
“Screening and Development of Antimalarial and Antifilarial Drugs of Herbal Origin through Target-Based Drug Development Approach”

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For the award of the degree of
Doctor of Philosophy (Ph. D)
In the Faculty of Science

By

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(Registration No: KAHER/Ph.D./2014-2015/DO1214023)

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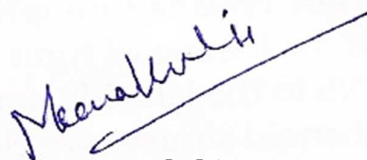
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*I hereby declare that the thesis entitled “**Screening and Development of Antimalarial and Antifilarial Drugs of Herbal Origin through Target-Based Drug Development Approach**” is a bonafide and original research carried out by me under the guidance of **Dr. S L Hoti, ICMR-Emeritus Scientist, Former Director-in-Charge, Scientist-G, Indian Council for Medical Research, National Institute of Traditional Medicine (ICMR-NITM), Belagavi, Karnataka, India.** The thesis or any part thereof has not formed the basis for the award of any degree/fellowship or similar title to any candidate of any University.*

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Thank you

Mrs. Meenakshi

LIST OF ABBREVIATIONS

ADP	:	Adenine diphosphate;
ANOVA	:	Analysis of variance;
AO	:	Anacardium Occidentale
BE	:	Binding energy;
BmHK	:	Brugia malayi hexokinase
BI%	:	Binding inhibition percentage;
CID	:	Compound identification number;
CFCF	:	Cell culture free filtrate;
CPCSEA	:	Committee for the purpose of control and supervision of experiments on animals;
cAMP	:	Cyclic adenylyate monophosphate;
DMSO	:	Dimethyl sulfoxide;
DNA	:	Deoxyribonucleic acid;
ICMR-NITM	:	Indian Council of Medical Research-National Institute of Traditional Medicine;
IC₅₀	:	The concentration of drug required for 50% inhibition;
MF	:	Molecular formula;
Mg	:	Milligram;
mL	:	Milliliter;
MDR	:	Multidrug resistance;
MD	:	Molecular dynamics simulation;
Na₂CO₃	:	Sodium carbonate;
NaOH	:	Sodium hydroxide;

EDTA	:	Ethylenediaminetetraacetic acid;
EA	:	Ellagic acid;
FBS	:	Fetal Bovine Serum;
GA	:	Gallic acid;
HPLC	:	High Performance Liquid Chromatography;
H₂SO₄	:	Sulphuric acid;
NaNO₂	:	Sodium nitrite;
NAD	:	Nicotinamide adenine dinucleotide;
PDB	:	Protein data bank;
PfTK	:	Plasmodium falciparum transketolase
pH	:	Potential of hydrogen;
PBS	:	Phosphate buffered saline;
SD	:	Standard deviation;
TPC	:	Total phenolic content;
TFC	:	Total flavonoid content;
%	:	Percentage
➤	:	greater than
~	:	approximate
°C	:	Degree Celsius
μF	:	microfarad
μl	:	microliter
μm	:	Micrometre
ATP	:	Adenosine triphosphate
BLAST	:	Basic Local Alignment Search Tool
Cm	:	Centimeter
cMCM	:	complete malaria culture medium
CO₂	:	Carbon dioxide
DHFR	:	Dihydrofolate reductase

DMSO	:	Dimethyl sulfoxide
EDTA	:	Ethylene Diamine Tetra Acetic Acid
Fig	:	Figure
H₂O₂	:	Hydrogen peroxide
HEPES	:	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
KDa	:	kilodaltons
kV	:	kilovolt
L	:	Litre
LB medium	:	Luria broth medium
ml	:	Milliliter
mM	:	Millimolar
N₂	:	Nitrogen
NCBI	:	National Center for Biotechnology Information
nm	:	nanometre
nt	:	nucleotides
P. ROS	:	Reactive Oxygen Species
rpm	:	Revolutions per minute
RPMI 1640	:	Roswell Park Memorial Institute medium 1640

ABSTRACT

Background: Among vector-borne diseases malaria affects nearly half of the world's population, as estimated by the WHO in 2022, and the disease was responsible for over 249 million people in 85 countries. Another global vector-borne threat is lymphatic filariasis that affects 859 million people in 50 countries, causing long lasting and severe morbidity. However, currently, while antimalarial campaigns are facing the problem of drug resistance, antifilarial programmes are facing the problem of less effective macrofilaricidal activity of available drugs. This means that they do not completely eradicate the adult parasites, allowing them to continue to produce microfilariae. In view of these facts, the goal of the current research is to develop anti-malarial and macrofilaricidal drugs of plant origin.

Aim and objectives: The Aim of the current study was to identify and investigate the antimalarial and macrofilaricidal activity of extracts of leaves of the plant *Anacardium occidentale* L., which has long been reported to have many biological activities.

Methodology: Six extracts *A. occidentale* leaves were prepared, and their ability to inhibit the recombinant *P. falciparum* transketolase (rPfTK) enzyme *in-vitro* was evaluated. Additionally, employing SYBR Green fluorescence-based *in-vitro* assays, growth inhibitory efficacy against grown blood stage *P. falciparum* parasites (3D7 strain) was investigated. Further, *A. occidentale* L. (Anacardiaceae) leaf extracts were investigated for their inhibitory efficacy against recombinant hexokinase (rBmHk) of *B. malayi*. This glycolytic pathway enzyme BmHK was selected as a target because it is essential for the survival and replication of filarial parasites. Acute toxicity of various doses of *A. occidentale* leaf hydroalcoholic extracts (HELA) was assessed in

mice and zebrafish embryos. The extract was subjected to GC-FID analysis and a preliminary phytochemical test.

Results: Among six extracts of *A. occidentale* leaves tested, hydroalcoholic extract of leaves of *A. occidentale* (HELA)), demonstrated $75.45 \pm 0.35\%$ inhibitory action against the rPfTk at the dose of 50 $\mu\text{g/ml}$ and $99.31 \pm 0.08\%$ growth inhibition of intra-erythrocytic stages of *P. falciparum* *in vitro*, with an IC_{50} of $4.17 \pm 0.22 \mu\text{g/ml}$. The HELA exhibited the highest level of inhibition of rBmHk activity ($65.80 \pm 1.60\%$), when compared to other extracts that showed less than 50% inhibition. The toxicity studies demonstrated that 0.1 to 10 $\mu\text{g/ml}$ of the HELA had no effect on the heartbeat, somite formation, tail detachment, or hatching of Zebra fish embryos. However, the fish embryos showed no heartbeat after 48 hours (1000 $\mu\text{g/ml}$) and 96 hours at the dose of 100 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ of HELA, respectively. Acute oral toxicity tests in mice showed that 2000 mg/kg body weight of HELA was safe.

Conclusion: Strong antimalarial activity was demonstrated by the hydro-alcoholic extract of *A. occidentale* L. leaves (HELA) against blood stage *P. falciparum* and recombinant transketolase of *P. falciparum* (PfTK), as also the against recombinant hexokinase of *B. malayi* (rBmHk) *in vitro*. It is necessary to investigate the promising antimalarial activity of purified chemicals derived from *A. occidentale* leaves in order to develop novel antimalarial and anti-filarial therapies.

Key words: Antimalarial activity, *Anacardium occidentale* L, Plasmodium falciparum, Transketolase, Malaria, Brugia malayi hexokinase, GC-FID, filariasis, Anti-filarial activity.

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1. INTRODUCTION

1. Malaria

Malaria, caused by protozoan parasite *Plasmodium* spp., is a serious public health concern, globally. It spreads through infected female *Anopheles* mosquito's bite.

Severe human infections are primarily caused by *Plasmodium falciparum* and *Plasmodium vivax*. Almost half of the world's population is at risk from malaria, and the disease continues to claim the lives of more than 0.6 million people worldwide, annually [1]. Although most nations outside of Sub-Saharan Africa have *P. vivax* as the primary malaria parasite, accounting for less than 2% of cases worldwide, the malarial parasite *P. falciparum* is the most common on the African continent [2].

Over the past 50 years, *P. falciparum* malaria has been more common in India. In 2022, an estimated 249 million individuals were reported to have malaria in 85 countries [1].

There are numerous antimalarial medications available for clinical use, such as Quinine, atovaquone-proguanil combination (Malarone), artemisinin, chloroquine, sulfadoxine and pyrimethamine combination, and primaquine. However, parasites have evolved resistance to these medications, rendering them ineffective against malaria [3]. There are currently no alternative options available as potent anti-malarial drugs, except from artemisinin and its derivatives [4].

However, South-East Asian countries have reported treatment failures with artemisinin combination treatments (ACTs) in recent years, and it has now been determined that there is a genetic foundation for artemisinin resistance [5]. There is

also a possibility that artemisinin resistance exists in South America, though more investigation is required to confirm this [1]. This scenario requires the development of innovative drugs against malarial parasites to support efforts towards control and elimination of malaria.

Adopting a targeted approach may help expedite the drug development process, particularly with the emergence of molecular and genomic techniques to examine proteins encoded by parasites, which has proven to be a fruitful approach [6]. Drug development efforts for malaria have identified transketolase as one of the parasite enzymes as a target [7]. The transketolase enzyme of *P. falciparum* catalyses the synthesis of pentose sugar in the non-oxidative section of the Pentose Phosphate Pathway (PPP), which is necessary for the synthesis of nucleic acids. *PfTK* is therefore a promising therapeutic target since it is crucial to the survival and reproduction of malaria parasites. It is also a selective target for drug research due to its minimal resemblance to human transketolase.

Herbal treatments have been utilised in traditional medicine to treat a wide range of diseases, including malaria. Traditional medicine uses a lot of herbal treatments made from *Anacardium occidentale* L., also called the "cashew," in Brazil, Colombia, Nigeria, Peru, and some tribal areas of India [8–9]. In traditional herbal therapy, cashew tree parts—particularly the leaves and stem bark—have been widely utilised [8]. As a result, the *A. occidentale* plant's medicinal qualities have been thoroughly researched during the past few decades [10].

The fruit of *A. occidentale* L., known as "cashew nuts," is a well-known source of alkylphenols. These substances exhibit a variety of biological properties, including molluscicidal, schistosomicidal, anti-cancer, and mosquito larvicidal effects

[11–15]. Additionally, it has been observed that these substances decrease the activity of several enzymes, including xanthine oxidase, glucosidase, aldose reductase, tyrosinase, acetylcholinesterase, invertase, and 15-lipoxygenase [16].

The antimalarial characteristics of *A. occidentale* leaves extracts were examined in this study for their ability to inhibit the transketolase enzyme and *P. falciparum* blood stage. Additionally, we evaluated the extract's acute toxicity using models of mice and zebrafish.

2. Filariasis

Lymphatic filariasis (LF) is a dangerous tropical disease which is neglected, and three worm parasites are responsible: *Brugia timori*, *Wuchereria bancrofti* and *Brugia malayi*. Around 90% cases are caused by *W. Bancrofti*, with the remaining 10% being caused by *B. malayi* and *B. timori*. Humans contract LF from infective mosquito bites, which results in progressive lymphatic dysfunction, irreversible swelling of the limbs, breasts, or genitalia, and other symptoms [17]. According to a 2022 World Health Organisation (WHO) report, 882 million people are at risk of LF in 44 countries. [18].

To halt the disease's spread, treatment and preventive treatments are needed. The Global Programme for Elimination of LF (GPELF) was launched in 2000 by the World Health Organisation (WHO) in consideration of the seriousness of the disease, the permanent damage it causes, control tools and vital diagnostic availability. The program's objective is to prevent the spread of LF by using a combination of albendazole and diethylcarbamazine, which is the Mass Drug Administration's (MDA) yearly plan [18]. However, Souza et al. [19] have reported that while adult worms can be killed by albendazole when administered for 21 days at a dose of 400

mg, which has side effects, DEC showed effect against the microfilaria stage but not against macrofilaria (adult worms) [20].

Therefore, these drugs are ineffective in reducing filarial transmission, which puts more pressure on the elimination program's efforts. Finding macrofilaricidal drug as a substitute is therefore felt to be necessary, particularly if they don't have any negative side effects. Natural compounds of plant origin are often used as treatments, because of their large safety margin.

Therefore, finding therapeutics of natural origin to treat neglected tropical diseases has recently attracted more attention. A few reports of previously discovered anti-helminthic plant-based medications exist. [21].

Also, there are reports of antifilarial activity in several herbal preparations derived from plants, including *Lantana camara*, *Andrographi spaniculata*, *Achyranthes aspera*, *Ocimum sanctum*, *Zingiber officinale*, *Solanum nigrum*, *Phyllanthus amarus*, *Azadirachta indica*, *Centella asiatica*, and *Aegle marmelos* and many more [22]. Find more anti-helminthic herbal leads as soon as possible to aid in the development of an anti-filarial medicine. The development of an anti-filarial medication may proceed more quickly if a target-based approach is used.

Hexokinase of *B. malayi* (*BmHk*) ATP is a major targeted substrate for the discovery of filarial treatment because it is an enzyme which is involved in the glycolytic pathway and is therefore necessary for the survival and reproduction of filarial parasites. Its low resemblance to human hexokinase further makes it a suitable target for anti-filarial medication research. Moreover, other hexokinases are either not affected by G6P nor inhibited; only *BmHk* directly activates G6P [23]. Therefore, investigating the ability of herbs to inhibit *B. malayi* hexokinase is worthwhile.

Known as the "cashew tree," *Anacardium occidentale* L. (Anacardiaceae) is grown as a large commercial plantation in various tropical nations, including Panama, Vietnam, Mozambique, India, Brazil, Nigeria, and Central America [24]. It is used to treat diabetes mellitus by traditional medicine practitioners in many tropical nations, including South Cameroon [25].

The phenolic contents of this plant are claimed to demonstrate a variety of pharmacological actions, including molluscicidal action, insecticidal action against *Aedes aegypti* and mushroom tyrosinase inhibition, antioxidant, anti-tumor, and anti-inflammatory properties [26; 27].

Given its widespread cultivation as a commercial crop in numerous nations and its purported bioactivity, this plant holds great promise as a source of antifilarial chemicals. Thus, the goal of the present was to investigate this plant for the creation of anti-filarial drugs.

2. AIM AND OBJECTIVES

Aim: To assess, the inhibitory effect of traditionally used and scientifically documented plants, as well as their bio-active fractions, against parasites that cause malaria and filariasis, using *in vitro*, *in vivo*, and *in silico* methods.

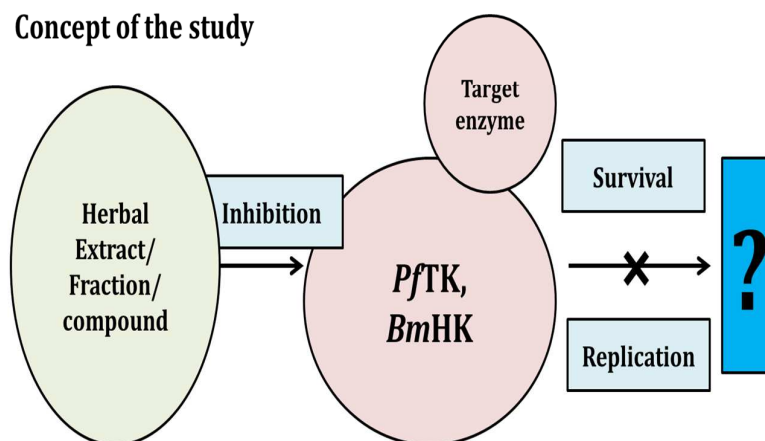
Objectives of the study

1. To explore the extracts of *Anacardium occidentale* L. (Anacardiaceae), active against malarial and filarial parasites.
2. To identify the *Anacardium occidentale* L. fractions active against malarial and filarial parasites.

Need for the study

- ✓ Available drugs – Partial effect, Resistance and ADR
- ✓ Herbal drugs against malaria and filariasis parasites have great promise
- ✓ Need to develop more anti-malarial and anti-filarial drugs.

Concept of the study



3. REVIEW OF LITERATURE

1. Background

1.1 Malaria

To be specific mosquito species can transmit the potentially deadly disease malaria to humans. Most of it is found in tropical nations. Both treatment and prevention are possible. The parasite that causes the illness is the only thing that can spread from person to person. There are symptoms that can be lethal or quite dangerous. Cough, fever, and chills are minor symptoms. Breathing problems, exhaustion, disorientation, and convulsions are examples of severe symptoms. Serious infections are more common in travellers, women carrying children under five, pregnant women, and those living with HIV/AIDS [28].

Medication and avoiding insect bites are two ways to prevent malaria. Mild cases can't go worse because of treatments. The primary method by which malaria is transmitted to humans is by female *Anopheles* mosquito bites. Malaria can also be spread by contaminated needles and blood transfusions. The initial signs and symptoms of malaria may be obscure at first, resembling many other feverish conditions. *P. falciparum* malaria can cause severe sickness and death in as little as 24 hours if left untreated [29].

Human malaria is caused by five different species of *Plasmodium* parasites, with *P. falciparum* and *P. vivax* presenting the highest dangers. The deadliest malaria parasite, *P. falciparum*, is also the most common in Africa. Outside of sub-Saharan Africa, *P. vivax* is the most prevalent malaria parasite. Other malaria species that can infect humans are *P. malariae*, *P. ovale*, and *P. knowlesi* [30].

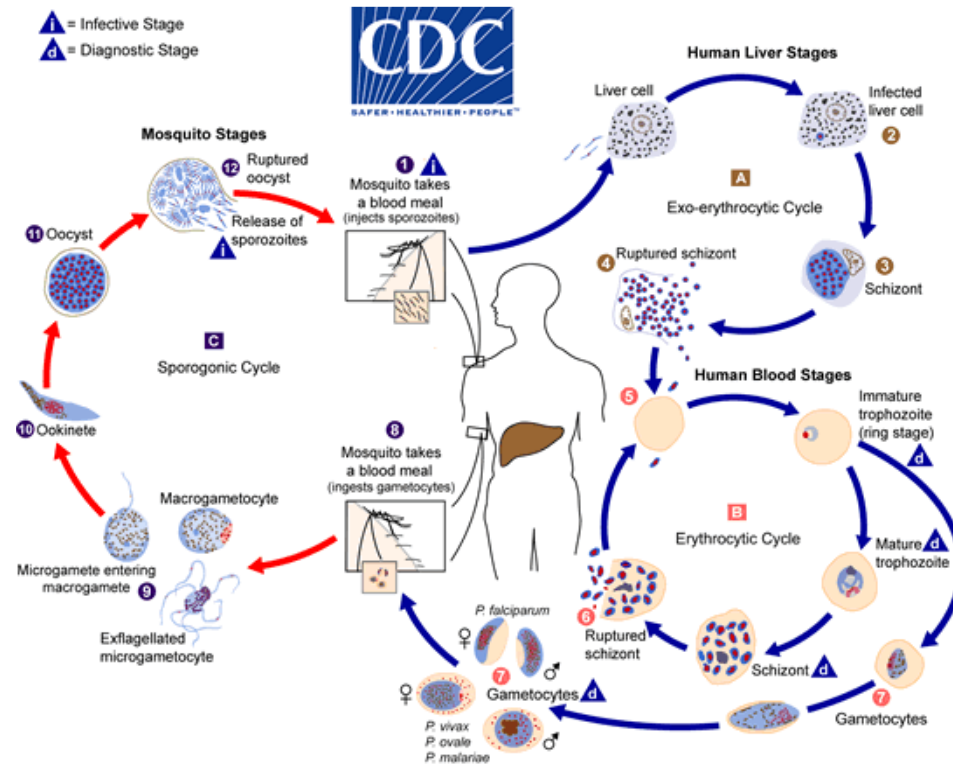


Fig 1 Lifecycle of Malaria [31]

The life cycle of malaria involves two hosts: humans (the primary host) and female *Anopheles* mosquitoes (the vector). The cycle begins when an infected mosquito bites human, injecting sporozoites into the bloodstream. These sporozoites travel to the liver, where they invade hepatocytes and undergo asexual replication (schizogony), forming schizonts that release thousands of merozoites. For certain species like *Plasmodium vivax* and *Plasmodium ovale*, some sporozoites become dormant hypnozoites, causing relapses later [32].

The released merozoites infect red blood cells (RBCs), initiating the erythrocytic cycle. Within RBCs, they develop from ring-stage trophozoites into mature schizonts, which multiply and cause the cells to burst, releasing more merozoites. Some

merozoites differentiate into sexual forms called gametocytes, which circulate in the bloodstream and are taken up by a mosquito during a blood meal.

In the mosquito's midgut, gametocytes mature into male and female gametes, which fuse to form a zygote. The zygote transforms into a motile ookinete, which penetrates the gut wall and develops into an oocyst. Inside the oocyst, the parasite undergoes sporogony, producing thousands of sporozoites. When the oocyst bursts, sporozoites migrate to the mosquito's salivary glands. During the mosquito's next bite, these sporozoites are injected into a new human host, completing the cycle.

The most common early symptoms of malaria include headache, chills, fever. Symptoms typically show up in 15 days following an infected mosquito bite. Some people, especially those who have already caught malaria, may only exhibit mild symptoms. Since many symptoms of malaria are vague, it is imperative to get tested as soon as possible [32].

- Some forms of malaria are exceedingly sickening or lethal. Those living with little children under five, pregnant women, HIV/AIDS, and travellers are among the most vulnerable groups.
- Extreme weariness and fatigue, irregular bleeding, diminished consciousness, recurrent convulsions, breathing difficulty, black or bloody urine, and jaundice (yellowing of the eyes and skin) are some of the severe symptoms.

Severe symptoms should be treated immediately with emergency care. If malaria is mild, therapy can prevent the infection from getting worse. A low birth weight or early delivery can also result from malaria infection during pregnancy [33].

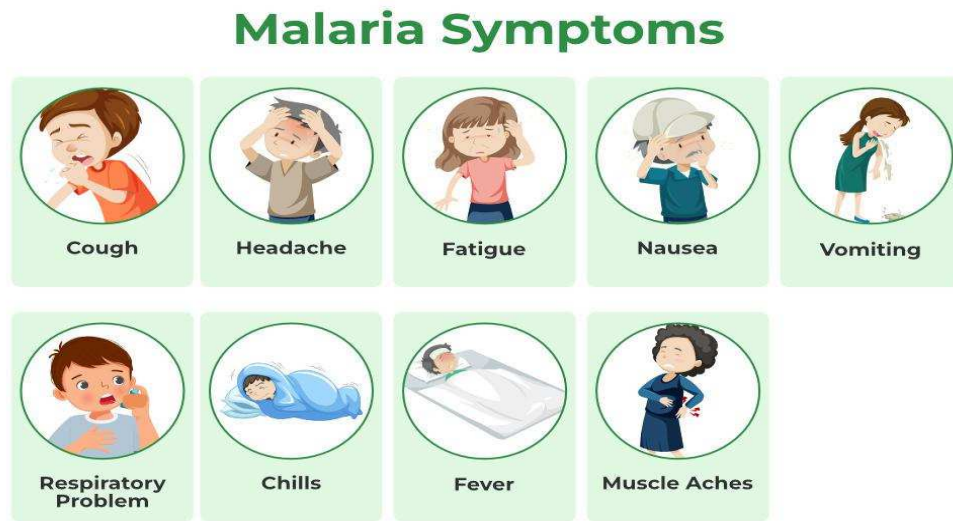


Fig 2 Symptoms of Malaria [34]

The most recent World Malaria Report states that there were 249 million cases of malaria in 2022 as opposed to 244 million cases in 2021. There were predicted to be 608 000 malaria deaths in 2022 as opposed to 610 000 in 2021[35].

World Health Organisation An unreasonably high percentage of malaria cases worldwide are still reported from the African Region. In 2022, the Region accounted for 94% of all malaria cases and 95% of deaths. Most malaria deaths in the region (78% of all deaths) were in children under five [31]. Slightly over half of all malaria deaths globally were reported from four African countries: Nigeria (26.8%), Uganda (5.1%), the Democratic Republic of the Congo (12.3%) and Mozambique (4.2%).

Malaria can be prevented by taking medicine and avoiding insect bites. Before travelling to areas where malaria is common, consult a doctor about taking drugs such as chemoprophylaxis [35].

By avoiding mosquito bites, you can reduce your chance of contracting malaria. Some preventive measures include using coils and vaporizers; using

mosquito repellents nightfall; and sleeping under mosquito nets in regions where malaria is common. Put on safety gear and make use of window screens [36].

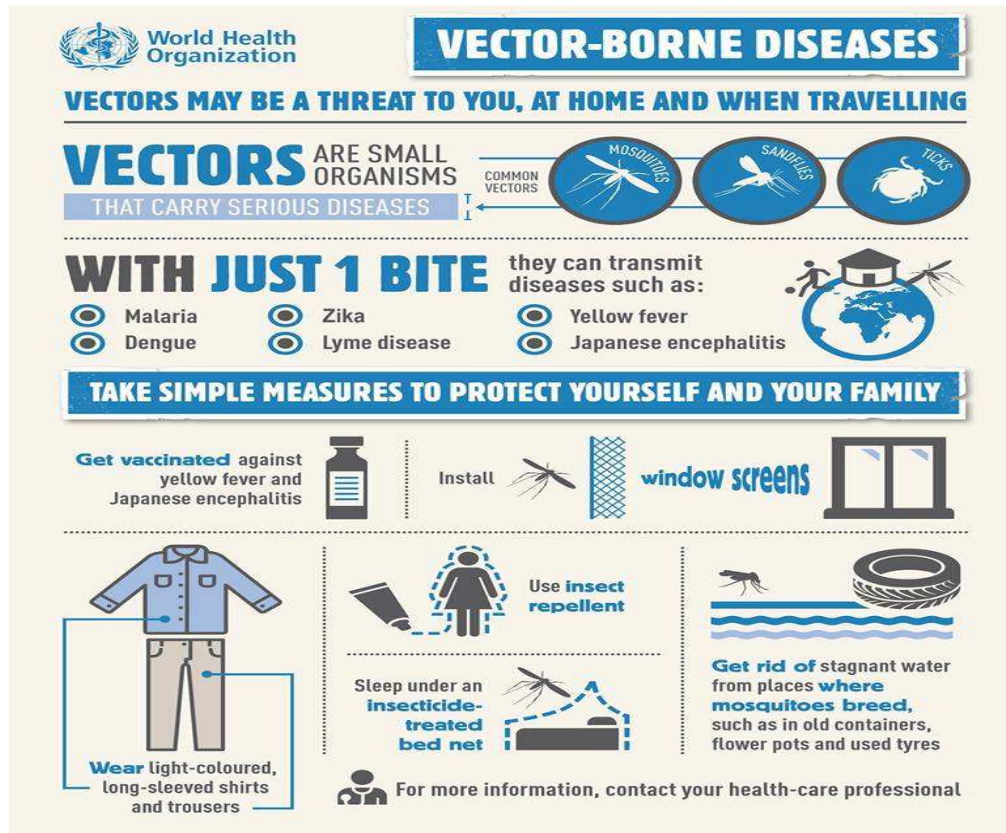


Fig 3 Prevention from Malaria Parasite [35]

Early diagnosis and treatment of malaria reduces disease, prevents deaths, and aids in reducing transmission. The World Health Organisation advises that parasite-based diagnostic tests, which may be completed swiftly, or microscopy be used to confirm any suspected cases of malaria [37].

As a dangerous infection, malaria always needs to be treated with medication. Malaria is treated and prevented with a variety of medications. Physicians will select one or more based on: The type of malaria; The age or weight of the patient; The

presence of medication resistance in the malaria parasite; and whether the patient is pregnant [38].

The following are the most often used medications for malaria: when treating *P. falciparum* malaria, the most successful method is combination therapy based on artemisinin: chloroquine is advised for *P. vivax* infection only in locations where the parasite is still susceptible to this medication. To avoid *P. vivax* and *P. ovale* infection relapses, primaquine should be administered to the primary course of treatment [39].

The bulk of medications are taken as pills. For injectable medications, some people might need to visit a hospital or health centre [40].



Fig 4 Treatments for Malaria [41]

Over the past ten years, partial artemisinin resistance has emerged as a threat to global malaria control efforts. The World Health Organisation is extremely concerned about reports of artemisinin partial resistance in Africa. These reports have

been confirmed in Uganda, Tanzania, Eritrea, and Rwanda most recently. To ensure early detection and control of drug resistance, as well as to inform treatment regimens in countries where malaria is endemic, the efficacy of antimalarial drugs needs to be routinely assessed. Furthermore, a strategy to address drug resistance in Africa has been developed by WHO [42].

1.2 Filariasis

Lymphatic filariasis, sometimes known as elephantiasis, is a neglected tropical disease. Infection occurs when filarial parasites are transmitted to humans by mosquitoes [43].

Typically, infections occur in childhood and harm the lymphatic system covertly. Later in adulthood, lymphoedema, elephantiasis, and scrotal swelling are the painful and severely disfiguring outward signs of the condition that can result in permanent impairment. In addition to their physical disabilities, these patients experience financial, social, and mental losses that exacerbate poverty and stigma [44].

To avoid the spread of infection, 882.5 million people in 44 nations needed preventative chemotherapy in 2022. Over 15 million persons with lymphoedema and 25 million men with hydrocele were estimated to be afflicted by lymphatic filariasis globally in the baseline estimate. At least 36 million people still experience these symptoms of chronic illness. Eliminating lymphatic filariasis can prevent unnecessary suffering and reduce the poverty rate [43].

Lymphatic filariasis is caused by an infection with parasites classified as nematodes (roundworms) of the family Filariodidea. There are three types of these filarial worms that resemble threads:

- *Wuchereria bancrofti*, the causative agent in 90% of instances
- *Brugia malayi*, the primary cause of most of the remaining instances
- *Brugia timori*, the disease's other cause [45]

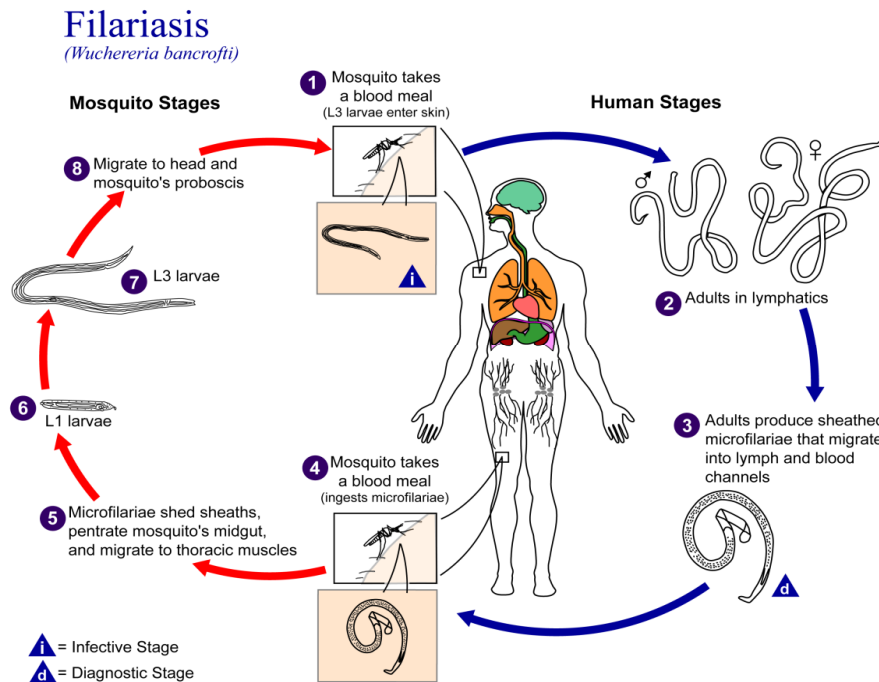


Fig 5 Lifecycle of Filariasis [43]

By constructing their nests in the lymphatic passages, adult worms wreak havoc on the immune system. Throughout their 6-to 8-year life cycle, the worms create millions of immature larvae, or microfilariae, which circulate throughout the blood. A mosquito can pick up microfilariae through blood when it attacks an affected host. Microfilariae grow into infectious larvae inside the mosquito. Mature parasite larvae are left behind after an infected mosquito bites the skin, where they can subsequently infiltrate the host. After that, the larvae go on to the lymphatic arteries, where they mature into adult worms and complete the transmission cycle [46].

Many mosquito species can transmit lymphatic filariasis. Among them are the *Aedes* mosquito, which is mainly found on indigenous islands in the Pacific, the *Anopheles* mosquito, which is typically found in rural areas, and the *Culex* mosquito, which is abundant in urban and semi-urban settings [47].

An infection with lymphatic filariasis can cause both acute and long-term asymptomatic symptoms. Most infections are asymptomatic, which means they contribute to the parasite's propagation without causing any obvious symptoms. These infections damage the kidneys, lymphatic system, and immune system even when there are no symptoms [48].

Lymphatic filariasis can become a chronic illness that leads to hydrocele (swollen scrotum) and lymphoedema (swollen limbs). Lymphatic filariasis also causes elephantiasis, which is characterised by thickening of the skin or tissue. Breasts and genital organs are often affected. These physical abnormalities often lead to social disgrace, poor mental health, lost opportunities for employment, and increased medical expenses for the sufferers and their careers. Poverty and isolation have serious negative societal effects [49].

Acute episodes of local inflammation affecting the skin, lymph nodes, and lymphatic veins are often associated with chronic lymphoedema or elephantiasis. Some of these episodes can be partially attributed to the immune system's response to the parasite. Most arise from secondary bacterial skin infections in which underlying lymphatic injury has partially compromised natural defences. The main reason lymphatic filariasis patients miss work is because of these crippling acute episodes, which can last for weeks [50].



Fig 6 Symptoms of Filariasis [51]

By using prophylactic chemotherapy to stop the infection's spread, lymphatic filariasis can be completely eradicated. The WHO recommends mass medication administration (MDA) as a preventive chemotherapeutic approach to eradicate lymphatic filariasis.

As part of MDA, all at-risk individuals receive a yearly dose of medication. The medications effectively lower the number of microfilariae in the blood and stop the parasites from spreading to mosquitoes, despite having minimal effect on adult parasites [52].

The co-endemicity of lymphatic filariasis with other filarial infections dictates the required MDA regimen. The following MDA regimens are advised by WHO: Twice a year, the treatment plan should be adjusted to include 400 mg of albendazole alone in areas where loiasis co-endemic; 200 mcg/kg of ivermectin combined with 400 mg of albendazole; 6 mg/kg of diethylcarbamazine citrate (DEC) and 400 mg of albendazole; and 200 mcg/kg of ivermectin combined with 600 mg of

diethylcarbamazine citrate (DEC) and 400 mg of albendazole in nations where onchocerciasis is prevalent [53].

2. *Plasmodium falciparum* Transketolase

In the Tropics, malaria is one of the main causes of disease and death. The parasite has become resistant to traditional therapies, therefore finding new pharmacological targets, and creating novel pharmacophores with distinct structures and modes of action is crucial.

One effective approach for developing novel anti-malarial drugs is to target the metabolic pathways of the parasite, which result in the production of the parasite's structural and functional components [54,55,56].

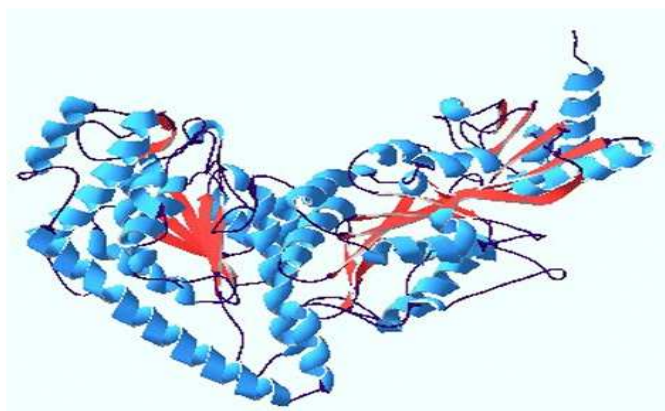


Fig 7 Structure of *Plasmodium falciparum* transketolase [57]

Following the publication of the genomic sequence for *Plasmodium falciparum* [58], it became evident that the parasite possesses a basic metabolic process called the pentose phosphate pathway (PPP). According to research, one of the most potential targets for the treatment of parasitic protozoa is the PPP enzymes [59].

It has been observed that PPP activity is present in erythrocytes infected with *P. falciparum* [60], and biochemical investigations have shown that the malarial parasite is active in the PPP's non-oxidative component [61].

Nevertheless, *P. falciparum*'s genome does not contain the transaldolase gene [62]. Crucial to the nonoxidative branch of PPP, transketolase creates fructose-6-phosphate (F6P), sedoheptulose-7-phosphate (SEDOP), and glyceraldehyde-3-phosphate (G3P) by transferring two carbon units from xylulose-5-phosphate to ribose-5-phosphate or erythrose-4-phosphate [63].

Transketolase requires thiamine diphosphate (ThDP) as a cofactor to perform its catalytic functions. The expression profile of *P. falciparum*'s genes encoding distinct PPP enzymes suggests that transketolase transcription peaks following the parasite's entry into human red blood cells [64]. The production of ribose-5-phosphate, a necessary component for nucleotide synthesis, in a malarial parasite depends on transketolase.

This enzyme uses the G3P and F6P generated by the glycolytic pathway by rotating the non-oxidative arm of PPP in the opposite direction [65]. The incorporation of radiolabelled carbon from glucose into nucleotides [66] demonstrates the critical role transketolase plays in the synthesis of nucleic acids.

These investigations have demonstrated that the non-oxidative portion of PPP is the direct or indirect source of over 80% of the nucleic acid within the parasite. Apart from its crucial function in the production of nucleic acids, transketolase appears to be the enzyme responsible for converting into erythrose-4-phosphate from G3P and F6P when transaldolase is not present [67].

Herbal drugs and natural products have shown potential as inhibitors of *Pf*TK. Several plant-derived bioactive compounds, such as flavonoids, alkaloids, terpenoids, and phenolics, possess kinase inhibitory activity. These compounds can bind to the active site or allosteric sites of *Pf*TK, interfering with its function. For example, flavonoids like quercetin and kaempferol are known to inhibit kinases through ATP-competitive mechanisms, making them potential candidates against *Pf*TK. Alkaloids from traditional antimalarial plants, such as artemisinin derivatives, also show promise due to their bioactivity against parasite-specific targets [68].

Research into herbal inhibitors of *Pf*TK involves bioassay-guided fractionation of plant extracts, virtual screening for kinase-specific binding, and molecular docking studies to predict binding affinities and interactions. Further in vitro and in vivo studies are essential to validate these findings, optimize the efficacy and specificity of herbal compounds, and minimize potential cytotoxicity to human cells. By focusing on *Pf*TK, herbal drug discovery offers an innovative approach to combat drug-resistant malaria strains [69].

The shikimate pathway needs erythrose-4-phosphate as a crucial metabolite to produce the aromatic precursor chorismate, which is subsequently broken down into a variety of aromatic chemicals, including folate [68]. The enzymes of the shikimate pathway are present in the mammalian parasite but are absent in mammals, suggesting that these enzymes may represent novel targets for therapeutic intervention [69].

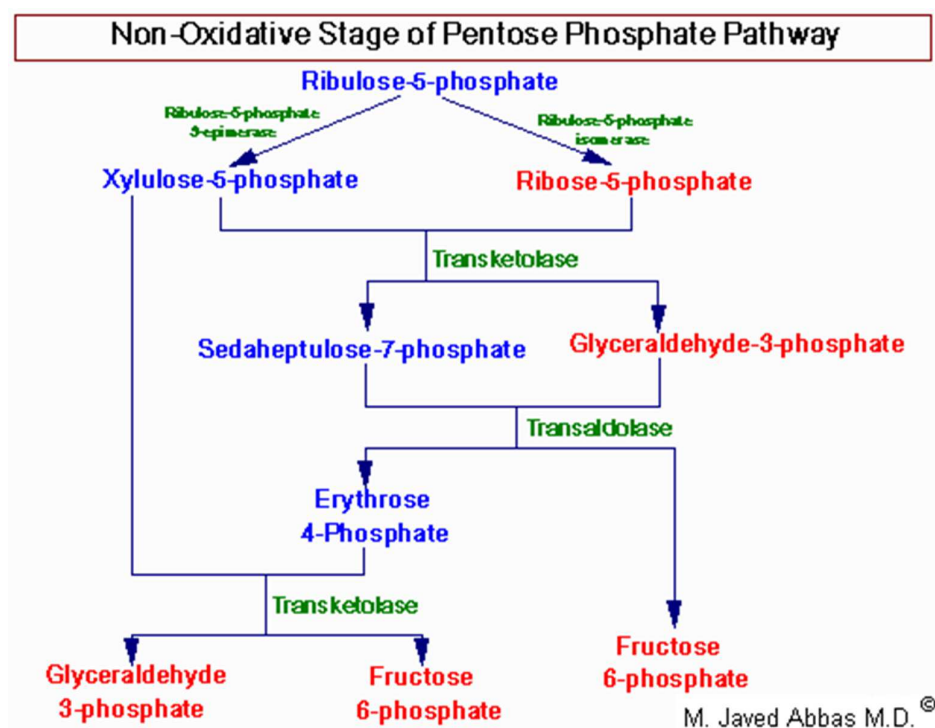


Fig 8 Mechanism of *Plasmodium falciparum* transketolase [70]

3. *Brugia malayi* Hexokinase

In tropical and sub-tropical regions, *Wuchereria bancrofti* and *Brugia malayi* are the primary causes of filariasis, often known as elephantiasis [71]. Diethylcarbamazine (DEC), an antifilarial agent, is most effective against the parasite during its microfilarial stage and has minimal effect on adult parasites [72, 73]. Finding new, parasite-specific chemotherapeutic targets is a necessary step towards developing new, potent antifilarial drugs for chemotherapeutic intervention. D-hexose-6-phosphotransferase (ATP: Hexokinase) is the first significant glycolytic route controlling enzyme [74].

The pentose phosphate pathway, which is catalysed by glucose-6-phosphate (G6P) as a byproduct, produces NADPH and pentose carbohydrates [75, 76]. The

latter is used in the production of nucleic acids, whilst the former preserves the redox potential [77]. Since glycolysis is the main energy metabolism of parasitic worms; hexokinase is a prospective target for the creation of antihelminthic medications [78].

Herbal drugs have shown potential as inhibitors of *BmHK*, leveraging the bioactivity of plant-derived metabolites. Flavonoids, alkaloids, and terpenoids, common in medicinal plants, can act as competitive or non-competitive inhibitors of hexokinase enzymes. For instance, flavonoids like quercetin and apigenin are known to interfere with ATP-binding sites, potentially inhibiting *BmHK* activity. Alkaloids such as berberine and phenolic compounds like catechins also exhibit enzyme-inhibitory properties that could be explored for their effects on *BmHK* [82].

The discovery of herbal inhibitors for *BmHK* involves a systematic approach, including the extraction and fractionation of bioactive compounds from traditional medicinal plants, molecular docking studies to predict their binding affinity and interaction with *BmHK*, and enzymatic assays to evaluate their inhibitory activity. Advanced techniques like high-throughput screening and structure-based drug design can further enhance the identification of potent inhibitors. Exploring *BmHK* as a target for herbal drugs offers a sustainable and innovative strategy to combat lymphatic filariasis while addressing the challenge of drug resistance and side effects associated with conventional treatments [83].

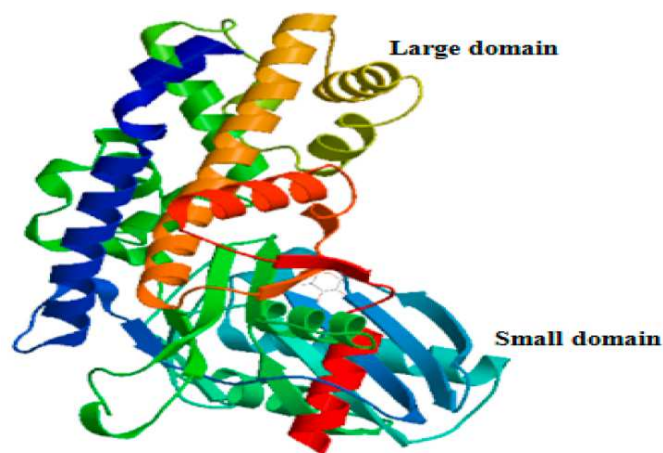


Fig 9 Structure of *Brugia malayi* hexokinase [79]

Hexokinases are characterised by their molecular weight and ability to be inhibited by the G6P product. The molecular weight of hexokinases originating from non-mammalian sources is typically approximately 50 kDa. Numerous marine species, including silkworms and the parasite *Schistosoma mansoni*, have hexokinases that exhibit physiologically significant levels of G6P inhibition [81]. However, some of these hexokinases, like yeast hexokinase, only exhibit little inhibition [80]. Plant hexokinases range in molecular mass from 38 to 68 kDa [82–83].

It has been demonstrated that hexokinase I, II, and III, three isozymes in mammals, are extremely sensitive to G6P inhibition [84]. Their molecular weights are approximately 100 kDa each.

It has been demonstrated that the duplication and fusion of a gene encoding a primitive 50 kDa G6P-sensitive hexokinase is responsible for the evolution of mammalian isozymes [77]. The cloning, expression, and characterisation of hexokinase from a human filarial parasite have never been reported before. The results of this work show that the hexokinase of the filarial parasite differs greatly

from that of the homologous host, suggesting that hexokinase could be a target for the development of customised inhibitors against *B. malayi*.

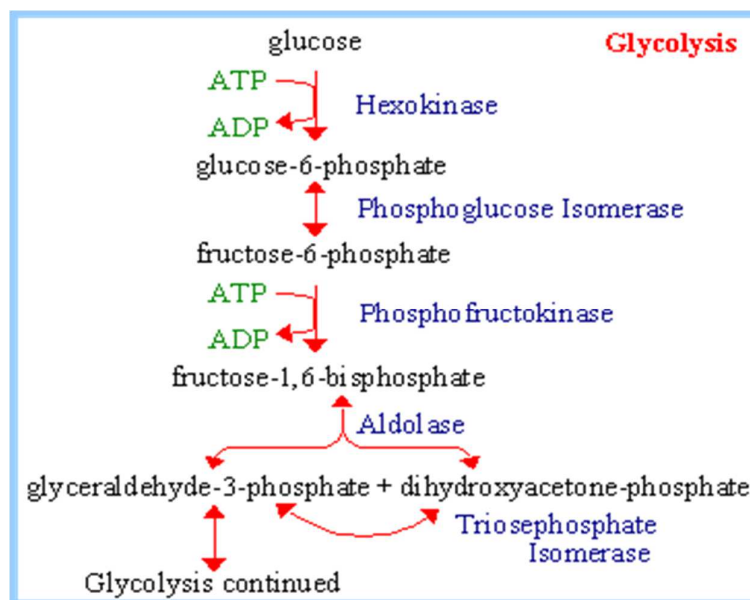


Fig 10 Mechanism of of *Brugia malayi* hexokinase [85]

4. *Anacardium Occidentale*

The Anacardiaceae family of plants, which has over 700 species and 70 genera, is mostly made up of tropical trees and is mostly found in tropical parts of Asia, Africa, and America [86]. In terms of phytochemistry, the Anacardiaceae family, which is known for producing xanthenes, alkylphenols, steroids, flavonoids, and triterpenes [87, 88, 89].

The "cashew," or *Anacardium occidentale* L., is recognised for its importance as an alkyl-phenols source which was isolated from its fruit, or "cashew nuts."

Numerous investigations [90, 91, 92] have shown various biological actions including cancer cell lines cytotoxicity, larvicidal, antioxidant, schistosomicidal and molluscicidal effects. They also showed inhibitory effects in vitro against α -

glucosidase, acetylcholinesterase, tyrosinase, invertase, xanthine oxidase, 15-lipoxygenase, and aldose reductase [93,94].

A. occidentale has been traditionally used in managing filariasis in some tropical regions due to its medicinal properties. The bark and leaves are prepared as decoctions or infusions and consumed to reduce inflammation, swelling, and pain associated with the disease, particularly lymphatic filariasis. Traditional healers attribute its effectiveness to its astringent, anti-inflammatory, and antimicrobial properties, which help alleviate symptoms and potentially target parasitic infections. Rich in bioactive compounds such as flavonoids, tannins, and phenolics, the plant is also used to boost immunity and support recovery. However, scientific studies are needed to validate its traditional use and elucidate its mechanisms against filarial parasites.

A. occidentale has been traditionally used in malaria treatment, particularly in tropical regions where the disease is prevalent. Decoctions of its bark and leaves are commonly consumed to reduce fever, alleviate chills, and manage inflammation associated with malaria. The plant is rich in bioactive compounds like flavonoids, tannins, saponins, and phenolics, which are believed to contribute to its antimalarial, antipyretic, and antioxidant properties. While traditional practices highlight its therapeutic potential, scientific validation through bioassay-guided studies is essential to confirm its efficacy and understand its mechanism of action against malaria parasites.

Due to the alkyl-phenols' promising activity against adult *Schistosoma mansoni* worms and the fact that in folk medicine seed oil of cashew is used to treat

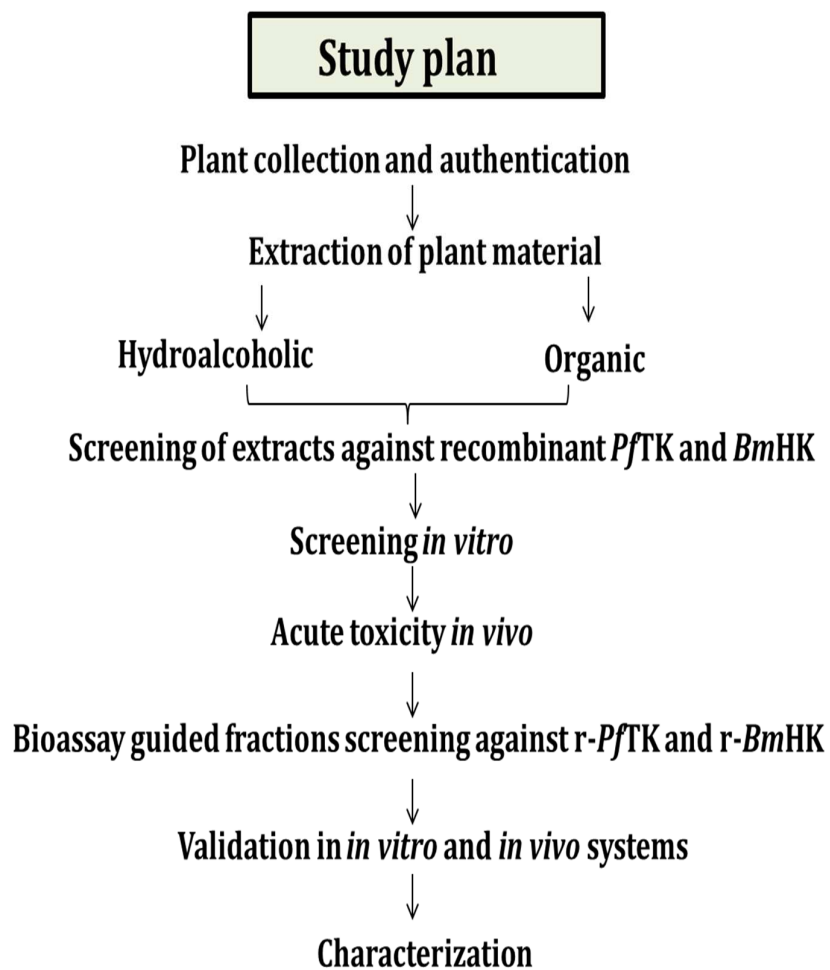
malaria, we set out to assess the *A. occidentale* extract alkyl-phenols active against *Plasmodium falciparum* [95].



Fig 11 *Anacardium Occidentale* [96]

Anacardium occidentale, a well-known plant belonging to the *Anacardiaceae* family, is found in tropical regions of the world, such as India, Vietnam, Central America, Brazil, Indonesia, Nigeria, Panama, and Mozambique [97]. Several biological features have been reported these include molluscicidal, antioxidant, anticancer, antibacterial, and insecticidal properties against *Aedes aegypti* (via inhibition of angiogenesis and histone acetyltransferase activity). Furthermore, exhibits a strong therapeutic promise as a mushroom tyrosinase inhibitor [98].

Fig 12 Workflow of the present study is represented as follows



4. METHODOLOGY

1. Collection of Plant Material

In the Belagavi district of Karnataka, India (N 15.88668; E 74.52353; around 800 metres above sea level), the leaves of *Simarouba glauca*, *Plumeria alba*, *Vitex negundo* and *Anacardium occidentale* were obtained from cashew groves. An herbarium specimen (RMRC-1356, RMRC-1357, RMRC-1358, RMRC-1359) of the plants has been deposited at the Indian Council of Medical Research- National Institute of Traditional Medicine (ICMR-NITM) Belagavi where it was identified.

2. Pilot Study – screening of extracts of different plants

Plants tested:

1. *Simarouba glauca*
2. *Anacardium occidentale*
3. *Plumeria alba*
4. *Vitex negundo*

Phytochemical analysis:

- The dried and powdered, leaves of *A. occidentale*, *S. glauca*, *V. negundo* and *P. alba* extracted with 6 different solvents.
- Phytochemical screening of extracts to detect tannins, terpenoids, amino acids, flavonoids, glycosides, and saponins.

The percentage yield of herbal extracts were calculated using the formula:
Percentage Yield=(Weight of the Extract Obtained/Weight of the Starting Material)×100

***BmHK* enzyme inhibitory assay**

This hexokinase inhibitory activity was determined by use of the methodology described by author Armstrong et al. (1996) [99]. The reaction mixture contained 1U of G6PDH, 10 mM ATP, 0.04 mM glucose, 100 mM Tris-HCL, 25 mM MgCl₂, and 0.25 mM NADP, 50 µg plant extract along with 10-20 µg hexokinase enzyme.

In the assay, pyruvate kinase and lactate dehydrogenase coexist with hexokinase to produce ADP, which measures the utilisation of NADH. The Kruskal-wallis test was used to compare the activity of various extracts while the reaction was carried out in triplicate.

3. Preparation of the *A. occidentale* leaves Extract and fractions, also Preliminary Phytochemical Test

Leaves of *A. occidentale* were dried in shade and then ground into a powder using an electric grinder. The extract was macerated cold for 72 hours using a range of solvents, such as hydroalcoholic solution (70:30 water: ethanol) petroleum ether, methanol, ethyl acetate, dichloromethane, chloroform. Each solvent was used to make three separate leaves extractions, which were then mixed and filtered through Whatman filter paper No. 1.

A rotary evaporator was used to evaporate the solvent. The obtained extracts were kept cold until they were needed again. Many qualitative phytochemical tests were performed, such as the Salkowski test for terpenoid and the Lead acetate test for favonoids, the Foam test for saponins, the Keller-Kiliani test for glycosides, the Dragendorff test for alkaloids, and the Ferric chloride test for tannins. Using silica gel column chromatography (60–120 mesh size) and several solvent systems, the extract

was fractionated using gradient elution with hexane:ethyl acetate. After elution, three fractions were gathered and examined. [100].

Collection of Plant Material, Extraction and Preliminary Phytochemical analysis

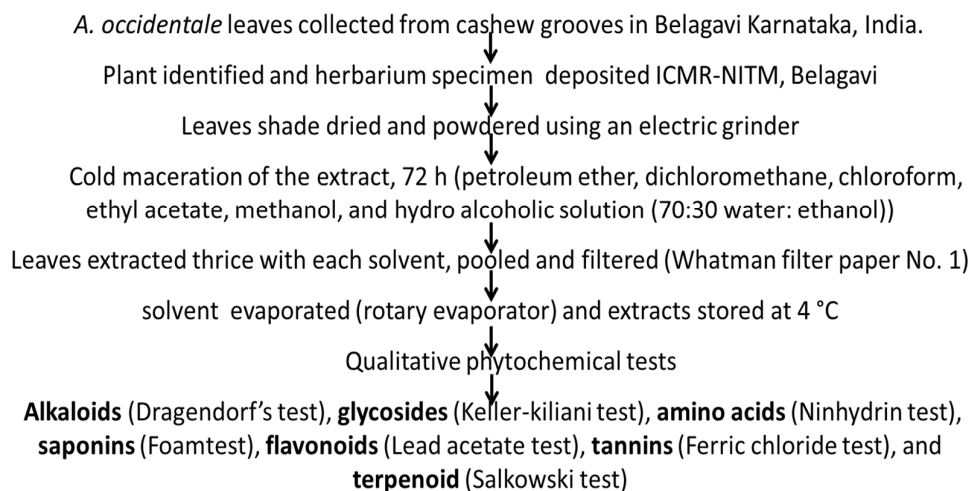


Fig 13 Preparation of the *A. occidentale* leaves Extract and Preliminary Phytochemical Test

4. Expression and Purification of enzyme *P. falciparum* Transketolase (*PfTk*)

The *PfTk* recombinant enzyme was purified, as was previously mentioned [101]. Recombinant plasmid BL21(DE3) containing the transketolase gene insert was grown on Luria Bertani media (LB) at 37 °C. It was given ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) until the optical density at 600 nm reached 0.6. The recombinant protein was activated using isopropyl-β-thiogalactopyranoside (IPTG) at a concentration of 1 mM. Furthermore, the culture was incubated in a shaker incubator for 20 hours at 18 °C. The cells were then separated by centrifuging the mixture for five minutes at 11,000 g.

The cell pellet in lysis bufer was suspended, a solution containing pH 8.0, 10% (v/v) glycerol, 10 mM imidazole, 300 mM NaCl, and 50 mM NaH₂PO₄ together with a protease cocktail inhibitor (Sigma). Next, ultrasonication was used to lyse the cells (Ultrasonic processor, Model-XL-2020, Germany). The lysate for 30 minutes at 11,000 g was centrifuged at 4 °C, and the supernatant was combined with Ni-NTA agarose resin that had been adjusted for lysis buffer.

After an hour of incubation at 4°C, the resin was separated from the cell lysate by centrifugation (5600 g for 5 minutes at 4°C), and the mixture was like the lysis buffer but with a washing buffer that has 20 mM imidazole in it.

The elution buffer containing 250 mM imidazole was then used to elute the enzyme recombinant PfTK. Ammonium sulphate precipitation (0–40% w/v) was employed to further purify the PfTK-containing elute. After the protein pellet was resuspended in storage buffer (50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, and 10% (v/v) glycerol), the protein concentration was determined using Lowry's technique [102]. Western blotting with anti-His tag antibodies and SDS-PAGE [103] were used to assess the purity of the protein.

Plasmodium falciparum Transketolase (PfTK)

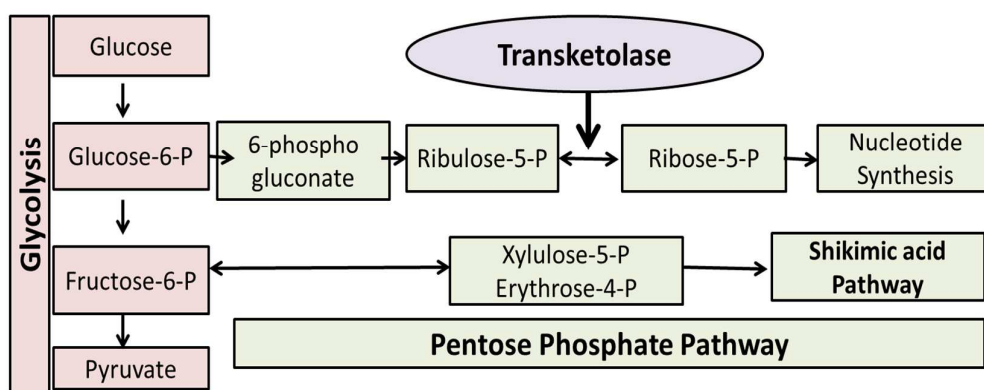
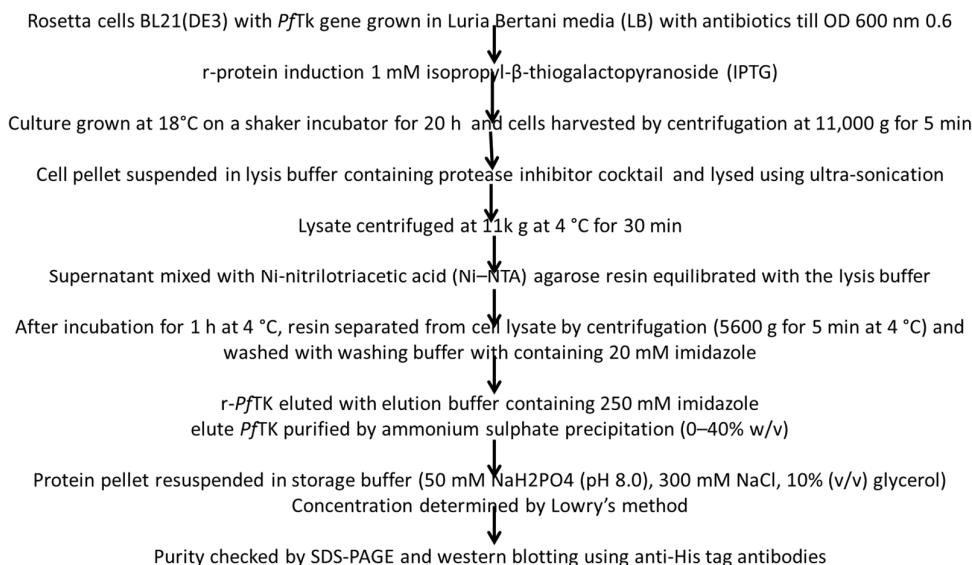


Fig 14 *Pf*TK enzyme**Expression and Purification of *P. falciparum* Transketolase (*Pf*Tk)**Fig 15 Purification of *Pf*TK**5. *Pf*TK assay**

The activity of the purified transketolase by measuring the rate at which the intermediate carbanion oxidised in presence ferricyanide was determined [104]. The 1.0 ml reaction mix contained the following ingredients: leaves extracts of *A. occidentale* L. in varying amounts; 0.1 mM thiamine pyrophosphate; 0.5 mM potassium ferricyanide; 2 mM magnesium chloride; 0.24 mg enzyme; 3 mM fructose-6-phosphates (F6P)/hydroxypyruvate (HP); and 50 mM glycine- glycyl bufer (pH 7.6).

The ferricyanide reduction was observed using a UV-1601 PC spectrophotometer (Shimadzu, Japan) at 420 nm after the *Pf*Tk enzyme and extract were added.

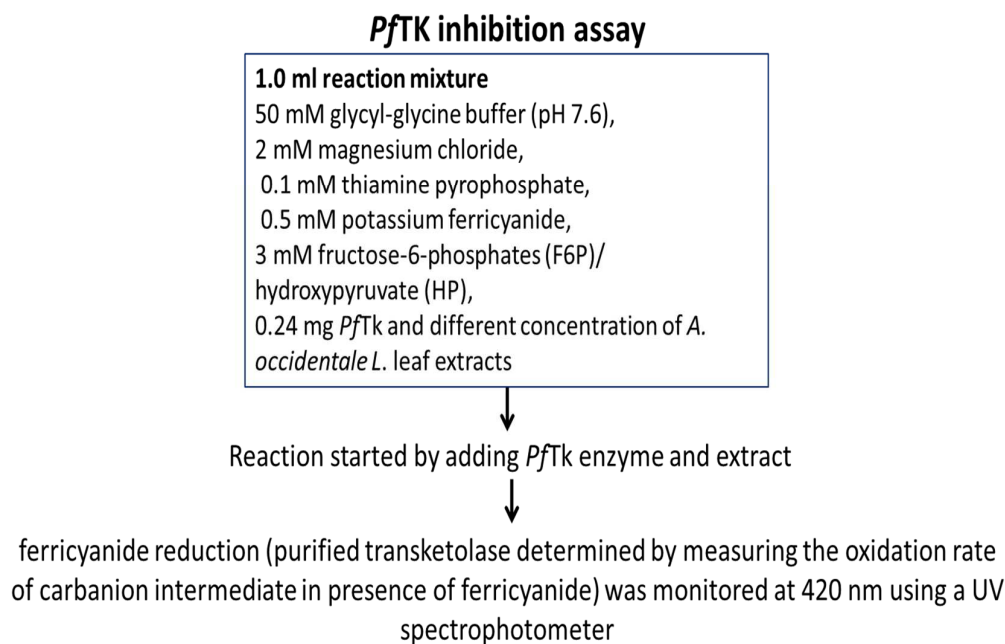


Fig 16 *P. falciparum* Transketolase (*Pf*Tk) assay

6. Cultivation of *P. falciparum* enzyme and Testing of Antimalarial Activity of Extracts against *P. falciparum* enzyme

Utilising the Trager and Jensen technique [105], the *P. falciparum* (3D7 strain) culture was expanded. In addition to 0.5% Albumax II (Gibco™ AlbuMAX™), 100 µM hypoxanthine, 0.15% sodium bicarbonate, 25 mM HEPES bufer, 2 mM glutamine, and 50 µg/ml gentamicin, the parasites were cultured in RPMI 1640. The best conditions for culture were 37 °C and 5% CO₂. Giemsa-stained thin blood smears were used to check for parasitaemia in routine cultures, and the level was kept between 2% and 5%. O+ve RBCs were used to keep the culture haematocrit at 2%. To enrich for ring stage parasites, parasites were synchronised with 5% sorbitol before to performing inhibitory assays.

To test the anti-malarial activity of the extracts, inhibition tests were set up in 96-well plates with 200 µl of culture per well at around 2% parasitaemia. Tests were carried out at a concentration of chloroquine 50 µg/ml to identify the extracts that inhibited the growth of malaria parasites in combination with the antimalarial drug. The extracts that exhibited growth inhibition of greater than 50% were then put through a dose response assay, wherein the extract's concentration was diluted twice, initially at 50 µg/ml and ultimately at around 50 ng/ml. For each assay, three runs were conducted.

After a 48-hour incubation period in optimal growth conditions, the plates were developed with 25 µl of SYBR Green I dye containing 10X lysis bufer. The plates were incubated for one hour in the dark at 485 nm and 530 nm, respectively, for excitation and emission wavelengths, before the fluorescence was measured using a multi-well plate reader.

Cultivation of *P. falciparum* and Testing of Antimalarial Activity of Extracts

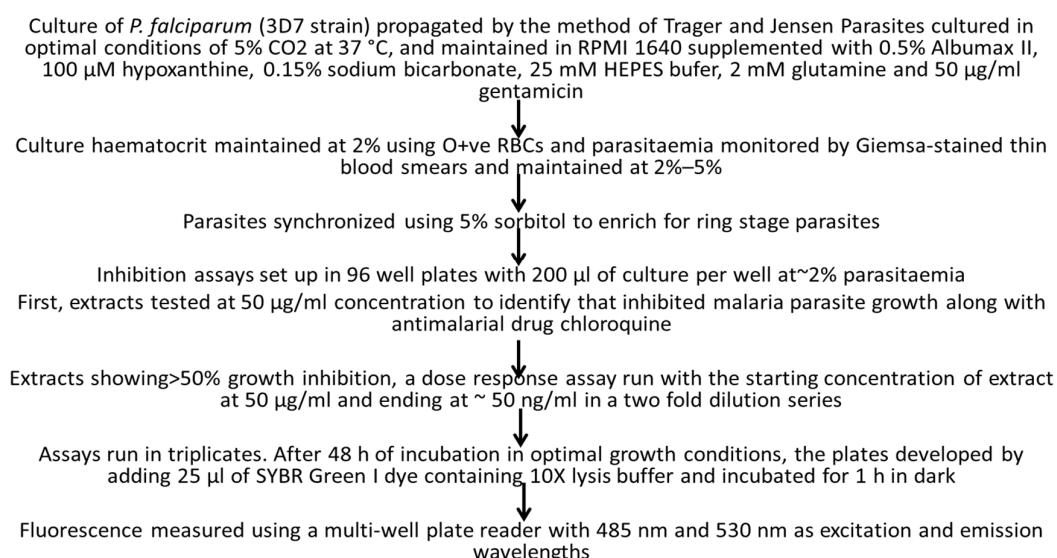


Fig 17 Cultivation of *P. falciparum* enzyme and Testing of Antimalarial Activity of Extracts against *P. falciparum* enzyme

7. Zebrafish Embryo Acute Toxicity

Since the 1930s, the South Asian zebrafish (*Danio rerio*), a fish belonging to the family Cyprinidae, has been utilised as a model organism in biological study. Zebrafish are inexpensive to maintain, simple to breed, and their embryos develop quickly (five days after fertilisation). The embryo's transparency makes it possible to view internal organs and morphological structures, such as the brain, eyes, heart, liver, and kidney, with light microscopy without requiring surgery.

Developmental toxicity in general and in specific organs can be measured quantitatively using dyes or evaluated visually. Due to its small size, one embryo can be chemically screened in a matter of days and maintained at fluorescent quantities of 100 µl in individual microtiter plate wells. More biological characteristics, including genes, developmental processes, anatomy, physiology, and behaviour, are shared by humans and zebrafish. Its genome shares a great deal of synteny with mammalian genomes, comprising about 50% of the mouse genome.

Additionally, its genes are orthologs for human genes and have an average homology of about 75%. There are known genes that contribute significantly to human illnesses. Because of these characteristics, the zebrafish is a special vertebrate model for high-throughput chemical screening, which is useful for toxicity assessment and preclinical drug discovery. In several of its most recent standards, the Organisation for Economic Co-operation and Development (OECD) has recommended zebrafish for aquatic toxicity evaluation (OECD, 1992a,c, 1998). (For additional details, see [106]). Using this paradigm, we examined the acute toxicity of leaves extracts from *A. occidentale*.

After being removed from the ICMR-NITM colony, adult zebra fish were grown in 3 L housing tanks (10–15) at 28.5 °C with a 14/10-hour light/dark cycle. A 24-well Corning costar TC tissue culture plate with two millilitres of sterile distilled water in each well was used to hold a recently fertilised embryo.

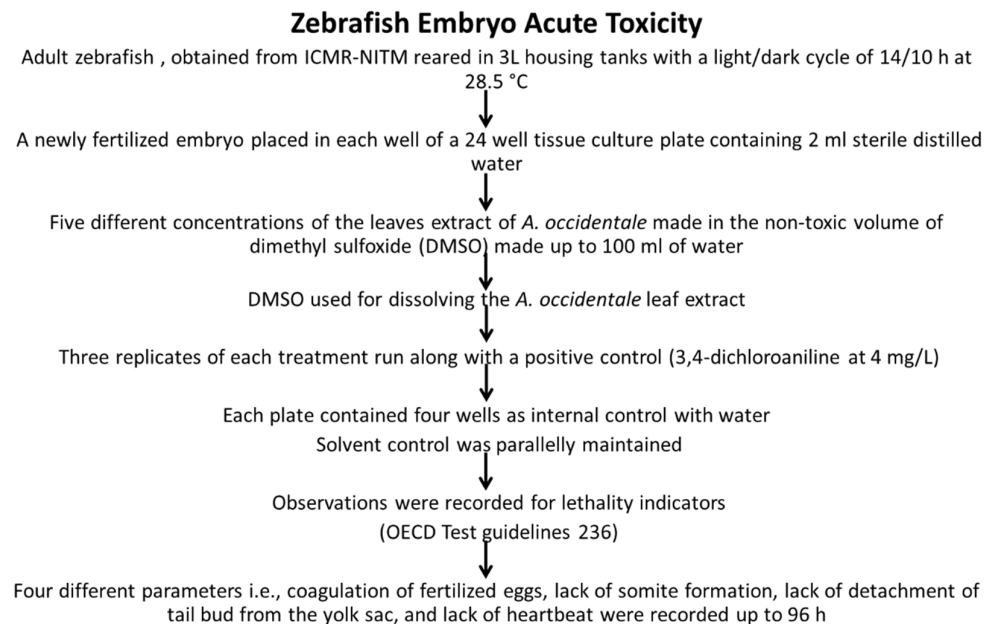


Fig 18 Zebrafish Embryo Acute Toxicity

One hundred millilitres of water were added to a non-toxic dimethyl sulfoxide (DMSO) container to create five different strengths of *A. occidentale* leaves extract. DMSO was used to dissolve the leaves extract of *A. occidentale*. Three duplicates of each treatment were carried out, and a positive control (3,4-dichloroaniline at 4 mg/L) was also examined. For internal water control, each plate included four wells. Solvent control in parallel was maintained. Observations of lethality indicators were documented.

In compliance with the recommendations of the Organisation for Economic Cooperation and Development guidelines (OECD Test guidelines 236) [107], four distinct parameters were recorded for a maximum of 96 hours: the coagulation of

fertilised eggs, the absence of somite formation, the detachment of the tail bud from the yolk sac, and the absence of a heartbeat.

8. Acute Oral Toxicity of Extracts on Mice Model

The safety of the *A. occidentale* leaves extract was assessed using acute oral toxicity in BALB/c mice, following approval by the Institutional Animal Ethics Committee of the ICMR-NITM (IAEC/ICMR-NTM BGM/2018/1). The toxicity test was conducted in accordance with OECD test recommendations No. 423 [108]. The animals were purchased from the ICMR-National Institute of Nutrition in Hyderabad, India, and housed in the ICMR-NITM animal house facility.

There were four groups of mice, each including six female mice. Three groups were designated as test groups and administered oral treatments containing different amounts of the extracts after a 12-hour overnight fast. Extractions and fractions were tested at 50, 300, and 2000 mg/kg body weight (b/w), with water serving as the vehicle treatment for the fourth control group.

Acute Oral Toxicity Study in Mice

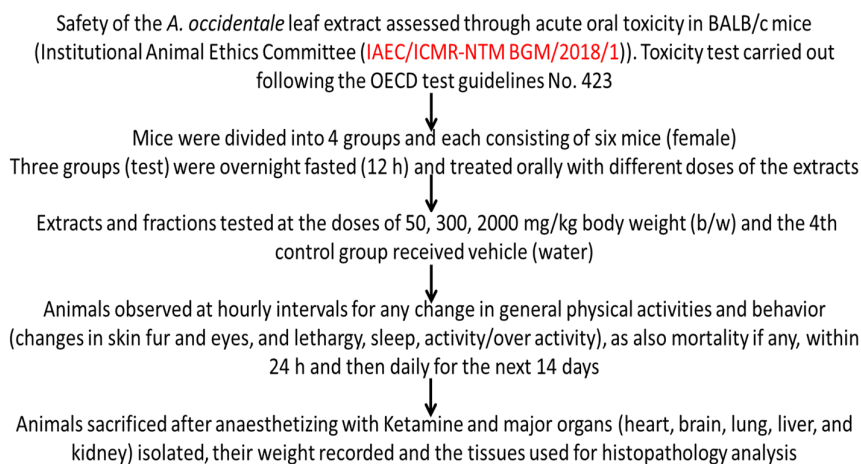


Fig 19 Acute Oral Toxicity of Extracts on Mice Model

From then on, the animals were checked hourly for 14 days for any changes in their general physical activities and behaviour (such as changes in their skin, fur, and eyes, as well as changes in their lethargy, sleep patterns, and level of activity or overactivity), as well as for any signs of mortality. The primary organs (liver, kidney, lung, heart, and brain) were removed from the animals at the end of the trial after they had been put to sleep with ketamine. Their weight was noted, and the tissues were used for histopathological investigation.

After the study's conclusion, the animals were sacrificed humanely with ketamine injections, and their brains, livers, kidneys, and lungs were kept for two days in 10% formalin. Different alcohol concentrations (70, 80, 90%, and 100% alcohol later) were used to dehydrate the organs for duration of 12 hours each. After being cleansed for 15 to 20 minutes with Xylene solution, the tissues were embedded in paraffin blocks. The tissue blocks were divided into five-micron slices using a microtome device. The sections were put on a microscope slide, and eosin and hematoxylin dyes were applied to them once they had dried. Under a microscope, the stained sections were inspected for immune cell infiltration, inflammation, and congestion, if any.

9. Cloning, expression, and purification of *BmHk*

In compliance with earlier reports, the recombinant *BmHk* was produced and cloned [109]. In summary, the cloned *BmHk* gene-containing plasmid pTriEx was transformed into the *E. coli* (DE3) Rosetta strain. Subsequently, one recombinant bacterial colony was incubated for 16 hours in 5 ml of LB medium supplemented with Chloramphenicol 40 µg/ml and ampicillin 100 µg/ml on a rotary shaker at 37 °C and 180 rpm.

It was injected 500 ml at 1% to of antibiotic-containing LB media and cultured till an OD600 of 0.6 was achieved. To induce the culture isopropyl-thiogalactopyranoside was used.

(IPTG) was used 0.5 mM for 16 hrs at 20 °C. The induced cells were next pelleted by centrifuging them for 10 minutes at 4 °C and 8000 g.

After the pellet was extracted, it was once more suspended in lysis buffer that contained a combination of protease inhibitors (5 mM imidazole, 300 mM NaCl, and 50 mM NaH₂PO₄) (Ultrasonic processor).

The lysate that had been sonicated was centrifuged at 10,000 g for 45 minutes, and then the *BmHk* was extracted from the supernatant using Ni²⁺-nitrilotriacetic acid resin (Ni²⁺-NTA) chromatography. Following the loading of the supernatant onto the column, imidazole was added four times, at concentrations varying from 10 to 40 mM, to clean the column. First, lysis buffer was used. The recombinant protein was eluted using the lysis buffer that included 450 mM imidazole.

Bradford's technique (1976) [110] was used to determine the concentration of the pure protein after it had been concentrated via a Centriprep-30 (Millipore) filter. 10% SDS-PAGE was used to verify the isolated protein's purity [111].

Brugia malayi Hexokinase (*BmHK*)

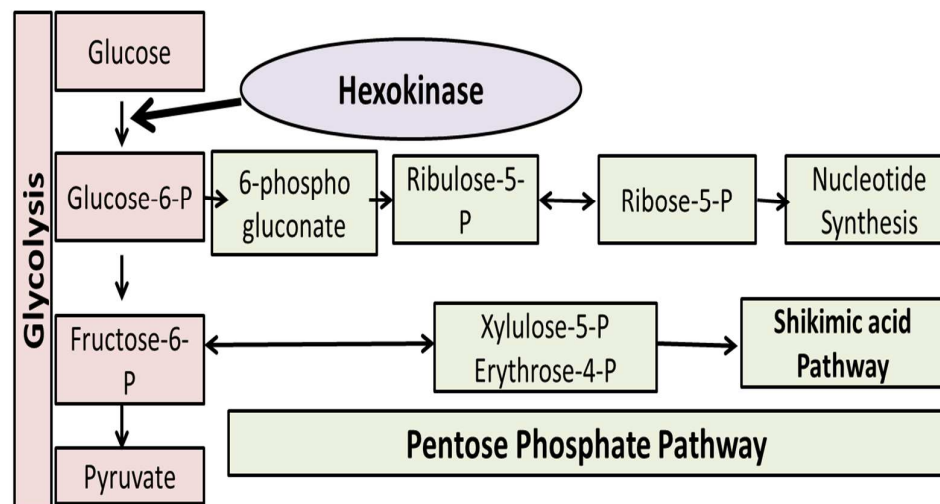


Fig 20 *BmHK*

Expression and Purification of *Brugia malayi* hexokinase (*BmHK*)

BmHK cloned into plasmid pTriEx and transformed into *E. coli* (DE3) Rosetta strain

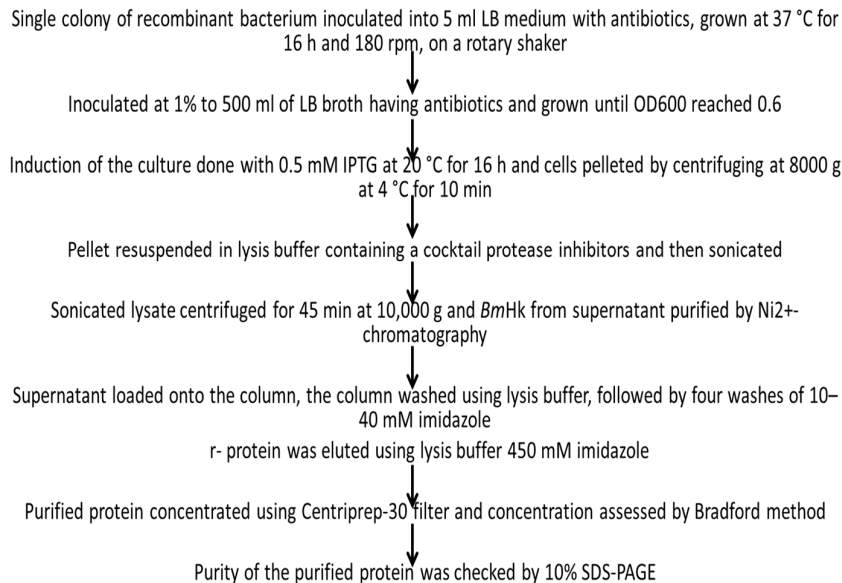


Fig 21 Cloning, expression, and purification of *BmHK* enzyme

10. Gas chromatography analysis

The Varian 450 gas chromatograph was utilised in conjunction with a stationary phase ZB-5 column to conduct a Gas Chromatographic (GC) examination of the extracts using previously documented experimental circumstances [113,114]. The carrier gas, nitrogen, flowed at a rate of 1.0 mL/min. The temperature of the apparatus was fixed at a rate of 3 °C per minute, between 80 and 280 °C/min. The temperatures of the injector and detector were set to 280 and 290 °C, respectively.

A. occidentale extract was injected at 1.0 mL volume with a split ratio of 1: 50. The GC peak area (FID response) was used to calculate the relative amounts of each component without the need for a correction factor.

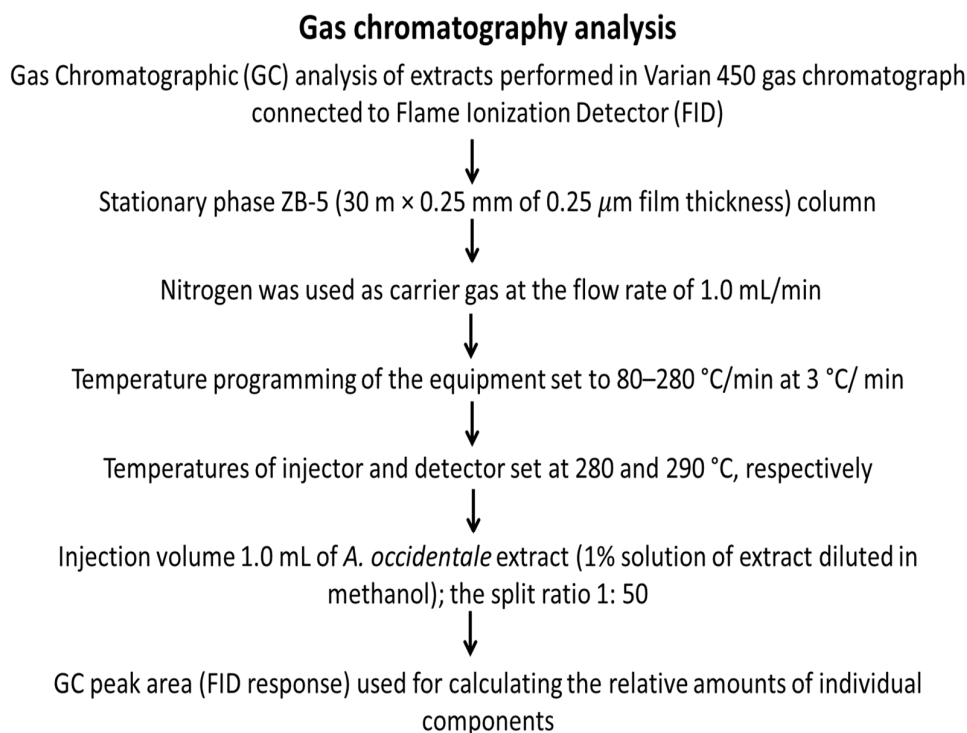


Fig 22 Gas chromatography analysis

11. HPLC analysis of extract of *A. occidentale* leaves

The mobile phase was prepared using solvent A 0.1% acetic acid in water and solvent B as acetonitrile, and degas using ultrasonication. Set up the HPLC with a C18 reverse-phase column at 38°C, a flow rate of 1.0 mL/min, and a detection wavelength suitable for your analyte (e.g., 220 nm). Equilibrate the column with the initial mobile phase composition (e.g., 90% A, 10% B) for 10–15 minutes. Filter the sample through a 0.45 µm syringe filter, inject 20 µL into the system, and run the analysis using an isocratic or gradient program depending on your analyte's properties. After the run, flush the column with 100% solvent B for 10–15 minutes, and store it in a 50:50 water-acetonitrile solution.

HPLC analysis of hydroalcoholic extract of *A. occidentale*

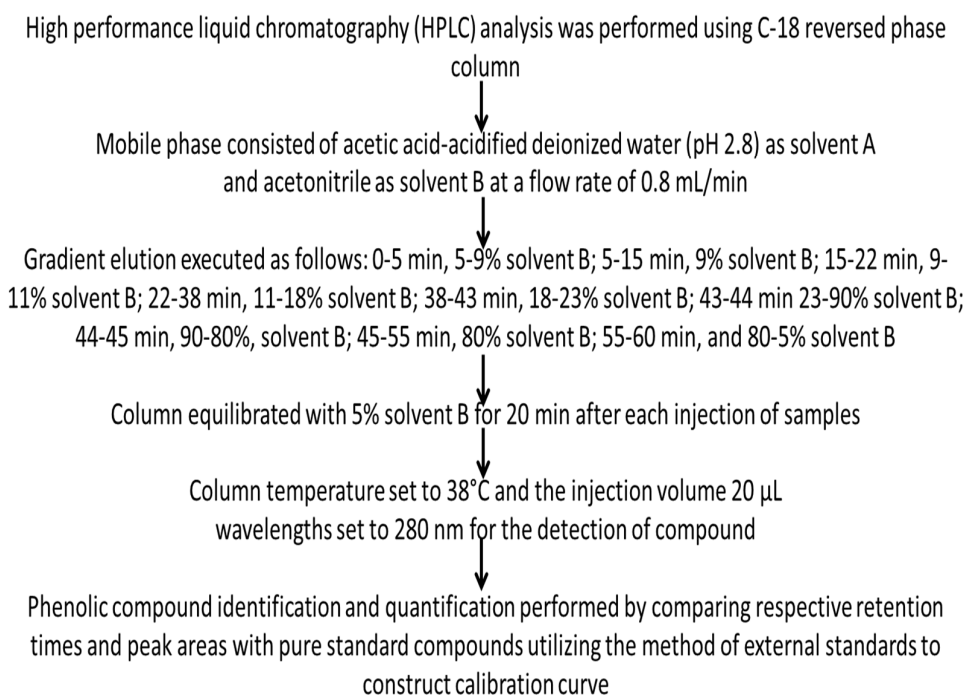


Fig 23 HPLC analysis of extract of *A. occidentale* leaves

12. *BmHK* enzyme inhibitory assay

The methods outlined by author Armstrong et al. (1996) [99] was utilised to ascertain the inhibitory action of hexokinase 50 µg of plant extract, 10 mM ATP, 0.04 mM glucose, 100 mM Tris-HCL (pH 7.6), 25 mM MgCl₂, 0.25 mM NADP, 1U of G6PDH, and 10–20 µg of hexokinase was all present in the reaction mixture.

In the assay, pyruvate kinase and lactate dehydrogenase coexist with hexokinase to produce ADP, which measures the utilisation of NADH. The Kruskal-wallis test was used to compare the activity of various extracts while the reaction was carried out in triplicate.

***BmHk* inhibitory assay**

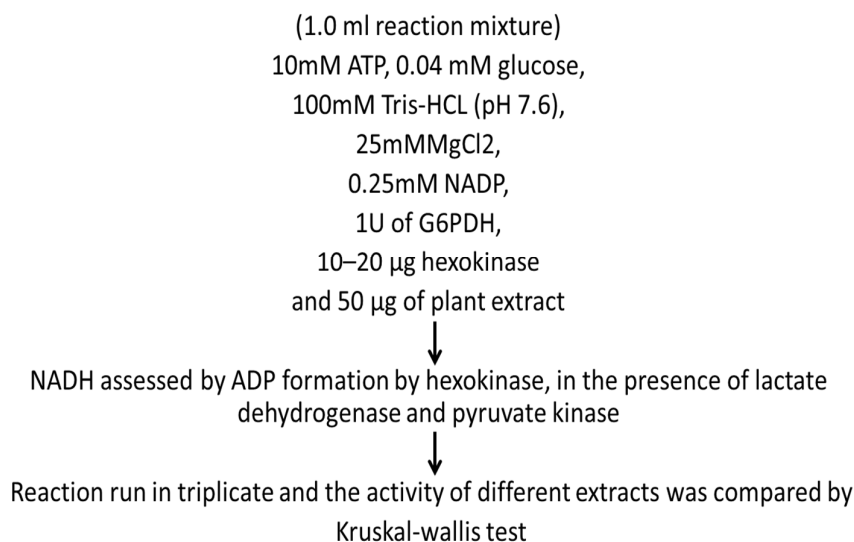


Fig 24 *BmHk* inhibitory assay

13. Molecular homology modeling, structure validation and Docking

The NCBI database provided the *B. Malayi* hexokinase protein's sequence. A homology model was produced by forwarding the recovered sequence to the I-TASSER (Iterative Threading ASSEmbly Refinement) server. Using Errat (<http://servicesn.mbi.ucla.edu/ERRAT/>) [116] and Verify 3D (<http://servicesn.mbi.ucla.edu/Verify3D/>) [115], the quality of the modelled structure was confirmed.

By assigning protonation states, completing residues, and fixing hydrogen atoms, structural artefacts in the created model were eliminated. The Schrodinger suite's protein preparation wizard was then used to force field OPLS_2005 to minimise the restraint.

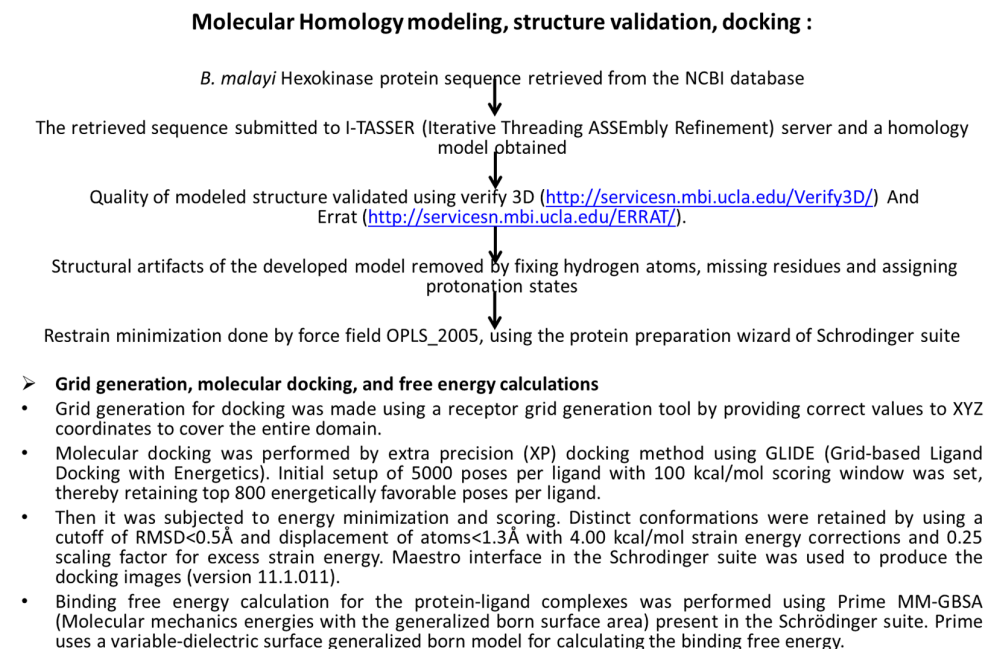


Fig 25 Molecular homology modeling, structure validation and docking

13.1 *BmHK* enzyme grid creation, molecular docking, and free energy calculations

The XYZ coordinates were selected to cover the whole domain to create the grid for docking using a receptor grid generating algorithm. Using GLIDE (Grid-based Ligand Docking with Energetics), the extreme precision (XP) docking technique was used to achieve molecular docking.

The top 800 energetically beneficial postures for each ligand were retained in the initial configuration, which had 5000 poses for each ligand and a 100 kcal/mol scoring window. Following that, energy minimization and scoring were used. Differentiable conformations were maintained with atom displacement of less than 1.3Å, a cutoff of RMSD<0.5Å, strain energy adjustments of 4.00 kcal/mol, and a scaling factor of 0.25 for surplus strain energy.

The docking images were constructed using the Maestro interface (version 11.1.011) for the Schrodinger suite [117]. Using the Prime MM-GBSA (Molecular mechanics energies with the generalised born surface area) from the Schrödinger suite, the binding free energy of the protein-ligand complexes was ascertained. Prime uses a generalised birth model with a variable-dielectric surface to calculate the binding free energy.

5. RESULTS

1. Phytochemical screening of extracts of different plants

The leaves of the selected four plants viz., *A. occidentale*, *Simarouba glauca*, *Plumeria alba* and *Vitex negundo*, were extracted with hydro-alcohol and subjected to phytochemical screening. The screening revealed the presence of alkaloids, glycosides, flavonoids, saponins, tannins, and terpenoids (Table 1). Flavonoids were found in high concentration (+++) in all extracts, highlighting their potential antioxidant properties. Alkaloids were present in all extracts, with varying levels from low (+) to moderate (++), while glycosides occurred in three of the four extracts, with a range of moderate (++) to low (+). Amino acids were absent in most extracts, except in the extracts of *Plumeria alba* (+). Saponins were found only in *P. alba* (++) . Tannins were present in high concentration (+++) in *A. occidentale*, but occurred either in low concentration (+) or absent in the other extracts. Terpenoids were present high concentration (+++) in *V. negundo* and moderate (++) in *A. occidentale*, but less prominent or absent in other extracts. (Table 1).

Table 1. Major phytoconstituents* of leaves extract of different plants.

Extracts	Alkaloids	Glycosides	Amino acids	Saponins	Flavonoids	Tannins	Terpenoid
<i>Anacardium occidentale</i>	++	+	-	-	+++	+++	++
<i>Simarouba glauca</i>	++	+	-	-	++	-	+++
<i>Plumeria alba</i>	++	++	-	+	+++	-	+++
<i>Vitex negundo</i>	+	+	-	-	+++	+	+++

* + + + High, + + average, + less, - absent

2. Purification of recombinant hexokinase of *Brugia malayi* (*BmHK*) and recombinant of transketolase of *Plasmodium falciparum* (*PfTK*).

The recombinant *BmHK* was produced and purified using Ni-NTI chromatography, and its purity was checked by 10% SDS-PAGE analysis. The SDS-PAGE showed the presence of a 72 kDa band (Fig. 26), which is accepted size of *BmHK* and demonstrating the electrophoretically homogeneous nature of the purified recombinant enzyme.

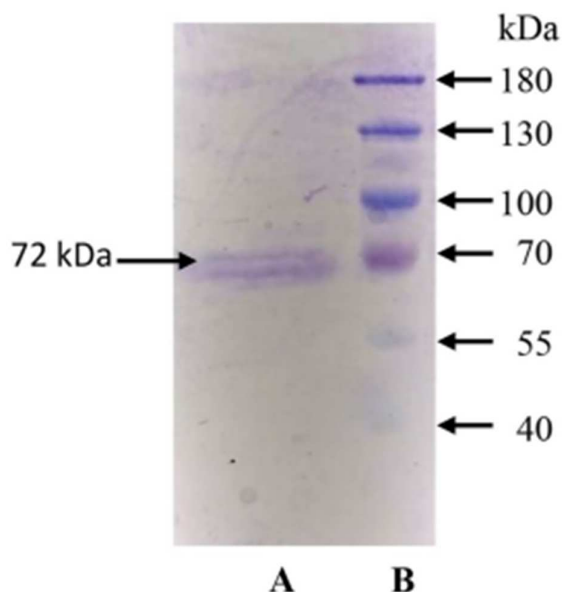


Fig. 26. The SDS-PAGE of the Ni-NTA column purified recombinant hexokinase of *B. malayi* (*BmHK*). Protein fraction was electrophoresed on a 10% SDS polyacrylamide gel under reducing conditions and stained with Coomassie Brilliant Blue R250 (Lane A – purified *PfTk* ~72 kDa, Lane B – Molecular weight marker with range 10-250 kDa).

Similarly, the recombinant *PfTK* enzyme of *P. falciparum* was produced and purified from the recombinant clone *E. coli* (Rosetta cells BL21 (DE3) expressing the His-tagged *PfTK* protein, as reported earlier [7]. The recombinant *PfTK* was purified using a Ni-NTA column. The SDS-PAGE analysis of the purified recombinant showed a single band of *PfTK* at 70 kDa (Fig. 27).

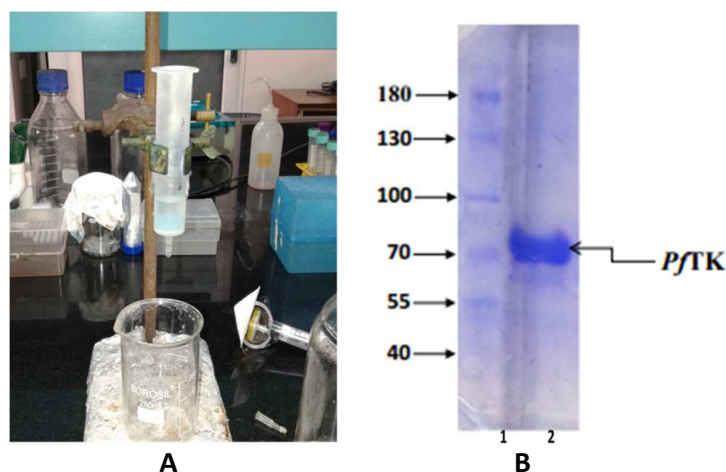


Fig. 27. Cloning, expression and purification of the recombinant *PfTK* enzyme of *P. falciparum*. A- Purification of *PfTK* by Ni-NTA column chromatography, B- SDS-PAGE analysis of *PfTK*. Protein fractions were electrophoresed on a 10% SDS polyacrylamide gel under reducing conditions and stained with Coomassie Brilliant Blue R250 (Lane 1 – Molecular weight marker with range 10-250 kDa, Lane 2 – purified *PfTK* ~70 kDa).

20. Inhibition of recombinant Hexokinase of *Brugia malayi* (*BmHK*) by the extracts of leaves of plants extracted with different solvents

Among all the extracts of different plant leaves, extracted with different solvents, hydro-alcoholic of *A. occidentale* leaves exhibited highest inhibition of the recombinant *BmHK* enzyme (65.80% w/w) and the dichloromethane extract showed lowest inhibition (12.25%) (Table 2). The methanol extract of *S. glauca* showed highest inhibition with (42.00%), while the petroleum ether effected the least inhibition (13.68%). The dichloromethane of *P. alba* demonstrated highest inhibition (30.55%) compared to other extracts, with petroleum ether giving the lowest inhibition (6.70%). Similarly, the hydro-alcoholic extract of *V. negundo* exhibited highest inhibition (40.90%), while petroleum ether showed the least (25.30%).

Table 2. Percentage inhibition of recombinant Hexokinase of *Brugia malayi* (BmHK) by the extracts of leaves of plants extracted with different solvents.

Plant	Extracts					
	Hydro-alcoholic	Methanol	Petroleum ether	Chloroform	Ethyl acetate	Dichloro methane
<i>Anacardium occidentale</i>	65.80	33.60	49.88	28.00	45.50	12.25
<i>Simarouba glauca</i>	34.55	42.00	13.68	20.16	39.10	26.85
<i>Plumeria alba</i>	13.90	37.59	6.70	16.40	20.15	30.55
<i>Vitex negundo</i>	40.90	38.50	25.30	23.85	35.60	19.89

21. Yield of extracts of *A. occidentale* leaves

As hydro-alcoholic extract of *A. occidentale* L. leaves exhibited the highest inhibitory effect against recombinant hexokinase enzyme of *B. malayi* several solvents were explored for the preparation of its extracts. The yield of extract (% w/w) was highest with methanol, followed by hydro-alcohol, while those with other solvents ranged from 13.0% - 5.2% (Table 3).

Table 3. Extraction of *A. occidentale* L. leaves with various solvents.

Extracts (solvent)	Yield (% w/w)
Methanol	22.25%
Hydro-alcoholic	17.32%
Ethyl acetate	12.50%
Chloroform	13.00%
Dichloromethane	9.20%
Petroleum ether	5.2%

22. Inhibition assay of recombinant *PfTK* enzyme by *A. occidentale* L. leaves extracts

In order to assess the inhibitory effect of different extracts of *A. occidentale* L. leaves, inhibition assays were conducted using the purified recombinant *PfTK*. The hydro-alcoholic extract exhibited highest inhibition of the recombinant *PfTK* ($75.45 \pm 0.35\%$), while the extracts from petroleum ether, methanol, and chloroform demonstrated moderate to low levels of inhibitory activity ($53.00 \pm 0.09\%$, $35.00 \pm 0.80\%$) (Table 4). The lowest inhibitory activity was observed with the dichloromethane ($22.00 \pm 1.50\%$) and ethylacetate ($18.88 \pm 0.20\%$) extracts. The bioactivity guided fractionation of the hydro-alcoholic extract was done and the fractions were assessed for their efficacy to suppress *P. falciparum* blood stage parasites *in vitro*, as described later.

Table 4. Inhibition assay of recombinant *PfTK* enzyme by *A. occidentale* leaves extracts.

Extract	% Inhibition
Hydroalcoholic	$75.45 \pm 0.35\%$
Chloroform	$53.00 \pm 0.80\%$
Methanol	$52.85 \pm 1.85\%$
Petroleum ether	$35.00 \pm 0.09\%$
Dichloromethane	$22.00 \pm 1.50\%$
Ethyl acetate	$18.88 \pm 0.20\%$

23. In-vitro inhibition of *P. falciparum* strain 3D7 growth by hydro-alcoholic extract of *A. occidentale* L. leaves.

To determine the anti-malarial activity, in terms of growth inhibition of *P. falciparum* blood stage parasites by the hydro-alcoholic extract, assays were conducted at various concentrations of then extract (50 $\mu\text{g/ml}$ to 50 ng/ml). The highest concentration (50 $\mu\text{g/ml}$) produced $99.31 \pm 0.08\%$ growth inhibition of *P. falciparum*, according to the dose response inhibition data. The IC_{50} was estimated to be $4.17 \pm 0.22 \mu\text{g/ml}$ (Fig. 28).

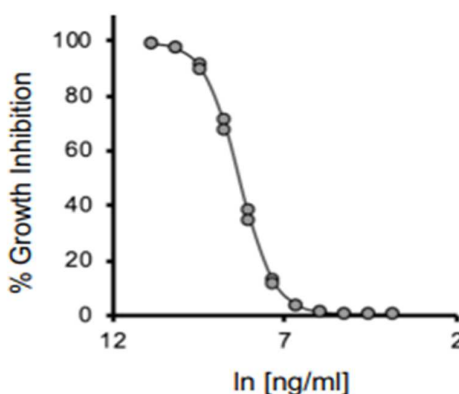


Fig. 28. Anti-malarial activity of hydro-alcoholic extract of *A. occidentale* L. leaves against *P. falciparum* strain 3D7. The extracts were tested as twofold serial dilution starting from 50 $\mu\text{g/ml}$. The data are from a representative test that had duplicate tests which were plotted independently.

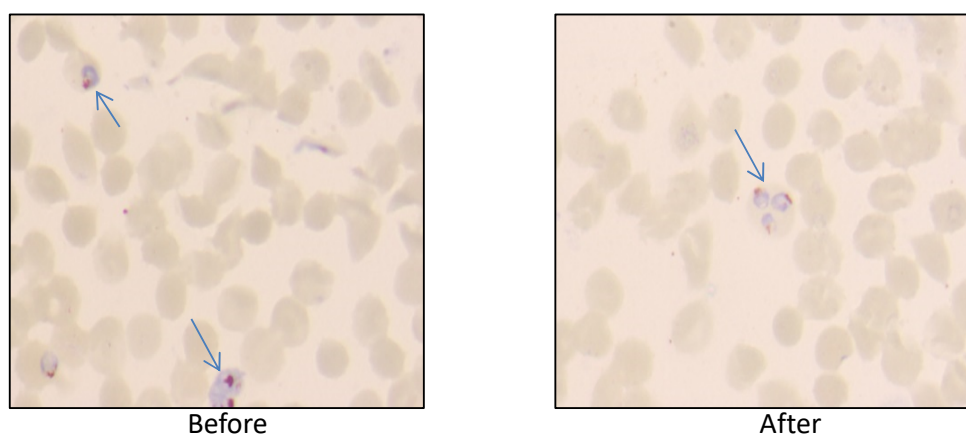


Fig. 29. Microscope observations showing the growth inhibition of *P. falciparum* growth by *A. occidentale* L. leaves hydro-alcoholic extract.

Bioactivity guided fractionation yielded 6 active fractions and their ant-malarial activity is presented in Table 5 and Fig. 30. Of the 6 fractions, fractions AO2 and AO6 exhibited maximum growth inhibition ($99.97\% \pm 0.004$ and $99.96\% \pm 0.005$, respectively) of *P. falciparum* strain 3D7, with the IC_{50} values of 0.56 ± 0.03 and $0.63 \pm 0.03 \mu\text{g/ml}$. Fractions AO1 and AO5 effected slightly lower growth inhibition ($89.30\% \pm 0.08$ and $84.56\% \pm 4.61$, respectively), than fractions AO2 and AO6, and had IC_{50} values of 10.29 ± 1.67 and $12.56 \pm 0.03 \mu\text{g/ml}$. The remaining 2 fractions exhibited moderate level of growth inhibition of the parasites.

Table 5. Percentage growth inhibition of *P. falciparum* strain 3D7 by the fractions of hydro-alcoholic extract of *A. occidentale* L. using fluorescence-based assay.

Fraction	% growth inhibition at 30 $\mu\text{g/ml}$	IC_{50} ($\mu\text{g/ml}$)
AO1	89.30 ± 0.08	10.29 ± 1.67
AO2	99.97 ± 0.004	0.56 ± 0.03
AO3	54.30 ± 2.01	27.28 ± 1.17
AO4	66.27 ± 8.60	21.27 ± 3.87
AO5	84.56 ± 4.61	12.56 ± 2.25
AO6	99.96 ± 0.005	0.63 ± 0.03

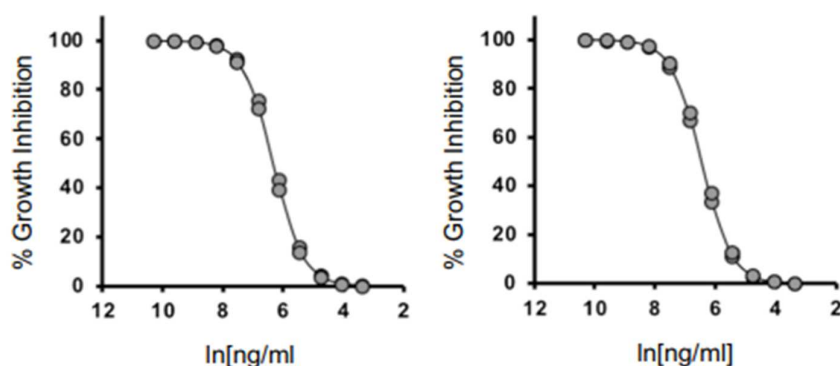


Fig. 30. Fractions AO2 (left) and AO6 (right) of hydro-alcoholic extracts of *A. occidentale* L. leaves exhibit anti-malarial efficacy against *P. falciparum*. The methodology for this experiment was the same as that of Fig. 2, with the exception that 30 $\mu\text{g/ml}$ was the maximum concentration. When AO2 and AO6 were added to untreated *P. falciparum*, the growth inhibition was $99.97 \pm 0.004\%$ and $99.96 \pm 0.005\%$, respectively, at 30 $\mu\text{g/ml}$ concentration. The results showed that the IC_{50} values were $0.56 \pm 0.03 \mu\text{g/ml}$ and $0.63 \pm 0.03 \mu\text{g/ml}$, respectively.

24. Inhibition of *BmHK* by different extracts of *A. occidentale* L. leaves

A. occidentale L. leaves extracts were tested for their inhibitory activity against the purified recombinant *BmHK* enzyme. The percent inhibition of extracts varied from $65.80 \pm 1.60\%$ to $12.25 \pm 2.50\%$ (Figure 34 & Table 31). The hydro-alcoholic extract showed highest inhibition against *BmHK* ($65.80 \pm 1.60\%$; $p=0.0053$), followed by the extracts of petroleum ether ($49.88 \pm 0.80\%$) and ethyl acetate ($45.50 \pm 1.90\%$). The methanol ($33.60 \pm 1.20\%$), chloroform ($28.00 \pm 1.00\%$), and dichloromethane ($12.25 \pm 2.50\%$) extracts exhibited lowest inhibition. Further investigation was focused on the hydro-alcoholic extract of *A. occidentale* L. leaves due to its superior inhibitory effect against *BmHK* in comparison with other extracts.

Table 6. Inhibition assay of *A. occidentale* L. leaves extracts against recombinant *BmHK* enzyme.

Extract	% Inhibition
Hydro-alcoholic	$65.80 \pm 1.60\%$
Petroleum ether	$49.88 \pm 0.80\%$
Ethyl acetate	$45.50 \pm 1.90\%$
Methanol	$33.60 \pm 1.20\%$
Chloroform	$28.00 \pm 1.00\%$
Dichloromethane	$12.25 \pm 2.50\%$

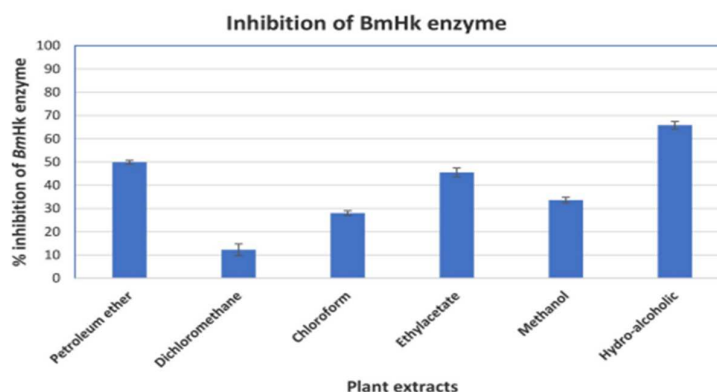


Fig. 31. Inhibition assay of *A. occidentale* leaves extracts against recombinant *BmHK* enzyme.

25. Acute toxicity study of hydro-alcoholic extract of *A. occidentale* leaves against Zebra fish embryo model

Varying concentrations of leaves extract of *A. occidentale* L. were tested for acute toxicity tests in zebra fish embryos. 3,4-dichloroaniline and water were included as positive and negative controls, respectively. The water control group and the hydro-alcoholic extract of *A. occidentale* L. leaves treatment groups had normal heartbeats, somite formation, tail separation, and embryo hatching after 24 hours of treatment, but the positive control group showed no somite development or tail detachment (Table 10 & Fig. 32).

However, 96 hours after treatment of 100 $\mu\text{g/ml}$ of extract, the fish embryos exhibited no heartbeat. Fish embryos treated with 1000 $\mu\text{g/ml}$ of extract showed no heartbeat after just 48 hours. None of the embryos in the control group showed signs of death even 96 hours later. The LC_{50} of the extract was determined to be 9.60 $\mu\text{g/ml}$ (Fig. 33).

Table 7. Acute toxicity study of hydroalcoholic extract of *A. occidentale* L. leaves against Zebrafish embryos model.

Time	Negative control*	Positive control**	Test compound (<i>A. occidentale</i> extract)				
			0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
0 h	–	–	–	–	–	–	–
24 h	SF, TD	NSF, NTD	SF, TD	SF, TD	SF, TD	SF, TD	SF, TD
48 h	HB	NA	HB	HB	HB	HB	NHB
72 h	HB	NA	HB	HB	HB	HB	NHB
96 h	H	NA	H	H	H	NHB	NA

SF – Somite formation, NSF – No somite formation, TD – Tail detachment, HB – heartbeat, NHB – no heartbeat, H – hatching, NA – not applicable, *Negative control (water), **Positive control (3,4-DCA 4mg/ml).

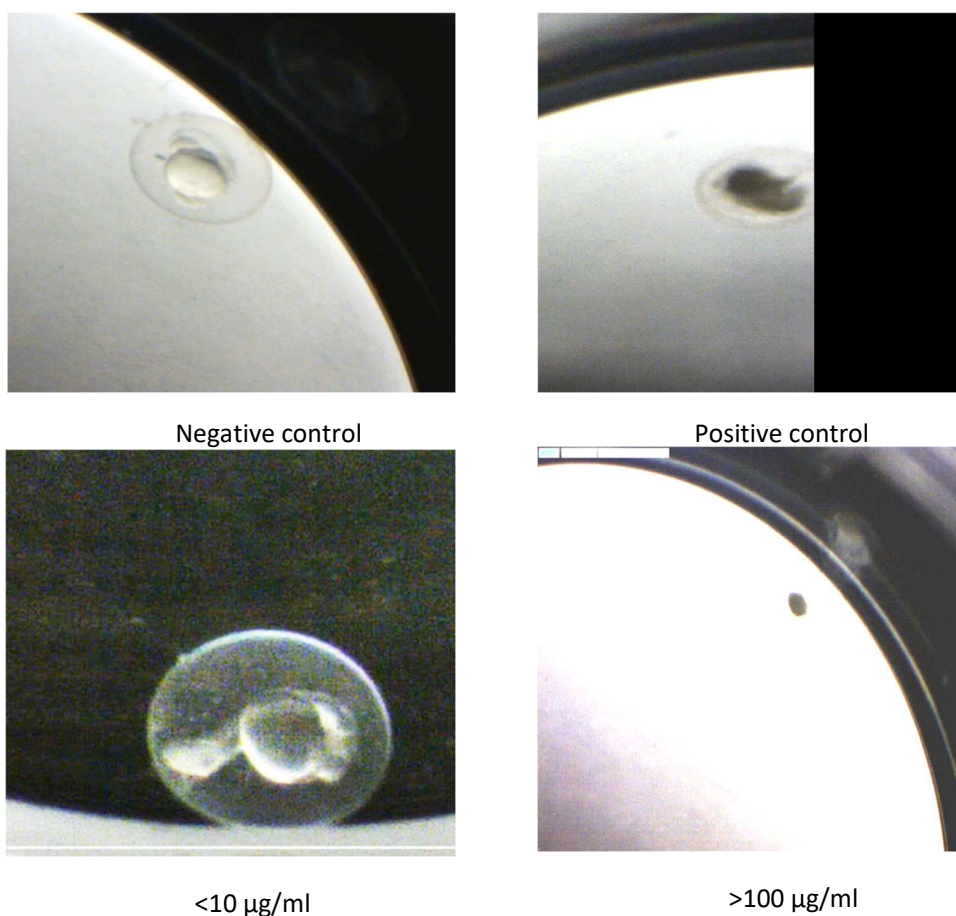


Fig. 32. Acute toxicity test of hydro-alcoholic extract of *A. occidentale* L. leaves against Zebra fish embryo model.

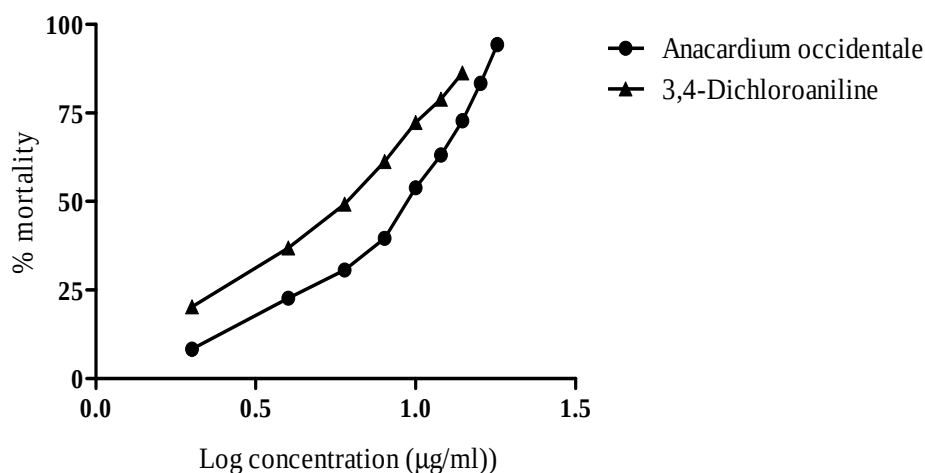


Fig. 33. Effect of different concentration hydro-alcoholic extract of *A. occidentale* L. leaves against Zebra fish embryo model. The activity of the extract was compared with the +ve control, 3,4-dichloroaniline.

26. Acute oral toxicity of hydro-alcoholic extract of *A. occidentale* leaves in mice model

The acute oral toxicity of *A. occidentale* L. leaves hydro-alcoholic extract was tested in BALB/c mice at doses of 50, 300, and 2000 mg/kg body weight. The results demonstrated that none of the three distinct extract dosages investigated resulted in any fatalities or serious illnesses in the animals. The behaviour and physical characteristics of experimental mice were found to be normal with no outward indications of abnormalities (Table 8).

Table 8. Bodyweight, general observations and histopathological analysis of BALB/c mice treated with different doses of hydro-alcoholic extract of the leaves of *A. occidentale* L.

Animal groups	Dose (mg/kg)	Parameters		
		Bodyweight (g)*	General observations	Histopathological analysis
<i>A. occidentale</i> L. hydroalcoholic extract	50	20.7 ± 1.80	Normal	No changes
	300	22.5 ± 0.40	Normal	No changes
	2000	23.0 ± 0.90	Normal	Slight congestion in lungs
Control	Vehicle (water)	24.0 ± 0.20	Normal	Normal

The histopathological examination of the of heart, liver, kidney, and brain of mice administered with varying doses of the extract did not exhibit any significant alterations. Nonetheless, at 2000 mg/kg, minor lung congestion was noted (Fig. 35). Mice administered with the extract at doses of 50 mg, 300 mg, and 2000 mg displayed normal histological structures in the brain sections showing several neurons in the striatum, hippocampus, and cerebral cortex. The ventricles and atrium of mice administered with the extract at doses of 50 mg, 300 mg, and 2000 mg are evidently normal.

Normal sinusoidal space, central vein, and hepatocytes were observed at 40x magnification after treatment with the extract. Additionally, the glomeruli and renal tubules of the renal corpuscles were seen to be in their normal configuration. The liver of mice treated with the extract 50 mg and 300 mg, showed no haemorrhage or visible tissue damage. However, in the mice treated with 2000 mg extract, there was focal lung haemorrhage (congestion) and vessel wall deterioration. Thus, the hydro-alcoholic extract of the leaves of *A. occidentale* L. was found to be safe, at least at 300 mg/kg.

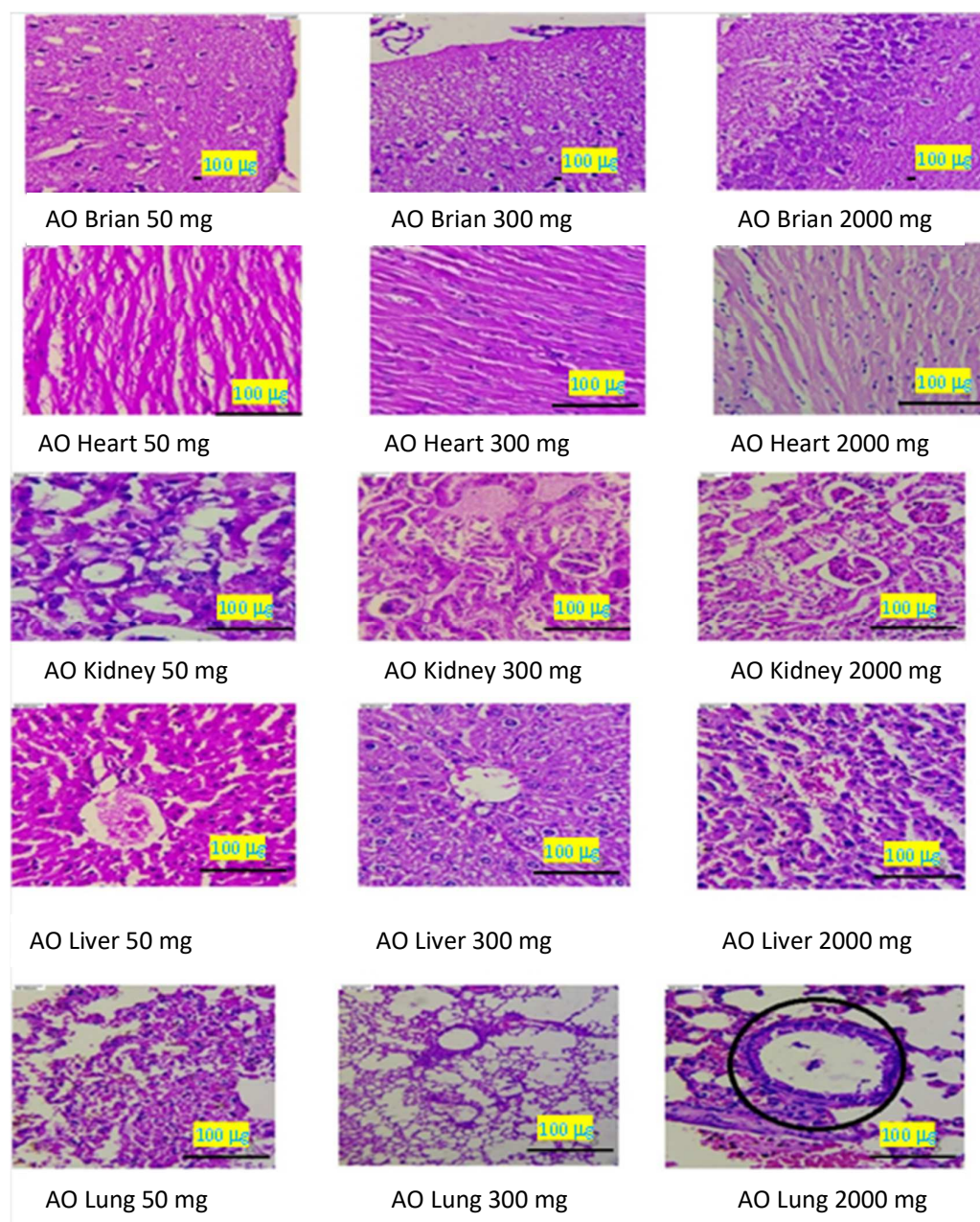


Fig. 34. The toxicity of *A. occidentale* L. leaf hydro-alcoholic extract in mice. The heart, liver, kidney, lung and brain of mice were investigated after treatment with different doses (50, 300 and 2000 mg/kg body weight of Balb/c mice) of the extract. Then ring shows the focal haemorrhage (congestion) of lung and vessel wall destruction at 2000mg/kg body weight dose.

27. GC-FID of *A. occidentale* leaves extract analysis.

The results of a phytochemical analysis using GC-FID of the extract of leaves from *A. occidentale* L. and other plants are displayed in Fig. 36. In this investigation, 0.1% of

anacardic acid was found. The hydroalcoholic extract of *A. occidentale* L. leaves contained relatively little anacardic acid, which is soluble in nonpolar solvents.

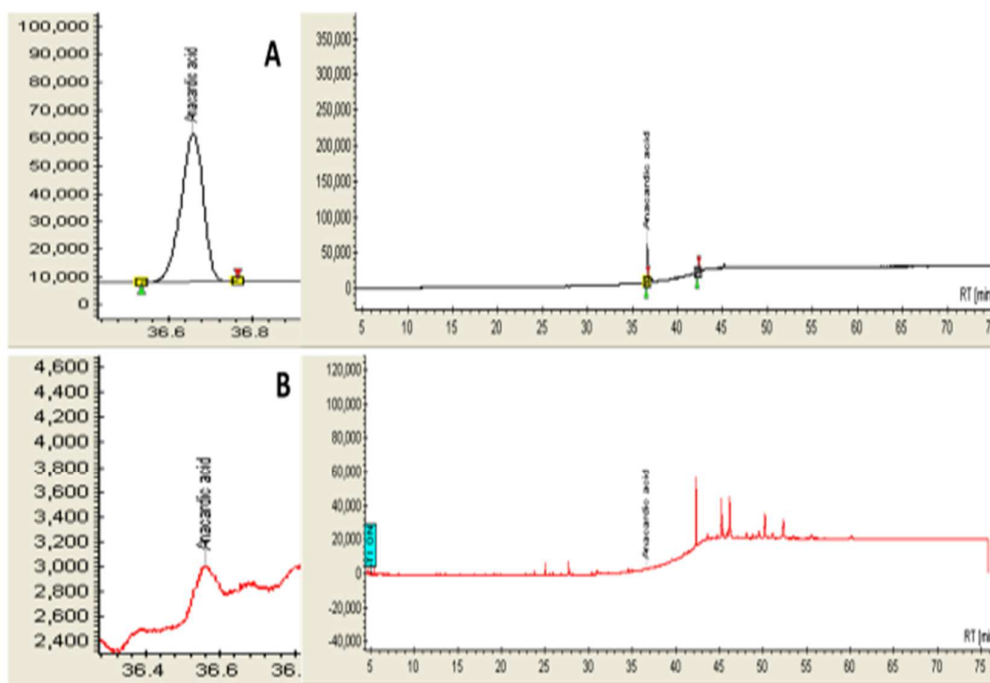
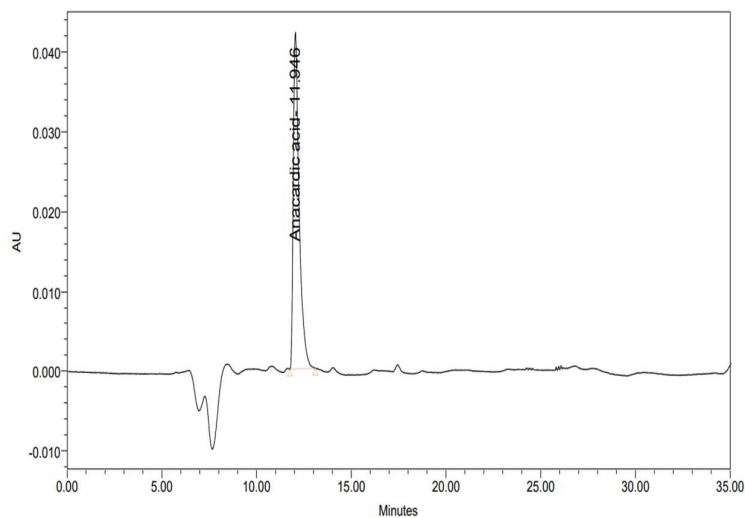


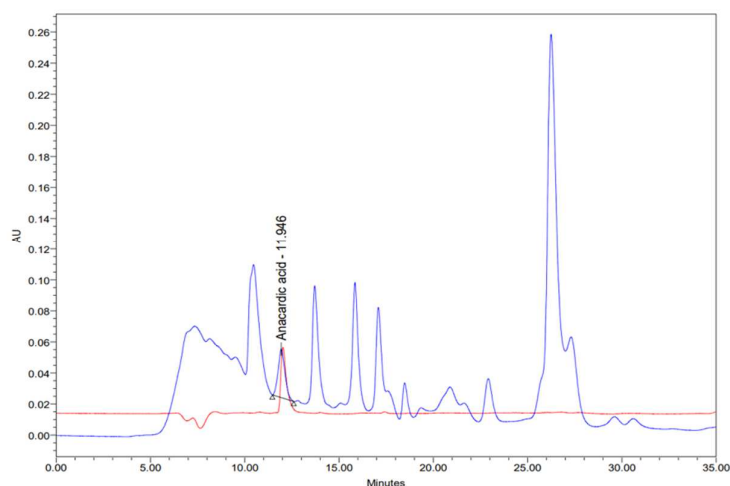
Fig. 35. GC-FID analysis of *A. occidentale* leaves hydro-alcoholic extract showing the presence of anacardic acid. A- Anacardic acid standard, B- *A. occidentale* leaves hydro-alcoholic extract.

28. HPLC analysis of *A. occidentale* L. leaves hydro-alcoholic extract

When the standard anacardic acid was run in the HPLC a single peak at 11.946 RT was observed (Fig. 37A). When the *A. occidentale* L. leaves hydro-alcoholic extract was subjected to HPLC, the profile showed the presence of other compounds in addition to anacardic acid at 11.946 RT (Fig. 37B). However, the profile showed that amount of anacardic acid present in the extract small.



Peak Name	RT	Area	% Area	Height
Anacardic acid	11.946	779670	100.00	31234

B

Peak Name	RT	Area	% Area	Height
Anacardic acid	11.946	908863	100.00	42083

Fig. 36. HPLC analysis of *A. occidentale* L. leaves hydro-alcoholic extract showing the presence of anacardic acid.

A- standard anacardic acid standard.

B- Red line – Anacardic acid standard, Blue line - *A. occidentale* L. leaves hydro-alcoholic extract, and RT of anacardic acid-11.946.

29. Molecular homology modeling, structure validation and Docking

On the I-TASSER server, homology modelling of *BmHK* was carried out. *BmHK* has ATP, glucose, and hexokinase binding activities and is composed of 572 amino acids. There were two sub-domains identified in the tertiary structure of *BmHK*: the small (105-237) and big (238-481) sub-domains. Four beta sheets and two beta sheets combined with two alpha helices make up the tiny domain, whereas four beta sheets combined with eight alpha helices were discovered in the large sub-domain. Furthermore, because it plays a dual role in glycolysis and sugar sensing for crucial metabolic and physiological processes, two domains for glucose 6 phosphate (G6P-DLGGTNLRV & IIATG), three domains for glucose (GLU-GFTFSYP, WTKGF & INDTVIG), and two domains for adenosine tri phosphate (ATP-DGSTYKYHPFF & GSGKGAALITA) binding were also discovered (Fig. 38).

The four ligands in the extract viz., Anacardic acid, salicylic acid, methyl salicylate, and palmitic acid were found to bind to the enzyme via molecular docking of these molecules with *BmHK*. Table 11 displays the Docking, MM-GBSA and E-model scores for these four compounds.

The four chemical ligands that the amino acids interact with are as follows: palmitic acid with ASN 238; methyl salicylate with ASN 238 and LYS 203; salicylic acid with ASN 238, LYS 203, ASP 239, and ASN 266; and anacardic acid with ASN 238 (Fig. 39). The ligand interacting amino acids of *BmHK* with anacardic acid (-5.789, -41.885, -43.411 and 2), salicylic acid (-5.219, -45.487, -38.146 and 4), methyl salicylate (-5.898, -32.299, -28.36 and 2), and palmitic acid (-3.095, -38.148, -41.83 and 2), respectively, are the docking score, Emodel score, MMGBSA score, and *BmHK*. Thus, the findings imply that these chemicals may be able to block hexokinase.

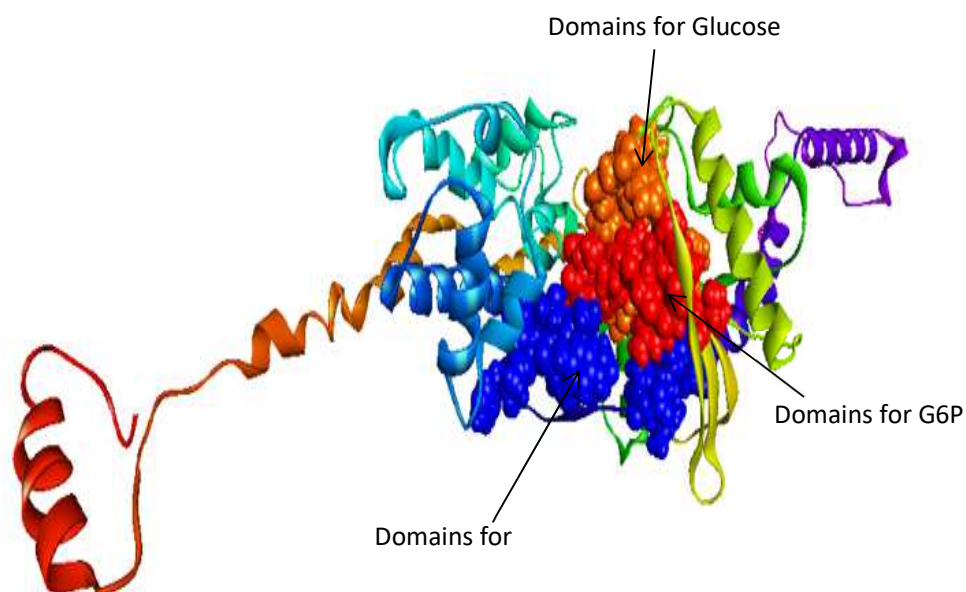


Fig. 37. Tertiary structure Hexokinase of *Brugia malayi* (BmHK), modelled to check interaction with compounds in the herbal extract.

Table 9. Docking score, Emodel score, MMGBSA score and ligand interacting amino acids of *BmHK* with anacardic acid, salicylic acid, methyl salicylate, and palmitic acid.

Name	Interacting amino acids	Docking score	Emodel score	MMGBSA score	HBonds
Anacardic acid	ASN 238	-5.789	-41.885	-43.411	2
Salicylic acid	ASN 238 LYS 203 ASP 239 ASN 266	-5.219	-45.487	-38.146	4
Methyl salicylate	ASN 238 LYS 203	-5.898	-32.299	-28.36	2
Palmitic acid	ASN 238	-3.095	-38.148	-41.83	2

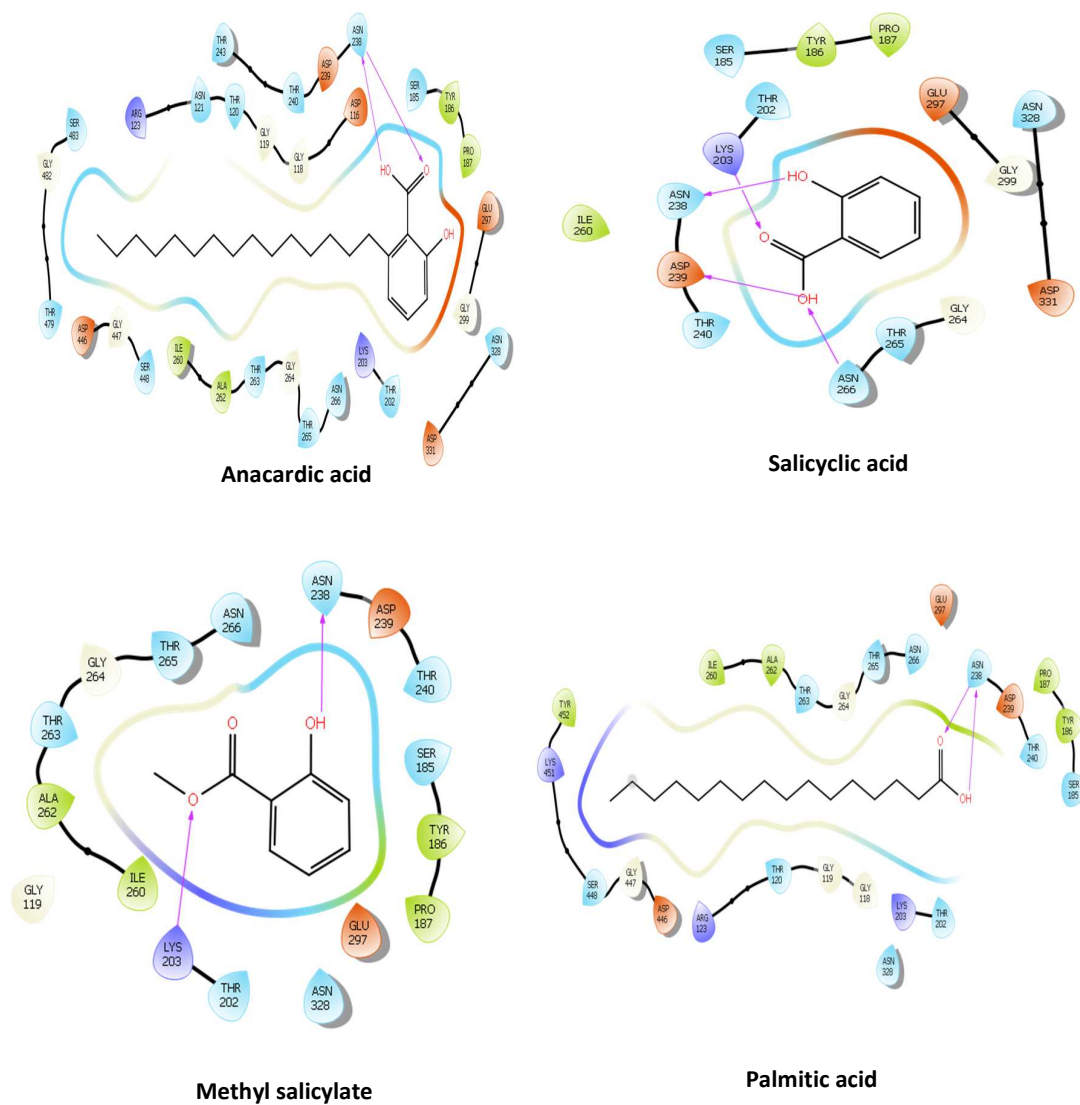


Fig. 38. Docking of the ligands with amino acid residues of *BmHK*. MM - GBSA, and E -model scores of anacardic acid, salicylic acid, methyl salicylate and palmitic acid.

6. DISCUSSION

Clinical malaria cases have been treated with a variety of antimalarial medications, including artemether, artesunate, arteether, amodiaquine, quinine, proguanil, sulfadoxine and pyrimethamine, primaquine, and mefloquine, which target different stages of the malaria parasite. However, malarial parasites have become resistant to a number of these medications, leaving arteether as the only available treatment at the moment. The compound artemisinin, which was first found in Chinese herb *Artemisia annua*, first showed promise as a plant source for chemotherapeutics. Currently, artemisinin-based combination therapies (ACT) are used in endemic places throughout the world.

Over the past ten years, unprecedented progress has been made in reducing malaria morbidity and mortality [118]. Nevertheless, the management of malaria and its long-term prospects for eradication have been severely harmed by the parasites' developing resistance to ACTs and insecticides utilised in the control mosquito vectors [119].

P. falciparum has grown resistant to the majority of antimalarial medications now in use as well as the current ACT [120, 121]. The development of drug resistance appears to be mostly caused by genetic drug influences, cross-resistance, parasite selection, and heavy drug use. Given the rise in treatment resistance, it is concerning that pharmaceutical corporations are not interested in creating medications to treat malaria [122].

There aren't many medications in different stages of testing among the limited pool of potential antimalarial medications to treat malaria. To strengthen the malaria control programme, it is therefore imperative to create medications that target malaria parasites. Traditional medicine has employed herbal remedies to treat a variety of

illnesses, including malaria. Investigating herbal sources might produce novel anti-malarial medications, and a focused strategy can speed up the discovery process.

Consequently, we tried a method that comprised using an herbal source and a pharmacological target in the malarial parasite to find a therapeutic lead or drugs for malaria. We looked into the possibility of employing *A. occidentale* leaves as a source of anti-malarial medication or pharmaceuticals, focusing on the parasite transketolase enzyme.

Since *P. falciparum* transketolase has been shown to be essential to the survival and reproduction of malaria parasites, we selected it as our target [123].

It is an essential enzyme in the non-oxidative portion of the pentose phosphate pathway (PPP), a metabolic system that generates reducing agents like NADPH and the pentose sugar needed for nucleic acid synthesis. It also bears minimal resemblance to the homologue of the human host, making it a prime candidate for the creation of anti-malarial medications. Dihydroorotate dehydrogenase (DHOD), cytochrome b (CYTb), myosin a tail domain-interacting protein (MTIP), and dihydrofolate reductase (DHFR) are significant *P. falciparum* pharmacological targets that have been exploited to identify possible treatment options.

Notably, cladospurin and halofuginone, two strong natural antimalarial compounds, were identified with the help of three tRNA synthetases [124]. Because of its purported therapeutic benefits and usage in traditional medicine, *A. occidentale* (cashew) leaves have been the subject of our investigation here [125]. Unlike other studies that looked at fruit, bark, and other plant parts, ours has focused on the antimalarial compounds present in leaves as they are readily available in large quantities, renewable, and usually thrown away as waste.

The idea of "green chemistry" states that it is preferable to use a cheap and plentiful source of raw materials, particularly natural waste products, as this guarantees environmental friendliness. For a very long time, folk medicine has employed cashew leaf or nutshell extracts to treat inflammation as well as ailments like cancer, ulcers, and asthma.

Using the recombinant *Pf*Tk enzyme as a target, we initially screened extracts/fractions of *A. occidentale* leaves for inhibitory activity in this work. Out of the six extracts prepared using different solvents, the hydroalcoholic extract was found to have the highest level of enzyme inhibition activity. As a result, an in vitro assay based on fluorescence was employed to assess the extract against the late-ring and early trophozoite stages of the *P. falciparum* 3D7 strain. The outcomes showed that the extract had considerable activity in terms of IC₅₀ (4.17±0.22 µg/ml) and growth inhibition (99.31±0.08% at 50 µg/ml).

The percentage inhibitory activity is greater than that of 80% ethanol extracts of various Tanzanian plants and *Berasama abyssinica* extract, which produced a maximum of 86.67±11.32% activity when evaluated at 100 µg/ml against *P. falciparum* Dd2 strain [126]. An ethanol extract of *B. abyssinica* leaves was demonstrated to have good in vitro anti-plasmodial effect [128], although a methanolic extract of the same plant's stem bark showed an IC₅₀ of 11.00 µg/ml [127]. With an IC₅₀ of 2.5 µg/ml, *maesa lanceolata* chloroform extract was found to have a very potent anti-plasmodial effect against clinical isolates of *P. falciparum*[129].

Plant extracts' anti-plasmodial properties can differ according on the type of plant, its components, its location, etc. Traditional medicine has employed decoctions, infusions, bark, and leaves as anti-malarial remedies [130]. Many tribes in Bankura,

West Bengal, India, use a variety of medicinal herbs, including *A. occidentale* L., to treat malaria [131]. However, Lima et al. showed that *A. occidentale* L. extracts were ineffective in vitro against *P. falciparum* [130].

Although Lemma et al. [132] classified the herbs as very good, good, moderate, and inactive if the IC₅₀ values were 1–5 µg/ml and >5 µg/ml, respectively, the hydroalcoholic extract of *A. occidentale* leaves demonstrated anti-malarial action against *P. falciparum* in vitro in the current investigation. Based on the IC₅₀, its activity can be considered moderate.

The hydroalcoholic extract of *A. occidentale* leaves did not affect zebra fish egg hatching, somite development, heartbeat, or tail detachment in an acute toxicity test. This implies the safety of the extract used in this animal model. Similarly, at doses ranging from 50–2000 mg/kg body weight, the extract was shown to be safe in BALB/c mice, with no changes in behaviour or physical activity seen. The lung of the mice treated with extract exhibited congestion at a relatively high dose (2000 mg/kg), but otherwise the primary organs showed no changes in histology.

TNF- α and IL-1 are reportedly downregulated by the ethanolic extract of *A. occidentale* stem bark, which controls the inflammatory response [133]. In this work, mice administered an extract at a dose of 2000 gm/kg body weight, which may have resulted in significant immunomodulation and lung congestion. However, it should be noted that studies have demonstrated the safety of *A. occidentale* leaf hydroalcoholic extract at doses as high as 300 mg/kg.

Testing on this extract indicated that two of the fractions (AO2 & AO6) demonstrated very promising antimalarial activity against blood stage parasites. The extract was fractionated utilising a bioactivity-guided technique. Alkyl-phenols, which have a range of biological roles, are abundant in *A. occidentale* L., as was

previously stated [15]. Further investigation is required to identify the compound responsible for the antimalarial effect seen in *A. occidentale* leaves as well as the mechanism directing the suppression of PfTK enzyme activity.

Finding the components in the extracts that have anti-malarial activity will aid in the creation of appropriate formulations for phytopharmaceuticals, which can subsequently be used in preclinical and clinical research and, in the long run, in clinical practice via both allopathic and traditional routes (like Ayurveda or Siddha).

A global eradication project was initiated in 2000 (WHO 2023) [134] to fulfil the 2020 target for the elimination of LF, is an urgent public health hazard in tropical nations. The method used for eradicating LF involves administering diethylcarbamazine and albendazole in six rounds of Mass Drug Administration (MDA) to the endemic populations. Even after more than six rounds of MDA were implemented, hotspots for the spread of infections continue to exist in a number of nations [135,136].

Drug resistance by the parasite, operational concerns, and challenges with drug coverage and intake are also potential causes. Of the two drugs that were administered, DEC's microfilaricidal rather than macrofilaricidal activity will cause adult parasites to continue producing microfilariae, which will sustain the transmission [137]. Veterinarian parasites have been known to become resistant to albendazole, and filarial parasites in hotspots might have done the same.

Additionally, these drug types have severe adverse responses and low intestinal absorption, which restricts their use [138]. More effective medications are needed in this condition, particularly macrofilaricidal ones.

Because there aren't any such medications on the market at the moment, research is being done to find and create macrofilaricidal medications that are both safe and effective.

Herbal-based treatment instruments have recently attracted fresh interest due to their high level of safety margin and bio-efficacy. Using a target-based methodology, we investigated the possible anti-filarial activity of (A) *occidentale* leaf extracts in this work. The hexokinase of (B) *malayi* has been chosen as the study's target. The glycolytic process is started by hexokinases (HK) [139]. Because they are essential components for trematode survival, enzymes from this pathway, like phosphoglycerate kinase [140] and enolase [141], have been investigated for use in vaccine and medication development.

The diseases *Clonorchis sinensis* [139], *Hhaemonchus contortus* [142], and *Schistosoma mansoni* [143] offer well-characterized prospective targets for vaccine and treatment development. A prior study [22] determined that *B. malayi*'s hexokinase (BmHk) was a viable target for drug discovery. Due to the fact that parasitic worms predominantly rely on this pathway for energy metabolism, the participation of hexokinase in the glycolysis route presents a significant target for the creation of anthelmintic medications. BmHk shows little identity when it comes to 50 kDa hexokinase IV and 100 kDa mammalian hexokinase I, II, III.

According to Singh et al. (2008) [23], BmHK showed homology with human hexokinases, *Haemonchus contortus*, and *Caenorhabditis elegans* of 59,43, and 37%, respectively, on BLAST analysis.

The sequence homology and kinetic differences between BmHk and the host enzymes suggest that BmHk would be a good target for the creation of novel herbal drugs that combat filarial parasites, according to [22]. Notably, previous studies have

shown that BmHk is expressed at critical chemotherapeutic life stages, including adult parasites and microfilariae [23].

Glutathione S-Transferase (GST) in *Seteria digitata* was examined by Mathew et al. (2011) [144] as a possible target for antifilarial medication development. On the other hand, Matutino et al. (2019) [145] discussed how *A. occidentale* chemicals could be used in target-based drug development to potentially create anti-*Trypanosoma cruzi* medications and sirtuin inhibitors.

Plants from thirteen families were investigated for their effectiveness against filarial parasite known as *Onchocerca* sp. [22]. The major active species were, *A. occidentale*, *Euphorbia hirta* and *Acacia nilotica*, with LC₅₀ values of 2.76, 6.25 and 1.2 µg/mL. *A. occidentale* was selected for this study and its inhibitory potential against BmHk was examined based on a review of the literature and its historical use. This enzyme already cloned as a recombinant enzyme [23].

Of the several extracts that were assessed, the hydroalcoholic extract exhibited the high inhibitory action against BmHk. A phytochemical analysis carried out by other researchers has identified several compounds present in the *A. occidentale* leaf extract [146]. In the current study's early phytochemical investigation, the extract tested positive for saponins, amino acids, tannins, alkaloids, terpenoids, and glycosides. More tannins and flavonoids than alkaloids and terpenoids were discovered. Anacardic acid was detected in the extract by GC-FID analysis.

Initially, this naturally occurring material was separated from cashew nut shells. A double bond chain was found at position eight in the components that are diene, monoene and triene. It has a side chain which is long at position six and a helpful salicylic acid [147].

In the current study, anacardic acid was found in leaves, albeit in less quantities. An abundance of research has been conducted on anacardic acid because of its numerous biological effects, which include its antifungal, anti-parasitic, antimicrobial, anti-tumoral, gastroprotective, and insectidal properties [148,149,150] as well as its inhibition of enzymes such as lipoxygenase [151], histone acetyltransferases and tyrosinase [152].

Anacardic acid reportedly demonstrates superior anti-protoscoleces activity in vitro compared to dihydroartemisinin and albendazole against *Echinococcus granulosus* protoscoleces and metacestodes of *E. multilocularis*. Additionally, it has no observable toxicity and efficiently prevents the latter's growth in vivo at a level similar to that of albendazole.

It was found to mainly suppress angiogenesis in *E. multilocularis* metacestodes via reducing VEGF-induced Src/FAK and Akt/PKB expression. A dose of 300 mg/kg was found to be risk free and safe in BALB/c mice [24]. HepG2 cells and Chang liver cells were used to establish the safety of anacardic acid in vitro, with IC₅₀ values of 49.0±8.0 µM and 70.0±3.0 µM respectively. The compound's substantial safety margin is indicated by these findings, which are significantly high than the EC₅₀ value on *E. protoscoleces* (1.9±2 µM) [153]. Recent research has shown that anacardic acid has excellent anti-dengue action [154].

Contrary to Cameroonian traditional medicine, research utilising hexane *extract of A. occidentale* leaf showed that the adverse effects at higher concentrations [155]. Alkyl-phenols from *A. occidentale*, namely 2-methylcardol diene and cardol diene, were shown to be effective in vitro against adult *Schistosoma mansoni* worms, with corresponding selectivity indices of 6.1 and 21.2 and LC₅₀ values of 32.2 and 14.5 µM [15]. This is an interesting finding. This study evaluated the hydroalcoholic

extract against *BmHk* enzyme and found that it exhibited promising antienzyme action.

The BOILED-Egg model indicates that anacardic acid (AA) derivatives have a high probability of being absorbed passively by the Gastrointestinal tract. The percentage of anacardic acid derivatives absorbed by human digestive system was calculated using the pkCSM web server application. This study demonstrated that all AA derivatives, with the exception of two (AA99 and AA100), were well absorbed via the digestive tract in humans. Moreover, phenols have been the subject of clinical investigation for almost 20 years [156].

In zebrafish embryos, which are currently utilised as models to examine acute toxicity of drugs, we conducted a toxicity examination on *A. occidentale* hydroalcoholic leaf extract and found that the treatment was safe. Furthermore, using the MTT assay to assess the extract's acute oral toxicity in BALB/c mice, we found that it was safe for up to 2000 mg/kg body weight [157]. This finding supports the safety of the extract. *A. occidentale* leaf extract tested for cytotoxicity by treating RAW 264.7 cells at doses ranging from 0.625 to 10 mg/mL. At a concentration of 2.5 mg/mL, the extract showed more than 90% cell viability after a 24-hour treatment.

7. CONCLUSION

The hydroalcoholic extract of *A. occidentale* leaves has shown strong antiplasmodial activity against *Plasmodium falciparum*, with a targeted inhibitory effect on the enzyme transketolase in in vitro studies. In vitro experiments revealed significant activity of both the extract and its two fractions against *P. falciparum*. Mice and Zebra fish embryos used in early toxicity tests revealed that the extract was not harmful. Consequently, it makes sense to investigate *A. occidentale*'s potential as a natural source of anti-malarial medication due to its anti-malarial properties and the fact that it can be collected in great numbers year-round.

Collectively, the results of this study suggest that the hydroalcoholic extract from *A. occidentale* leaves may have anti-*Bm*HK activity. *Bm*HK is the main enzyme in the glycolytic pathway that the worms rely on. In light of this, further investigation is necessary to ascertain the hydroalcoholic leaves extract of *A. occidentale*'s potential for the development of antifilarial drug(s).

LIMITATION

Although initial studies have demonstrated promising antimalarial and antifilarial activities, the precise bioactive compounds responsible for these effects remain unidentified. To address this gap, further research is essential to isolate, characterize, and elucidate the chemical structures of these compounds. Advanced analytical techniques such as HPLC, GC-MS, and NMR can be employed for compound identification and purification. Moreover, bioassay-guided fractionation will be crucial to pinpoint the active fractions. These studies will not only advance our understanding of the underlying mechanisms of action but also pave the way for the development of novel therapeutic agents targeting malaria and filariasis.

8. SUMMARY

The present study set out to identify herbal based drug leads for the two major vector borne diseases viz., malaria and lymphatic filariasis, with the former inflicting significant mortality and the latter causing lifelong disability among millions of people globally. Exploring the antimalarial and anti-filarial potential of plant extracts derived from the leaves of *A. occidentale* L., (Anacardiaceae) a plant that has long been used to treat malaria, was the goal of the current study. Different leaves extracts of *A. occidentale* L. were prepared and their *in-vitro* inhibitory activity against the target enzyme, rPfTK was assessed. Additionally, the growth inhibitory efficiency against established blood stage *P. falciparum* parasites (3D7 strain) was examined using SYBR Green fluorescence-based *in-vitro* assays. Mice and zebrafish embryos models were used to test the acute toxicity of different doses of hydro-alcoholic extract of leaves of *A. occidentale* L.

With an IC₅₀ of 4.17±0.22 µg/ml, the hydro-alcoholic extract of leaves of *A. occidentale* L showed 75.45±0.35% inhibitory effect against the recombinant PfTK and 99.31±0.08% growth inhibition against *P. falciparum* intra-erythrocytic stages at the maximum dose (50 µg/ml). The results of the toxicity test showed that there was no influence on the heartbeat, somite formation, tail detachment, or hatching of Zebra fish embryos when tested at 0.1 to 10 µg/ml of the extract. On the other hand, at higher dosages of the extract, the fish embryos did not exhibit heartbeat at 48 hours (1000 µg/ml) and 96 hours (100 µg/ml and 1000 µg/ml respectively). In acute oral toxicity tests, mice treated with the extract showed no toxicity up to 300 mg/kg body weight.

For filariasis, the majority of therapies that are now in use are microfilaricidal, meaning that the adult parasites are not entirely eliminated, allowing them to continue producing microfilariae. Therefore, this study aimed to explore for macrofilaricidal drug of plant origin. The inhibitory effect of leaves extracts of *A. occidentale* L. against *BmHK*, the selected target enzyme of *B. malayi* was studied. The target *BmHK* is a vital enzyme of the glycolytic pathway and essential for the survival and replication of filarial parasites. The extract exhibited significant inhibitory activity against *BmHK*. Compared to other extracts of *A. occidentale* L., which showed less than 50% inhibition, hydroalcoholic extract exhibited the greatest degree of inhibition ($65.80 \pm 1.60\%$) of *BmHk* activity.

The extract was subjected to GC-FID analysis, HPLC and a preliminary phytochemical test, and one chemical compound, anacradic acid, which is reported to have anti-parasitic activity, was detected.

Thus, the anti-malarial and anti-filarial activity of *A. occidentale* L. leaves extracts was indicated by the tests against *PfTK* and *BmHK* and its safety in Zebra fish and Balb/c animal models was evident. Further, work on its anti-parasitic activity against the two parasites, both *in-vitro* and *in-vivo*, will throw more light on its potential as a source of anti-parasitic drug. Also, identification of bioactive compounds from the *A. occidentale* L. hydro-alcoholic leaves extracts will paving way for development of a new drug molecule of herbal origin for lymphatic filariasis.

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ANNEXURE - A

Animal Ethical Approval certificate

- Ethical approval for animal studies

This animal experiments were performed in the present study after receiving the ethical clearance from Institutional Animal Ethics Committee (IAEC) of ICMR-NITM Belagavi; resolution number IAEC/ICMR-NTM BGM/2018/1, Reg. No. (Reg no:1388/GO/Re/S/10/CPCSEA) dated 21-07-2018.



ICMR-National Institute of Traditional Medicine
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Nehru Nagar, Belgaum - 590 010

Institutional Animal Ethics Committee
Reg. No. 1388/GO/Re/S/10/CPCSEA
Resolution No. IAEC/ICMR-NTM BGM/2018/1

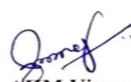
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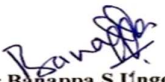
CERTIFICATE

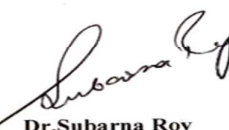
This is to certify that the project proposed by Dr. S L Hoti entitled "Screening & development of anti-malarial drugs of herbal origin through target based drug development approach" has been approved by the IAEC. The approval granted for:

Species	Gender	Number
Mice (Balb/C)	Female	99

The Investigator must report the outcome of the experiment to IAEC/CPCSEA. The approved number/ species/ gender of animals should not be changed without prior permission of CPCSEA/IAEC.


Dr. AHM Viswanath Swamy
CPCSEA Main Nominee


Dr. Banappa S Unger
Member Secretary


Dr. Subarna Roy
Chairperson

ANNEXURE - B

PUBLICATIONS

1. Antimalarial Activity of *Anacardium occidentale* Leaf Extracts Against *Plasmodium falciparum* Transketolase (PfTK)

Doi.org/10.1007/s11686-023-00718-6

2. Assessment of the inhibitory activity of hydroalcoholic extract from *Anacardium occidentale* L. leaves against *Brugia malayi* hexokinase, a promising target for the development of anti-filarial drug

Doi.org/10.1007/s42535-024-00837-z



Antimalarial Activity of *Anacardium occidentale* Leaf Extracts Against *Plasmodium falciparum* Transketolase (PfTK)

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Abstract

Background As per estimates by WHO in 2021 almost half of the world's population was at risk of malaria and > 0.6 million deaths were attributed to malaria. Therefore, the present study was aimed to explore the antimalarial activity of extracts derived from the leaves of the plant *Anacardium occidentale* L., which has been used traditionally for the treatment of malaria. Different extracts of *A. occidentale* leaves were prepared and tested for their inhibitory activity against recombinant *P. falciparum* transketolase (rPfTK) enzyme, *in vitro*. Further, growth inhibitory activity against cultivated blood stage *P. falciparum* parasites (3D7 strain), was studied using SYBR Green fluorescence-based *in vitro* assays. Acute toxicity of the hydro alcoholic extracts of leaves of *A. occidentale* (HELA) at different concentrations was evaluated on mice and Zebra fish embryos. HELA showed $75.45 \pm 0.35\%$ inhibitory activity against the recombinant PfTK and $99.31 \pm 0.08\%$ growth inhibition against intra-erythrocytic stages of *P. falciparum* at the maximum concentration (50 µg/ml) with IC_{50} of 4.17 ± 0.22 µg/ml. The toxicity test results showed that the heartbeat, somite formation, tail detachment and hatching of embryos were not affected when Zebra fish embryos were treated with 0.1 to 10 µg/ml of the extract. However, at higher concentrations of the extract, at 48 h (1000 µg/ml) and 96 h (100 µg/ml and 1000 µg/ml, respectively) there was no heartbeat in the fish embryos. In the acute oral toxicity tests performed on mice, the extract showed no toxicity up to 300 mg/kg body weight in mice.

Conclusion The hydro-alcoholic extract of leaves of *A. occidentale* L. showed potent antimalarial activity against blood stage *P. falciparum*. Based on the observed inhibitory activity on the transketolase enzyme of *P. falciparum* it is likely that this enzyme is the target for the development of bioactive molecules present in the plant extracts. The promising anti-malarial activity of purified compounds from leaves of *A. occidentale* needs to be further explored for development of new anti-malarial therapy.

Keywords Antimalarial activity · *Anacardium occidentale* L. · *Plasmodium falciparum* · Transketolase · Malaria

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Introduction

Malaria is a major public health problem caused by protozoan parasite belonging to *Plasmodium* spp. transmitted by the infective bite of female *Anopheles* mosquitoes. *Plasmodium falciparum* and *Plasmodium vivax* are mainly responsible for severe infection in humans. Almost half of the world's population is at risk of acquiring malaria and the total number of deaths due to malaria remains high at more than 0.6 million [1]. *P. falciparum* is the most prevalent malarial parasite in the African continent [2] and *P. vivax* is the dominant malaria parasite in most countries outside Sub-Saharan Africa, although it accounts for < 2% of global malaria cases. There has been a rising trend of *P. falciparum* malaria in India over the last five decades, and in 2021 an estimated 247 million people in India were affected by malaria, resulting in 619 000 deaths [1].

Many antimalarial drugs like artemisinin, chloroquine, sulfadoxine and pyrimethamine combination, quinine, atovaquone-proguanil combination (Malarone) and primaquine are available for clinical use, but parasites have developed resistance against these drugs, thereby rendering them ineffective against malaria [3]. Currently, there are no alternate options as effective anti-malarial drugs, other than artemisinin and its derivatives [4]. However, in recent years, treatment failures with artemisinin combination therapies (ACTs) have been reported from South-East Asian countries and genetic basis for resistance to artemisinin has now been established [5]. Occurrence of artemisinin resistance is also suspected in South America but confirmatory studies are still going on [1]. This scenario, therefore, calls for the development of newer drugs against malarial parasites to augment the malaria control and elimination programs.

The drug discovery process can be hastened by adopting a targeted approach, especially with the dawn of molecular and genomic approaches to study parasite encoded proteins which has evolved as a promising approach [6]. Certain enzymes of parasites, such as transketolase have been identified as target for drug development [7]. Transketolase of *P. falciparum* is involved in the non-oxidative arm of the pentose phosphate pathway (PPP), catalysing the production of pentose sugar needed for nucleic acid synthesis. Hence, PfkFB plays a very important role in the replication and survival of malaria parasites and therefore is a potential drug target. Further, it also has very low similarity with human transketolase thus making it a selective target for drug development.

Herbal preparations have been in traditional use for treating various infections, including malaria. Herbal treatments derived from *Anacardium occidentale* L. (the "cashew") are popular forms of traditional medicine in

some countries like Brazil, Colombia, Nigeria and Peru [8] and also in some tribal areas of India [9]. All parts of the cashew tree (especially leaf and stem bark) have been extensively used in traditional herbal medicine [8]. Thus, during the last few decades, medicinal properties of *A. occidentale* plant, have been extensively studied [10]. *A. occidentale* L. is well recognized as a source of alkyl-phenols, which are isolated from its fruit (the "cashew nuts"). These compounds have several biological activities such as antioxidant, mosquito larvicidal, anti-cancer, antibacterial, molluscicidal and schistosomicidal [11–15]. These compounds are also reported to have inhibitory activities against enzymes such as tyrosinase, acetylcholinesterase, glucosidase, aldose reductase, invertase, 15-lipoxygenase, and xanthine oxidase [16]. In the present study, we have explored the antimalarial activity of leaves extracts of *A. occidentale* L. for their inhibitory activity against transketolase enzyme and blood stage of *P. falciparum*. Also, we assessed the acute toxicity of the extract in Zebra fish and mice models.

Materials and Methods

Collection of Plant Material

The leaves of *A. occidentale* were collected from cashew grooves in Belagavi district (N 15.88668; E 74.52353; altitude ~800 m from sea level), Karnataka, India. The plant was identified at the ICMR-National Institute of Traditional Medicine (ICMR-NITM), Belagavi, and an herbarium specimen (RMRC-1356) has been deposited at the institute.

Preparation of the Leaf Extract and Preliminary Phytochemical Test

The leaves of *A. occidentale* were shade dried and powdered using an electric grinder. Cold maceration of the extract was carried out for 72 h using different solvents viz., petroleum ether, dichloromethane, chloroform, ethyl acetate, methanol, and hydro alcoholic solution (70:30 water: ethanol). Leaves were extracted thrice with each solvent, pooled and then filtered through Whatman filter paper No. 1. The solvent was evaporated using a rotary evaporator. The extracts obtained were stored at 4 °C until further used. Various qualitative phytochemical tests for alkaloids (Dragendorff's test), glycosides (Keller-kiliani test), amino acids (Ninhydrin test), saponins (Foam test), flavonoids (Lead acetate test), tannins (Ferric chloride test), and terpenoid (Salkowski test) were carried out [17].

Expression and Purification of *P. falciparum* Transketolase (PfTk)

The recombinant PfTk was purified as described earlier [7]. Rosetta cells BL21(DE3) harboring the recombinant plasmid with transketolase gene insert were grown in Luria Bertani media (LB) at 37 °C, supplemented with Ampicillin (100 µg/ml) and Chloramphenicol (34 µg/ml), till OD 600 nm reached 0.6. The recombinant protein was induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). Further, the culture was grown at 18 °C on a shaker incubator for 20 h and the cells were harvested by centrifugation at 11,000 g for 5 min. The cell pellet was suspended in lysis buffer (50 mM NaH₂PO₄, pH 8.0), 300 mM NaCl, 10 mM imidazole, and 10% (v/v) glycerol) containing protease inhibitor cocktail (Sigma, USA) and the cells were lysed using ultra-sonication (Ultrasonic processor, Model-XL-2020, Germany). The lysate was centrifuged at 11,000 g at 4 °C for 30 min and the supernatant obtained was mixed with Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Germany) resin equilibrated with the lysis buffer. After incubation for 1 h at 4 °C, the resin was separated from the cell lysate by centrifugation (5600 g for 5 min at 4 °C) and washed with washing buffer with similar composition to the lysis buffer but containing 20 mM imidazole. The recombinant PfTK enzyme was then eluted with elution buffer containing 250 mM imidazole. The elute containing PfTK was further purified by ammonium sulfate precipitation (0–40% w/v). The protein pellet was resuspended in storage buffer (50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10% (v/v) glycerol) and protein concentration was determined by Lowry's method [18]. The purity of the protein was checked by SDS-PAGE [19] and western blotting using anti-His tag antibodies.

PfTKassay

The activity of the purified transketolase was determined by measuring the oxidation rate of carbanion intermediate in presence of ferricyanide [20]. The 1.0 ml reaction mix contained 50 mM glycyl-glycine buffer (pH 7.6), 2 mM magnesium chloride, 0.1 mM thiamine pyrophosphate, 0.5 mM potassium ferricyanide, 3 mM fructose-6-phosphates (F6P)/hydroxypyruvate (HP), 0.24 mg enzyme and different concentration of *A. occidentale* L. leaf extracts. The reaction was started by adding PfTk enzyme and extract, and then the ferricyanide reduction was monitored at 420 nm using a UV-1601 PC spectrophotometer (Shimadzu, Japan).

Cultivation of *P. falciparum* and Testing of Antimalarial Activity of Extracts

The culture of *P. falciparum* (3D7 strain) was propagated by the method of Trager and Jensen [21]. The parasites were

cultured in optimal conditions of 5% CO₂ at 37 °C, and maintained in RPMI 1640 supplemented with 0.5% Albumax II (Gibco™ Albumax™), 100 µM hypoxanthine, 0.15% sodium bicarbonate, 25 mM HEPES buffer, 2 mM glutamine and 50 µg/ml gentamicin. The culture haematocrit was maintained at 2% using O+ve RBCs and in routine cultures the parasitaemia was monitored by Giemsa-stained thin blood smears and maintained at 2%–5%. Parasites were synchronized using 5% sorbitol to enrich for ring stage parasites before carrying out inhibition assays.

To test the anti-malarial activity of the extracts, inhibition assays were set up in 96 well plates with 200 µl of culture per well at ~2% parasitaemia. First the extracts were tested at 50 µg/ml concentration to identify those that inhibited malaria parasite growth along with the antimalarial drug chloroquine. Subsequently, for the extracts showing > 50% growth inhibition, a dose response assay was run with the starting concentration of the extract at 50 µg/ml and ending at ~50 ng/ml in a twofold dilution series. All assays were run in triplicates. After 48 h of incubation in optimal growth conditions, the plates were developed by adding 25 µl of SYBR Green I dye containing 10X lysis buffer. The plates were incubated for 1 h in dark and the fluorescence was measured using a multi-well plate reader with 485 nm and 530 nm as excitation and emission wavelengths.

Zebrafish Embryo Acute Toxicity

Zebrafish (*Danio rerio*), a member of the Cyprinidae family, originating from South Asia has been used as a model organism for biological research since the 1930s. Zebrafish are easy to breed and inexpensive to maintain and its embryos develop rapidly (5 days post-fertilization). Because the embryo is transparent, morphological structures and internal organs, including brain, eyes, heart, liver, and kidney can be easily visualized using light microscopy, without the need for surgery. Organ-specific and overall developmental toxicity can be assessed visually or quantified using dyes. Because of its small size, a single embryo can be maintained in fluid volumes of 100 µl in individual wells of microtiter plates and compound screening can be completed in a few days. Zebrafish are more closely related to humans and share many biological traits, genes, developmental processes, anatomy, physiology, and behavior. Its genome is ~50% of the mouse genome and has extensive synteny with mammalian genomes. Further, its genes are ~75% homologous to human genes on average and its orthologs for some genes that are known to play key roles in human diseases have been identified. These features make the zebrafish a unique vertebrate model for high-throughput chemical screening, which is useful for preclinical drug discovery and toxicological evaluation. A number of recent guidelines from the Organisation of Economic Co-operation and Development

(OECD; Guidelines 203, 210, and 212; OECD, 1992a,c, 1998) recommended zebrafish for aquatic toxicity testing (For more information please refer [22]).

We used this model for acute toxicity testing of extracts of *A. occidentale* leaves. Adult zebra fish, obtained from the colony of ICMR-NITM were reared in 3 L housing tanks (10–15) with a light/dark cycle of 14/10 h at 28.5 °C. A newly fertilized embryo was placed in each well of a 24 well tissue culture plate (Corning costar TC) containing 2 ml sterile distilled water per well. Five different concentrations of the leaves extract of *A. occidentale* were made in the non-toxic volume of dimethyl sulfoxide (DMSO) made up to 100 ml of water. DMSO was used for dissolving the *A. occidentale* leaf extract. Three replicates of each treatment were run along with a positive control (3,4-dichloroaniline at 4 mg/L). Each plate contained four wells as internal control with water. Solvent control was parallelly maintained. Observations were recorded for lethality indicators. As per the recommendation of the Organization for Economic Co-operation and Development guidelines (OECD Test guidelines 236), four different parameters i.e., coagulation of fertilized eggs, lack of somite formation, lack of detachment of tail bud from the yolk sac, and lack of heartbeat were recorded up to 96 h.

Acute Oral Toxicity Study in Mice

The safety of the *A. occidentale* leaf extract was assessed through acute oral toxicity in BALB/c mice, after obtaining the approval by the Institutional Animal Ethics Committee (IAEC/ICMR-NTM BGM/2018/1) of the ICMR-NITM. The toxicity test was carried out following the OECD test guidelines No. 423. The animals were obtained from the ICMR-National Institute of Nutrition, Hyderabad, India, and maintained at the ICMR-NITM animal house facility. The mice were divided into 4 groups and each consisting of six mice (female). Three groups were overnight fasted (12 h) after which they were treated orally with different doses of the extracts and designated as test groups. The extracts and fractions were tested at the doses of 50, 300, 2000 mg/kg body weight (bw) and the 4th control group received vehicle (water) treatment. Thereafter, the animals were observed at hourly intervals for any change in general physical activities and behavior (changes in skin fur and eyes, and lethargy, sleep, activity/over activity), as also mortality if any, within 24 h and then daily for the next 14 days. At the end of the study, animals were sacrificed after anaesthetizing with

Ketamine and major organs (heart, brain, lung, liver, and kidney) were isolated, their weight was recorded and the tissues were used for histopathology analysis. At the end of the study, animals were sacrificed humanly using ketamin injection and the brain, liver, kidney and lungs were preserved and held in 10% formalin for 2 days. Organs were dehydrated using different concentrations of the alcohol (70, 80, 90%, and absolute alcohol subsequently) for 12 h each. The tissues were cleaned using Xylene solution for 15–20 min and embedded in paraffin blocks. Five-micron sections of the tissue blocks were cut using microtome unit. The sections were placed on a microscopic slide and after drying they were stained with eosin and hematoxylin dyes. The stained sections were examined for infiltration of immune cells, inflammation and congestion if any, under a microscope.

Results

Yield of Extracts of Leaves of *A. occidentale* and Preliminary Phytochemical Analysis

Leaves of *A. occidentale* L. were processed and extracted with various solvents. The yield of extracts (% w/w) with various solvents viz., petroleum ether, dichloromethane, chloroform, ethyl acetate, methanol and hydroalcoholic (70: 30 water: ethanol) solvents was 5.2%; 9.20%; 13.00%; 12.50%; 22.25% and 17.32%, respectively. Preliminary phytochemical analysis was carried out for the hydro alcoholic extract, since it showed highest inhibition activity against the recombinant transketolase of *P. falciparum* (as given below). The analysis revealed the presence of alkaloids, glycosides, flavonoids, tannins, and terpenoids at varying amounts (Table 1).

Inhibition of Recombinant Pfk Enzyme by *A. occidentale* Leaf Extracts

The cloning and expression of recombinant Pfk enzyme was reported in a previous study [7]. The recombinant Pfk, expressed as a His-tagged protein in *E. coli* (Rosetta cells BL21(DE3)), was purified using Ni-NTA column. The purified protein was analyzed by SDS PAGE to confirm its size as ~ 70 kDa (Fig. 1) and used in inhibition assays for testing the inhibitory activity of different extracts. The hydroalcoholic extract (75.45 ± 0.35%) showed highest inhibitory activity against the enzyme, while chloroform,

Table 1 The phytoconstituents of *A. occidentale* leaf hydroalcoholic extract

Phytoconstituent	Alkaloids	Glycosides	Amino acids	Saponins	Flavonoids	Tannins	Terpenoids
Quantity*	++	+	–	–	+++	+++	++

* + + + High, + + average, + less, and – not present

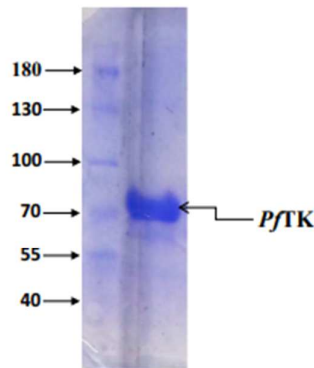


Fig. 1 The SDS-polyacrylamide gel of the Ni-NTA purified recombinant *P. falciparum* transketolase (PfTK). Protein fractions were electrophoresed on a 10% (w/v) SDS polyacrylamide gel under reducing conditions and stained with Coomassie Brilliant Blue R250. Lane 1—Molecular weight marker (Thermo Scientific, range 10–250 kDa), Lane 2–6—purified PfTK

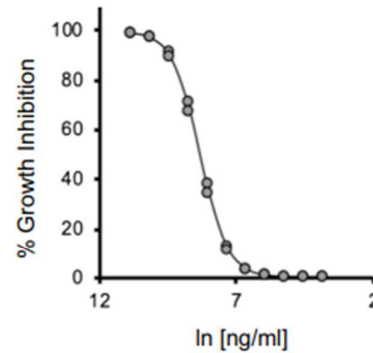


Fig. 2 Anti-malarial activity of hydroalcoholic extract of leaves of *A. occidentale*. The extracts were tested in a dose assay starting with a concentration of 50 µg/ml and going lower by twofold serial dilution. Data from representative assay with two replicates (both plotted separately) are shown. The growth inhibition obtained at 50 µg/ml concentration is $99.31 \pm 0.08\%$ with respect to control untreated *P. falciparum*. The IC_{50} obtained is 4.17 ± 0.22 µg/ml

methanol and petroleum ether extracts showed moderate levels of inhibitory activity ($53.00 \pm 0.80\%$, $52.85 \pm 1.85\%$ and $35.00 \pm 0.09\%$, respectively). The inhibitory activity of dichloro-methane ($22.00 \pm 1.50\%$) and ethyl acetate ($18.88 \pm 0.20\%$) extracts was very poor. Based on the inhibitory activity obtained, the hydroalcoholic extract was fractionated and the fractions were tested for activity against *P. falciparum* blood stage parasites *in vitro*.

Inhibition of *P. falciparum* Growth by Hydroalcoholic Extract of *A. occidentale* Leaf

To evaluate the antimalarial activity, the hydroalcoholic extract was tested at different concentrations (from 50 µg/ml to 50 ng/ml). At the highest concentration tested the inhibition was $99.31 \pm 0.08\%$ from the fit of the dose response inhibition data, the IC_{50} was estimated as 4.17 ± 0.22 µg/ml (Fig. 2). Among the fractions of the hydroalcoholic extract tested, six (AO1 to AO6) showed significant inhibition activity (Table 2). Two of the fractions, AO2 and AO6, showed the highest activity, with close to 100% inhibition of parasite growth at 30 µg/ml concentration. A dose response assay was carried out for all the extracts to find out the IC_{50} values. Interestingly, the fractions AO2 and AO6 showed potent antimalarial activity with the estimated IC_{50} values in the ng/ml range (Fig. 3).

Acute Toxicity Study of *A. occidentale* Leaves Hydroalcoholic Extract Against Zebra Fish Embryo

The Zebra fish embryo acute toxicity test was performed using different concentrations of the hydroalcoholic extract

of leaves of *A. occidentale* along with positive (3,4-dichloroaniline) and negative (water) controls. In positive control group there was no somite formation and tail detachment after 24 h of treatment, whereas in *A. occidentale* leaf extract treatment and water control groups normal heartbeat, somite formation, tail detachment and hatching of embryos was observed (Table 3). However, in fish embryos treated with 100 µg/ml of extract, there was no heartbeat at 96 h. In fish embryos treated with 1000 µg/ml of extract, heartbeat was absent after 48 h only. All the embryos in control group showed no mortality even after 96 h. The LC_{50} of the extract was determined as 9.60 µg/ml.

Acute Oral Toxicity of *A. occidentale* Leaves Hydroalcoholic Extract in Mice

The acute oral toxicity of the hydroalcoholic extract of leaves of *A. occidentale* was tested at doses 50, 300, and 2000 mg/

Table 2 Percent growth inhibition of late-ring and early trophozoite stage of *P. falciparum* strain 3D7 by the fractions of hydro-alcoholic extract of *A. occidentale* L. by fluorescence-based assay

Fraction	% growth inhibition at 30 µg/ml	IC_{50} (µg/ml)
AO1	89.30 ± 0.08	10.29 ± 1.67
AO2	99.97 ± 0.004	0.56 ± 0.03
AO3	54.30 ± 2.01	27.28 ± 1.17
AO4	66.27 ± 8.60	21.27 ± 3.87
AO5	84.56 ± 4.61	12.56 ± 2.25
AO6	99.96 ± 0.005	0.63 ± 0.03

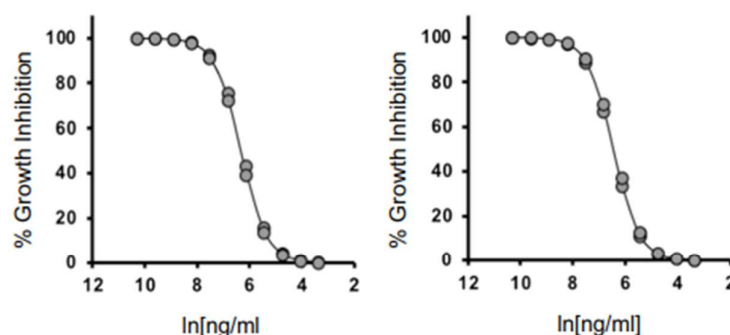


Fig. 3 Anti-malarial activity for the fractions AO2 (left) & AO6 (right) of hydroalcoholic extracts of *A. occidentale* leaves against blood stage of *P. falciparum* strain 3D7. This experiment was carried out in similar manner to that shown in Fig. 2 with the exception that the highest concentration was 30 µg/ml. The growth inhi-

bition obtained at 30 µg/ml concentration for AO2 and AO6 was $99.97 \pm 0.004\%$ and $99.96 \pm 0.005\%$, respectively, with respect to control untreated *P. falciparum*. The IC_{50} values obtained were 0.56 ± 0.03 µg/ml and 0.63 ± 0.03 µg/ml, respectively

Table 3 Acute toxicity study of *A. occidentale* leaves hydroalcoholic extract against Zebra fish embryos

Time	Negative control*	Positive control**	Test compound (<i>A. occidentale</i> extract)				
			0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
0 h	–	–	–	–	–	–	–
24 h	SF, TD	NSF, NTD	SF, TD	SF, TD	SF, TD	SF, TD	SF, TD
48 h	HB	NA	HB	HB	HB	HB	NHB
72 h	HB	NA	HB	HB	HB	HB	NHB
96 h	H	NA	H	H	H	NHB	NA

SF Somite formation, NSF no somite formation, TD tail detachment, NTD no tail detachment, HB heart beat, NHB no heart beat, H hatching, NA not applicable

*Negative control–water

**Positive control–3,4-DCA 4 mg/L

kg body weight in BALB/c mice. The results revealed that 3 different doses of extract tested did not incite any mortality and morbidity throughout the experimental period. Behavioral and physical observations of the experimental mice were found normal and did not exhibit any visible signs of abnormality (Table 4). Histopathological analysis of different organs of mice treated with different doses of the extract did not show any significant changes in the heart, liver, kidney, and brain. However, slight congestion of the lung was observed at 2000 mg/kg (Fig. 4). Sections of mice Brain, heart, kidney, liver, lung were stained with haematoxylin and eosin (scalebar = 100 µm). Mice receiving AO 50 mg, 300 mg, 2000 mg brain showed a normal histological structure of different neurons in the cerebral cortex, hippocampus and striatum. Normal atrium and ventricle are evident from mice treated with AO 50 mg, 300 mg, 2000 mg. Normal architecture of renal corpuscles with their glomeruli and renal tubules was seen after treatment with AO. Normal hepatocytes, central vein and sinusoidal space at magnification 40 × were observed. No haemorrhage or apparent

tissue damage was found in the liver of mice in AO 50 mg and 300 mg treated groups, but in AO 2000 mg destruction of the vessel wall and focal haemorrhage (congestion) of lung were observed. Thus, the extract was found to be safe atleast up to 300 mg/kg.

Discussion

Many antimalarial drugs such as chloroquine, mefloquine, amodiaquine, quinine, proguanil, sulfadoxine and pyrimethamine, primaquine, artemether, artesunate, arteether that target different stages of malaria parasites have been in use for treating clinical malaria cases. But malarial parasites have developed resistance against several of these drugs and currently there are no alternate options other than arteether. Artemisinin, initially extracted from the Chinese herb *Artemisia annua*, highlights the potential of plants as a source of chemotherapeutics. Artemisinin-based combination therapies (ACT) are currently being used in endemic countries

Fig. 4 Toxicity of hydroalcoholic extracts of *A. occidentale* leaves in mice. Histopathological analysis of different organs of mice treated with different doses (50, 300 and 2000 mg/kg body weight in BALB/c mice) of the extract did not show any significant changes in the heart, liver, kidney, and brain. Destruction of the vessel wall and focal haemorrhage (congestion) (indicated by ring) of the lung was observed at the highest dose (2000 mg/kg)

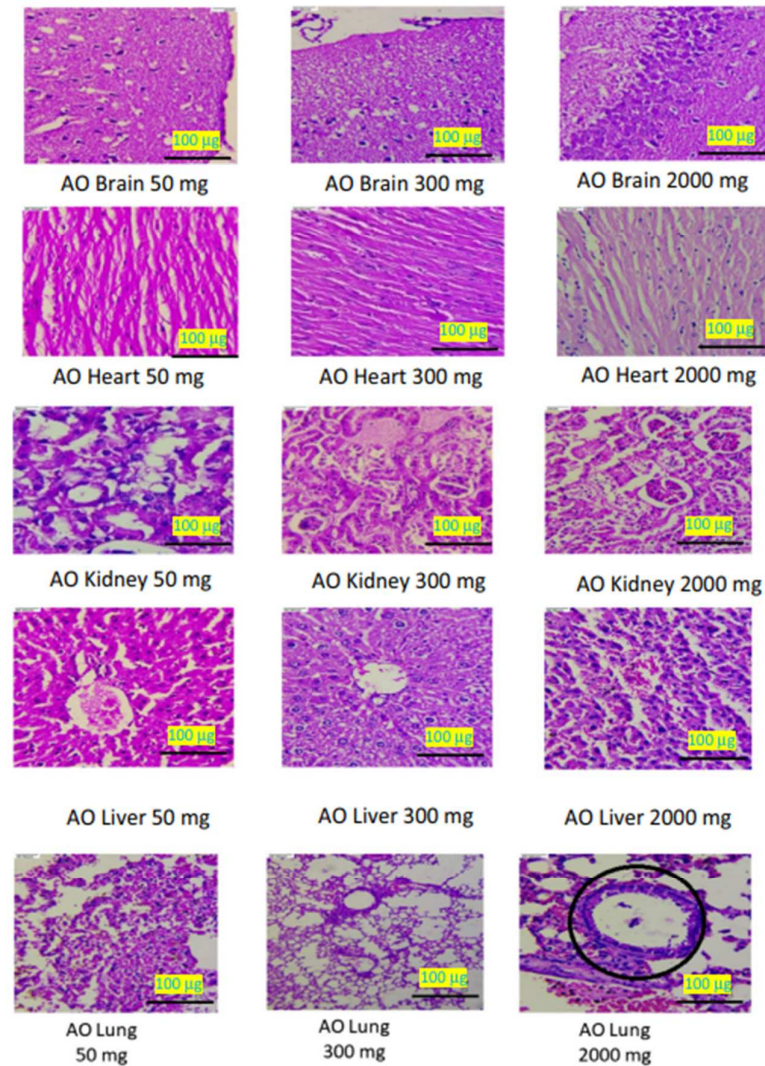


Table 4 Bodyweight, general observations and histopathological analysis of BALB-C mice treated with different doses of hydroalcoholic extract of the leaves of *A. occidentale* L.

Animal groups	Dose (mg/kg)	Parameters		
		Bodyweight (g)*	General observations	Histopathological analysis
<i>A. occidentale</i> L. hydroalcoholic extract	50	20.7 ± 1.80	Normal	No changes
	300	22.5 ± 0.40	Normal	No changes
	2000	23.0 ± 0.90	Normal	Slight congestion in lungs
Control	Vehicle (water)	24.0 ± 0.20	Normal	Normal

*Mean body weight post-treatment (14 days)

worldwide. Over the past decade, unprecedented progress has been made in reducing malaria morbidity and mortality [23]. However, growing resistance to ACTs by malarial parasites, coupled with resistance to insecticides used in the control mosquito vectors, have negatively impacted the malaria control and long-term prospects for elimination [24]. *P. falciparum* has developed resistance to most antimalarial drugs in use, as also to the current ACT [25, 26]. Drug resistance development appears to be due probably to heavy drug use, parasite selection, cross resistance and genetic influences of drugs. Pharmaceutical companies do not evince interest in developing drugs for malaria and this is a matter of great concern, in the face of increasing drug resistance [27]. There are very few drugs in various phases of trials in the thin pipeline of candidate antimalarials for treating malaria. Therefore, there is an urgent need to develop drugs against malaria parasites to augment the malaria control program. Herbal preparations have been traditionally used for treating certain infections, including malaria. Exploring herbal sources may yield new anti-malarial drugs and the discovery process can be hastened by adopting a targeted approach. Therefore, we attempted an approach which involved a drug target in malarial parasite and herbal source for discovering drug lead(s) for malaria.

We explored the possibility of *A. occidentale* leaves as source of anti-malarial drug(s), with the parasite transketolase enzyme as the target. We chose transketolase of *P. falciparum* as the target as it has been reported to play a vital role in the survival/replication of the malaria parasites [28]. It is a key enzyme of the non-oxidative arm of pentose phosphate pathway (PPP), which is an important metabolic pathway for yielding reducing agents in the form of NADPH and the production of pentose sugar needed for nucleic acid synthesis. Further, it exhibits very low similarity to the homologue in human host, making it a selective target for anti-malarial drug development. Key drug targets of *P. falciparum* such as dihydrofolate reductase (DHFR), Cytochrome b (CYTb), dihydroorotate dehydrogenase (DHOD) and Myosin A tail domain-interacting protein (MTIP) have been used for identifying drug leads. Notably, three tRNA synthetases were successfully used in identifying two potent antimalarial natural products viz., cladosporin and halofuginone [29].

Here, we have explored the leaves of *A. occidentale* (cashew) because of its reported medicinal properties and use in traditional medicine practice [10]. Unlike other studies in which the fruit, bark and other plant parts were tested, we have used the leaves for exploring the presence of anti-malarial compounds as it is readily available in large quantities, renewable and usually disposed off as a waste. According to the concept of green chemistry, it is desirable that an abundant and cheap source of raw material, especially natural waste product, should be utilized as it also ensures environmental friendliness. Leaves or nutshell extracts

from cashew have long been used as a folk medicine to treat inflammation and other conditions including asthma, ulcers, and cancer.

In this study, we utilized the recombinant PfTk enzyme as a target for initial screening for the inhibitory activity of the extracts/fractions of the leaves of *A. occidentale*. Out of six extracts of leaves of *A. occidentale* prepared using different solvents, the hydroalcoholic extract was found to have highest enzyme inhibition activity. Therefore, this extract was tested against late-ring and early trophozoite stages of *P. falciparum* 3D7 strain in fluorescence based *in vitro* assay, where the extract showed a very high activity, both in terms of growth inhibition ($99.31 \pm 0.08\%$ at $50 \mu\text{g/ml}$) and IC_{50} ($4.17 \pm 0.22 \mu\text{g/ml}$). The percentage inhibition activity is higher than that reported for 80% ethanol extracts of several plants of Tanzania and extract of *Berasama abyssinica*, which gave a maximum of $86.67 \pm 11.32\%$ activity when tested at $100 \mu\text{g/ml}$ against *P. falciparum* Dd2 strain [30]. An ethanol extract of *B. abyssinica* leaves was reported to have good *in vitro* anti-plasmodial activity with IC_{50} of $2.5 \mu\text{g/ml}$ [31], while methanolic extract of stem bark of the same plant had IC_{50} of $11.00 \mu\text{g/ml}$ [32]. A chloroform extract of *Maesa lanceolata* was found to have very good anti-plasmodial activity with IC_{50} of $2.5 \mu\text{g/ml}$ against clinical isolates of *P. falciparum* [33]. The anti-plasmodial activity of plant extracts may vary depending upon the plant and its parts, geographic location of the plant etc. Bark, leaves, fruit infusions and decoction have been used in traditional medicine as anti-malarial treatments [8]. Several medicinal plants, including *A. occidentale* L. are being used by various tribes of Bankura, West Bengal, India for the treatment of malaria [9]. But, extracts prepared from *A. occidentale* L. were reported to be inactive *in vitro* against *P. falciparum* by Lima *et al.* [8]. However, in the present study the hydroalcoholic extract of leaves of *A. occidentale* showed anti-malarial activity *in vitro* against *P. falciparum*. Based on the IC_{50} its activity can be considered as moderate as per the classification of Lemma *et al.* [34], who categorized the herbs as very good, good, moderate and inactive if the IC_{50} values were $< 0.1 \mu\text{g/ml}$, $0.1\text{--}1 \mu\text{g/ml}$, $1\text{--}5 \mu\text{g/ml}$ and $> 5 \mu\text{g/ml}$, respectively.

The hydroalcoholic extract of leaves of *A. occidentale* did not affect the heartbeat, somite formation, tail detachment and hatching of Zebra fish embryos in acute toxicity test. This indicates the safety of extract in this animal model. Similarly, the extract when tested at doses $50\text{--}2000 \text{ mg/kg}$ body weight in BALB/c mice, it was found to be safe to without causing any mortality and morbidity or change in behavioral and physical activities. Histopathological analysis of major organs of extract treated mice did not show any changes, except lung showing congestion at a very high dose (2000 mg/kg) (Fig. 4). The ethanolic extract of stem bark of *A. occidentale* was reported to play a modulatory role in the inflammatory response mediated

by the down regulation of IL-1 and TNF- α [35]. In the current study, the extract caused lung congestion in mice which may be due to excessive dose administered (2000 gm/kg body weight), which might have resulted in severe immunomodulation. However, it can be concluded that the hydroalcoholic extract of leaves of *A. occidentale* was found to be safe up to a dose of 300 mg/kg.

Bioactivity guided fractionation of this extract was done and upon testing, two of the fractions (AO2 & AO6) showed very promising antimalarial activity against blood stage parasites. As stated above *A. occidentale* L. is well recognized as a rich source of alkyl-phenols, which have several biological activities [15]. Further studies are required to identify the compound responsible for the observed antimalarial activity in the leaves of *A. occidentale* and delineate the mechanism by which the activity of PfTK enzyme is inhibited. Identifying the compounds from the extracts with anti-malarial activity will help to develop suitable formulations as a phytopharmaceutical drug which can then be further taken up for preclinical and clinical studies, and eventually for clinical practice either through traditional route (such as Ayurveda or Siddha) and allopathic route.

Conclusion

The *in vitro* assays showed that the hydroalcoholic extract of leaves of *A. occidentale* has promising activity against *P. falciparum* and its target enzyme Transketolase. The extract and its two fractions of the extract showed significant activity against *P. falciparum* in *in vitro* tests. The extract was found to be non-toxic in preliminary toxicity studies in Zebra fish embryos and mice. Therefore, because of the anti-malarial potential and that the leaves of *A. occidentale* can be obtained in large quantities perennially, it is worthwhile to explore it as a natural anti-malarial drug source.

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Data availability The raw data is available with the senior author (M.K.).

Declarations

Conflict of interest The authors declare no conflict of interest.

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RESEARCH ARTICLES



Assessment of the inhibitory activity of hydroalcoholic extract from *Anacardium occidentale* L. leaves against *Brugia malayi* hexokinase, a promising target for the development of anti-filarial drug

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Abstract

Globally, lymphatic filariasis poses a hazard to 859 million people in 50 nations. Because of its severity, the majority of medications that are now on the market are microfilaricidal, meaning that they do not effectively kill the adult parasites, which continue to create microfilariae. Therefore, the present study aims to develop a plant-based macrofilaricidal drug. The inhibitory activity of various solvent extracts of leaves of *Anacardium occidentale* L. (Anacardiaceae) was tested against the recombinant hexokinase of *B. malayi* (BmHK). BmHK, an enzyme of the glycolytic pathway that plays a vital key in the replication/survival of filarial parasites and hence was chosen as a target. A preliminary phytochemical test and GC-FID analysis of the extract were carried out. The hydroalcoholic extract of *A. occidentale* showed the highest inhibition ($65.80 \pm 1.60\%$) of activity of BmHK, compared to other extracts, which showed less than 50% inhibition. The present study showed that the hydroalcoholic extract of *A. occidentale* has inhibitory activity against BmHK and can be explored further for developing the herbal-based anti-filarial drug.

Keywords BmHK · GC-FID · *Anacardium occidentale* · Anti-filarial activity

Introduction

Lymphatic filariasis (LF), is a major neglected tropical disease caused by 3 nematode parasites viz., *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. *W. Bancrofti* is responsible for 90% of the cases, while *B. malayi* and *B. timori* are cause the remaining 10% percent of cases. LF is transmitted to humans through infective mosquito bites and causes lymphatic dysfunction, limbs, breasts, or genitals irreversible swelling, resulting in progressive (Paily et al. 2009). According to World Health Organization (WHO) report 2019 (WHO 2023), 859 million people in 50 countries are at risk of LF and there is a need for a preventive therapy to interrupt the transmission as well as for treatment (WHO 2023). Realizing the severity and permanent disability caused by this disease and taking into account the availability of necessary diagnostic and control tools, the WHO launched the Global Programme for Elimination of LF (GPELF) in the year 2000. The aim of the programme is to stop the transmission of LF employing the strategy of Mass Drug Administration (MDA) of a combination of

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diethylcarbamazine (DEC) and albendazole for 5 years, annually (WHO 2023). However, DEC is effective against the microfilaria stage but ineffective in killing adult worms (Souza et al. 2018), whereas albendazole can kill adult worms only when treated for 21 days with 400 mg which causes adverse effects (Zhu et al. 2005). Hence, these drugs are not effective in cutting down the filarial transmission, thus straining the efforts of the elimination program. Therefore, there is a felt need to find alternative drugs that are macrofilaricidal, especially those without adverse effects. The use of natural products as therapeutics is well known, especially because of their high safety margin. Hence, there is a renewed interest recently in sourcing therapeutic drugs from natural products for treating neglected tropical diseases. There are a few reports of plant-based anti-helminthic drugs identified earlier (Cheuka et al. 2016).

Several herbal preparations of plants such as *Phyllanthus amarus*, *Aegle marmelos*, *Centella asiatica*, *Azadirachta indica*, *Lantana camara*, *Ocimum sanctum*, *Andrographis paniculata*, *Achyranthes aspera*, *Solanum nigrum*, *Zingiber officinale*, and many more, are reported as having antifilarial activity (Ndjonka et al. 2018). There is a necessity for accelerating the pace of identifying more anti-helminthic herbal leads to develop an anti-filarial drug and a target-based approach for anti-filarial drug development may yield a quicker expected outcome. Hexokinase of *B. malayi* (*BmHk*) (EC 2.7.1.1, ATP: D-hexose-6-phosphotransferase) is an ideal target for identifying antifilarial drug leads, since it is a glycolytic pathway key enzyme and hence plays a major role in the survival/reproduction of filarial parasites. Further, it has least homology with human hexokinase thus making it a good target for anti-filarial drug discovery. Further, *BmHk* uniquely activates G6P, unlike other hexokinases which are not affected or inhibited by G6P (Singh et al. 2008). Hence, it is worthwhile to explore the inhibitory activity of herbals against *B. malayi* hexokinase.

Anacardium occidentale L. (Anacardiaceae), commonly called 'cashew tree' is cultivated as a major commercial plantation in several tropical countries like India, Brazil, Nigeria, Central America, Mozambique, Indonesia, Vietnam and Panama (Morais et al. 2010). It is used by traditional medicine practitioners as a remedy for treating of diabetes mellitus in many tropical countries like South Cameroon (Ha and Kubo 2005). Various parts of this plant are reported to have various biological activities such as anti-tumour, anti-inflammatory, antioxidant, insecticidal against *Aedes aegypti* and mushroom tyrosinase inhibition, and molluscicidal activity, and hence have great therapeutic potential due to its phenolic constituents (Kubo et al. 1994; Carvalho et al. 2011). Since this plant is grown in vast areas as a commercial crop in many countries and is reported to have bioactivity, it appears to be a promising source for

antifilarial compounds. Therefore, the present was aimed at exploring this plant for anti-filarial drug development.

Materials and methods

Cloning, expression and purification of *BmHk*

Cloning and expression of the recombinant *BmHk* were done as reported earlier (Singh et al. 2008). In brief, the *BmHk* gene was cloned into plasmid pTriEx and the recombinant plasmid was transformed into *E. coli* (DE3) Rosetta strain. Then single colony of the recombinant bacterium was inoculated into 5 ml Luria-Bertani (LB) medium containing 100 µg/ml ampicillin and 40 µg/ml chloramphenicol and grown at 37 °C for 16 h and 180 rpm, on a rotary shaker. It was inoculated at 1% to 500 ml of LB broth having antibiotics and grown until OD₆₀₀ reached at 0.6. The induction of the culture was done with 0.5 mM isopropyl-thiogalactopyranoside (IPTG) at 20 °C for 16 h and the induced cells were pelleted by centrifuging at 8000 g at 4 °C for 10 min. The pellet obtained was resuspended in lysis buffer (5 mM imidazole, 300 mM NaCl, 50 mM NaH₂PO₄), which contained a cocktail protease inhibitors (Sigma) and then sonicated (Ultrasonic processor, Model-XL-2020, Germany). The sonicated lysate was centrifuged for 45 min at 10,000 g and the *BmHk* from the supernatant was purified by Ni²⁺-nitrilotriacetic acid resin (Ni²⁺-NTA) chromatography. The supernatant was loaded onto the column, the column washed using lysis buffer, followed by four washes of 10–40 mM imidazole. The recombinant protein was eluted using lysis buffer having 450 mM imidazole. The purified protein was concentrated by passing through Centrprep-30 (Millipore) filter and its concentration was assessed by the method of Bradford (1976). The purity of the purified protein was checked by 10% SDS-PAGE (Ndjonka et al. 2018).

Collection of study plant material

Leaves of *A. occidentale* plant were collected during 2018 from cultivation areas in Belagavi (N 15.88668; E 74.52353; from sea level altitude ~800 m), Karnataka state, India. The plant was identified by a botanist at the ICMR-National Institute of Traditional Medicine (ICMR-NITM), Belagavi and a herbarium specimen (RMRC-1356) was deposited in the institute.

Preparation of extract and preliminary phytochemical test

Leaves of *A. occidentale* were dried in shade, powdered using an electric grinder and extracted using different

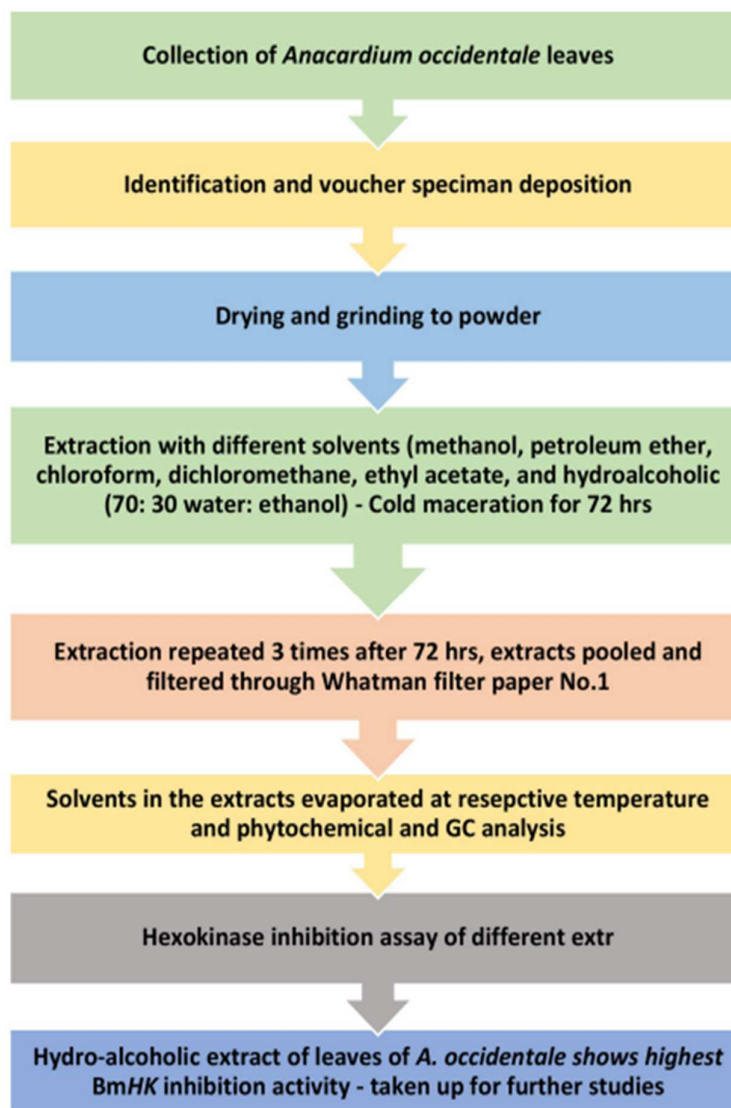
solvents viz., Whatman filter paper No. 1 by cold maceration (for 72 h) method. Each solvent extract of *A. accidental* was again extracted three times after 72 h, filtered through Whatman filter paper No.1 and evaporated using a rotary evaporator set at different temperatures (as per the solvent property). The extracts obtained were stored until further use at -4 °C. Preliminary qualitative phytochemical tests such as alkaloids (Dragendorff's test), amino acids (Ninhydrin test), saponins (Foam test), glycosides (Keller-kiliani test), flavonoids (Lead acetate test), tannins (Ferric chloride

test), and terpenoid (Salkowski test) were conducted as per standard procedure. The bio-activity guided solvent extraction of leaves of *A. occidentale* for inhibition of hexokinase of *Brugia malayi* has been mentioned in Fig. 1.

Gas chromatography analysis

The Gas Chromatographic (GC) analysis of extracts was performed in Varian 450 gas chromatograph connected to Flame Ionization Detector (FID), and stationary phase ZB-5

Fig. 1 Bio-activity guided solvent extraction of leaves of *Anacardium occidentale* for inhibition of hexokinase of *Brugia malayi*



(30 m × 0.25 mm i.d., 0.25 µm film thickness) column, under experimental conditions reported earlier (Bouazzi et al. 2020; Joshi 2021;2022). Nitrogen was used as carrier gas at the flow rate of 1.0 mL/min. The temperature programming of the equipment was set to 80–280 °C/min at 3 °C/min. The temperatures of injector and detector were set at 280 and 290 °C, respectively. The injection volume was 1.0 mL of *A. occidentale* extract (1% solution of extract diluted in methanol); the split ratio was 1: 50. The GC peak area (FID response) was used for calculating the relative amounts of individual components, but without using a correction factor.

BmHk inhibitory assay

Hexokinase inhibition activity was determined by the method reported by Armstrong et al. (1996). The reaction mixture contained 10mM ATP, 0.04 mM glucose, 100mM Tris-HCL (pH 7.6), 25mM MgCl₂, 0.25mM NADP, 1U of G6PDH, 10–20 µg hexokinase and 50 µg of plant extract. In this assay, the utilization of NADH is assessed by the ADP formation by the hexokinase, in the presence of lactate dehydrogenase and pyruvate kinase. The reaction was run in triplicate and the activity of different extracts was compared by Kruskal-wallis test.

Results and discussion

Preliminary phytochemical analysis

The yield (% w/w) of petroleum ether, dichloromethane, chloroform, ethyl acetate, methanol, and hydroalcoholic (70: 30 water: ethanol) extract was 5.2%; 9.20%; 13.00%; 12.50%; 22.25% and 17.32%, respectively. The preliminary phytochemical testing of the extract showed the presence of amino acids, flavonoids, glycosides, alkaloids, terpenoids saponins, and tannins in different amounts. Flavonoids and tannins were in higher quantities, followed by alkaloids and terpenoids (Matutino et al. 2019).

GC-FID analysis of a hydroalcoholic extract of *A. occidentale*

Phytochemical analysis of the extract of leaves of *A. occidentale* among others by GC-FID is presented in Fig. 2. In this analysis anacardic acid was found to be present at 0.1% concentration. The anacardic acid is more soluble in non-polar solvents, and very minor quantity was identified in hydroalcoholic extract of *A. occidentale* leaves.

Expression and purification of BmHk by IMAC (Ni-NTA column) and inhibition assay with herbal extracts

The recombinant BmHk was purified using Ni-NTA column and the purity was checked by 10% SDS-PAGE.

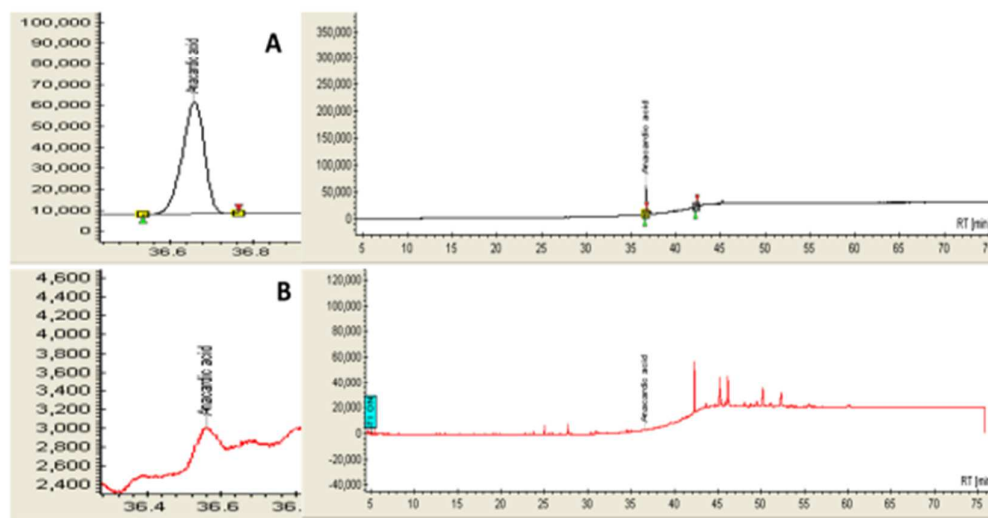


Fig. 2 GC-FID analysis of *A. occidentale* hydroalcoholic extract showed the presence of 0.1% anacardic acid. A-Anacardic acid standard, B- *A. occidentale* leaves hydroalcoholic extract

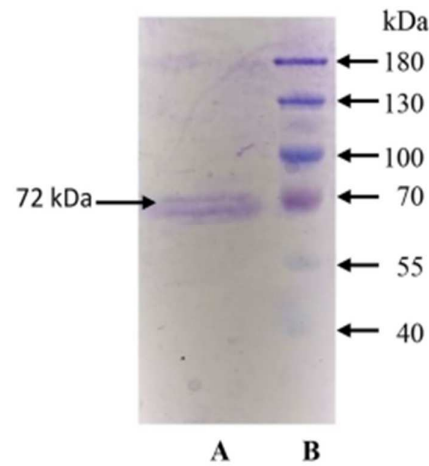


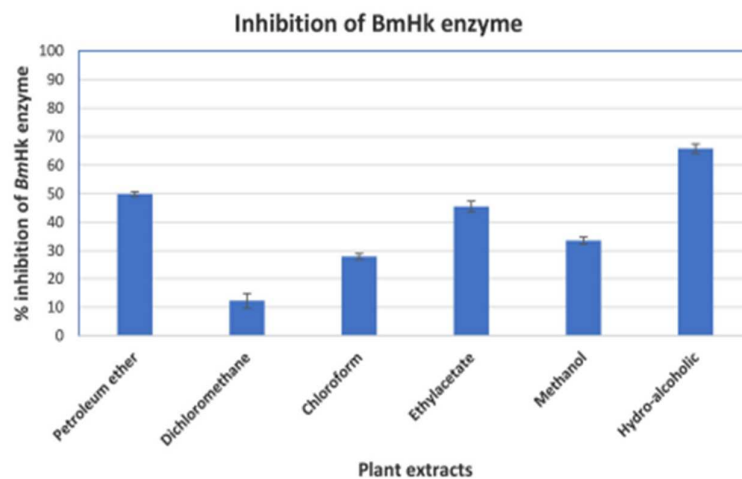
Fig. 3 The SDS-polyacrylamide gel of the Ni-NTA recombinant *B. malayi* hexokinase (*BmHk*) purified. Protein fraction electrophoresis was done on a 10% (w/v) SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R250. (A) Lane 1 Purified *BmHk*, (B) Lane 2 prestained protein ladder

The SDS-PAGE of the purified enzyme showed the presence of a expected band of 72 kDa (Fig. 3), indicating the purified recombinant *BmHk* enzyme is electrophoretically homogenous.

Inhibition of *BmHk* by the different extracts of the leaves of *A. occidentale*

The inhibitory activity of different extracts of the leaves of *A. occidentale* was tested against purified recombinant *BmHk* enzyme. Figure 4 shows the percentage inhibition

Fig. 4 Percent inhibition of *BmHk* enzyme by various extracts of the leaves of *A. occidentale*



of *BmHk* by different extracts of leaves of *A. occidentale*. The percent inhibition by different extracts ranged from 12.25 ± 2.50% to 65.80 ± 1.60%. The hydro-alcoholic extract of leaves of *A. occidentale* exhibited the highest inhibition (65.80 ± 1.60%) ($p = 0.0053$) against *BmHk*, followed by petroleum ether (49.88 ± 0.80%) and ethyl acetate (45.50 ± 1.90%) extracts which showed moderate inhibition, while low inhibition activity was found with methanol (33.60 ± 1.20%), chloroform (28.00 ± 1.00%) and dichloromethane (12.25 ± 2.50%) extracts against *BmHk*. The hydro-alcoholic extract of leaves of *A. occidentale* was thus had higher inhibitory activity against *BmHk* compared to other extracts and hence it was taken up for further studies.

LF, a major public health problem in developing tropical countries, was targeted for elimination by 2020, and a global program for elimination was launched in the year 2000 to achieve the target (WHO 2023). The strategy for elimination of LF involves implementation of 6 rounds of Mass Drug Administration (MDA) of a combination of Diethylcarbamazine and albendazole to the LF endemic communities. Although more than 6 rounds of MDA have taken place, hotspots with infection transmission are sustained in several countries (Rao et al. 2018; Subramanian et al. 2017). Possible reasons could be problems of drug coverage and consumption, operational issues, or drug resistance by the parasite. Among the two drugs administered, DEC has microfilaricidal activity and not macrofilaricidal activity and hence adult parasites continue to produce microfilariae which will sustain the transmission (Adu Mensah et al. 2022). Resistance to albendazole by several veterinary parasites has been reported earlier and filarial parasites in the hot spots may have developed resistance. Besides, these classes of drugs suffer from poor intestinal absorption and severe adverse reactions, which limit their use (Zhu et al.

2005). This situation calls for the deployment of more efficacious, especially, macrofilaricidal drugs.

Currently, no such drugs are available in the drug pipeline and there is a felt need to identify and develop safe and effective macrofilaricidal drugs. Of late, there is a renewed interest in herbal-based therapeutic tools because of their bio-efficacy and especially high level of safety margin. In this study we explored the potential of extracts of leaves of *A. occidentale* for anti-filarial activity, through a target-based approach. The target selected for the study is hexokinase of *B. malayi*. Hexokinases (HK) initiate the glycolytic pathway (Chen et al. 2015). Enzymes such as enolase (Tielens et al. 1994) and phosphoglycerate kinase (Lee et al. 1995) of this pathway have been explored for vaccine and drug development, as they are crucial molecules for trematode survival. In *Clonorchis sinensis* (Chen et al. 2015), *Hhaemonchus contortus* (Schmitt-Wrede et al. 1999), and *Schistosoma mansoni* (Shoemaker 1995) HKs have been well characterized as potential targets for vaccine and drug development. In an earlier study, hexokinase of *B. malayi* (BmHK) was identified as a target for drug discovery (Ndjonka et al. 2018). As parasitic nematodes are dependant mainly on glycolysis pathway for energy metabolism, involvement of hexokinase in glycolysis pathway represents as an important target for anthelmintic drug development. BmHK has a low identity with both 50 kDa type hexokinase IV and 100 kDa type mammalian hexokinase I, II, III. Upon BLAST analysis BmHK exhibited a homology of 59, 43 and 37%, with *Caenorhabditis elegans*, *Haemonchus contortus* and human hexokinases, respectively (Singh et al. 2008). The differences in sequence homology and kinetic properties as compared to the host enzymes suggest that BmHK can be an appropriate drug target for identification of novel herbal drugs for filarial parasites (Ndjonka et al. 2018). It is of interest to note that earlier studies have reported expression of BmHK in chemotherapeutically important life stages viz., microfilariae and adult parasites (Singh et al. 2008). Mathew et al. 2011 explored Glutathione S-Transferase (GST) of *Seteria digitata* as a target for identifying anti-filarial compounds. Similarly, in the target based drug development approach (Matutino et al. 2019) reported the potential of *A. occidentale* compounds for the development of potential sirtuin inhibitors and anti-*Trypanosoma cruzi* agents.

Plants belonging to 13 families were investigated for activity against another filarial parasite, *Onchocerca* sp., in vitro and most active species were *Euphorbia hirta*, *A. occidentale*, and *Acacia nilotica*, each with an LC₅₀ value of 6.25, 2.76, and 1.2 µg/mL, respectively (Ndjonka et al. 2018). In the present study, *A. occidentale* was selected based on the literature search and traditional use and explored for its inhibition activity against BmHK. This enzyme was cloned and

expressed as a recombinant molecule, earlier (Singh et al. 2008).

Among various *A. occidentale* leaves extracts tested, the hydroalcoholic extract exhibited the highest inhibition activity against BmHK. Phytochemical analysis of the leaf extract of the *A. occidentale* by other workers have shown the presence of several compounds (Fadaye et al. 2015). In the present study, the preliminary phytochemical testing of the extract was exhibited the presence of glycosides, alkaloids, amino acids, flavonoids, terpenoids, tannins, and saponins with flavonoids and tannins being in higher quantities, followed by alkaloids and terpenoids. Anacardic acid was found in the extract when GC-FID analysis was done. This compound, a natural product which was isolated initially from the cashew-nut shell, presents a long side-chain at the 6-position and convenient salicylic acid system, in which a double bond was found at C-8 in the monoene, diene, and triene components (Schultz et al. 2006). In the present study, anacardic acid was found to be present even in leaves, although in smaller quantity. Anacardic acid has been of considerable attraction because of its diverse biological effects such as anti-fungal, anti-parasitic, antimicrobial, anti-tumoral, gastroprotection, insecticidal activities (Harris et al. 2013; Davis et al. 2016; Jaiswal et al. 2017), and inhibition of enzymes such as lipoxigenase (Ha and Kubo 2005), tyrosinase (Kubo et al. 1994), and histone acetyltransferases (Sun et al. 2006). Anacardic acid has been reported to have excellent activities against protozoal parasites of *Echinococcus granulosus* and metacystodes of *E. multilocularis* than that of dihydroartemisinin and albendazole in vitro, and in vivo growth inhibition of the latter as effective same as albendazole, with no obvious toxicity. Anacardic acid was found to inhibit angiogenesis in *E. multilocularis* metacystodes through the inhibition of VEGF-induced Akt/PKB and Src/FAK expression, mainly (Kubo et al. 1986). It was found safe with no adverse effects at a dosage of 300 mg/kg in BALB/c mice (Morais et al. 2010). In vitro studies using Chang liver cells and HepG2 cells exhibited safety of anacardic acid with IC₅₀ values 70.0 ± 3.0 µM, and 49.0 ± 8.0 µM respectively, which is much more higher than the EC₅₀ value on *E. protoscoleces* (1.9 ± 2 µM), thus indicating the high safety margin of this compound (Yuan et al. 2019). We have recently reported excellent anti-dengue activity of Anacardic acid (Alagarasu et al. 2022). Studies with *A. occidentale* hexane leaf extract showed that the toxic effects occurred at higher doses compared to Cameroon folk medicine (Carvalho et al. 2011). It is interesting to note that alkyl-phenols from the *A. occidentale* such as cardol diene and 2-methylcardol diene, were found to be active against adult worms of *Schistosoma mansoni* in vitro, with LC₅₀ values of 32.2 and 14.5 µM and selectivity indices of 6.1 and 21.2, respectively (Alvarenga et al. 2016). In the present

study, the hydroalcoholic extract of leaves of *A. occidentale* was tested against BmHk and found to have promising anti-enzyme activity. BOILED-Egg model has shown the high probability of passive absorption of anacardic acid (AA) derivatives by the GI tract. The human intestinal absorption percentage (%) of the anacardic acid (AA) derivatives was analyzed using an online server tool, pkCSM. This study reported that all the AA derivatives, except two (AA99 and AA100), showed good human intestinal absorption. Also, phenols have been subjected to clinical trials for over 20 years (Chen et al. 2015; Marino et al. 2020).

We have done the toxicity study of the hydroalcoholic leaves extract of *A. occidentale* in Zebrafish embryos, which are updated models to study acute toxicity of compounds and found it to be safe. We have also performed acute oral toxicity test of the extract and found it to be safe up to 2000 mg/kg body weight in BALB/c mice (data not shown). The safety of the hydroalcoholic extract of leaves of *A. occidentale* as assessed by the MTT assay, has been reported by Wattanathorn et al. (2019). They assessed the cytotoxicity of the extract against RAW 264.7 cells by treating *A. occidentale* leaf extract in the concentration range between 0.625 and 10 mg/mL. The extract showed more than 90% cell viability at the concentration of 2.5 mg/mL after 24-hour exposure.

Conclusion

Taken together, the present study showed that *A. occidentale* leaf hydroalcoholic extract was found to have potential activity against BmHk, the key enzyme in the glycolytic pathway upon which the nematodes have a dependency. The extract was found to be safe to Zebra fish and mice models. In view of this, the potential of the hydroalcoholic leaves extract of *A. occidentale* need to be explored further for developing antifilarial drug(s).

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Declarations

Disclosure No potential conflict of interest was reported by the author.

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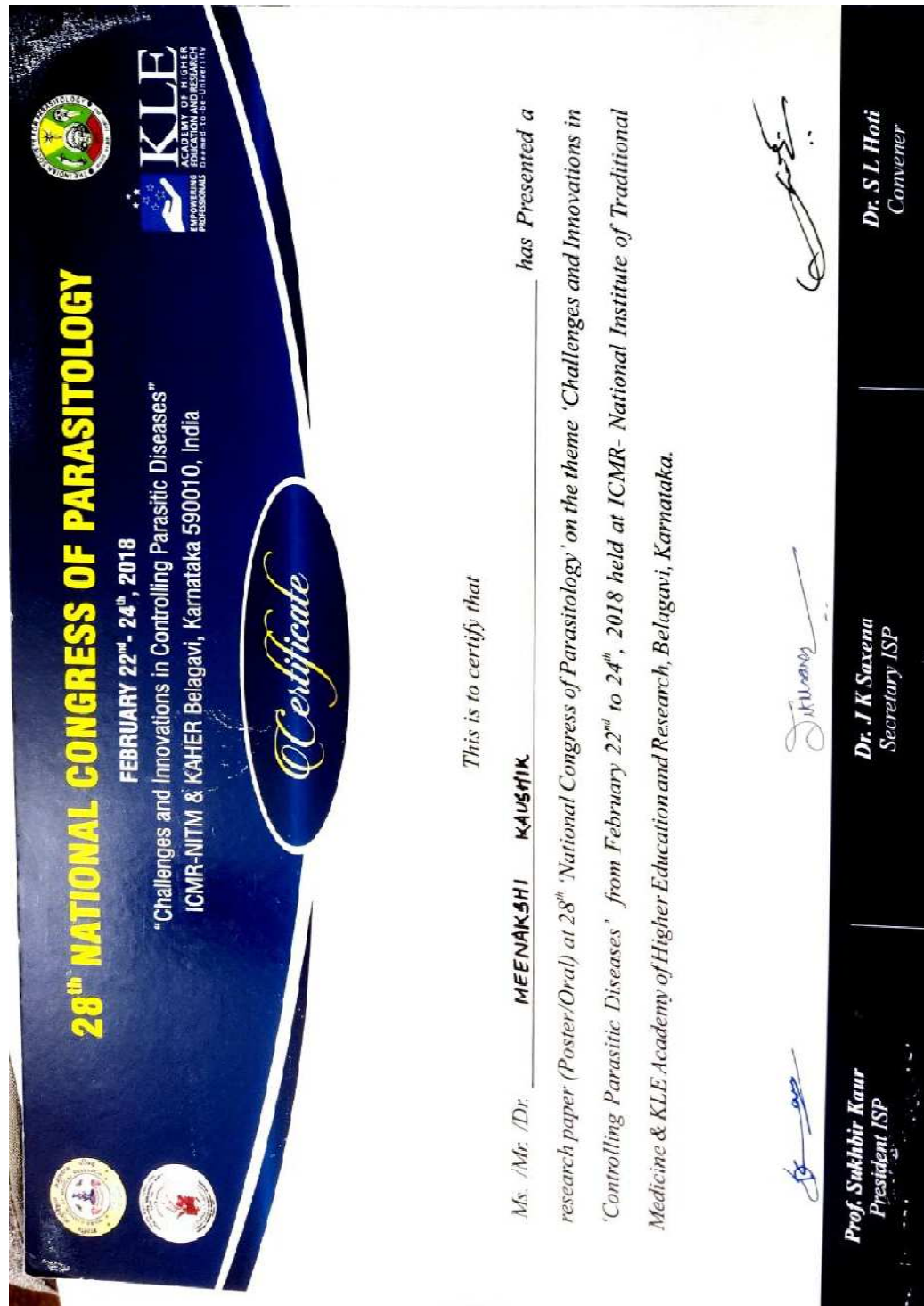
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ANNEXURE - C

- **List of Presentations**

1. Oral/poster presentations: Meenakshi Kaushik, Y.Nazeer, Dr.S.L.Hoti, 'Screening of antimalarial drugs of herbal origin through target based drug development approach' in 28th National congress of parasitology 2018 held at KLE Academy of Higher Education and Research, Belagavi.



ANNEXURE D

1. Women Scientist Award



Dr Vandana Singh
Officer-in-charge
Women Scientists Scheme-A (WOS-A)
Phone: 011-26590273
E-mail: vandana.singh@nic.in

GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
Department of Science & Technology
Technology Bhavan
New Delhi-110016

File No.: SR/WOS-A/LS-456/2016 13-12-2016

Sub: The project entitled "Screening and development of antimalarial drugs of herbal origin through target based drug development approach".

Dear Ms Kaushik,

Please refer to the proposal mentioned above which you had submitted for consideration of possible funding under the Women Scientists Scheme (WOS) for Research in Basic/Applied Sciences.

We are glad to inform you that the above-mentioned proposal has been technically recommended for financial support for 3 years. The recommended budget is as follows:

Fellowship to PI:	₹ 14,40,000/-
Consumables:	₹ 6,00,000/-
Equipments:	Nil

Some funds for travel and contingencies have also been recommended.

In order to further process your case for issue of financial sanction, you are requested to submit the following papers at the earliest:

- Certificate from the Head of the Institution where the said project will be implemented (in the attached format);
- Name of the official in the host institution in whose name the demand draft should be sent;
- Bank details of the host institute including Institute name as per bank record, Saving Account No., Bank name, Branch and IFSC code endorsed by the Registrar/Finance Officer/Competent authority;
- Estimates of consumables required in the project (name, rate, quantity and amount);
- Copy of your highest science/engineering degree;
- Name, address and bio-data of the scientist who is willing to technically help you as a mentor or guide.

It is expected that you would not undertake a visit abroad for a period exceeding 3 months. And, any visit abroad except for the purpose of attending workshops/conferences/schools etc. should be with the prior permission of DST.

If you have got an employment after presentation before the Expert Committee, and would like to continue the same, please let us know.

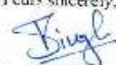
We shall process your case for financial sanction once we receive the above documents/clarifications from you. **Please note that a project starts only after it is accorded financial sanction by DST.**

If you have received/are receiving research support under any other scheme of DST or other agencies, please send the details of the same.

We would request you to please note that if you are not able to send the required papers within 30 days of the date of this letter, the project file would be closed. No correspondence in this regard would be entertained subsequently.

Looking forward to receiving your reply and with best regards,

Ms Meenakshi Kaushik
Regional Medical Research Centre (ICMR),
Nehru Nagar,
Belagavi - 590010

Yours sincerely,

(Vandana Singh)