.	Methanol (ME)	2.37 ± 0.01	0.43
3.	Petroleum ether	2.09 ± 0.01	0.44
4.	Water (WT)	1.79 ± 0.01	0.40

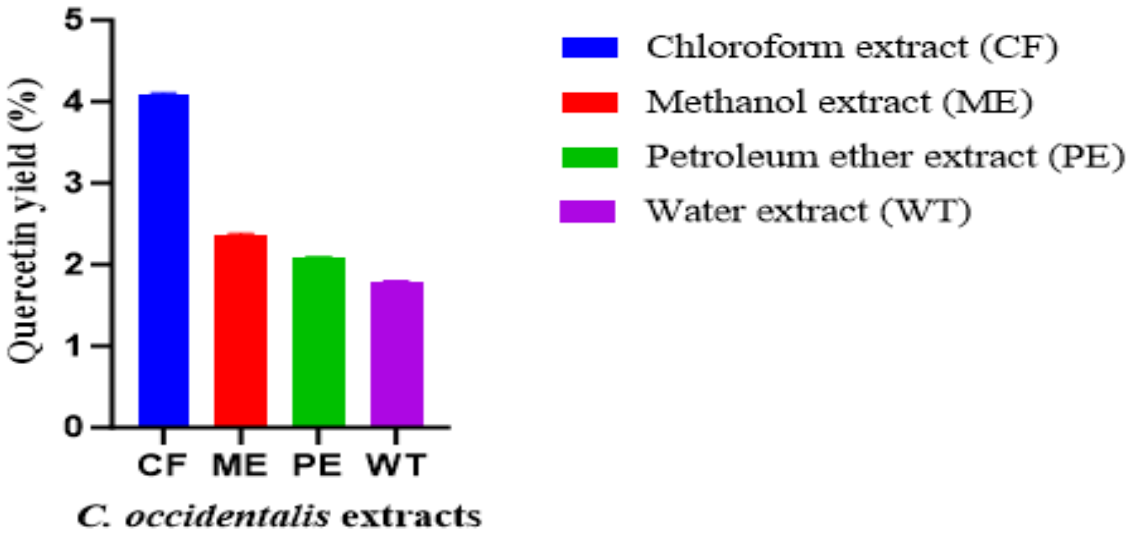


Figure 4.32: Quercetin content in different extracts of *C. occidentalis* plant

4.5.4 Linearity (Quercetin)

To assess the linearity, various concentrations of working standard solutions of quercetin were applied. The correlation coefficient (r^2) was ascertained by performing a linear least square regression analysis on the resultant data. The correlation coefficient (r^2) of 0.9843 was obtained for quercetin standard (**Figure 4.33**) indicating a robust linear association between the concentration of the standard and those of the corresponding responses. The linearity range of the calibration curve for quercetin was established as 5-20 µg/spot. **Figure 4.33** illustrates the calibration curve graph for quercetin showing their linearity range.

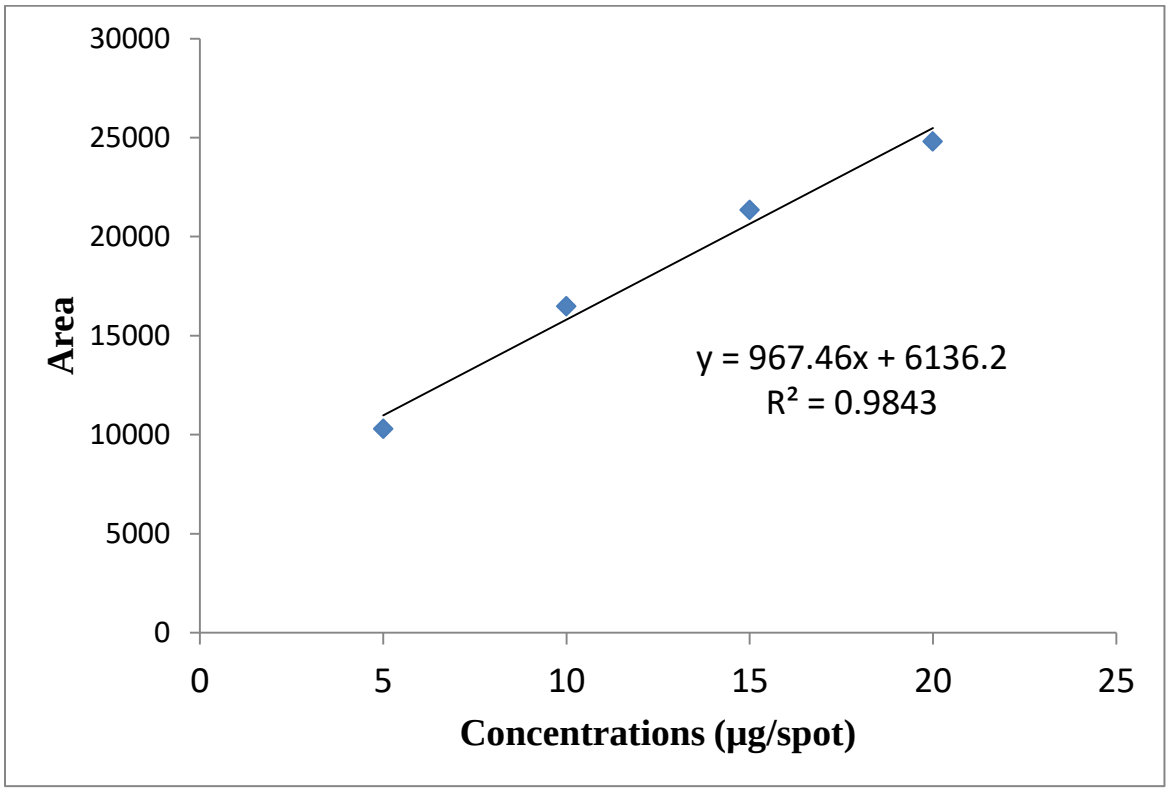


Figure 4.33: Calibration curve of Quercetin standard

4.5.5 Intra-day and inter-day precision (Quercetin)

An intra-day and inter-day precision analysis method were carefully evaluated in four separate determinations of quercetin (n = 4) on distinct tracks. Each track underwent scanning, and the resultant chromatograms were recorded. The consistency of these measurements was quantified using the percent coefficient of variation (RSD%) for the peak area of the standard compounds integrated into the extracts. Intra-day precision was found to be 1.46% ± 0.01 while inter-day precision was found to be 1.52% ± 0.01 for quercetin. **Figures 4.34** represent the 3D chromatogram data of both standard and extracts for quercetin while **figure 4.35** represents the spectral comparisons of quercetin standards and that of the extracts. The results obtained affirmed that the method showcases a remarkable degree of precision for the analysis of quercetin in *C. occidentalis* respectively. The presence of low RSD% values implies negligible variation in peak areas, underscoring the method's exceptional measurement repeatability.

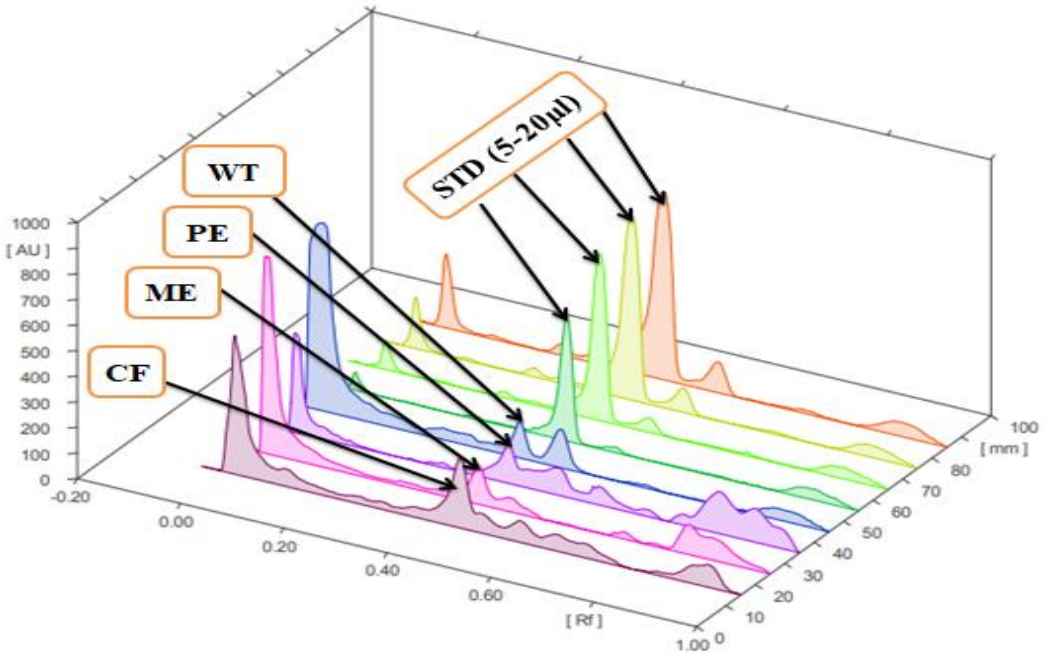


Figure 4.34: 3D Chromatogram of quercetin in four different extract of *Cassia occidentalis*

Key: **CF** = Chloroform extract; **ME** = Methanol extract; **PE** = Petroleum ether extract; **WT** = Water extract, **STD =** (Standard)

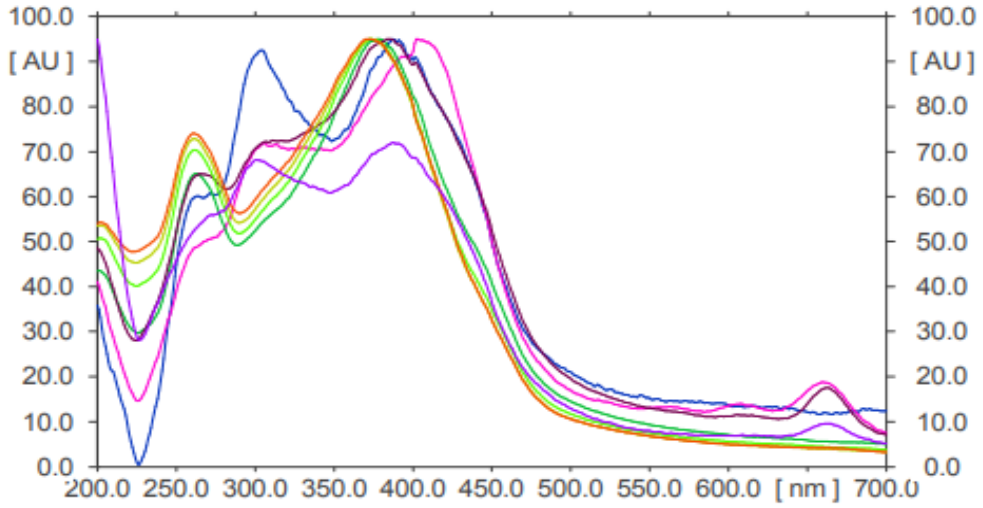


Figure 4.35: Overlay spectral comparison of extracts and quercetin standards

4.5.6 Limit of Detection and Limit of Quantitation of quercetin

Table 4.9 illustrates that the limit of detection (LOD) for quercetin was determined to be 3.29 µg/spot, while the limit of quantitation (LOQ) was established at 9.97 µg/spot. These values were computed based on the standard error (SE) of the response and slope. Consequently, this finding underscores the sensitivity of the method and its ability to accurately detect and quantify quercetin compounds even at low concentrations, demonstrating a high level of reliability and precision.

Table 4.9: HPTLC validated parameters for quercetin estimation

Parameters	Quercetin
Specificity	Specific
Regression equation	Y = 967.46X + 6136.2
Correlation coefficient (r^2)	0.9843
Correlation coefficient (r)	0.9921
Slope	967.46
Standard error of the slope	965.14
Linearity range (µg/spot)	5-20
Limit of detection (LOD) µg/spot	3.29
Limit of quantitation (LOQ) µg/spot	9.97

4.6 Qualitative and quantitative estimation of Quinine using HPTLC-densitometry method

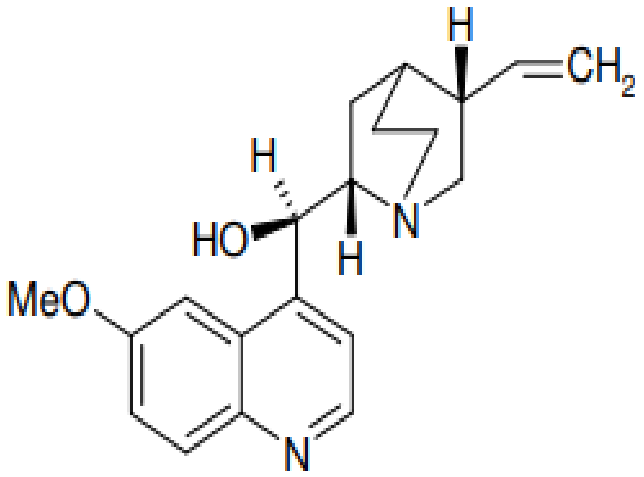


Figure 4.36: Chemical structure of quinine

4.6.1 Development of Optimum Mobile Phase ratio for Quinine separation

We achieved the proper separation of quinine by using the solvent system of ethyl acetate: diethyl amine in the ratio (88 : 12, v/v) as shown in **Figure 4.37.**

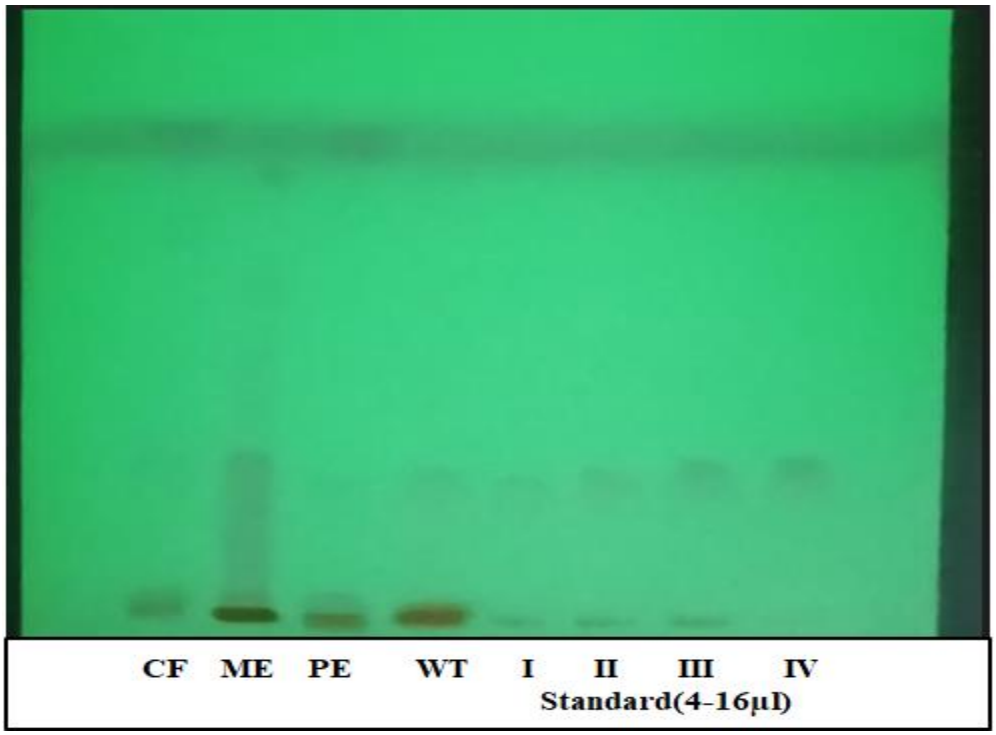
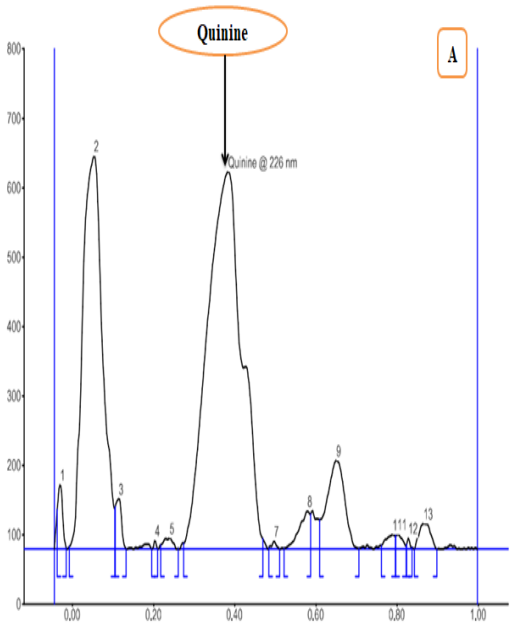
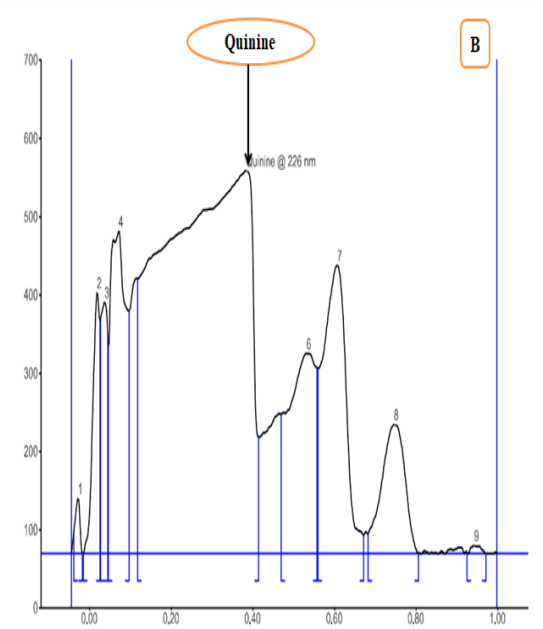


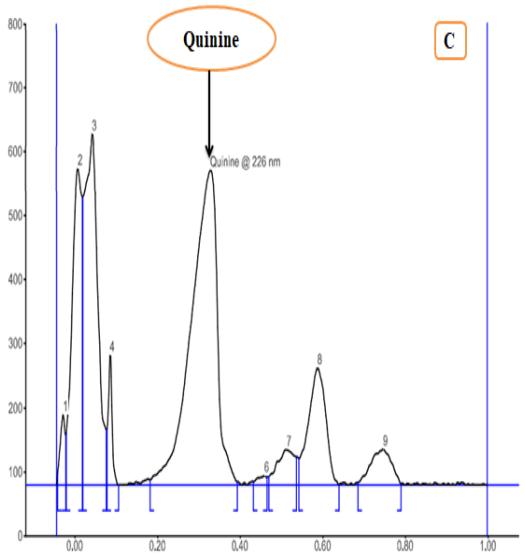
Figure 4.37; HPTLC fingerprinting of four different extracts of *Cinchona officinalis*
Key: **CF** = Chloroform extract; **ME** = Methanol extract; **PE** = Petroleum ether extract; **WT** = Water extract;
Accessions (I-1V) = Quinine Standard (4-16µl)

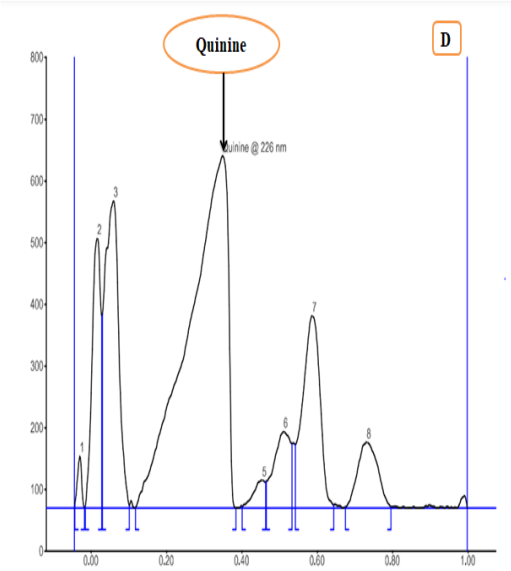
4.6.2 Specificity (quinine)

To confirm the specificity of quinine compounds in the extracts, a comparison was made between the Rf (retention factor) values of the extract and that of the standard bands. These comparisons were depicted in **Figure 4.37**. The results indicated that the method employed was specific, as it facilitated the clear separation of quinine compound from *C. officinalis* bark extracts. However, a well-defined, symmetrical peak were obtained in petroleum ether extract and chloroform extract indicating the good separation and high purity quinine compound better than the other two solvents which appears to have impurities or co-elution of other compounds as shown in **Figure 4.38.**


Quinine
A
Quinine @ 226 nm


Quinine
B
Quinine @ 226 nm


Quinine
C
Quinine @ 226 nm


Quinine
D
Quinine @ 226 nm

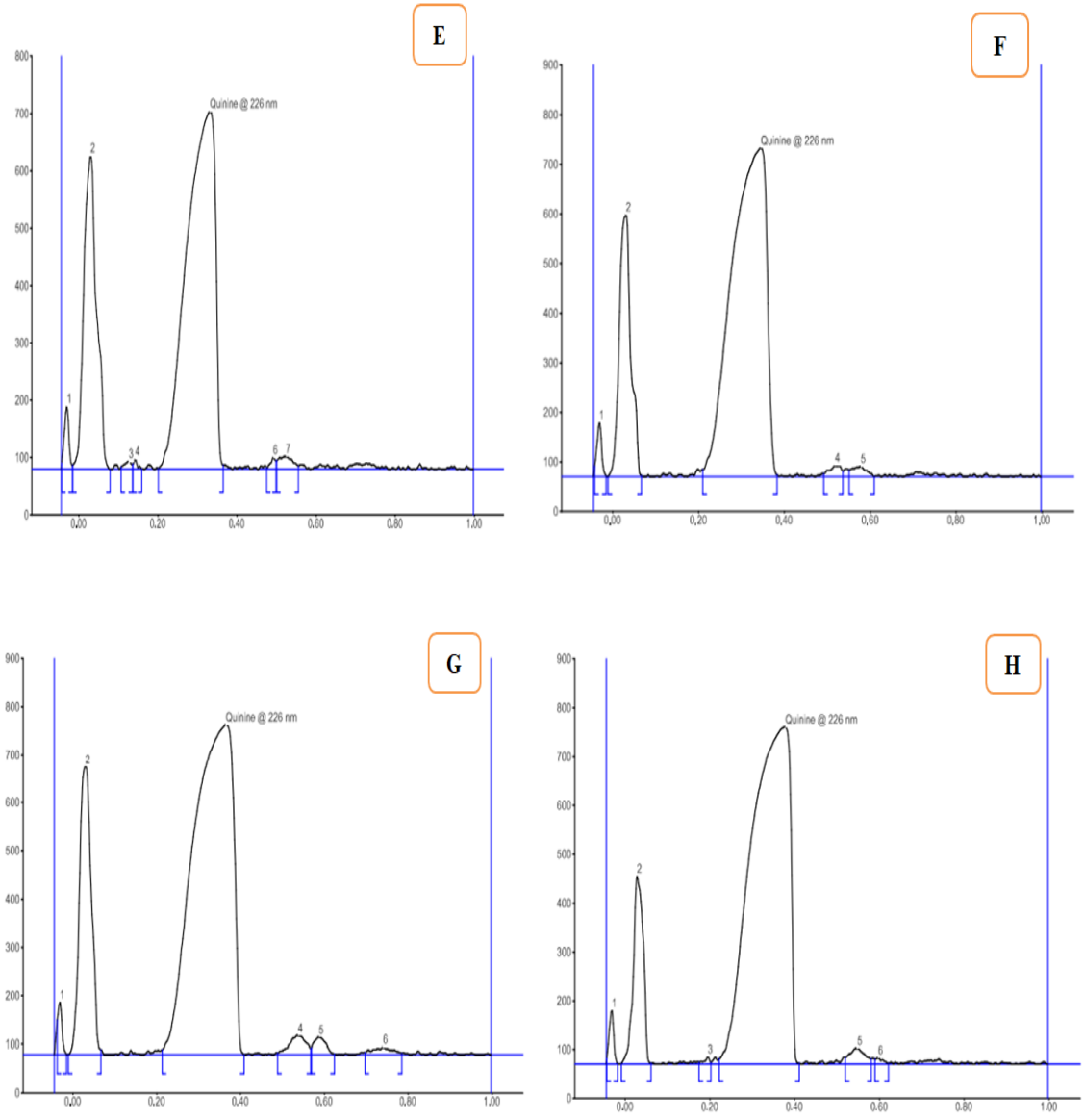


Figure 4.38: Densitometry graph showing isolation of Quinine compound from (**A)** Chloroform extract; **(B)** Methanol extract; **(C)** Petroleum ether extract; **(D)** Aqueous extract of *Cinchona officinalis;* (**E-H) =** Quinine standard (4-16µl) at 226nm.

4.6.3 Influence of solvents on quinine yields

The choice of an appropriate solvent system stands as a pivotal determinant significantly influencing the separation of components on an HPTLC plate. In this study, the primary objective was to isolate and quantify the high-quality quinine compound from four distinct extracts of *C. officinalis* using high-performance thin-layer chromatography. To accomplish this, four solvents with varying polarities; petroleum ether, chloroform, methanol, and water were employed. The amount of quinine compounds present was determined through the utilization of peak area parameters and mean percentages based on dry weight, as detailed in **Table 4.10**. Methanol extract yield the highest quinine content followed by water and chloroform extract. However, very little amount was obtained in chloroform extract. Methanol extract of *C. officinalis* contain maximum content of quinine of about (**2.60% ± 0.036**), water extract (1.44% 0.034), chloroform extract (1.13% ± 0.03), and petroleum ether extract (0.76% ± 0.014) as shown in (**Table 9)** below.

The solvent used for quinine extraction is an important component that influences its extraction efficiency. The selection of solvent for the extraction of quinine is very vital as it significantly affect the yield and purity of the extracted compound. A wide range of solvents have been used for this purpose, including hexane, ethanol, methanol, chloroform, petroleum ether and ethyl acetate. Methanol is a polar solvent that has been used for the extraction of quinine from Cinchona bark. Several studies have investigated the efficiency of methanol in the extraction of quinine, which is an alkaloid that has been used for the treatment of malaria.

A study by (Misra *et al.,* 2008) on the screening of quinine extraction using different solvents. Methanol was found to showed good recovery of analyte quinine. Therefore, methanol is an efficient solvent for quinine extraction. Another study by (Ndiomu *et al.*, 1988) investigated the extraction of quinine from Cinchona bark using different solvents, including methanol, ethanol, and water. The study found that methanol was able to extract quinine sufficient amount of quinine.

Putting all together, methanol extract was found to be an excellent solvent for the extraction of high quality quinine from *Cinchona* bark. Methanol has been shown to extract quinine with high yields and high purity compared to ± 0.036 other solvents like ethanol and water. Further studies are needed to optimize the use of methanol in the extraction of quinine and other alkaloids from *Cinchona* bark.

Table 4.10: Influence of solvents on Quinine yields

S/no.	Extraction solvents	Mean % of Quinine ± SD (dry wt. basis)	Rf value
1.	Chloroform (CF)	1.13 ± 0.03	0.39
2.	Methanol (ME)	**2.60 ± 0.036**	0.39
3.	Petroleum ether	0.76 ± 0.014	0.33
4.	Water (WT)	1.44 ± 0.034	0.35

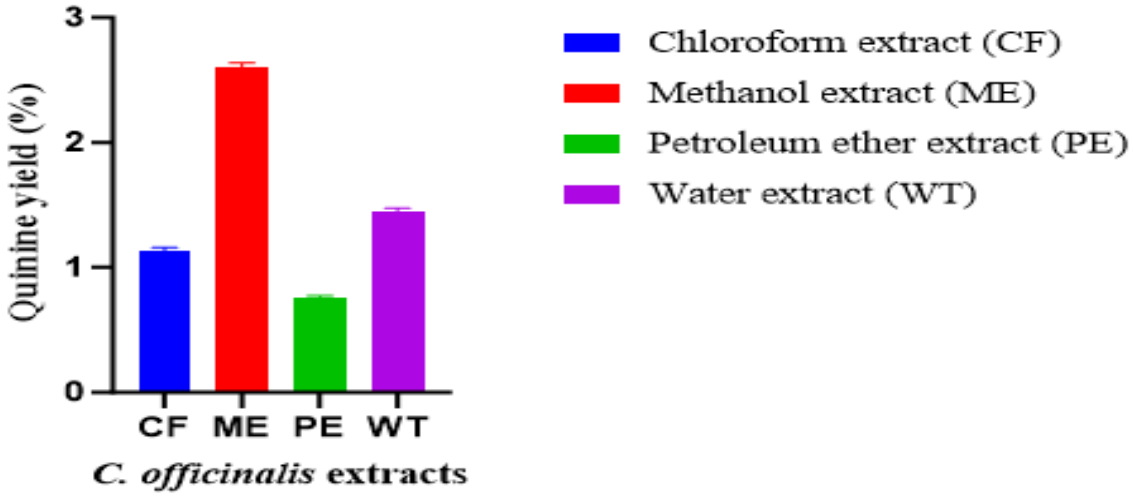


Figure 4.39: Quinine content in different extracts of *C. officinalis* plant

4.6.4. Linearity (quinine)

To assess the linearity, various concentrations of working standard solutions of quinine were applied. The correlation coefficient (r^2) was ascertained by performing a linear least square regression analysis on the resultant data. For Quinine, the correlation coefficient (r^2) was found to be 0.9974 (**Figure 4.40**) indicating a robust linear association between the concentration of the standard and those of the corresponding responses. The linearity range of the calibration curve for quinine was determined to be 400-1600 ng/spot. The calibration curve graph, demonstrating the linearity range of quinine is presented in the **Figure 4.40.**

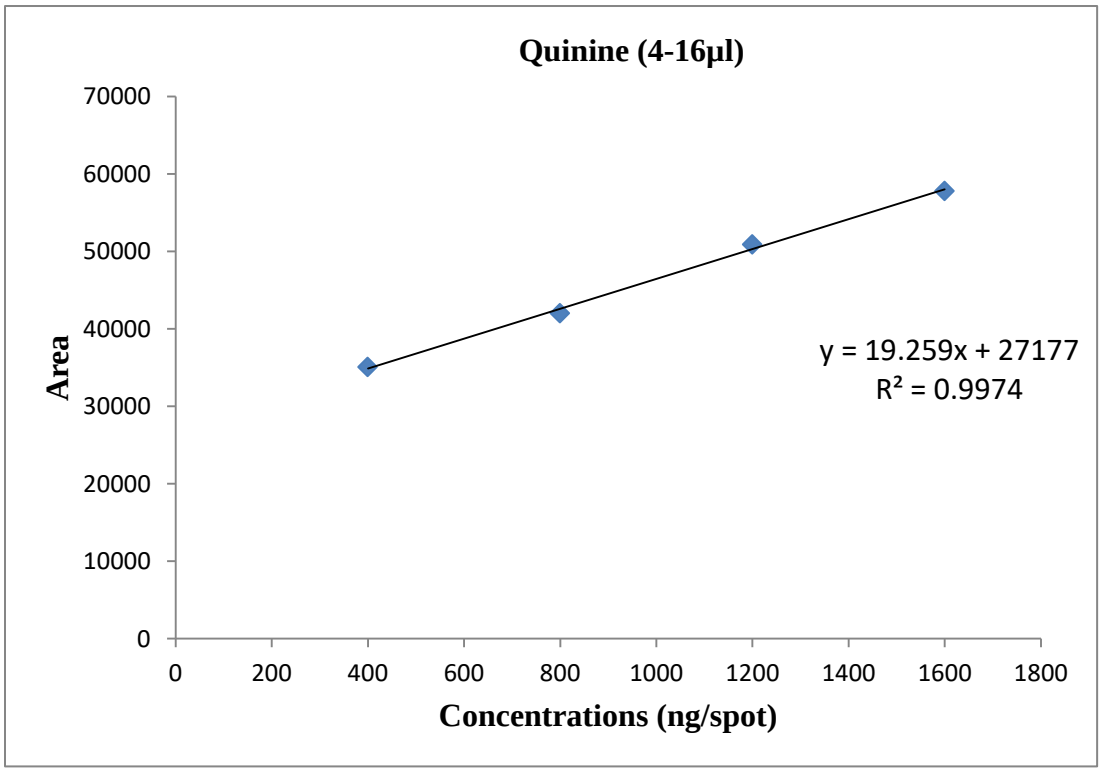


Figure 4.40: Calibration curve of quinine standard

4.6.6 Precision (quinine)

The precision or repeatability of the analysis method was assessed in eight determinations of quinine of the same concentration on different tracks. Each track was scanned, and the resulting chromatograms were recorded. The repeatability of the measurements was expressed as the percent coefficient of variation (RSD %) for the peak area of the standards. The obtained results, presented in **Figure 4.41** confirm that the method exhibits high precision for the analysis of quinine in *C. officinalis*. Intra-day precision was found to be 0.34% ± 0.001, and inter-day precision was found to be 0.37% ± 0.001. The results obtained affirm that the method showcases a remarkable degree of precision for the analysis of quinine in *Cinchona officinalis* plant. The presence of low RSD% values implies negligible variation in peak areas, underscoring the method's exceptional measurement repeatability.

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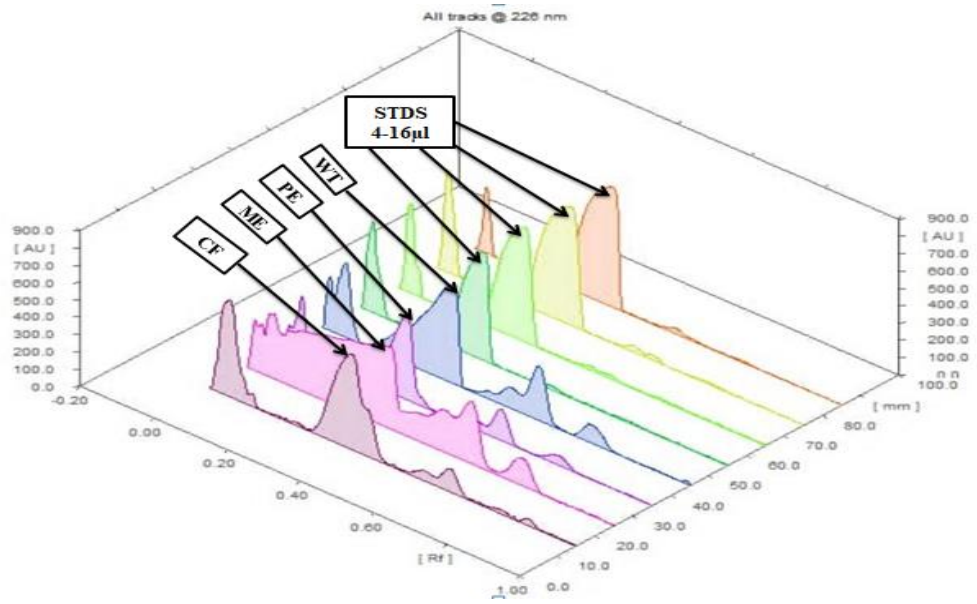


Figure 4.41: 3D Chromatogram of the extracts of *Cinchona officinalis* and standard

Key: **CF** = Chloroform extract; **ME** = Methanol extract; **PE** = Petroleum ether extract; **WT** = Water extract;

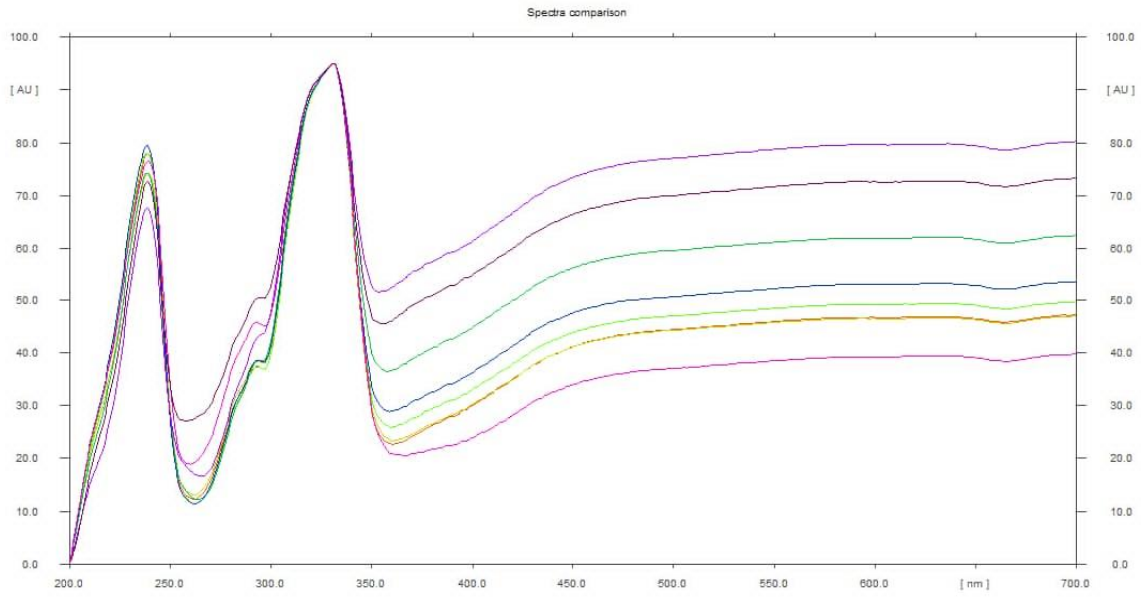


Figure 4.42: Overlay spectral comparisons of the extracts quinine standards

4.6.7 Limit of Detection and Limit of Quantitation of Quinine

The limit of detection (LOD) of quinine was found to be 1.2 µg/spot while the limit of quantitation (LOQ) was found to be 3.65 µg/spot as shown in **Table 4.11**.

Table 4.11: HPTLC validated parameters for quinine estimation

S/n	Parameters	Quinine
1.	Specificity	Specific
2.	Regression equation	y = 19.259x + 27177
3.	Correlation coefficient(r^2)	0.9974
4.	Correlation coefficient (r)	0.9987
5.	Slope	19.259
6.	Standard error	623.013
7.	Linearity range (ng/spot)	400-1600
8.	Limit of detection (LOD) µg/spot	1.20
9.	Limit of quantitation (LOQ) µg/spot	3.65

4.7 Gas chromatography-mass spectrometry

The GC-MS analysis of the methanol extract of *S. thomsonii* revealed a variety of phytocompounds contained in the extract studied. The chemicals identified were cross-referenced with the NIST database, renowned for its extensive collection of known compounds. This database provides detailed information on the structure and mass spectra of each compound, facilitating precise identification. Utilizing Gas Chromatography-Mass Spectrometry (GC-MS) which is one of the most widely utilized and valuable techniques for volatile chemical analysis, enabled accurate identification of the compounds. The compounds were identified from the database using particular parameters such as molecular weight, retention duration, and peak area, which were determined during the identification procedure. Because just a small percentage of the compounds are dissolved in highly volatile organic solvents, the majority of the compounds imbedded in the extracts turn volatile when subjected to high temperatures (Rukshana *et al.,* 2017).

4.7.1 GC-MS analysis of methanol extract of *S. thomsonii*

The crude methanol extract of *S. thomsonii* underwent gas chromatography-mass spectrometry analysis for approximately 38.5 minutes, generating a gas chromatogram displaying peaks at various retention times. These peaks were scrutinized and identified to align with known compounds in the NIST database, as depicted in **Figure 4.43.**

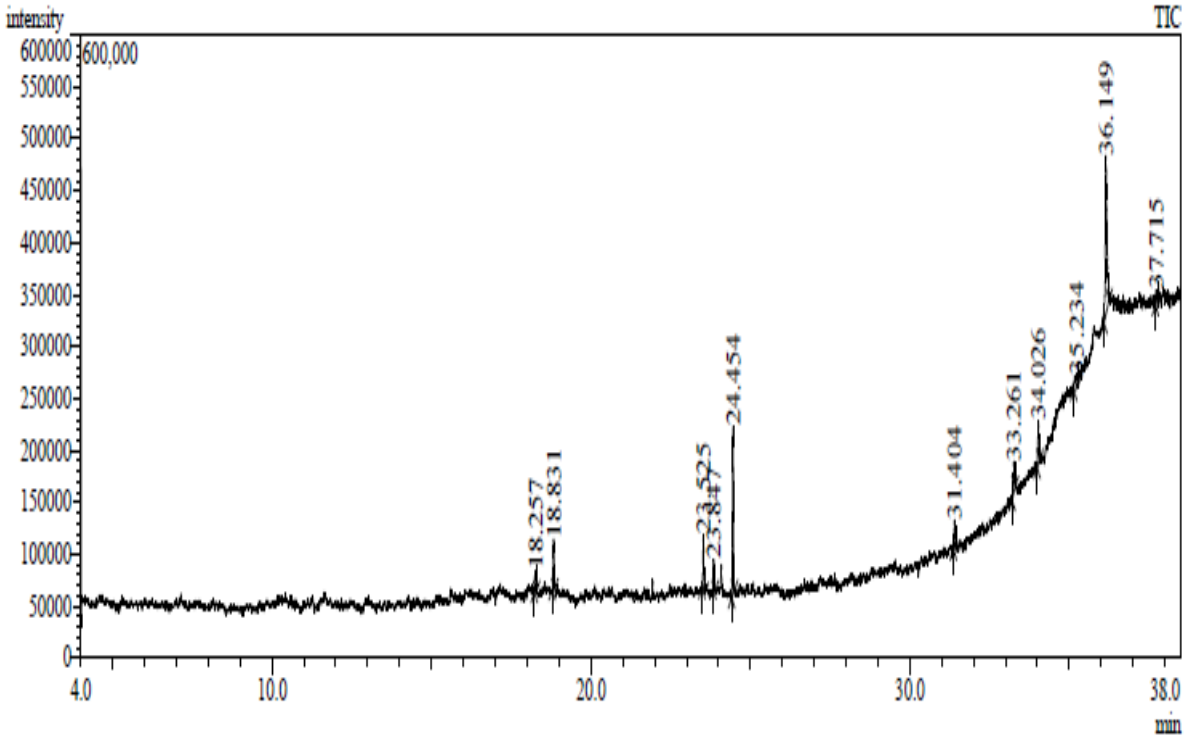


Figure 4.43: *S. thomsonii's* methanol gas chromatogram

The gas chromatography-mass spectrometric technique examination of *S. thomsonii's* methanol extract showed several chemicals, with the most notable ones being: Methyl 4-methoxysalicylate, TMS derivative, 2,4-Di-tert-butylphenol, Neophytadiene, Phthalic acid, butyl undecyl ester, 7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione, Bis(2-ethylhexyl) phthalate, Flavone, 5-hydroxy-7,8-dimethoxy-, Squalene, 4'-Hydroxy-3'-methoxyflavanone trimethylsilyl ether, Desogestrel, Methyl 3-bromo-1-and adamantaneacetate. This corresponds to 100% of the entire chromatogram percentage peak area. The different compounds' percentage peak area, retention time, and molecular weight is provided in (**Table 4.12**). *Swertia spp.* is a diverse genus with several bioactive components, including: xanthones, iridoids, seco-iridoids, triterpenoids. These compounds contribute to the medicinal properties of *Swertia spp*. The genus has gained industrial importance due to its market value, which rises by 10% annually (Kaur *et al.*, 2020).

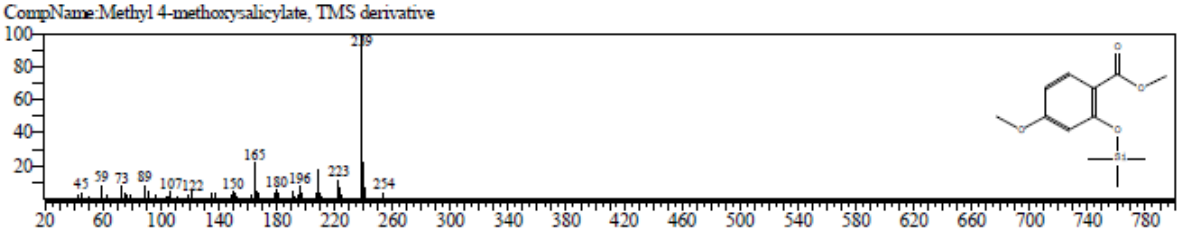
The aforementioned compounds identified from methanol extract of *S. thomsonii* were s virtually screen through molecular docking and molecular interaction study and Flavone, 5-hydroxy-7,8-dimethoxy- were found to have excellently interact with the three target receptors of *P. falciparum* employed in this study. The present finding corroborated with the previous study by (Sachdeva, 2011) which reported the antimalarial activity of andrographolide including the 5-hydroxy-7, 8-dimethoxyflavone and found to inhibit the parasite growth with (15.4 ± 2.9%). The results of this study's GC-MS analysis were compatible with prior work that employed GC-MS to discover several chemical contents in the aerial part of *S. thomsonii*, despite the fact that the initial extraction was performed with a different solvent system. **Figure 4.44** shows the varied mass spectra of the discovered bioactive chemicals.

Table 4.12: Compounds identified in methanol extract of *S. thomsonii* by GC-MS

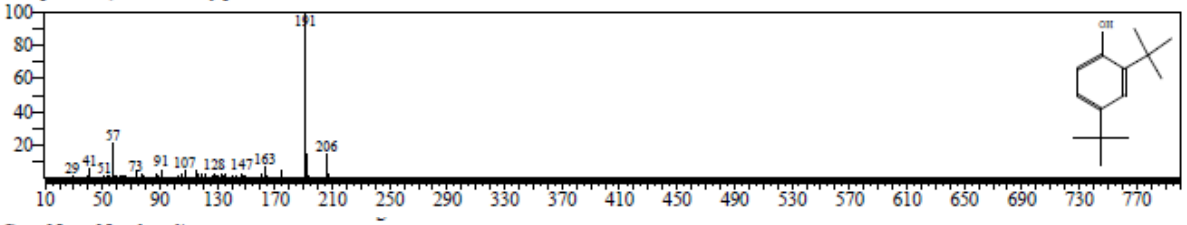
RT	Name of compound	Peak area (%)	Molecular formular	MW
18.257	Methyl 4-methoxysalicylate, TMS derivative	2.53	$C_{12}H_{18}O_4Si$	254
18.831	2,4-Di-tert-butylphenol	5.71	$C_{14}H_{22}O$	206
23.525	Neophytadiene	6.72	$C_{20}H_{38}$	278
23.847	Phthalic acid, butyl undecyl ester	3.31	$C_{23}H_{36}O_4$	378
24.454	7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	20.17	$C_{17}H_{24}O_3$	276
31.404	Bis(2-ethylhexyl) phthalate	4.56	$C_{24}H_{38}O_4$	390
33.261	Flavone, 5-hydroxy-7,8-dimethoxy-	5.27	$C_{17}H_{14}O_5$	298
34.026	Squalene	6.05	$C_{30}H_{50}$	410
35.234	4'-Hydroxy-3'-methoxyflavanone, trimethylsilyl ether	4.35	$C_{19}H_{22}O_4Si$	342
36.149	Desogestrel	37.46	$C_{22}H_{30}O$	310
37.715	Methyl 3-bromo-1-adamantaneacetate	3.87	$C_{13}H_{19}BrO_2$	286

Key: RT= retention time; **MW=** Molecular weight

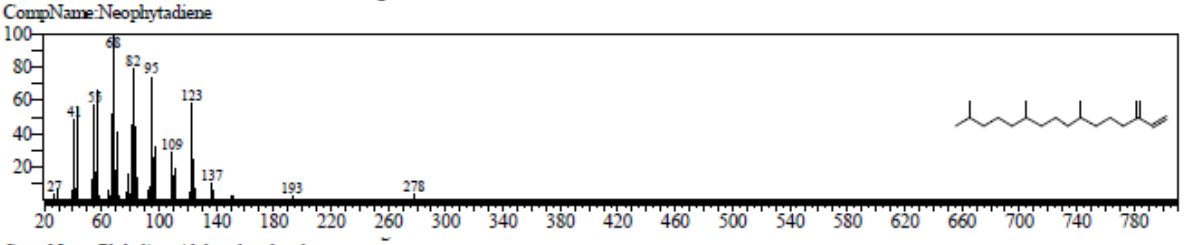
CompName:Methyl 4-methoxysalicylate, TMS derivative



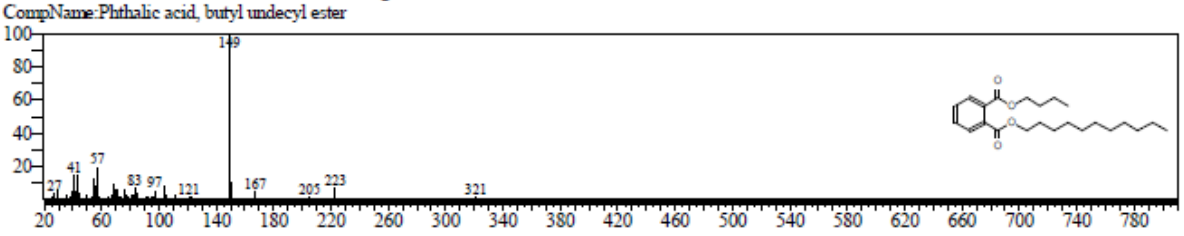
CompName:2,4-Di-tert-butylphenol



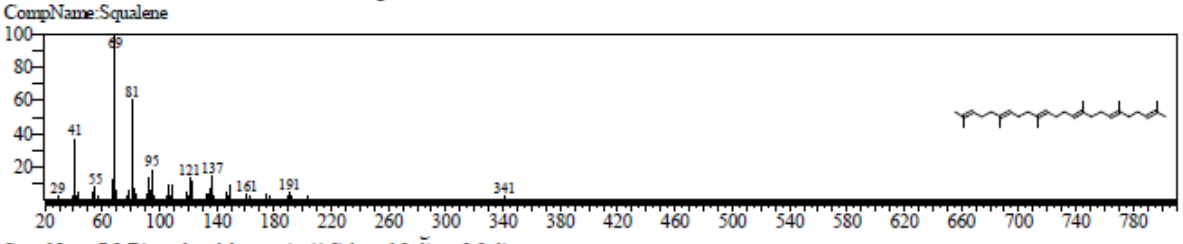
CompName:Neophytadiene



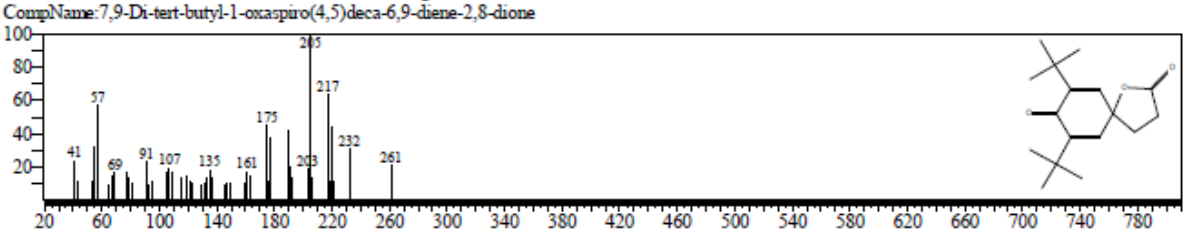
CompName:Phthalic acid, butyl undecyl ester



CompName:Squalene



CompName:7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione



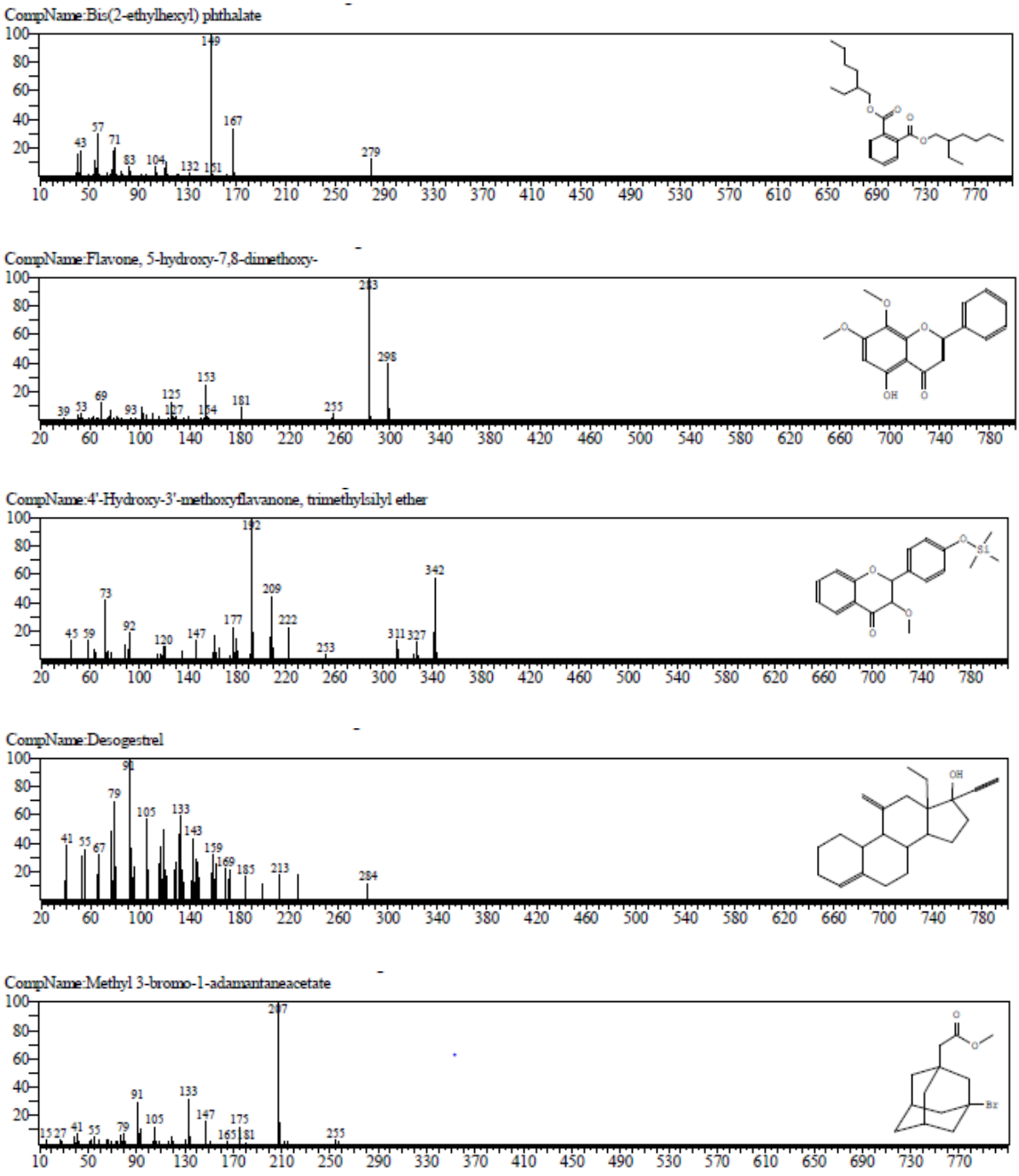


Figure 4.44: Mass spectra of bioactive compounds found using GC-MS in *S. thomsonii* methanol extract.

4.8 In vitro antimalarial efficacy of the selected extracts from the four selected medicinal plants & determination of IC50 values

In two separate trials, the *Plasmodium falciparum* was cultured in triplicate for 72 hours, utilizing both the drug-sensitive clone (3D7) and the chloroquine-resistant strain (Dd2) as targets. The asexual blood stage of *P. falciparum* was cultured continuously, following a slightly modified version of the procedure outlined by (Trager and Jensen in 1976). To quantify antiplasmodial activity *in vitro*, the parasite lactate dehydrogenase assay was followed as reported by (Makler *et al*. in 1993).

4.8.1 Efficacy of petroleum ether extract of *A. annua L* (PEARA) against the 3d7 and Dd2 strains of *Plasmodium falciparum*

From the *in vitro* study while evaluating the antimalarial efficacy of the selected extracts, the ***PEARA*** were found to be very active on Dd2 strain with average mean of inhibition and standard error of the mean of (0.920 ± 0.083 µg/mL) than in 3D7 strain with mean inhibition of (1.046 ± 0.101 µg/mL) as shown in **Table 4.13.** Therefore, the mean of inhibition of ***PEARA*** on the two isolates of *P. falciparum* were found largely comparable with that of the Chloroquine phosphate (with IC50 values of 0.009 ± 0.002µg/ml and 0.232 ± 0.015µg/ml against 3D7 and Dd2 respectively). It was also found comparable with that Artesunate drug with average mean of inhibition of approximately 0.005 ± 0.001µg/ml in both 3D7 and Dd2 strains of *P. falciparum.* Artemisinin is a strong antimalarial bioactive compound found in extracts of *A. annua* and is renowned for its action against *Plasmodium falciparum* malaria, including highly drug-resistant strains (Verma *et al.,* 2020). Artemisinin, a natural compound extracted from Artemisia annua, also known as sweet wormwood, has significantly transformed malaria treatment with its strong antimalarial effects. Its distinctive feature is an endoperoxide bridge, pivotal for its biological activity. Upon activation by reduced heme or ferrous iron, artemisinin generates cytotoxic carbon-centered radicals. These radicals selectively attack vital parasite molecules, resulting in the parasite's death. However, the exact primary target of artemisinin remains unclear (Dai *et al.,* 2017). Moreover, recent studies suggest that *A.*

annua whole-plant extracts was more effective than pure artemisinin in treating rodent malaria and reducing resistance development. These extracts could potentially benefit artesunate-resistant malaria patients (Czechowski *et al.,* 2019). The present finding also supported the previous finding as both the two strains shows no resistance to crude treatment of ***PEARA.*** Moreover, excellent efficacy was also observed while testing the ***PEARA*** against the two clones of *Plasmodium falciparum.*

Table 4.13: Antiplasmodial activity of Petroleum ether extract of *A. annua* **(*PEARA*)** fractions against *P. falciparum* strains

Test samples	**3d7**		**Dd2**	
	Mean (µg/mL)	SEM (µM)	Mean (µg/mL)	SEM (µM)
Petroleum ether extract of *A. annua* (*PEARA)*	1.046	0.101	0.920	0.083
Chloroquine phosphate (CQ)	0.009	0.002	0.232	0.015
Artesunate (Arts)	0.004	0.001	0.005	0.001
Dimethyl sulphate (*DMSO)*	NA	NA	NA	NA

Note; Data shown are the mean of two independent evaluations in triplicate, and are presented in µg/mL except where noted *Standard error on the Mean. **NA** = No activity detected.

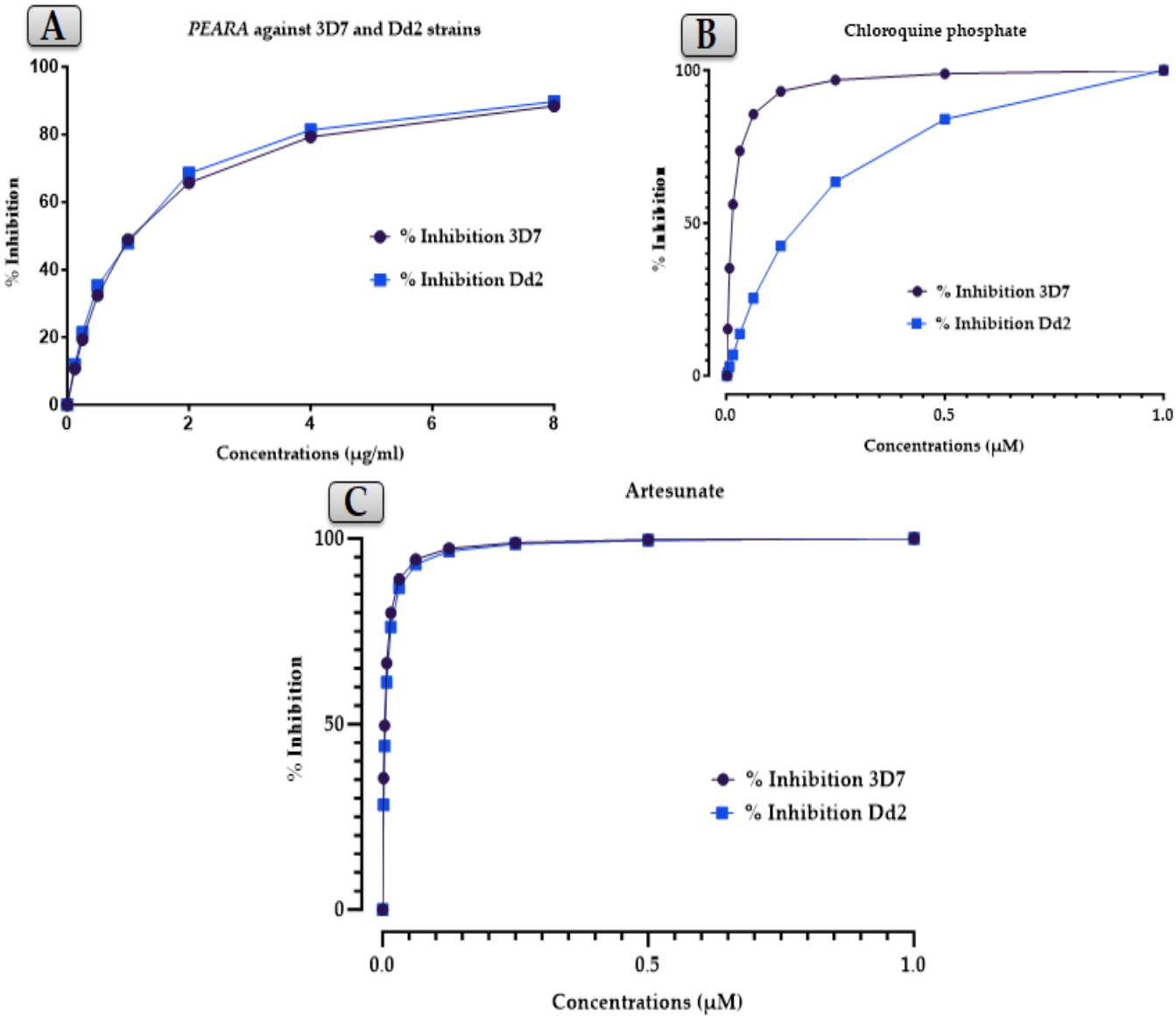


Figure 4.45: IC50 values calculated using nonlinear regression analysis using 10 distinct concentrations and a two-fold serial dilution. The *PEARA* **(A)** were diluted from 100µg/ml to 0.04875µg/ml, whereas the standard drugs **(B & C)** were diluted from 1µg/ml to 0.0156µg/mL. The experiment was carried out in triplicate, with each value representing the mean ± SEM of three replicates.

4.8.2 Efficacy of methanol extract of *C. officinalis* (MECIO) against the 3d7 and Dd2 strains of *Plasmodium falciparum*

Methanol extract of *C. officinalis (**MECIO**)* were found to be very active against the tested strains of *P. falciparum* with mean inhibition value of (0.741 ± 0.122 µg/mL in Dd2) and (0.882 ± 0.071 µg/mL in 3D7) as shown in **Table 4.14**. Therefore, the mean of inhibition of *(**MECIO**)* on the two isolates of *P. falciparum* were found largely comparable with that of the Chloroquine phosphate (with IC50 values of 0.009 ± 0.002µg/ml and 0.232 ± 0.015µg/ml against 3D7 and Dd2 respectively). It was also found comparable with that Artesunate drug with average mean of inhibition of approximately 0.005 ± 0.001µg/ml in both 3D7 and Dd2 strains of *P. falciparum.* According to the previous studies on the antimalarial efficacy of *C. officinalis,* the bark extract of *Cinchona officinalis* bark were tested for antimalaria using *in vivo* study with mother tincture and potencies demonstrated excellent chemo-suppression of about 97-100% against lethal murine malaria parasite (Rajan *et al.,* 2012). Another study by (Rajan and Bagai, 2012) using *in vitro* study on the potency of *Cinchona officinalis* against the *P. barghei* also showed 80% *schizonts* inhibition.

Table 4.14: Antiplasmodial activity of Methanol extract *C. officinalis (MECIO)* fractions against *P. falciparum* strains

Test samples	3d7		Dd2	
	Mean (µg/mL)	SEM (µM)	Mean (µg/mL)	SEM (µM)
Methanol extract *C. officinalis (**MECIO**)*	0.882	0.071	0.741	0.122
Chloroquine phosphate **(CQ)**	0.009	0.002	0.232	0.015
Artesunate (**Arts)**	0.004	0.001	0.005	0.001
Dimethyl sulphate **(*DMSO*)**	NA	NA	NA	NA

Note; Data shown are the mean of two independent evaluations in triplicate, and are presented in µg/mL except where noted *Standard error on the Mean. **NA** = No activity detected.

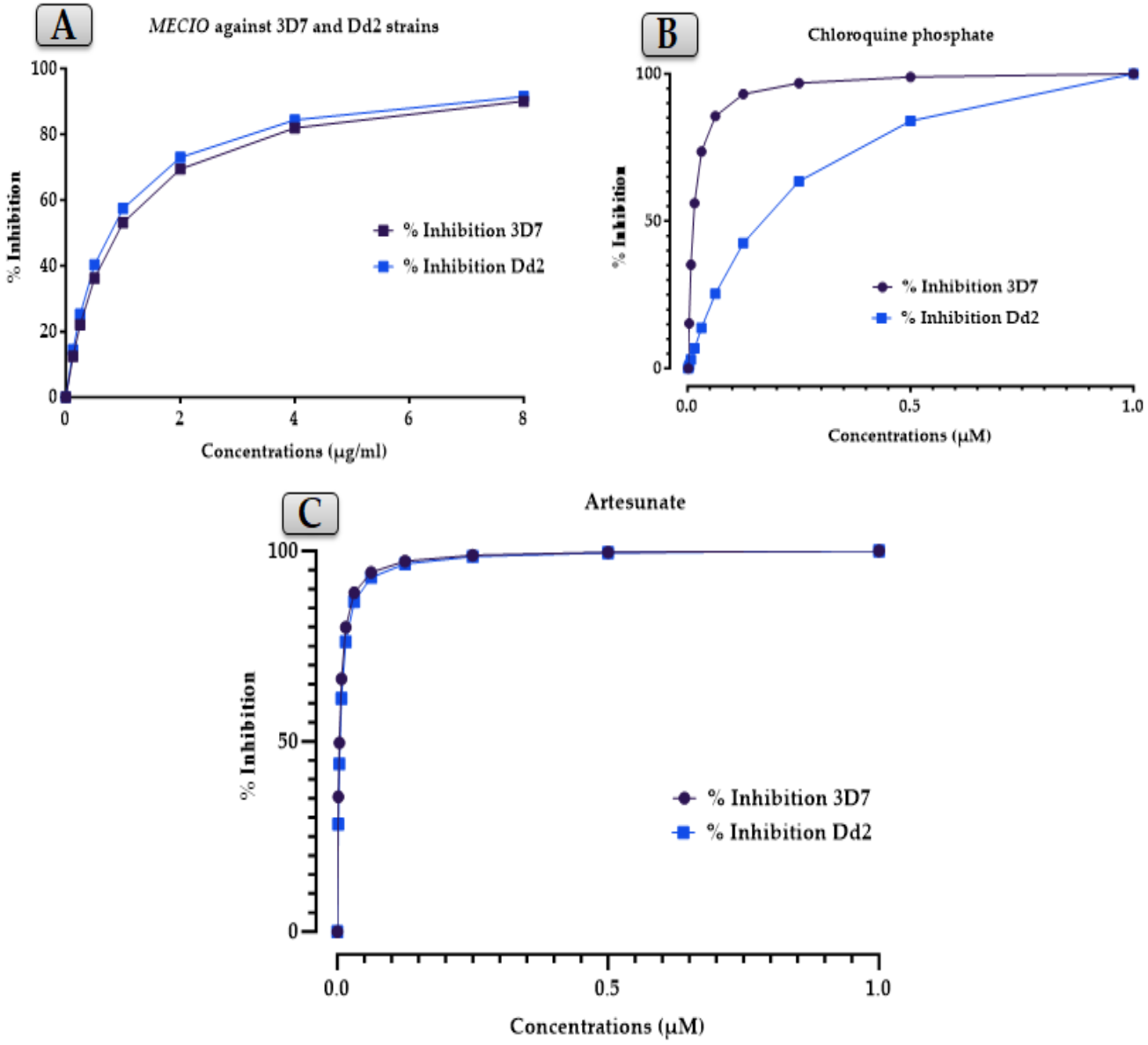


Figure 4.46: IC50 values calculated using nonlinear regression analysis using 10 distinct concentrations and a two-fold serial dilution. The *MECIO* **(A)** were diluted from 100µg/ml to 0.04875µg/ml, whereas the standard drugs **(B & C)** were diluted from 1µg/ml to0.0156µg/mL. The experiment was carried out in triplicate, with each value representing the mean ± SEM of three replicates.

4.8.3 Efficacy of Chloroform extract of *Cassia occidentalis L.* (CFCAO) against the 3d7 and Dd2 strains of *Plasmodium falciparum*

Chloroform extract of *Cassia occidentalis* L. shows good activity against the two strains with mean inhibition (10.67 ± 0.147 µg/mL in 3D7) and (11.22 ± 1.32µg/mL in Dd2) as shown in **Table 4.15**. Therefore, the mean of inhibition of ***(CFCAO)*** on the two isolates of *P. falciparum* were found largely comparable with that of the Chloroquine phosphate (with IC50 values of 0.009 ± 0.002µg/ml and 0.232 ± 0.015µg/ml against 3D7 and Dd2 respectively). It was also found comparable with that Artesunate drug with average mean of inhibition of approximately 0.005 ± 0.001µg/ml in both 3D7 and Dd2 strains of *P. falciparum.* This finding corroborated with previous study which reported the antiplasmodial activity of extracts from different parts of *Cassia occidentalis*. The extracts show promising activity in combating *plasmodium* parasites, which cause malaria (Singh *et al.,* 2016; Mogaka *eta al.,* 2023). (Yadav *et al.,* 2010) also disclosed that the extract from *C. occidentalis* possesses strong antimalarial properties. The antimalarial efficacy of various extracts derived from the root bark of *C. occidentalis* was assessed through in vivo 4-day suppressive test targeting *Plasmodium berghei* ANKA in murine models. Oral administration of ethanolic and dichloromethane extracts at a dose of 200 mg/kg resulted in significant chemosuppression of parasitemia, exceeding 60%. Notably, the lyophilized aqueous extract demonstrated comparatively lower activity than its ethanolic counterpart. Additionally, investigations into the antimalarial potential of C. occidentalis leaves revealed promising outcomes, with ethanol and chloroform extracts exhibiting notable activity. Specifically, these extracts demonstrated more than sixty percent inhibition of parasite growth *in vitro* at a concentration of 6µg/ml.

Table 4.15: Antiplasmodial activity of Chloroform extract of *C. occidentalis* **(*CFCAO*)** fractions against *P. falciparum* strains

Test samples	**3d7**		**Dd2**	
	Mean (μg/mL)	SEM (μM)	Mean (μg/mL)	SEM (μM)
Chloroform extract of *C. occidentalis* (***CFCAO*)**	10.67	0.147	11.22	1.32
Chloroquine phosphate **(CQ)**	0.009	0.002	0.232	0.015
Artesunate (**Arts)**	0.004	0.001	0.005	0.001
Dimethyl sulphate (***DMSO)***	NA	NA	NA	NA

Note; Data shown are the mean of two independent evaluations in triplicate, and are presented in μg/mL except where noted *Standard error on the Mean. **NA** = No activity detected.

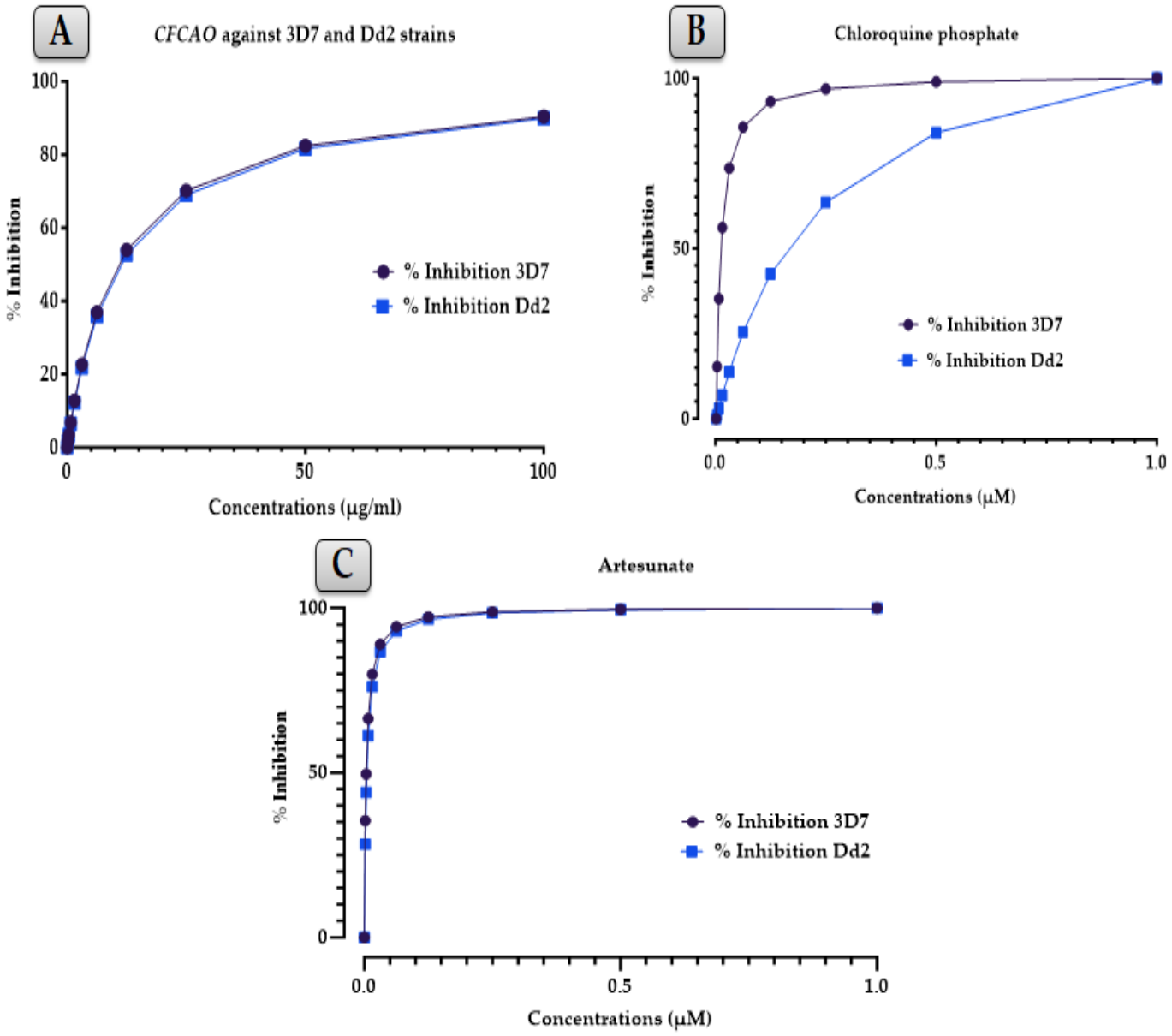


Figure 4.47: IC50 values calculated using nonlinear regression analysis using 10 distinct concentrations and a two-fold serial dilution. The *CFCAO* **(A)** were diluted from 100µg/ml to 0.04875µg/ml, whereas the standard drugs **(B & C)** were diluted from 1µg/ml to0.0156µg/mL. The experiment was carried out in triplicate, with each value representing the mean ± SEM of three replicates.

4.8.4 Efficacy of methanol extract of *Swertia thomsonii* L. against the 3d7 and Dd2 strains of *Plasmodium falciparum*

Finally, in methanol extract of *Swertia thomsonii L.* ***(MESWT)*** Moderate activity were found in 3D7 with mean inhibition value of (36.98 ± 11.57 µg/mL in 3D7) and very less activity in Dd2 strain with mean inhibition value of (86.19 ± 20.11 µg/mL) as shown in **Table 4.16.** Therefore, the mean of inhibition of ***(MESWT)*** on the two isolates of *P. falciparum* were found less comparable with that of the Chloroquine phosphate (with IC50 values of 0.009 ± 0.002µg/ml and 0.232 ± 0.015µg/ml against 3D7 and Dd2 respectively). It was also found less comparable with that Artesunate drug with average mean of inhibition of approximately 0.005 ± 0.001µg/ml in both 3D7 and Dd2 strains of *P. falciparum.* However, the result is still deemed satisfactory at the crude extract level. As per the *in vitro* and *in vivo* previous investigations of antiplasmodial activity of *Swertia spp.* a promising activity were reported and pure compound isolated from the *Swertia alata* like demonstrated antimalarial activity in both in vitro and in vivo studies (Kaur *et al.,* 2020).

Table 4.16: Antiplasmodial activity of methanol extract of S. thomsonii L. ***(MESWT)*** fractions against *P. falciparum* strains

Test samples	3d7		Dd2	
	Mean (µg/mL)	SEM (µM)	Mean (µg/mL)	SEM (µM)
Methanol extract of *Swertia thomsonii* ***(MESWT)***	36.98	11.57	86.19	20.11
Chloroquine phosphate (**CQ)**	0.009	0.002	0.232	0.015
Artesunate (Arts)	0.004	0.001	0.005	0.001
Dimethyl sulphate (***DMSO)***	NA	NA	NA	NA

Note; Data shown are the mean of two independent evaluations in triplicate, and are presented in µg/mL except where noted *Standard error on the Mean. **NA** = No activity detected.

Overall, all the fractions of the selected extracts with the exception of ***MESWT*** were largely comparable between the two isolates (3D7 and Dd2) with around 2-fold activity difference at a maximum, which is not unusual for crude preparations given that they are a mixture of many individual compounds and thus resistance is not expected. Therefore, both the isolates show no resistance to the tested fractions of the crude extracts of the four selected medicinal plants. Fractions showed varying levels of activity from highly active with IC_{50} values less than 1μg/mL (*A. annua* and *C. officinalis*) to moderate active with IC50 value 10x higher in *C. occidentalis* and 30x higher in *S. thomsonii.* The control compounds chloroquine phosphate and artesunate are extremely active and showed IC_{50} values of 9 nM and 5 nM respectively against the sensitive isolate. As expected for the CQ-resistant strain of *P. falciparum* Dd2, the artesunate IC50 is around the same range at approximately 5nM (0.005μg/mL). Therefore, the Chloroquine (CQ) IC50 is significantly higher in Dd2 than in 3D7 strain.

Therefore, the mean of inhibition of the three tested extracts ***(PEARA, MECIO & CFCAO)*** on the two isolates of *P. falciparum* were found comparable with the control. Although the IC50 value of ***MESWT*** was not comparable to the standard drug (**CQ)** (IC50 values 0.005 ± 0.002μg/ml for 3D7 strain and 0.119 ± 0.015μg/ml for Dd2 strain, but the outcomes of the results were deemed satisfactory and warrant further investigation. This suggests potential for further research, as the extracts exhibited a degree of inhibition towards parasite growth; as a result, the herbal formulation made from these plant parts benefits malaria patients. **Figure 4.49** depicted photos taken during the observation under a light microscope at a magnification of 1000X displaying erythrocytes infected with several 3D7 and Dd2 strains of *P. falciparum.*

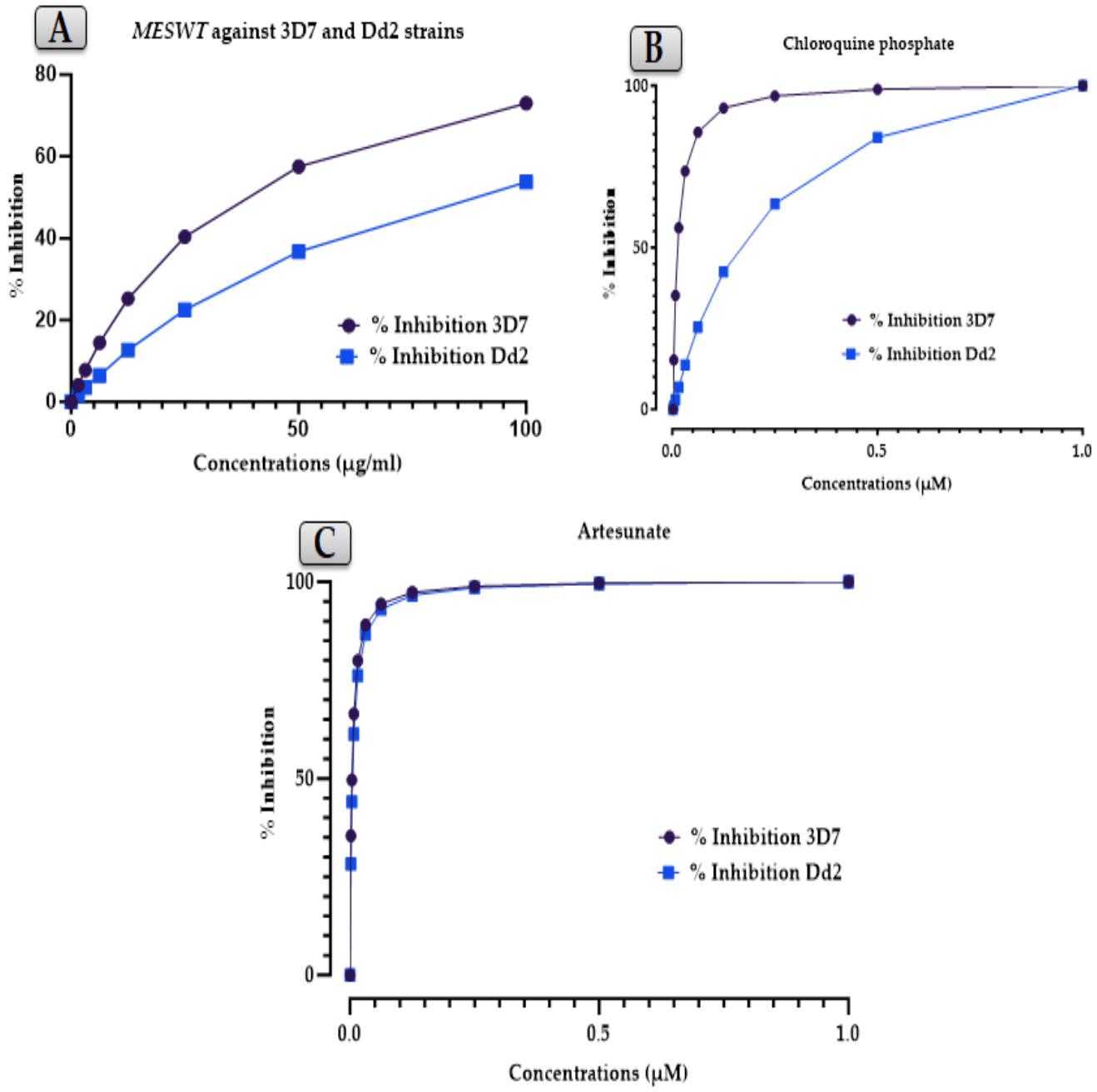


Figure 4.48: IC50 values calculated using non-linear regression analysis using 10 distinct concentrations and a two-fold serial dilution. The *MESWT* **(A)** were diluted from 100µg/ml to 0.04875µg/ml, whereas the standard drugs **(B & C)** were diluted from 1µg/ml to 0.0156µg/mL. The experiment was carried out in triplicate, with each value representing the mean ± SEM of three replicates.

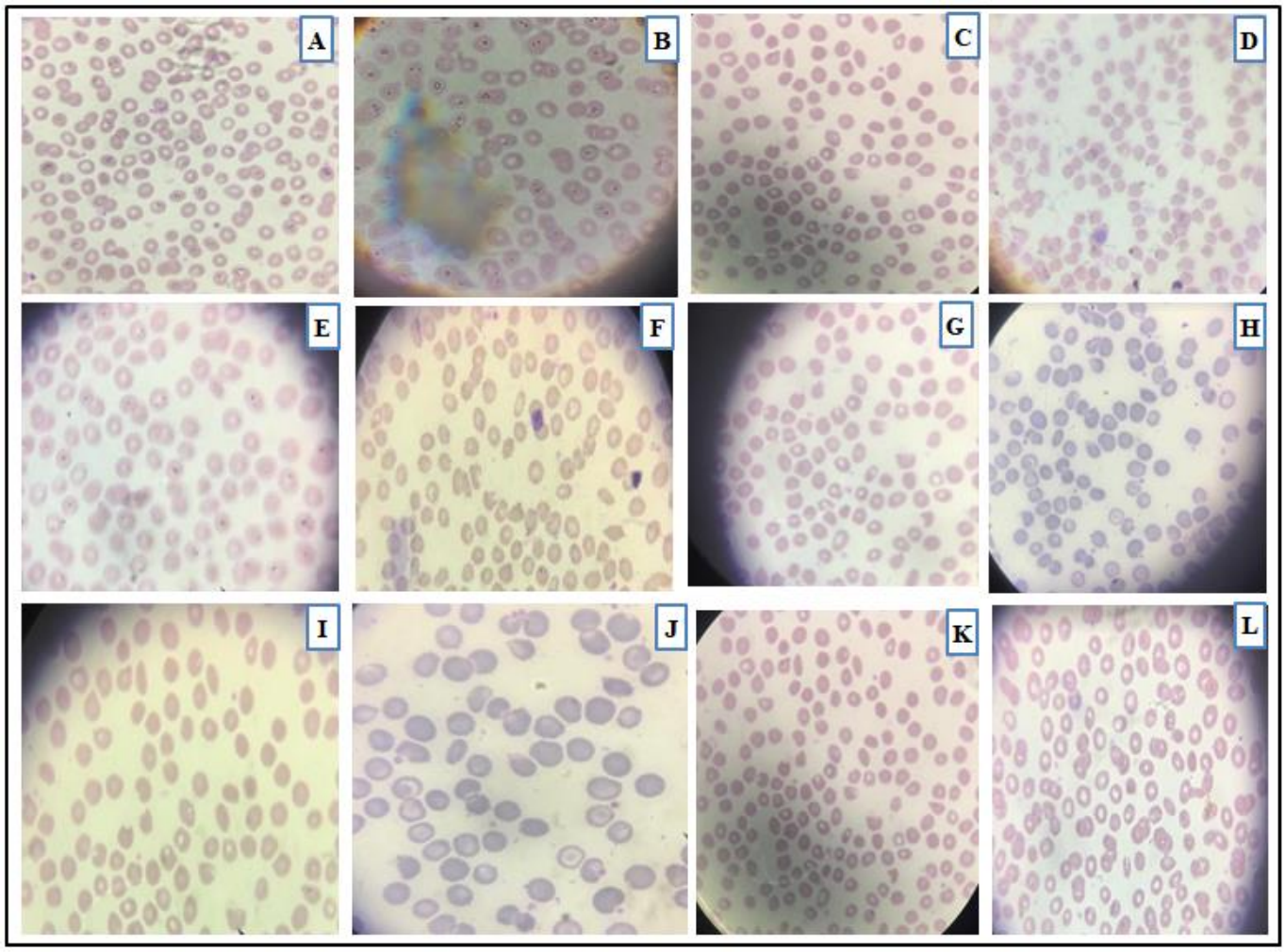


Figure 4.49: Some microscopy images observed under the light microscope at 1000X, revealing infected erythrocytes by distinct strains of 3D7 and Dd2 strains, respectively. [A,B]= 3D7 and Dd2 without treatment, [C,D]= 3D7 and Dd2 treated with CQ and ART. [E,F] = 3D7 and Dd2 treated with *MECIO*, [G,H] = 3D7 and Dd2 treated with *PEARA*, [I,J] = 3D7 and Dd2 treated with *CFCAO* and [K,L]= 3D7 and Dd2 treated with *MESWT*. The data are expressed as Mean ± SEM of two independent experiments.

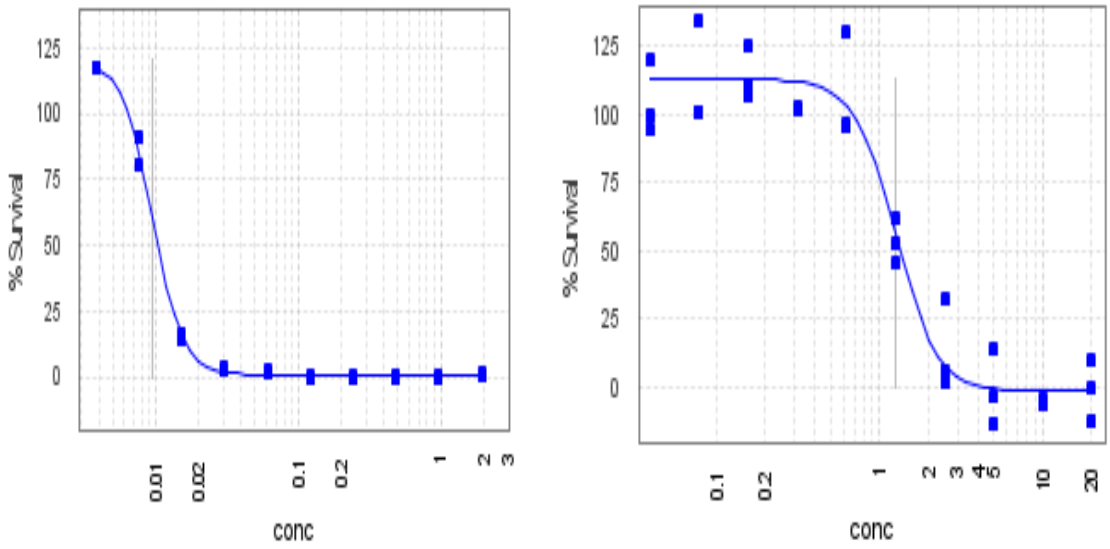


Figure 4.50: Dose-response curve of the control Chloroquine phosphate (left) and samples (right) against *P. falciparum* 3d7, showing %survival of the parasites at 10 different concentrations (in μM) of each compound. ***CFCAO*** is shown at scale of 1/10 and thus the calculated IC_{50} is 10x higher than shown.

4.9 *In-vitro* cytotoxicity study by MTT assay

4.9.1 Inhibition of Neonatal mouse dermal fibroblast by the test samples

The selected plant extracts were studied for cell cytotoxicity using MTT test on NMDF cell lines. The mitochondrial behavior was compared when treated with extracts at concentrations ranging from 50µg/ml to 500µg/ml. At increasing doses, the sample extracts inhibit the proliferation of NMDF cell line, as illustrated in **Figures 4.54-4.57.**

The outcomes of the cytotoxicity assay conducted with all chosen extracts revealed a cytotoxic effect dependent on concentrations. Chloroform extract of *C. occidentalis* revealed the highest percent cytotoxic effect of 2.367±1.14 at 500 µg/ml followed by petroleum ether extract of *A. annua* (2.23±0.87), then methanol extract of *C. officinalis* and *Swertia specie* with (2.094 ± 0.66) and (1.761±0.77) respectively. The LC_{50} values of all the tested extracts are recorded as ***CFCAO*** (104.51 µg/ml), ***PEARA*** (228.47 µg/ml), ***MESWT*** (273.79µg/ml) and ***MECIO*** (259.11 µg/ml) as shown in (**Table 4.17**) and **(Figure 4.51** and **4.52).** Therefore, from the results obtained, we concluded that the sample cause significant growth Inhibition of neonatal mouse dermal fibroblast cell line at 500µg/ml concentration. The chloroform extract of *C. occidentalis* is thus more toxic than the other three extracts; however, both plant extracts have acceptable toxicity values for future study and use in determining the initial doses for in vivo acute toxicity testing.

Although cows and oxen don't eat *C. occidentalis*, several conventional herbal treatments use portions of the plant. Fresh or dry beans are poisonous, as shown by several animal experiments. Large amounts consumed by grazing animals have resulted in fatal illnesses (Mensah *et al.,* 2019). The toxicological impact of *C. occidentalis* consumption in larger animals, rodents, and poultry manifests primarily in the skeletal muscles, liver, kidneys, and heart. The specific organ systems affected vary depending on the species of animal and the dosage of ingested beans. Cognitive functions are frequently impaired as a result of exposure. Gross lesions observed during necropsy typically include necrosis of skeletal muscle fibers and centrilobular necrosis in the liver, with renal tubular necrosis being less common. Biochemical irregularities suggestive of necrosis in muscle and liver cells are

frequently noted (Akbar, 2020). The LD50 in rats and mice is 1 g/kg. Numerous anthraquinones, derivatives, and alkaloids have been linked to toxicity; however, the specific toxic compounds are yet unknown.

Siddiqui et al. (2018) observed that oral administration of hydro-ethanolic extract from Artemisia annua to Swiss mice, at doses up to 5000 mg/kg body weight, did not result in mortality or adverse effects. This absence of toxicity signs suggests that *A. annua* was deemed safe and well-tolerated at the doses used in the study.

Prior studies have examined the safety of *Swertia* species extracts, finding them suitable for human consumption; however, their safety for pregnant and lactating mothers has not been investigated (Mazumder *et al.*, 2023; Tariq *et al.*, 2018). Furthermore, these extracts have demonstrated various hepatoprotective properties, as evidenced by previous research. The observed efficacy of Swertia species extracts against numerous free radicals appears to be linked to their ability to enhance protective cellular components, thereby potentially reducing their cytotoxicity (Naqvi *et al.*, 2013). Additionally, several studies have suggested the plant's potential as an alternative anticancer agent, with indications of its ability to inhibit cancerous cell lines (Saha *et al.*, 2004; Subedi and Karki, 2018).

The toxicity of crude extract of *Cinchona officinalis* on fibroblast cell lines has been the subject of several studies. The extract of *Cinchona officinalis* has been traditionally used for its antimalarial properties, but its toxicity on fibroblast cells has not been extensively studied.

Table 4.17: Cytotoxicity and calculated LC50 of selected extracts

Extracts	Mean absorbance ± Standard Deviations				
	50 (µg/ml)	100 (µg/ml)	200 (µg/ml)	500 (µg/ml)	(LC50) (µg/ml)
CFCAO	0.905±0.63	1.29±1.08	1.838±0.96	2.367±1.14	104.51
PEARA	0.311±0.33	1.04±0.58	1.28±0.82	2.23±0.87	228.47
MESWT	0.555±0.32	0.98±0.24	1.219±0.73	1.761±0.77	273.79
MECIO	0.247±0.22	0.847±0.35	1.22±0.83	2.094±0.66	259.11

KEY: A= Chloroform extract of *C. occidentalis* (CFCAO); **B**= Petroleum ether extract of *A. annua* (PEARA); **C**= Methanol extract of *Swertia sp.* (MESWT); **D**= Methanol extract of *C. officinalis* (MECIO); **Error bars** = SD. (n=3)

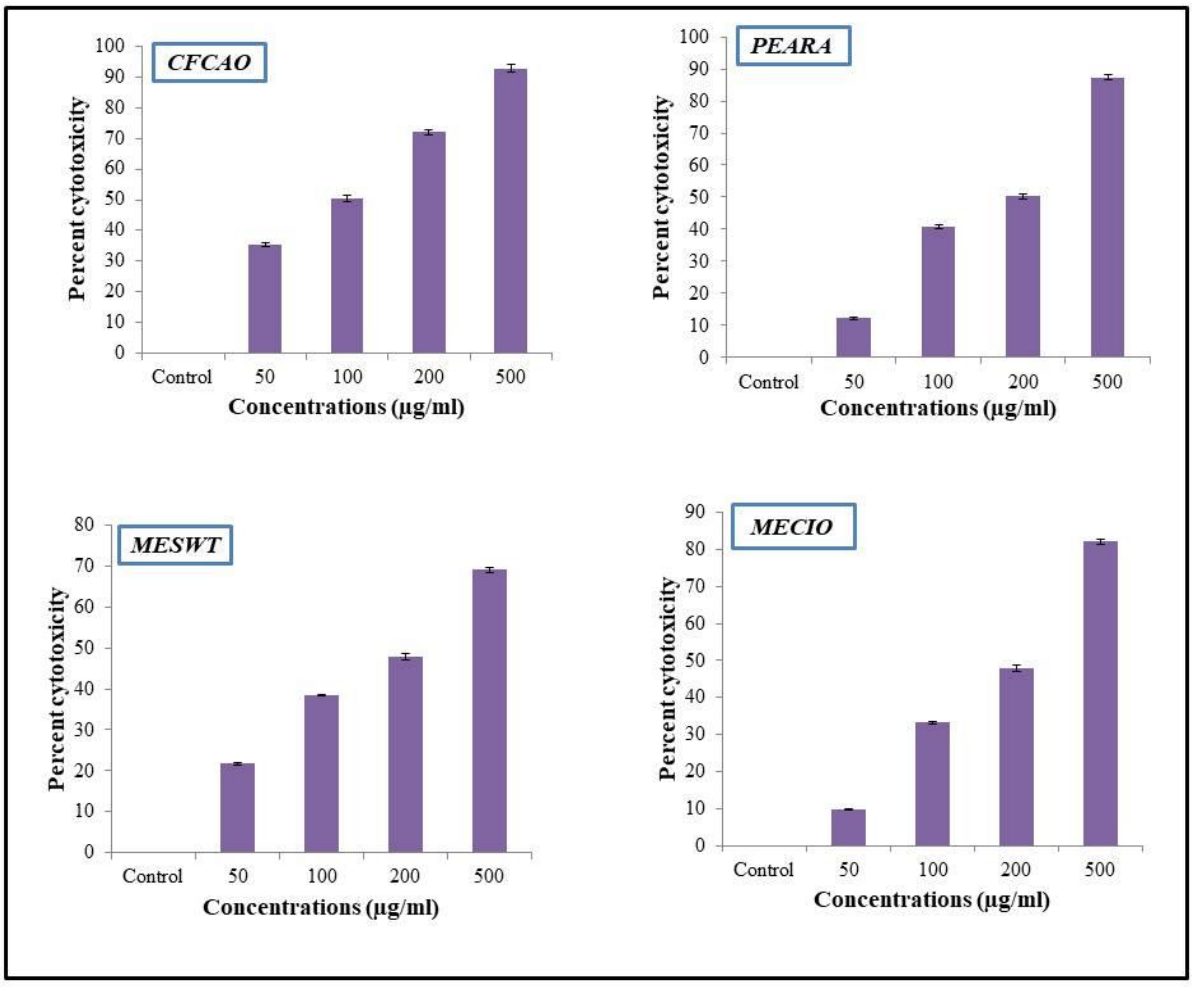


Figure 4.51: Cytotoxicity study of selected plants' extracts on NMDF cell lines

KEY: ***CFCAO***= Chloroform extract of *C. occidentalis* ; ***PEARA*** = Petroleum ether extract of *A. annua*; ***MESWT*** = Methanol extract of *Swertia thomsonii.* ; ***MECIO*** = Methanol extract of *C. officinalis*; Error bars = SD (n=3)

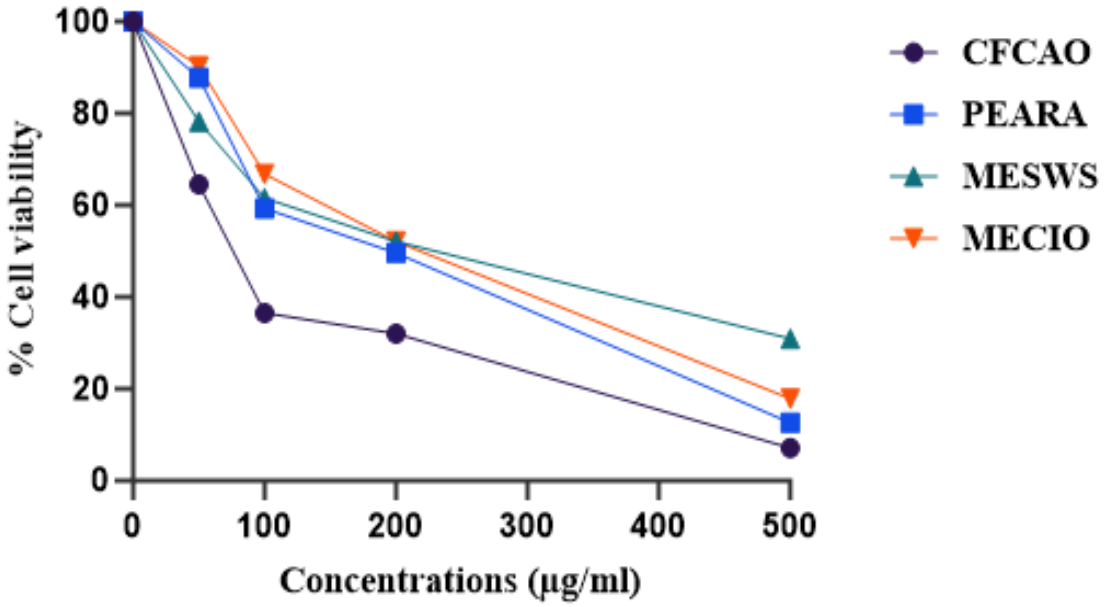


Figure 4.52: Percent cell viability on treatment with different concentrations (50–500µg/mL) of the selected extracts.

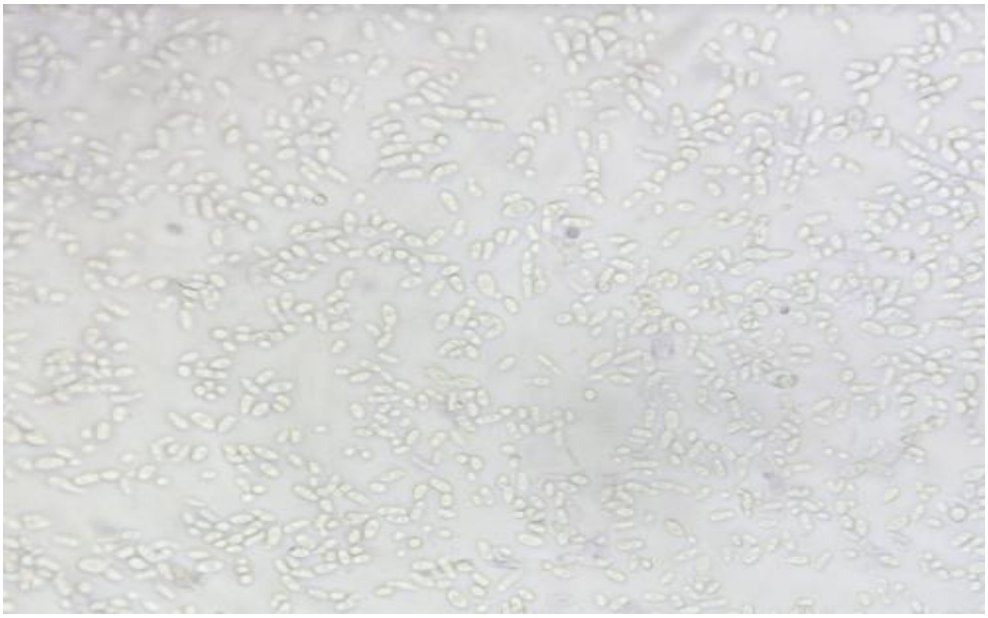


Figure 4.53: Normal Neonatal mouse dermal fibroblast (NMDF)

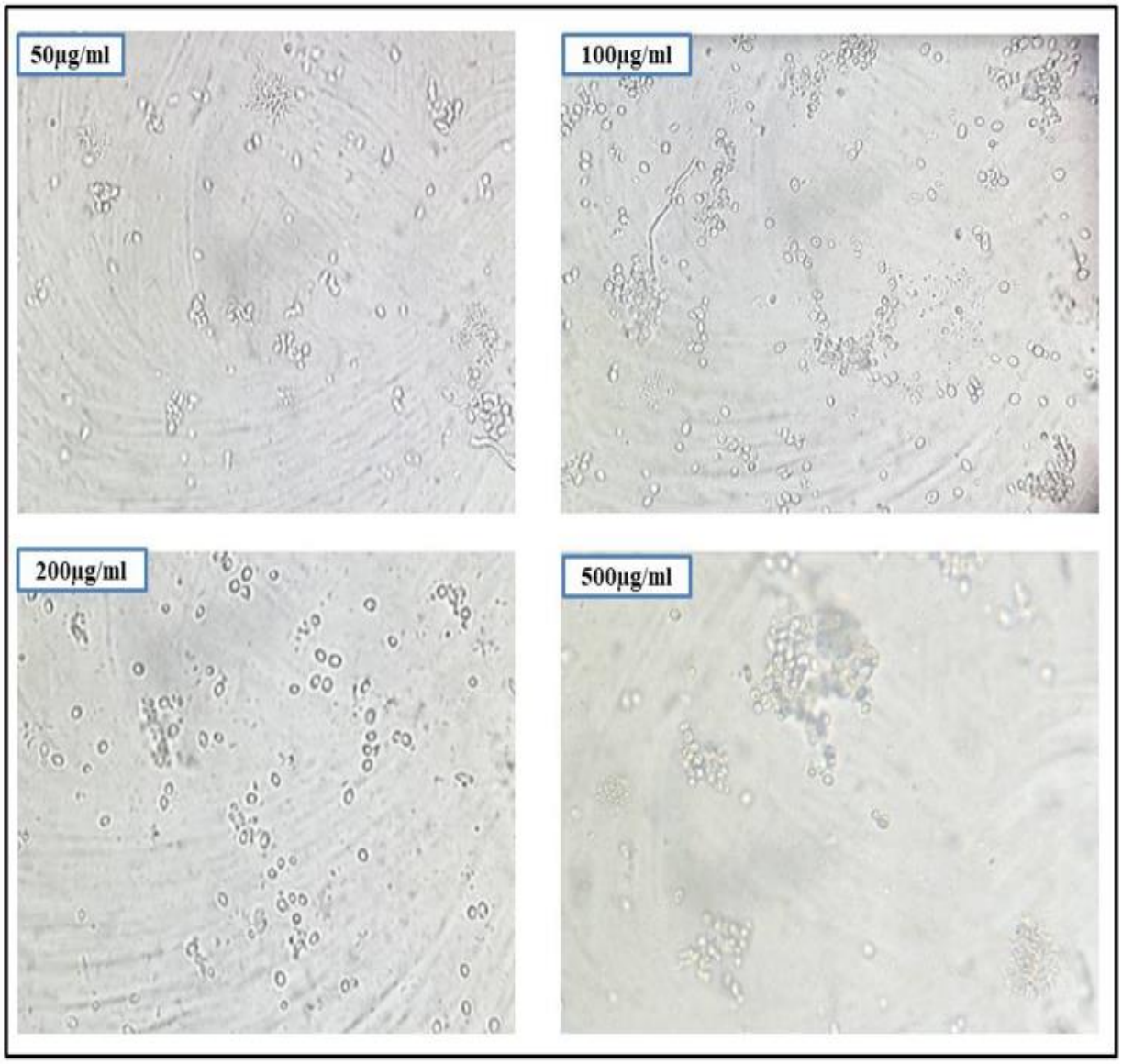


Figure 4.54: NMDF cell lines treated with different concentrations of ***PEARA***

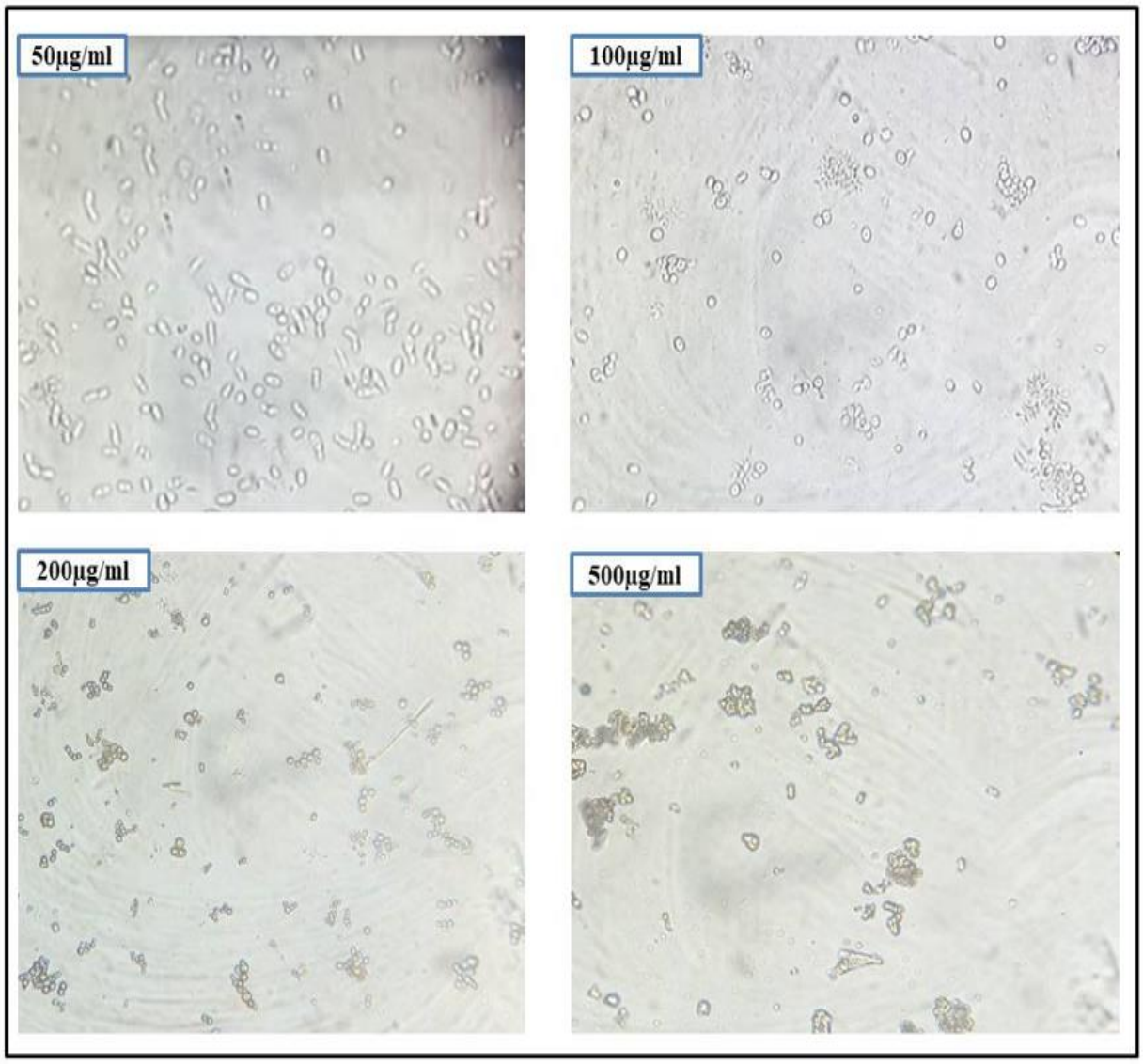


Figure 4.55: NMDF cell lines treated with different concentrations of ***MESWT.***

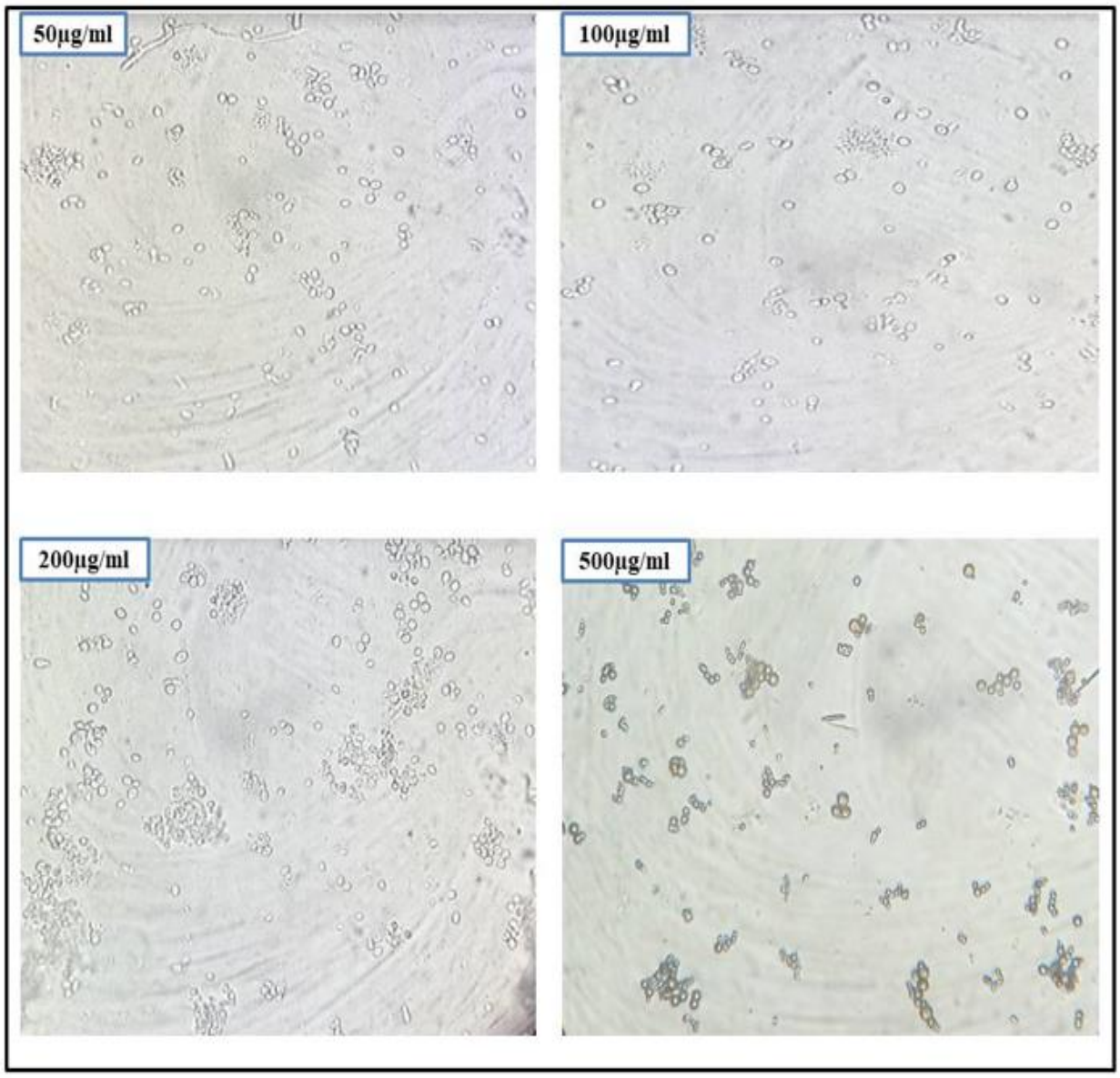


Figure 4.56: NMDF cell lines treated with different concentrations of ***CFCAO.***

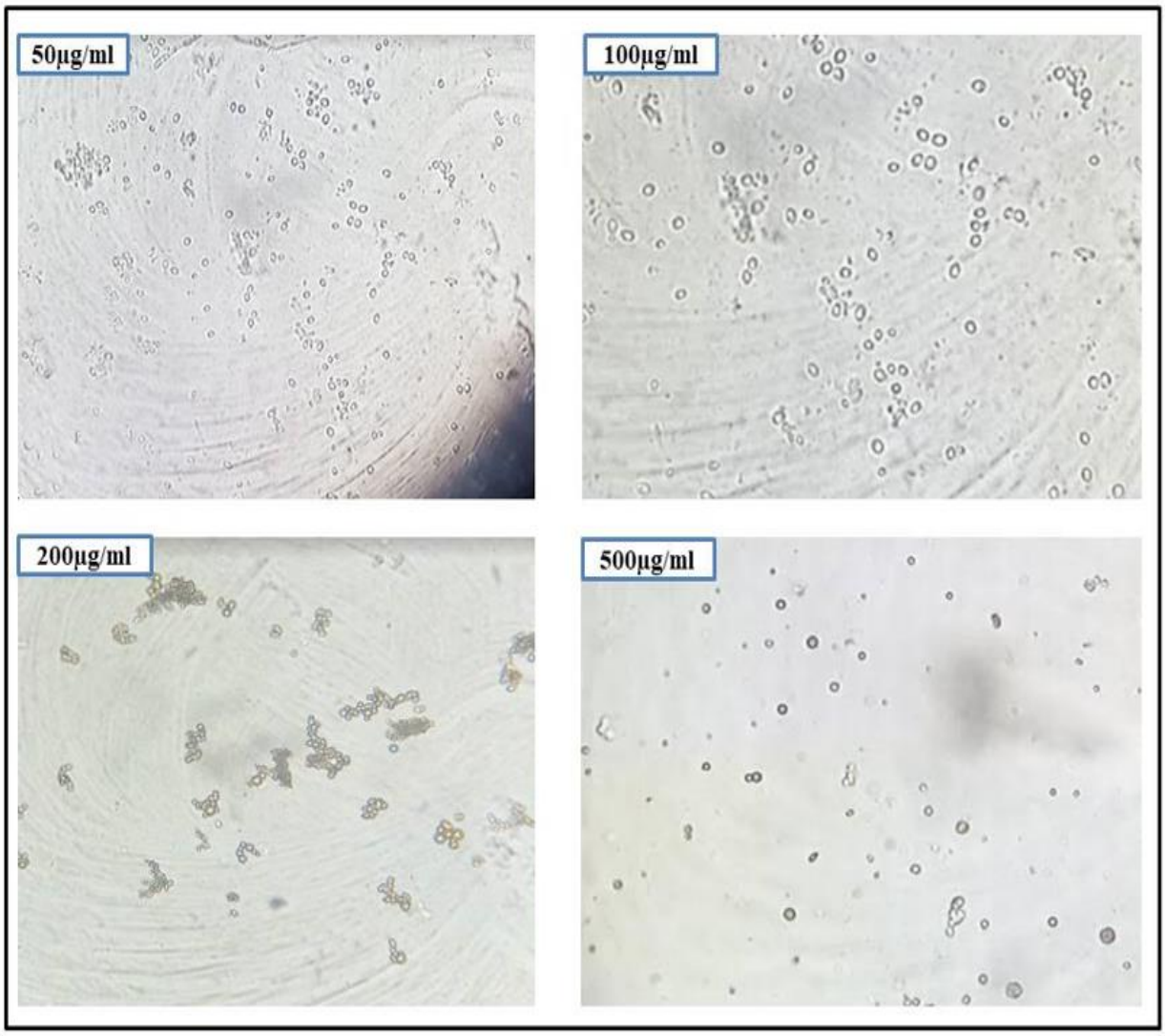


Figure 4.57: NMDF cell lines treated with different concentrations of ***MECIO***

4.10 Development of antimalarial herbal formulation

Table 4.18: Herbal composition, % of antimalarial agents and safety levels

Sno.	Extracts	Identified antimalarial agents	% of antimalarial agents	Safety levels
1	***PEARA***	Artemisinin	0.71	Highly safe
2	***CFCAO***	Quercetin	4.10	Moderately safe
3	***MECIO***	Quinine	2.60	Highly safe
4	***MESWT***	Flavone, 5-hydroxy-7,8-dimethoxy-	5.27	Highly safe

Figure 4.58: Antimalarial herbal formulation

Herbal medicines have been explored as potential alternatives for malaria treatment**,** especially in regions where conventional antimalarial drugs face challenges due to resistance. Malaria which is caused by *Plasmodium* parasites (particularly *Plasmodium falciparum)* continues to be a major public health concern worldwide. The advent of resistance to traditional antimalarial medications has spurred experts to look into herbal therapies as viable alternatives. Several herbal antimalarial medicines show synergistic advantages when combined with conventional antimalarial drugs. Numerous studies discovered antagonistic effects of combined treatment of herbal antimalarial and CAMDs, despite the fact that most associations were synergistic. Herbal remedies showing synergistic effects with CAMDs hold promise for developing standardized antimalarial-herbal combination therapies. Such approaches could help combat malaria resistance to conventional antimalarial treatments (Erhirhie *et al.,* 2021). However**,** It's important to note that while herbal medicines are commonly used in communities as alternatives to modern antimalarial agents, their efficacy and safety need further investigation (Ocan *et al.,* 2023). Therefore, ongoing research explores the potential of herbal drugs to enhance malaria treatment and address resistance issues. In this study, after evaluation of the antimalarial efficacy of the individual extracts of the selected medicinal plants, the herbal formulation was developed from the effective doses of all the four selected active extracts; ***PEARA*** (with 0.71% artemisinin) **,** ***CFCAO*** (with 4.1% quercetin)**,** ***MESWT*** (with 5.27% Flavone, 5-hydroxy-7,8-dimethoxy-) and ***MECIO*** (with 2.6% quinine) as shown in **Table 4.17**. The herbal remedy derived from these extracts may therefore serve as one of the excellent alternatives to the present resistance problem in *Plasmodium falciparum.*

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

The escalating challenge of antimalarial drug resistance poses a significant threat not only to African nations but also to several Asian countries. As per the World Health Organization (WHO) report, this phenomenon has contributed to a rise in global mortality, reaching 627,000 deaths as of 2021. *Plasmodium falciparum* has exhibited resistance to several existing antimalarial medications. This necessitates the rapid development of novel medications with distinct mechanisms of action. Moreover, most synthetically drugs were shown to contain more carcinogens than the herbal remedies. Therefore, herbal remedy could serves as alternative treatments for not only malaria but other ailments with less cancer risk. The current study uses high performance thin layer chromatography (HPTLC) and gas chromatography mass spectrophotometry (GC-MS) in developing antimalarial herbal formulation from the four chosen medicinal plants. Phytochemical screening revealed various secondary metabolites in the extracts with vast biological activity including antimalarial activity. *In silico* study identified bioactive compounds with promising activity on *P. falciparum* protease enzymes. *In vitro* evaluations of antimalarial activity of individual extracts demonstrated significant antimalarial activity on both tested strains of *P. falciparum*. The developed herbal remedy exhibited notable efficacy and safety profiles, suggesting its potential as an alternative to combat malaria resistance.

5.2. Conclusion

The escalation of antimalarial drug resistance by *P. falciparum* to numerous antimalarial medications has had a significant impact on people worldwide for several decades, leading to increased mortality from malaria infections caused by resistant strains of *P. falciparum*. The current study was conducted to identify alternate methods of tackling the issue of antimalarial drug resistance demonstrated by *P. falciparum* by creating the herbal cure as an alternative malaria treatment. *In silico* study of bioactive compounds from the four selected medicinal plants were carried out against the three protease enzymes of *P. falciparum.* The derivatives of quercetin, artemisinin, quinine and Flavone, 5-hydroxy-7,8-dimethoxy- from the selected medicinal plants revealed the excellent binding affinity, convenient molecular interactions and ADMET properties. The lead compounds were characterized using HPTLC and GC-MS in developing antimalarial herbal remedy from the four selected medicinal plants namely; *Artemesia annua, Cinchona officinalis, Cassia occidentalis* and *Swertia thomsonii.*

The *in vitro* antimalarial activity test carried out with the selected active extracts namely petroleum ether of *Artemesia annua*. Chloroform extracts of *Cassia occidentalis,* methanol extracts of *Cinchona officinalis,* and *Swertia thomsonii* were tested against 3D7 and Dd2 strains of *P. falciparum* and found to be significantly very effective against both the drug sensitive and resistant strain of *P. falciparum*. Both extracts except ***MESWT*** had lower IC50 values, which justifies their long-term usage as herbal cure for malaria sickness. The results indicated that the formulation developed from these extracts might be used as an alternative treatment to battle malaria drug resistance. The toxicity studies performed on the selected extracts of *Artemesia annua, Cinchona officinalis, Cassia occidentalis,* and *Swertia thomsonii* using MTT assay revealed a favorable safety profile for use.

From the result obtained, it can be concluded that, the herbal remedy developed from these medicinal plants can served as the excellent malaria treatment with zero resistant against the *P. falciparum* strains.

5.3. Recommendations

Based on the findings presented in this study, the following recommendations were raised:

1. There is a need for continued research and development of novel antimalarial medications, particularly herbal formulations, to combat the growing threat of drug resistance. This includes exploring the efficacy of different plant extracts and their combinations to identify optimal treatments.

2. The developed herbal formulation should further be evaluated for antimalarial activity using *in vivo* approach to validate the present findings

3. Synergistic effects and enhanced antimalarial activity of the developed formulation should further be evaluated using in vivo approach

4. Education and awareness programs should be implemented to inform communities about the availability and benefits of herbal antimalarial treatments. This can help promote acceptance and utilization of herbal medicines as viable alternatives to conventional drugs.

5. Cytotoxicity study of the developed formulation should be carried out to evaluate its safety profile

By following these recommendations, researchers and healthcare practitioners can contribute to the development and implementation of effective herbal antimalarial treatments, addressing the urgent need to combat drug resistance in malaria-endemic regions.

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Appendices

List of publications

1. **Ibrahim Isyaku Muhammad,** Devendra Kumar Pandey (2024). A validated HPTLC quantification of artemisinin from different extracts ofArtemisia annua L. and its inhibitory activity against serine hydroxyl methyltransferase (SHMT). *Journal of applied biology and biotechnology*. 12(6), 156-165. DOI: 10.7324/JABB.2024.178566

2. **Ibrahim Isyaku Muhammad,** Devendra Kumar Pandey (2024). HPTLC quantification of quercetin from leaf extracts of C. occidentalis L. and its inhibitory activity against protease enzyme of P. falciparum. *Vegetos.* 37, 1804-1816. *https://doi.org/10.1007/s42535-024-00934-z*

Vegetos
https://doi.org/10.1007/s42535-024-00934-z

RESEARCH ARTICLES

HPTLC quantification of quercetin from leaf extracts of *C. occidentalis* L. and its inhibitory activity against protease enzyme of *P. falciparum*

Ibrahim Isyaku Muhammad[1] · **Devendra Kumar Pandey**[1]

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Abstract

Most of the therapeutically relevant antimalarial medications target the parasite during the blood stage of its life cycle. As a result, new antimalarial medicines have focused on inhibiting the breakdown of host-cell hemoglobin. *C. occidentalis* was reported to have antimalarial activity and proved very effective in the conventional Indian medical system. This study aimed to analyze the quercetin content of four different leaf extracts of *C. occidentalis* using a validated HPTLC method and to evaluate the inhibitory activity of quercetin against Plasmepsin-II of *P. falciparum* using in silico approach. HPTLC results revealed that the CF extract of *C. occidentalis* had the highest quercetin content with 4.10% (w/w), followed by the ME extract with 2.37% (w/w), PE extract with 2.09% (w/w), and WT extract with 1.79% (w/w). In silico investigations of quercetin with the Plasmepsin-II enzyme of *P. falciparum* portrayed excellent binding affinities of (− 7.5 kcal/mol) and convenient molecular interactions with important amino acid residues of the target receptor as compared with control. Furthermore, MD simulation study confirmed the finding of molecular docking by depicting the stability of the simulated system both in water and in vacuum. Finally, the study suggested that the chloroform extract of *C. occidentalis* is the most suitable and efficient solvent for extracting high-quantity and quality quercetin. The findings of the molecular docking and MD simulation analysis further supported the potential inhibitory activity of quercetin against Plasmepsin-II. This research contributes to the understanding of quercetin and its potential applications in combating antimalarial drug resistance.

Keywords HPTLC · Quercetin · Inhibitory activity · Plasmepsin-II · Molecular docking · MD simulation

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A validated HPTLC quantification of artemisinin from different extracts of *Artemisia annua* L. and its inhibitory activity against serine hydroxyl methyltransferase (SHMT)

Ibrahim Isyaku Muhammad, Devendra Kumar Pandey*

Lovely Professional University, Phagwara, Punjab, India.

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Molecular docking,
ADMET analysis.

ABSTRACT

The present study aimed to analyze the artemisinin content in various extracts of *Artemisia annua* L. using a validated HPTLC densitometry method and evaluate the inhibitory activity of artemisinin against serine hydroxyl methyltransferase (SHMT) of *Plasmodium falciparum* using *in silico* approach. High performance thin layer chromatography revealed that the petroleum ether extract of A. annua had the highest artemisinin content (0.71% ± 0.02 w/w), methanol extract (0.25% ± 0.02 w/w), chloroform extract (0.14% ± 0.01 w/w), and water extract (0.06% ± 0.01 w/w). Densitometry result indicated a well-defined and symmetrical peak in the petroleum ether extract, indicating excellent separation of high-purity artemisinin. *In silico* study of artemisinin with SHMT enzyme portrayed an excellent binding affinity (–9.1 kcal/mol) and convenient interactions compared with control. Moreover, both artemisinin and control compound showed good absorption, distribution, metabolism, excretion and toxicity properties. In conclusion, the study suggested that the petroleum ether extract of *A. annua* is the most suitable and efficient solvent for extracting high-quantity artemisinin. The findings of the molecular docking analysis further supported the potential inhibitory activity of artemisinin against the SHTM of *P. falciparum*. This research contributes to the understanding of artemisinin's antimalarial properties and optimized method of its production efficiently.

List of conferences attended

1. **Isyaku Ibrahim Muhammad**, Devendra Kumar Pandey (2023). A research paper entitle "***Virtual screening, in silico prediction of inhibitory activity and pharmacokinetic properties of herbal derivatives of Artemesia annua L. as potential inhibitors against P. falciparum's hemoglobinases"*** presented at 2nd international conference on plant physiology and biotechnology (ICPPB) held from 20-21 April, 2023. Organized by school of bioengineering and biosciences, under the aegis of lovely professional university, Punjab.

2. **Isyaku Ibrahim Muhammad**, Devendra Kumar Pandey (2023). A research paper entitle "***Qualitative and quantitative screening of Artemisinin from different extracts of Artemisia annua L. (whole plant) using HPTLC and densitometry analysis"*** presented at 6th international conference on advances in agriculture technology and allied sciences (ICAATAS 2023) organized by society of agriculture research and social development (New delhi) and (Loyola academy), Secundrabad, Telangana, 2023


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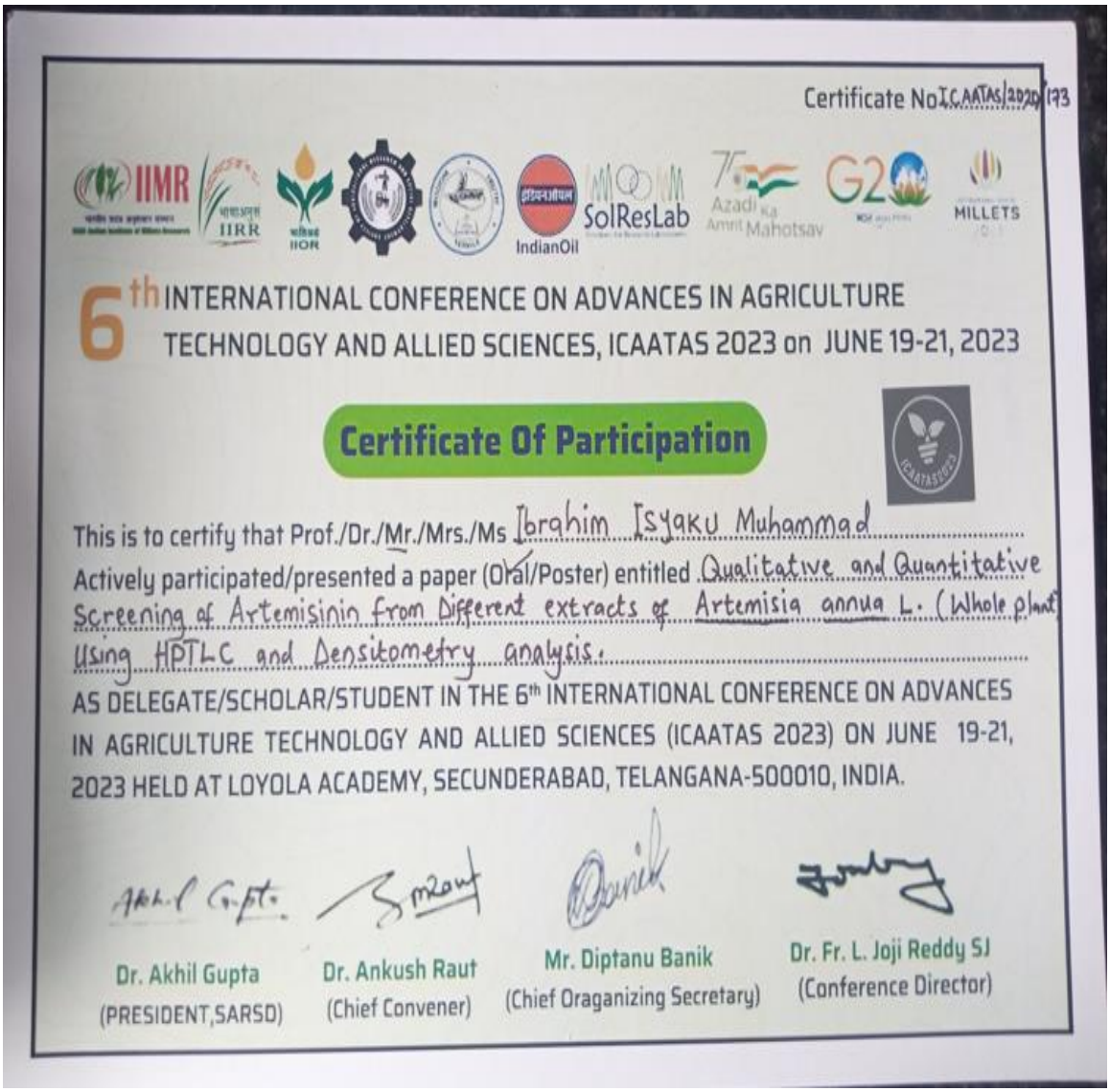
CERTIFICATE

This is to certify that Prof./Dr./Mr./Ms. Isyaku Ibrahim Muhammad
of Lovely Professional University has participated in Poster Presentation/Oral Presentation on the topic entitled... Virtual Screening, in silico prediction of inhibitory activity and pharmacokinetic properties of herbal derivatives Artemesia annua L. as potential inhibitors against P. falciparum's hemoglobinases
in 2nd International Conference on Plant Physiology and Biotechnology (ICPPB) held from 20-21 April 2023 organized by School of Bio-engineering and Biosciences, under the aegis of Lovely Professional University, Punjab.

Dr. Vijay Kumar
Organizing Secretary

Dr. Umesh Goutam
Co-Convener

Dr. Neeta Raj Sharma
Convener



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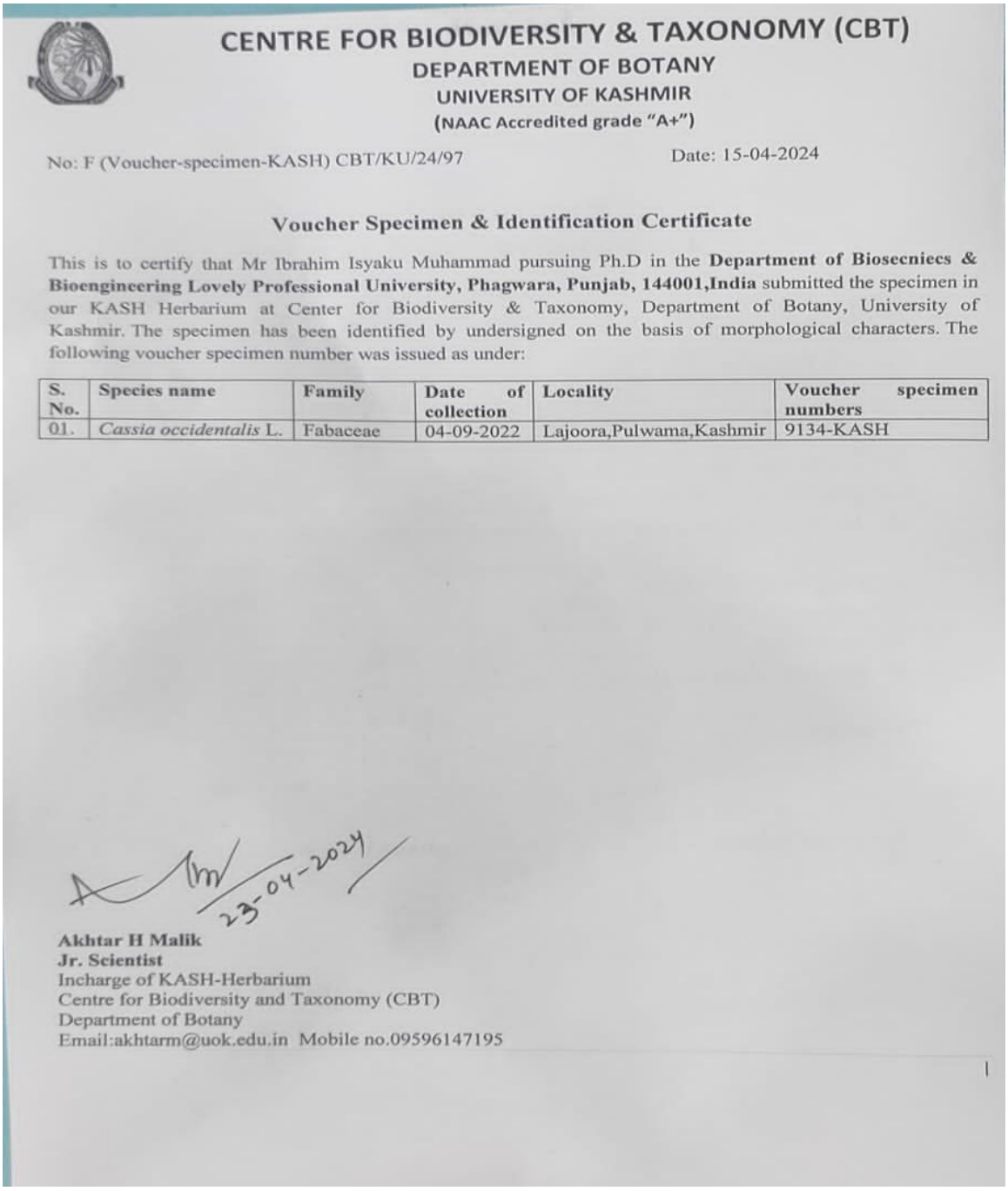
6th INTERNATIONAL CONFERENCE ON ADVANCES IN AGRICULTURE TECHNOLOGY AND ALLIED SCIENCES, ICAATAS 2023 on JUNE 19-21, 2023

Certificate Of Participation

This is to certify that Prof./Dr./Mr./Mrs./Ms Ibrahim Isyaku Muhammad
Actively participated/presented a paper (Oral/Poster) entitled Qualitative and Quantitative Screening of Artemisinin from Different extracts of Artemisia annua L. (Whole plant) Using HPTLC and Densitometry analysis.
AS DELEGATE/SCHOLAR/STUDENT IN THE 6th INTERNATIONAL CONFERENCE ON ADVANCES IN AGRICULTURE TECHNOLOGY AND ALLIED SCIENCES (ICAATAS 2023) ON JUNE 19-21, 2023 HELD AT LOYOLA ACADEMY, SECUNDERABAD, TELANGANA-500010, INDIA.

Dr. Akhil Gupta	Dr. Ankush Raut	Mr. Diptanu Banik	Dr. Fr. L. Joji Reddy SJ
(PRESIDENT,SARSD)	(Chief Convener)	(Chief Oraganizing Secretary)	(Conference Director)

Certificate of authentication of collected plant



CENTRE FOR BIODIVERSITY & TAXONOMY (CBT)
DEPARTMENT OF BOTANY
UNIVERSITY OF KASHMIR
(NAAC Accredited grade "A+")

No: F (Voucher-specimen-KASH) CBT/KU/24/97 Date: 15-04-2024

Voucher Specimen & Identification Certificate

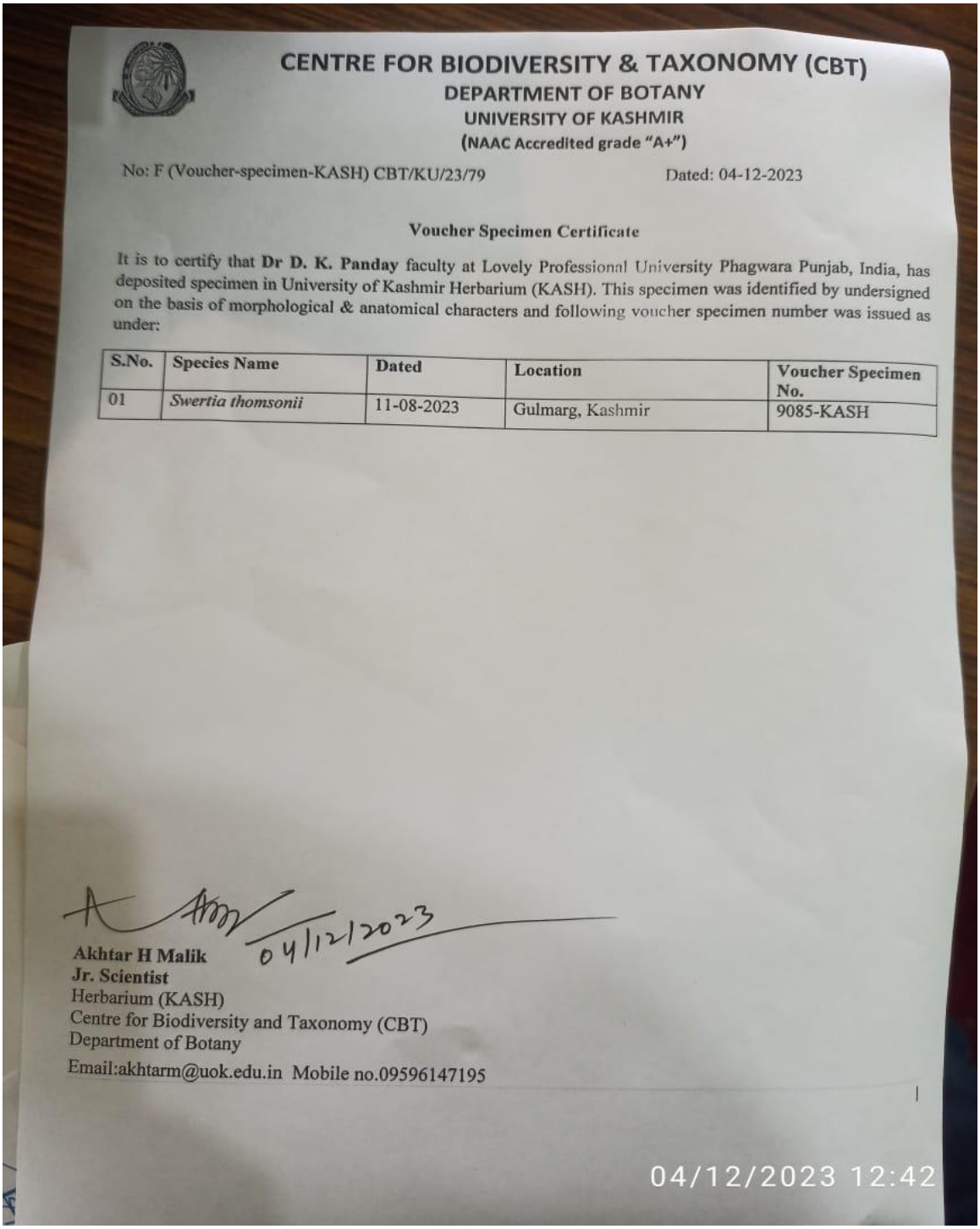
This is to certify that Mr Ibrahim Isyaku Muhammad pursuing Ph.D in the **Department of Biosecniecs & Bioengineering Lovely Professional University, Phagwara, Punjab, 144001,India** submitted the specimen in our KASH Herbarium at Center for Biodiversity & Taxonomy, Department of Botany, University of Kashmir. The specimen has been identified by undersigned on the basis of morphological characters. The following voucher specimen number was issued as under:

S. No.	Species name	Family	Date of collection	Locality	Voucher specimen numbers
01.	*Cassia occidentalis* L.	Fabaceae	04-09-2022	Lajoora,Pulwama,Kashmir	9134-KASH

23-04-2024

Akhtar H Malik
Jr. Scientist
Incharge of KASH-Herbarium
Centre for Biodiversity and Taxonomy (CBT)
Department of Botany
Email:akhtarm@uok.edu.in Mobile no.09596147195

Certificate of authentication of *C. occidentalis L.*



CENTRE FOR BIODIVERSITY & TAXONOMY (CBT)
DEPARTMENT OF BOTANY
UNIVERSITY OF KASHMIR
(NAAC Accredited grade "A+")

No: F (Voucher-specimen-KASH) CBT/KU/23/79 Dated: 04-12-2023

Voucher Specimen Certificate

It is to certify that **Dr D. K. Panday** faculty at Lovely Professional University Phagwara Punjab, India, has deposited specimen in University of Kashmir Herbarium (KASH). This specimen was identified by undersigned on the basis of morphological & anatomical characters and following voucher specimen number was issued as under:

S.No.	Species Name	Dated	Location	Voucher Specimen No.
01	*Swertia thomsonii*	11-08-2023	Gulmarg, Kashmir	9085-KASH

04/12/2023

Akhtar H Malik
Jr. Scientist
Herbarium (KASH)
Centre for Biodiversity and Taxonomy (CBT)
Department of Botany
Email:akhtarm@uok.edu.in Mobile no.09596147195

04/12/2023 12:42

Certificate of authentication of *S. thomsonii*