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Antifilarial effect of ursolic acid from *Nyctanthes arbortristis*: Molecular and biochemical evidences

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ABSTRACT

A bio-assay guided fractionation and purification approach was used to examine *in vitro* antifilarial activities of the crude methanolic extract of *Nyctanthes arbortristis* as well as fractions and isolated compound. From ethyl-acetate fraction we isolated and identified a triterpenoid compound which has been characterized as ursolic acid (UA) by HPLC and NMR data. We are reporting for the first time isolation and identification of UA from the leaves of *N. arbortristis*. The crude extract and UA showed significant micro- as well as macrofilaricidal activities against the oocyte, microfilaria and adult of *Setaria cervi* (*S. cervi*) by dye exclusion test and MTT reduction assay. Significant microfilaricidal activity of UA was further proved against mf of *W. bancrofti* by viability assay. The findings thus provide a new lead for development of a suitable filaricide from natural products. The molecular mechanism of UA was investigated by performing TUNEL, Hoechst staining, Annexin V-Cy3, flow cytometric analysis and DNA fragmentation assay. Differential expressions of pro- and anti-apoptotic genes were observed at the transcription and translational levels in a dose-dependent manner. Depletion in the worm GSH level and elevation in the parasite GST, SOD and super oxide anion indicated the generation of ROS. In this investigation we are reporting for the first time that UA acts its antifilarial effect through induction of apoptosis and by down-regulating and altering the level of some key antioxidants like GSH, GST and SOD of *S. cervi*.

1. Introduction

Lymphatic filariasis is a major tropical disease and one of the major common causes of global disability. Two filarial worms, namely, *Wuchereria bancrofti* and *Brugia malayi* cause lymphatic filariasis. The World Health Organization (WHO) considers lymphatic filariasis to be a global health problem affecting 120 million people in over 80 tropical countries. One-third of affected persons are from South Asia and another one-third from Africa. One-sixth of the world population is at risk of infection [1]. It is a disease of mostly the poor and it significantly affects their ability to earn an income. The WHO has outlined two objectives for its campaign of mass drug administration (MDA): to interrupt transmission and to reduce morbidity of the disease [1]. The first line choice of drugs are diethylcarbamazine (DEC), ivermectin and albendazole. DEC and ivermectin are effective at killing microfilariae (mf) but are associated with systemic and inflammatory adverse reactions. Albendazole increases the efficacy of DEC and ivermectin and is used in combination with either of the drugs as the basis of long-term intervention programme. One factor linked to resurgence of infection following MDA is the resistance to drugs. It is not possible to assess the resistance to DEC because its mode of action is not yet fully understood. In 2004, resistance to ivermectin was reported in *Onchocerca volvulus*, a filarial parasite affecting human(s) [2]. The resistance to albendazole (benzimidazoles) is seen in many nematode parasites due to single nucleotide polymorphism (SNP) [3]. Schwab et al. [4] have demonstrated that one of the SNPs exists in *W. bancrofti* in untreated populations studied in Ghana and Burkina Fasco, Africa. However, such mutations (a beta-tubulin allele associated with benzimidazole resistance) are selected for higher frequencies in these populations after mass treatment with combination therapy of albendazole and ivermectin [5]. Thus, there is an urgent need to develop a cheap, non-toxic novel antifilarial drug with long term microfilaricidal and macrofilaricidal activity. More than 80% of the people around the world, for their day-to-day medicinal needs, rely on traditional medicine, which has been around for centuries. Even modern medicine including drugs active against infectious agents in most instances relies on natural products or from structures

Abbreviations: Mf, microfilariae; HPLC, high performance liquid chromatography; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; GSH, glutathione; GST, glutathione-S-transferase; SOD, superoxide dismutase; NBT, nitroblue tetrazolium; DEC, diethylcarbamazine; DMSO, dimethyl sulfoxide; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; HRP, horseradish peroxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CED, cell-death abnormal; EGL, egg laying defective; RT-PCR, reverse transcriptase polymerase chain reaction.

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suggested by natural products. Compounds from natural sources have an advantage in that they are usually multi-targeted. With the recent emphasis of the WHO on the development of antifilarial drugs from natural products, we are engaged in the screening of a large number of extracts obtained from terrestrial plants. The bovine filarial parasite *Setaria cervi* (*S. cervi*), which resembles the human bancroftian parasite in its nocturnal periodicity and antigenic patterns [6], was used as a model organism for drug development research. The easy availability of the adult worms makes them more convenient for preliminary screening of antifilarials and molecular studies.

Several natural products had earlier proved themselves against many species of filarial infections [7]. We have observed filaricidal properties of two triterpenoid saponins acaciaside A and acaciaside B isolated from the funicles of *Acacia auriculiformis*. The saponins when tested on *S. cervi* transplanted in rats were found effective against both microfilaria and adult worm [8]. An ethanolic extract obtained from the funicles of the plant, which contains saponins, when administered orally to pariah dogs naturally infected with *Dirofilaria immitis* proved effective against both microfilaria and adult worm [9]. Recently we have shown micro- and macrofilaricidal activity of curcumin and ferulic acid [10,11].

Nyctanthes arbortristis Linn. (Order: Lamiales; Family: Oleaceae), commonly known as "Harsingar" or "Night jasmine", is a well documented plant. It is a native of India, distributed wild in the sub-Himalayan region and also found in Indian gardens as an ornamental plant. The indigenous people of Chittoor district (Andhra Pradesh, India) widely use the whole plant for treatment of cancer, root for fever, sciatica, and anorexia; bark as expectorant, leaf to control fever and diabetes and as a cholagogue, diaphoretic and anthelmintic. The decoction is used to treat arthritis [12] and malaria, to purge intestinal worms, and as a tonic and laxative [13]. Antileishmanial [14], anti-inflammatory [15] and antioxidant [16] activities have also been exhibited by the various extracts of the plant. Earlier workers have reported the isolation of polysaccharides, iridoid glycosides, phenylpropanoid glycoside, ß-sitosterol, ß-amyrin, hentri-acontane, benzoic acid, glycosides, nyctanthoside-a iridoid, nyctanthic acid, friedelin, lupeol, oleanolic acid, 6ß-hydroxylonganin, iridoid glucoside-arborside A, B and C, alkaloids, phlobatanins, terpenoids and cardiac glycosides from this plant [14,17].

Our preliminary observations reveal that the leaves of *N. arbortristis* possess strong antifilarial activity. This has prompted us to evaluate the activity in the crude extract and the active principle obtained from the leaves. The study confirmed strong antifilarial effect on adults and mf of *S. cervi* and mf of *W. bancrofti*. In this paper we present several molecular evidences that suggest the macrofilaricidal activity is mediated through induction of apoptosis possibly by ROS (reactive oxygen species).

2. Materials and methods

2.1. Plant materials

Plant leaves were collected from the laterite soil of Birbhum district (23.68°N and 87.68°E) of West Bengal, India and authenticated by a botanist from Botanical Gardens, Howrah, India. A voucher specimen has been deposited at the herbarium of the National Institute of Pharmaceutical Education and Research, Kolkata for future studies (NIP-K/BCP/005).

2.2. Chemicals and reagents

HPLC grade methanol, acetic acid, other chemicals and solvents of highest purity grade were purchased from Merck India. Milli-Q water (Milli-Q Academic with 0.22 μm Millipak R-40) was used for the assays and HPLC analysis. FBS (fetal bovine serum), HEPES buffer, ivermectin, streptomycin, penicillin, amphotericin-B, Hoechst 33258, Annexin V-Cy3 and AnnexinV-FITC apoptosis detection kit were purchased from Sigma Aldrich, St. Louis MO, USA. RPMI-1640, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), NBT (Nitroblue tetrazolium) and reagents required for GST, GSH assay were obtained from Hi-Media Laboratories, Mumbai, India. SOD assay kit was procured from Cayman Chemical, Ann Arbor, USA. Primary antibodies; egg laying defective-1 (EGL-1), cell death abnormal (CED)-3, 4 and 9, β-tubulin and horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

2.2.1. Extraction and isolation

The freshly collected leaves were shade-dried and crushed to a coarse powder using a mechanical grinder. The coarse powder (3 kg) was subjected to extraction using methanol (10 L × 6) at room temperature for 48 h. The extract was filtered and the methanol was evaporated under reduced pressure using a rotary evaporator at 45 °C. The resultant extract was lyophilized to yield the crude methanol extract (148.45 g). A part (70 g) of the extract was then suspended in milli-Q water and partitioned successively with ethylacetate and n-butanol [18]. Each fraction was evaporated under vacuum to yield the ethylacetate fraction (40.31 g), n-butanol fraction (16.96 g) and aqueous fraction (12.0 g). All the fractions were stored at 4 °C till further use. The ethylacetate fraction (25 g) was chromatographed over silica gel (250 g) using step gradient of petroleum ether (1000 mL, fr-1), petroleum ether-chloroform 1:1 (2000 mL, fr-2), chloroform (2000 mL, fr-3), chloroform-methanol 9:1 (2000 mL, fr-4), 8:2 (2000 mL, fr-5) and 7:3 (2000 mL, fr-6), to get a total of 6 fractions. Fr-4 (8 g) was further eluted, over a bed of silica gel (80 g), with increasing polarity of solvents, petroleum ether:chloroform (1:1 to 0:1 v/v), chloroform-methanol (9.5:0.5 to 7:3 v/v) to give a total of 50 fractions (fr-4-1 to fr-4-50). Out of all the fractions tested, fraction 4 revealed maximum inhibition of the parasite. The active fractions (Fr-4-29 to fr-4-36) were mixed together, based on TLC analysis, and crystallized by methanol and chloroform to give a compound (110 mg), which was identified as ursolic acid by comparison of its spectroscopic data (mass, ^{1}H-NMR and ^{13}C-NMR) with those reported earlier [19].

2.2.2. Quantitative analysis by HPLC

HPLC analyses were performed at 25 ± 1 °C using sample solutions filtered through 0.45 μm membrane (Whatman's syringe filter) and analyzed (20 μL injected volume). Separation was achieved using Water's XTerra™ RP column (C18, 4.6 × 250 mm, 5 μm particle size) and isocratic elution was done using mobile phase 0.2% acetic acid in methanol: water (90:10 v/v) at a flow rate of 1 mL/min. The eluate was monitored at 210 nm. Quantitative estimation of the constituents present in the methanolic extract and fractions (10 mg/mL) was done by using the calibration curve of the standard solutions (1 mg/mL). The experiment was performed in triplicate.

2.3. Sample preparation

The crude extract, fractions and UA obtained from the leaves of *N. arbortristis* and the standard drug ivermectin were dissolved in DMSO to make a stock solution and diluted in RPMI-1640 to obtain the desired concentrations.

2.4. Ethical clearance

The protocol of this study was approved by the Institutional Animal Ethics Committee (Visva-Bharati University, Santiniketan, West Bengal, India) and the Human Ethical Committee of the Sub Divisional Hospital, Bolpur, West Bengal, India. Before taking blood samples, each volunteer (men and women) was read a consent form and required to sign it.