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Antifilarial Activity of *Eucalyptus globulus* Labill. Leaves Against *Brugia malayi*

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Abstract

The present study is aimed to evaluate the antifilarial activity of *Eucalyptus globules* Labill. (*Myrtaceae*) against human lymphatic filarial parasite *Brugia malayi in vitro* and *in vivo*. The ethanolic extract of the leaves was tested *in vitro* on adult worms and microfilariae (mf) of *B. malayi* and the active sample was further evaluated *in vivo* in *B. malayi* intraperitoneally (i.p.) transplanted in the jird model (*Meriones unguiculatus)* and *Mastomys coucha* subcutaneously infected with infective larvae (L3). The ethanolic extract of the leaves of the *E. globulus* was tested *in vitro* on adult worms and microfilariae (mf) of *B. malayi* and the active sample was further evaluated *in vivo* in *B. malayi.* The ethanolic extract was active *in vitro* (IC_{50}: adult = 62.5 µg/ml; mf = 31.2. µg/ml) where it demonstrated 66.7% adulticidal and embryostatic effect on *B. malayi* in *Mastomys* at a dose of 5 × 100 mg/kg by oral route. The antifilarial test conducted was at 5 × 100 mg/kg by subcutaneous route revealed excellent adulticidal efficacy resulting in to the death of 66.7% transplanted adult *B. malayi* in the peritoneal cavity of jirds in addition to noticeable microfilaricidal action on the day of autopsy. The findings revealed that the extract from the leaves of *E. globulus* contains promising *in vitro* and *in vivo* antifilarial activity against human lymphatic filarial parasite *B. malayi* which may be further explored to new antifilarial agents.

Key words: *E. globulus*, antifilarial activity, *in vitro, in vivo, B. malayi*

Introduction

Over the past few years, the interest in natural medicine has been increasing in industrialized societies because of the ever growing problem of side effects and high cost of synthetic medicines. *Eucalyptus* is a native genus from Australia belonging to *Myrtaceae* family and comprises about 900 species (Booker *et al.*, 2004). More than 300 species of this genus contain volatile oils in their leaves. Some 20 species are known for their high content of 1,8-cineole (more than 70%) which have been commercially used for the production of essential oils in pharmaceutical and cosmetic industries (Pino *et al.*, 2002). In Tunisian folk medicine, inhalation of essential oil *Eucalyptus* sp. has traditionally been used to treat respiratory tract disorders such as pharyngitis, bronchitis, and sinusitis (Boukef, 1986). Consequently, the scientific interest in this field has been expanding. Researchers have demonstrated some efficacy of *E. globulus* essential oil against *Haemophilus influenzae* and *Stenotrophomonas maltophilla* (Cermelli *et al.*; 2008 *et al.* and Fabio *et al.*, 2007). Few studies have reported the antiviral activity of *Eucalyptus* essential oils against Adenovirus, mumps and herpes simplex viruses (Schnitzler *et al.*, 2001).

Eucaliptus globulus

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Materials and Methods

Plant material: Leaves of the *E. globulus* (500g.) were purchased from the local market of Uttar Pradesh,, India and was authenticated at the Botany Department of the Central Drug Research Institute, Lucknow, India,where voucher specimen have been preserved with the code number 2191.

Extraction: The air dried leaves of the *E. globulus* (500 g) were powdered and percolated in 95% ethanol at room temperature for 24 hours, filtered and the process was repeated four times. All the extracts were mixed and filtered. Mixed ethanolic extract was concentrated under reduced pressure below 50 ºC in a rotavapour to a green viscous mass, which was dried under high vacuum for 2 hours to remove the last traces of the solvent. Weight of the dried ethanolic extract 18.5 g which was used for the screening of antifilarial activity against *B. malayi*.

Antifilarial activity: *in vitro* assays

Sample preparation: 1 mM stock solution of the ethanol extract of the *E. globulus* was prepared in dimethylsulfoxide.

Parasite isolation: The live adult *B. malayi* worms were isolated from the peritoneal cavity of jird (*Meriones unguiculatus*) infected 100-150 days earlier by intraperitoneal inoculation of 150-200 infective larvae (L3) of *B. malayi* recovered from experimentally infected mosquitoes, *Aedes aegypti* (McCall *et al*. 1973 After isolating the adult parasites, the peritoneal washing was passed through a membrane filter (pore size 5.0 mm) and the microfilariae were pelleted by centrifugation (Singh *et al*., 1985). All the animals and experimental procedures were duly approved by the Animal Ethics Committee of CDRI, duly constituted under the provisions of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India. This study bears the approval no. IAEC/2011/120/Renew 01/dated 14/08/2012.

Primary in vitro screening: The actively motile female worms were placed individually wells of 48 well culture plate containing RPMI 1640 medium fortified with antibiotics (penicillin 100 units/mL, streptomycin sulfate 100 mg/mL, and neomycin mixture; Sigma, USA). Each well contained one female worm in 1 mL of the medium. Simultaneously, 10 microfilariae were suspended in 200 mL medium in each well of a 96 well culture plate (NUNC). The parasites were incubated at 37 °C in 5% CO_2 in air for 5 days in the presence of 10 mM concentration of the ethanol extract of leaves and the motility of parasites was monitored microscopically at regular time intervals. At the end of the experiment, adult parasites were transferred to fresh drug free medium for one hour at 37 °C to observe reversal, if any, in the worm motility. The worms were later processed individually for MTT [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as published earlier (Mukherjee *et al*., 1988) for checking their metabolic viability. The experiments were carried out in duplicate and the degree of loss in the motility as well as percent inhibition in MTT reduction in treated parasites over the untreated controls was assessed. The extract which consistently demonstrated their lethal effects on the parasites at 10 mM (highest concentration tested) with 50% inhibition in MTT reduction as compared to untreated respective controls were considered as active extract (Mukherjee *et al*., 1988) while those bringing about 100% irreversible inhibition in motility of microfilariae were considered microfilaricidal.

(iv) *Evaluation of IC_{50} and CC_{50}:* The test samples found active in primary *in vitro* screen were followed for IC_{50} using four serial two fold dilutions of each sample starting from MIC in the same way as mentioned above. IC_{50} values were determined by Excel based line graphic template after plotting concentration values of each sample versus percent motility inhibition of parasite on x- and y-axis. *In vitro* CC_{50} assay on Verocells (monkey kidney cell line) was performed as mentioned earlier (Misra *et al*. 2011). In brief, Vero cells (104/well/100 ml) in 96 well plate were exposed to seven three-fold serial dilutions of active test samples starting from 100 mM at 37 °C in a CO_2 incubator. After 72 h, resazurin dye was added and the plate was re-incubated for 3-4 h. The reaction was monitored fluorometrically at an excitation wavelength of 536 nm and emission at 588 nm in a fluorometric plate reader.

Scoring and activity evaluation criterion: The motility of the adult worms and microfilariae was

scored as 0% motility reduction (4þ); 1-49% motility reduction (3þ); 50-74% motility reduction (2þ); 75-99% motility reduction (1þ) and 100% motility reduction (dead) (Misra *et al*. 2011).

Determination of selectivity index (SI): The safety of the active samples was determined by assessing SI values (CC_{50}/IC_{50}) The extract with SI values of 10 were considered safe and therefore further followed in vivo.

In vivo antifilarial activity

In vivo screening model: Intraperitoneal (i.p) transplantations of 10 females and five male adult worms of *B. malayi* were carried out in 6-8 week old male jirds. The jirds were anaesthetized by ketamine (50 mg/kg, i.p), animals were quickly shaved and a small incision was made on latero-ventral region of abdomen to introduce worms into the peritoneal cavity. The success of transplantation was affirmed by the presence of live microfilaria in a drop of peritoneal fluid aspired on day 4 and these jirds were selected to screen *in vivo* hits.

Treatment schedule: The ethanol extract of the leaves of *E. globulus* and the standard drug, diethylcarbamazine were administered subcutaneously at a dose of 100 mg/kg for five consecutive days to seven groups of transplanted jirds and each group had three animals. Three jirds received vehicle only and served as control group.

Assessment of antifilarial activity in the primary jird model: The treatment was initiated from day 5 post worm transplantation and the observations were continued till 45 days. On 45th day, the jirds were euthanized along with the untreated controls to recover worms by peritoneal washings. The recovered parasites were counted and examined for motility, death or calcification. Live female worms were teased in a drop of phosphate buffer saline (PBS, pH 7.2) to dissect out the uterus for observing the uterine contents microscopically to assess the embryostatic effect of test samples, if any (Bajpai *et al*., 2005). The peritoneal washing collected on autopsy was microscopically observed to assess the effect of test sample on released microfilariae.

Statistical analysis: The statistical analysis of the data was carried out by PRISM 3.0 using one way ANNOVA (nonparametric). Dunnett's multiple (Bajpai *et al*., 2005) comparison test was applied to assess the statistical significance of the values between treated and control group. Values were expressed as mean ± SE. $P < 0.05$ was considered as of low significance (*) while $P < 0.01/0.001$ were considered as highly significant (**/***).

Results

The ethanolic extract of the leaves of *E. globulus* was tested *in vitro* on adult worms and microfilariae (mf) of *B. malayi* and the active sample was further evaluated *in vivo* in *B. malayi* intraperitoneally transplanted in the jird model and *Mastomys coucha* subcutaneously infected with infective larvae (L3). The ethanolic extract was found to be active *in vitro* (IC_{50}: adult = 62.5 µg/ml; mf = 31.2. µg/ml) and demonstrated 66.7% adulticidal and embryostatic effect on *B. malayi*, respectively, in Mastomys at a dose of 5 × 100 mg/kg. The antifilarial activity at 5 × 100 mg/kg by subcutaneous route revealed excellent adulticidal efficacy resulting in the death of 66.7% transplanted adult *B. malayi* in the peritoneal cavity of jirds in addition to noticeable microfilaricidal action on the day of autopsy.

Conclusion

The present findings revealed that the ethanol extract of the leaves of *E. globulus* contains promising *in vitro* and *in vivo* antifilarial activity against human lymphatic filarial parasite *B. malayi* which can be further explored to isolate and characterize the active molecules to provide new antifilarial agents.

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