Inhibitory activity of hydroalcoholic extract of Anancardium occidentale L. leaves against Brugia malayi hexokinase, a target for anti-filarial drug development

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Article

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Abstract

**Purpose:** Globally, 859 million people in 50 countries are threatened by lymphatic filariasis. Due to its severity, the available drugs are mainly used for microfilaricidal and do not kill efficiently the adult parasites, which continue to produce microfilariae. Therefore, the present study aims to develop a plant-based macrofilaricidal drug.

**Methods:** The inhibitory activity of the various solvent extracts of the 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 role in the replication/survival of filarial parasites, was chosen as a target. A preliminary phytochemical test and GC-FID analysis of the extract were carried out.

**Results:** The hydroalcoholic extract of *A. occidentale* showed the highest inhibition (65.80±1.60%) of activity of *BmHk*, compared to other extracts, which showed less than 50% inhibition.

**Conclusion:** The present study showed that hydroalcoholic extract of *A. occidentale* has inhibitory activity against *BmHk* and can be explored further for developing the herbal-based anti-filarial drug.

Introduction

*Lymphatic filariasis* (LF), commonly known as elephantiasis which is a major neglected tropical disease caused by 3 nematode parasites viz., *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori*. *W. Bancrofti* causes 90% of the cases while *B. malayi* and *B. timori* are responsible for the remaining 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 [1]. According to the World Health Organization (WHO) report 2019 [2], 859 million people in 50 countries are at the risk of LF and there is a need for a preventive therapy to interrupt the transmission as well as for treatment [3]. 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 interrupt the transmission of infection using the strategy of Mass Drug Administration (MDA) annually of a combination of diethylcarbamazine (DEC) and albendazole for 5 years [2]. However, DEC is effective against the microfilaria stage but ineffective in killing adult worms [4], whereas albendazole can kill adult worms only when treated for 21 days with 400mg which causes adverse effects [5]. 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 therapeutics from natural products for treating neglected tropical diseases. There are a few reports of plant-based anti-helminthic drugs identified earlier [6]. Several herbal preparations of plants such as *Phyllanthus amarus, Aegle marmelos, Centella asiatica, Azadirachta indica, Lantana camara, Ocimum sanctum, Andrographis paniculata, Achyranthes aspera,* and *Solanum*
*nigrum*, *Zingiber officinale*, and many more, are reported as having antilarial activity [7]. 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 a promising target for identifying antilarial drug leads, as 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, *B. malayi* hexokinase has a unique property of activation by G6P, unlike all other hexokinases which are either unaffected or inhibited by G6P [8]. Hence, it is worthwhile to explore the inhibitory activity of herbals against *B. malayi* hexokinase.

*Anancardium occidentale* L. (Anacardiaceae), commonly called ‘cashew tree’ is cultivated as a major commercial plantation in several tropical countries such as India, Brazil, Nigeria, Central America, Mozambique, Indonesia, Vietnam and Panama [9]. It is used by traditional practitioners as a folk remedy for the treatment of diabetes mellitus in South Cameroon as well as in other tropical countries [10]. Various parts of plant reported to have several bio-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 [11, 12]. 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 reported earlier [8] (Singh et al. 2008). In brief, the *BmHk* gene was cloned into plasmid pTriEx and then transformed into *E. coli* (DE3) Rosetta strain. Then single colony of the recombinant *BmHk* was inoculated into 5 ml broth of Luria-Bertani (LB) medium containing 100 µg/ml ampicillin and 40 µg/ml chloramphenicol and grown at 37°C for 16 hrs and 180 rpm on a rotary shaker. The culture was inoculated at 1% into 500 ml of LB broth containing antibiotics and grown until OD\textsubscript{600} reached at 0.6. The culture was induced with 0.5 mM isopropyl-thiogalactopyranoside (IPTG) at 20°C for 16 hrs and the induced cells were harvested by centrifugating 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 NaH\textsubscript{2}PO\textsubscript{4}) containing protease inhibitor cocktail (Sigma) and then lysed by sonication (Ultrasonic processor, Model-XL-2020, Germany). The sonicated lysate was centrifuged for 45 min at 10000 g and the *BmHk* was purified by using Ni\textsuperscript{2+}-nitrilotriacetic acid resin (Ni\textsuperscript{2+}-NTA) from the supernatant. The supernatant was loaded onto the column and then column was washed with lysis buffer, followed by four washes of 10–40 mM imidazole. The recombinant protein was eluted as fractions of 1 ml using lysis buffer containing 450 mM imidazole. The recombinant protein was
concentrated by Centriprep-30 (Millipore) and then concentration was determined by method of Bradford [13]. The purity of the recombinant protein was checked by 10% SDS-PAGE [7].

Collection of study plant material

Leaves of A. occidental plant were collected during 2018 from cultivation areas in Belagavi (N 15.88668; E 74.52353; from sea level altitude ∼ 800 m), Karnataka, India. The plant was identified by 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. accidental were dried in shade, powdered using an electric grinder and extracted using different solvents viz., methanol, petroleum ether, chloroform, dichloromethane, ethyl acetate, and hydroalcoholic (70: 30 water: ethanol) by cold maceration (for 72hrs) method. Each solvent extract was extracted three times after 72 hrs. The A. accidental extract was filtered through Whatman filter paper No.1. The solvent was evaporated using a rotary evaporator at different temperatures (as per the solvent property). The extracts obtained were stored at -4°C until further use. Preliminary qualitative phytochemical tests such as alkaloids (Dragendorff’s test), amino acids (Ninhydrin test), saponins (Foam test), glycosides (Keller-kiliian test), flavonoids (Lead acetate test), tannins (Ferric chloride test), and terpenoid (Salkowski test) were carried out as per standard procedure.

Gas chromatography analysis

The gas chromatography (GC) analysis of the extracts was performed on Varian 450 gas chromatograph equipped with Flame Ionization Detector (FID), using stationary phase ZB-5 (30 m x 0.25 mm i.d., 0.25 μm film thickness) column under the experimental conditions reported earlier [14, 15]. Nitrogen was used as carrier gas at a 1.0 mL/min flow rate. The experiment temperature programming was set to 80–280°C/min at 3°C/min and the injector and detector temperatures were 280°C 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 relative amounts of individual components were calculated based on the GC peak area (FID response), without using a correction factor.

BmHk inhibitory assay

Hexokinase inhibition activity was estimated by the method of Armstrong et al. [16]. 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, ADP formation by the hexokinase reaction was coupled to utilization of NADH 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

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 revealed the presence of amino acids, glycosides, alkaloids, saponins, flavonoids, terpenoids, and tannins in different amounts. Flavonoids and tannins were in higher quantities, followed by alkaloids and terpenoids.

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. 1. The analysis showed the presence of anacardic acid 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*.

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

Recombinant *BmHk* was purified using Ni-NTA column and the purity was checked by 10% SDS-PAGE. The SDS-PAGE analysis of the purified enzyme showed the presence of a single expected band of 72kDa (Figs. 2 and 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. Table 1 shows the percentage inhibition 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 found to have higher inhibitory activity against *BmHk* compared to other extracts and hence it was taken up for further studies.
Table 1
Percent inhibition of $BmHk$ enzyme by various extracts of leaves of $A. occidentale$.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroalcoholic</td>
<td>65.80 (0.01)</td>
<td>0.0053*</td>
</tr>
<tr>
<td>Methanol</td>
<td>33.61 (0.06)</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>45.51 (0.01)</td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>28.03 (0.15)</td>
<td></td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>12.25 (0.01)</td>
<td></td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>49.88 (0.01)</td>
<td></td>
</tr>
</tbody>
</table>

*Kruskal-wallis test

Discussion

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 [2]. The strategy for elimination is implementation of 6 rounds of MDA of a combination of DEC 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 [17, 18]. 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 [19]. 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 [5]. 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. The present study explored the potential of extracts of $A. occidentale$ for anti-filarial activity, through a target-based approach. The target selected for the study is hexokinase of $B. malayi$. Hexokinase (HK) is the first key enzyme of the glycolytic pathway [20]. Glycolytic enzymes such as enolase [21] and phosphoglycerate kinase [22] have been targeted for vaccine and drug development as they are recognized as crucial molecules for trematode survival. In $Clonorchis sinensis$ [20], $Hhaemonchus contortus$ [23], and $Schistosoma mansoni$ [24]. 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 [7]. Since parasitic nematodes depend mainly on glycolysis pathway for the metabolism of their energy,
involvement of hexokinase in glycolysis pathway represents it an important putative 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. BLAST analysis showed BmHK homology with Caenorhabditis elegans, Haemonchus contortus and human hexokinases by 59, 43 and 37%, respectively [8]. The differences in sequence homology and kinetic properties as compared to the host enzymes suggest that BmHk may contribute as an interesting drug target for the identification of novel herbal inhibitors for filarial parasites [7]. It is of interest to note that earlier studies have reported expression of BmHk in chemotherapeutically important life stages viz., microfilariae and adult parasites [8]. Mathew et al. [25] explored Glutathione S-Transferase (GST) of Seteria digitata as a target for identifying anti-filarial compounds.

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 [7]. 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 [8].

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 reported earlier by other workers revealed the presence of several compounds [26]. The preliminary phytochemical testing of the extract was revealed 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. GC-FID analysis of the extract showed the presence of anacardic acid. This compound, a natural product which was isolated from the cashew-nut shell liquid, 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 [27]. In this study, this compound was found to be present even in leaves although in smaller quantities. This compound has been considerable attractive due to its diverse biological effects such as anti-fungal, anti-parasitic, anti-microbial, anti-tumoral, gastroprotection, insectidal activities [28–30], and enzymes inhibition such as lipoygenase [10], tyrosinase [11], and histone acetyltransferases [31]. Anacardic acid has been reported to have excellent activities against Echinococcus granulosus protoscoleces and E. Multilocularis metacestodes than that of dihydroartemisinin and albendazole in vitro, and growth inhibition of the E. metacestode as effective same as albendazole in vivo, with no obvious toxicity. Anacardic acid was found to inhibit angiogenesis in E. metacestode mainly through the inhibition of VEGF-induced Akt/PKB and Src/FAK expression [32]. It was found safe with no adverse effects at a dosage of 300 mg/kg in BALB/c mice [33]. 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 was much higher than the EC50 value on E. protoscoleces (1.9 ± 2 µM), thus indicating the high safety margin of this compound [34]. We have recently reported excellent anti-dengue activity of Ananrcardic acid [35]. Studies with A. occidentale hexane leaf extract showed that the toxic effects occurred at higher doses compared to Cameroon folk medicine 12]. 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 LC$_{50}$ values of 32.2 and 14.5 µM and selectivity indices of 6.1 and 21.2, respectively [36]. 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 with the using an online server tool, pkCSM. This study reported that all the AA derivatives, except two AA molecules (AA99 and AA100), showed good human intestinal absorption. Also, phenols have been subjected to clinical trials for over 20 years [20, 37]. We have done the toxicity study of the hydroalcoholic extract of leaves 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 upto 2000mg/kg body weight in BALB/c mice (data not shown).

**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. The potential of the hydroalcoholic extract of *A. occidentale* leaves need to be explored further for developing antilarial drug(s).

**Declarations**

**Acknowledgements**

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**Data availability**

All data generated or analysed during this study are included in this article. Further inquiries can be directed to the M.K., and S.LH. as one of the corresponding authors.

**Competing interests**

The authors declare no competing interests.

**Ethics approval**

No animal or human study was involved. The bioassay was performed in accordance with the relevant guidelines and regulations.
Experimental research and field studies on plant

The plant material was collected from the medicinal garden of ICMR-NITM Belagavi and performed all the experiments related to plant material in ICMR-NITM Belagavi, and it complies with the institutional and national guidelines.

Author contributions

M.K., research work and performed all the assays and wrote the manuscript; S.L.H., designed the study and wrote the manuscript and management of funds; J.K.S., provided enzyme and helped in enzyme purification and inhibition assays; R.K.J., provided and performed the phytochemical analysis of the plant and wrote the manuscript; I.S., helped in plant extraction and purification of enzyme; S.M., Helped in research work, H.V.H., plant identification and wrote the manuscript

References


**Figures**
Figure 1

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.
Figure 2

The SDS-polyacrylamide gel of the Ni-NTA recombinant *B. malayi* hexokinase (*Bm*Hk) purified. Protein fraction electrophoresis was done on a 10% (w/v) SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R250. Lane 1 Purified *Bm*Hk (A), Lane 2 prestained protein ladder (B). The original gel picture is presented in Figure 3, under Supplementary figure.
Figure 3

The SDS-polyacrylamide gel of the Ni-NTA recombinant *B. malayi* hexokinase (*BmHk*) purified. Lane 1- Molecular weight marker, Lane 2.