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Research Article
Ethyl Acetate Root Extract of *Terminalia glaucescens* Protects *Drosophila melanogaster* Against Virulent *Aspergillus* Species

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Abstract

Background and Objective: Fungal pathogens especially, *Aspergillus fumigatus* and *Aspergillus flavus* are the main fungi implicated in many fungal diseases of plants and animals. The present study determined the antifungal activity of ethyl acetate root extract of *Terminalia glaucescens in vitro* against *Aspergillus fumigatus* and *Aspergillus flavus* and *in vivo* against *A. fumigates* in *Drosophuila melanogaster*. **Materials and Methods:** In the *in vitro* evaluation, antifungal potentials of the extract were measured using the agar well diffusion method. Survival of *Drosophila melanogaster* (Drs-LacZ) challenged with *A. fumigatus* by ingestion method was used to evaluate *in vivo* activity. **Results:** Antifungal activity increased in a concentration dependent manner. There was increased activity with increased concentrations. *Aspergillus fumigatus* was more susceptible to ethyl acetate extract of the roots of *T. glaucescens* with zones of inhibition when compared with *A. flavus* at the same concentrations. Zones of inhibition recorded with Itraconazole at different doses against *A. flavus* and *A. fumigatus*. MIC of 12.5 mg mL^{-1} was recorded against both organisms with Itraconazole and ranged from 62.25-125 mg mL^{-1} with the ethyl acetate root extract. Survival rate increased by more than 50% in flies treated with ethyl acetate root extract of *T. glaucescens*. **Conclusion:** The antifungal results *in vitro* and *in vivo* validate the use of this plant in traditional medicine in the treatment of fungal infections. These results have stressed the need for isolation and characterization of the active compound and the elucidation of the mechanisms of action of secondary metabolites on the immune response of *Drosophila melanogaster*.

Key words: *Aspergillus fumigatus*, *Aspergillus flavus*, *Drosophila melanogaster*, *Terminalia glaucescens*, ethyl acetate root extract, secondary metabolites, fungal infection

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The incidence of opportunistic fungal infections has been on the increase in recent times especially among people with compromised immunity in HIV infections, cancers and transplant recipients. The high morbidity and mortality rates associated with invasive fungal infections are a cause for concern. Fungal pathogens are becoming prospective agents of bioterrorism; there is widespread emergence of fungi other than *Aspergillus* species being implicated in invasive diseases, increased use of antifungal prophylaxis[1] and resistance to standard antifungal drugs[2]. Invasive fungal infections pose a threat to public health in spite of the advancements in human medicine and more so in developing countries where availability of medicines is relatively low coupled with the toxic effects and high cost of antifungal drugs. Current approaches are focused on finding potent, safe and effective antifungal drugs from plants based on their availability and ethno-medicinal use[3]. There are many methodological problems associated with evaluation of antifungal activity *in vitro*, resulting in discrepancies with clinical outcomes[4]. Thus *in vivo* studies carried out in animal models are preferred only those models are costly, labour intensive and not conducive for rapid testing of antifungal potency[5].

Consequently, non-vertebrate mini hosts such as *D. melanogaster*, *Caenorhabditis elegans* are used to study the pathogenesis of *Aspergillus* spp. and other fungi as well as Gram-positive bacteria and yeast[6]. The edge with *D. melanogaster* is advantageous because it has a short life cycle, can be studied in large numbers with relative ease and at lower cost. *D. melanogaster* was used in this study as a model host to evaluate the antifungal potentials of *Terminalia glaucescens* ethanolic root extract against virulent *Aspergillus* species. The Imd and Toll are the two conserved signaling pathways activated during immune response to pathogen challenge. The Toll pathway confers protection in *D. melanogaster* against fungi and with it mutations, the fly becomes easily infected to *Aspergillus* species and other fungi[7].The purpose of this study was to investigate the antifungal potentials in the extracts of *Terminalia glaucescens* and its protective role against virulent *Aspergillus* species infection in *Drosophila melanogaster*.

MATERIALS AND METHODS

Study area: The study was carried out between June, 2018 to August, 2019, at the Drosophila Laboratory: Fungal pathogens and Plant Bioactive Compounds, Department of Plant Science and Biotechnology, University of Jos, Nigeria.

Plant collection and authentication: The fresh leaves and fruits of, *Terminalia glaucescens* Planch ex. Benth was identified in the Department of Pharmacognosy and Drug Development, Kaduna State University, Kaduna State, Nigeria with the voucher number KASU/PCG/097. The plant specimen (roots) was then collected based on ethno-botanical uses by the community.

The roots of *Terminalia glaucescens* were obtained and washed with clean tap water to rid them of all debris and dust particles. The clean roots were then shade dried to a constant weight (over three weeks) and size reduced to a coarse powder using pestle and mortar. One kilogram of coarse powder was successively macerated with petroleum ether, dichloromethane and ethyl acetate, in order of increasing polarity) in a sealed vessel at room temperature and frequently stirred with a sterile glass rod for over 72 h with frequent stirring using a sterile glass rod[8]. A muslin cloth was then used to filter the mixture, the filtrate allowed to stand for some time, decanted and further filtered through Whatman No. 1 filter paper. The filtrate was then concentrated in vacuum under reduced pressure in a rotary evaporator maintained at a temperature of 40°C. The concentrated extract was then evaporated to dryness on a thermostatic water bath maintained at a temperature of 50°C, weighed, the percentage yield calculated, stored in an air tight bottle and kept in a refrigerator maintained at a temperature 40°C until required for further use. The marc was always shade dried at room temperature completely before a successive solvent was added for extraction.

Phytochemical screening: Phytochemical screening was carried out to determine the presence of phytochemical constituents according to standard methods[9-11].

Preparation of fungal inoculums: Stock cultures of *A. fumigatus* and *A. flavus* were streaked on yeast agar glucose (YAG) and PDA respectively and incubated at 29°C for 3 days. Colonies produced from the subcultures were identified through their macroscopic and microscopic morphological characters and the ability to grow at 48°C for *A. fumigatus*[12]. A sterile inoculating needle was used to collect spores from the agar plates, suspended in 4 mL of sterile water and homogenized. Heavy particles were allowed to settle and the homogeneous suspension was adjusted to 0.5 McFarland standards equivalent to the turbidity of the suspension adjusted with a spectrophotometer at 530 nm to obtain a final concentration to match that of a 0.5 McFarland standard for mould[13] (0.4×10^6) CFU mL^{-1}.

In vitro antifungal assay

Antifungal susceptibility of *Aspergillus* isolates: The antifungal susceptibility of *Aspergillus* isolates was carried out[14]. In the procedure, five equidistant wells of about 2.5 mm deep and some millimeters away from the edge of the plate were made using a sterile 4 mm cork borer into *A. fumigatus* and *A. flavus* seeded Sabouraud dextrose agar plates. About 50 µL of the varying concentrations of constituted extract were placed in the wells. The plates were allowed to stand for an hour for optimum initial diffusion of the extract into the medium[15]. This was incubated for 72 h at 29°C. Fungal susceptibility was determined by measuring the zones of inhibition around the wells on the agar surface (including the diameters of the wells) with a meter rule in millimeters. Sterile distilled water and Itraconazole, served as the negative and positive controls respectively.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC): The minimum inhibitory concentration was determined according to the method described elsewhere[16]. In the procedure, a 5 mL double strength potato dextrose broth was prepared and introduced in the first of 7 sterilized test tubes (T_1-T_7) while the remaining test tubes had 5 mL of the nutrient broth each. Then 5 mL of 500 mg mL^{-1} of the extract was added into T1 and mixed thoroughly. From T1, 5 mL was taken and transferred into T2 and well mixed before transferring 5 mL from T2 into T3 and done continuously up to T7, discarding the last withdrawn 5 mL from T7. Into each of these tubes, about 0.1 mL of the test organism was introduced and shaken to mix. The test tubes were then plugged and incubated at 28°C for 72 h. The minimum inhibitory concentration was the first tube without visible growth. This procedure was done in triplicates. The MIC was identified as the lowest concentration that inhibited the growth of the test organism.

Media from each tube showing no visible growth was sub-cultured on media plates and incubated at 29°C until there was visible growth on the control plates[14,17]. The MFC is the consequent concentration required to kill 99% of the cells.

In vivo antifungal assay

***Drosophila melanogaster* stock selection, sexing and sorting:** *Drosophila melanogaster* (Drs-LacZ) was obtained from UPLEM-Lemaitre Laboratory-EPFL and used for all experiments. They were maintained and bred in fly basal medium at constant temperature and relative humidity of 23±1°C and 60% respectively under natural day/night lighting conditions. Newly emerged virgin flies were anaesthetized on ice to enable for gender separation. Virgin female flies were used because they have been found to display lesser mortality rate compared to 10-15 day old following *Aspergillus* infection[5,6]. Female flies also have a larger size and the selection of only one gender reduces potential sex-dependent infection susceptibility effects.

Acute toxicity testing of *T. glaucescens* roots extracts and Itraconazole in *D. melanogaster*: Basal fly food was prepared, 4 mL placed in each feeding vial and allowed to dry over 5 h. Burrows were made on its surface with a paint brush. Then, 1 mL of each of serial concentrations (90, 80, 70, 60, 50, 40, 30, 20 and 10 mg mL^{-1}, respectively) of the extract was introduced in the prepared food surface and allowed to stand for some time for optimal absorption into the meal. A little amount of yeast was spread over the surface of the food to absorb residual extract and to prevent moistening of the surface. Fifteen virgin female flies (earlier starved for 8 h) were transferred into each vial. The flies were monitored at 3 and 6 h and every day for 14 days for signs of toxicity and any mortality. Two controls were used. The positive control was Itraconazole while the negative control were, starved flies placed in vials containing only basal fly food. Each experiment was carried out in triplicate.

Preparation of virulent fungal inoculum establishment of infection: Using a sterile loop, frozen glycerol stock of *A. fumigatus* was streaked onto YAG agar plates and incubated at 37°C for 24 h. A single colony from this was inoculated onto a fresh YAG agar plate and incubated at 37°C for 72 h. A uniform lawn of *A. fumigatus* formed on the agar surface. About 0.5 mL of autoclaved water was added to the surface of the agar to collect the conidia. Virulence for *A. fumigatus* is ability to grow at 37°C. A sterile glass spreader was used to spread 100 µL of the conidia solution onto special fly food vials containing YAG medium and incubated at 37°C for 72 h to obtain a uniform lawn of conidia on the surface. Fifteen female flies which had been starved for 6 h were then transferred onto the conidial carpet and allowed to feed on it for 8 h.

Preparation of drug containing food vials and assessment of fly survival rate: Basal fly food was prepared as earlier described. Fifteen infected virgin female flies were placed into the feeding vials and allowed to feed. After every 2 days, the flies were transferred into fresh fly food vials containing the plant extract for 14 days and maintained at 29°C. Each experiment was carried out in triplicate. The infected flies were

observed at 3 and 6 h post infection. Monitoring of live flies and recording the survival rate of the infected flies treated with various concentrations of the extract was done every day for 14 days. Three controls were used. These consisted of infected flies treated with Itraconazole at the same concentration, infected flies that were not treated and uninfected flies fed with extracts in glucose containing fly meal.

Statistical analysis: All experimental data was analyzed using GraphPad Prism Software, version 8.0. Comparison between treatment groups was done using Kruskal-Wallis test and survival rates differences between treatment groups were analyzed using log-rank test 8.0) where $p \geq 0.05$ is significant.

RESULTS

Extract yield, characteristics and phytochemical screening: The yield of *T. glaucescens* Planch ex Benth roots in ethyl acetate was 1.3% w/w in the ethyl acetate extract was observed to be a light golden brown colored powder. The phytochemical investigation of the ethyl acetate extract showed the presence of cardiac glycosides, flavonoids, tannins, triterpenes, saponins, steroids and alkaloids. The results obtained are shown in Table 1.

Susceptibility of *Aspergillus* isolates to ethyl acetate extract: Evaluation of the antifungal activity of the root extracts of *T. glaucescens* was measured by the zones of inhibition of mycelia growth of *Aspergillus* species observed around the wells containing varying concentrations of the extracts (ranging from 31.25-500 mg mL^{-1}) as well as the positive and negative controls. At 500 mg mL^{-1}, the zones of inhibition against *A. flavus* was 24.83±0.29 mm with ethyl acetate root extract Ethyl acetate root extract however, inhibited *A. fumigates* with zones of 28.83±0.29 mm at 500 mg mL^{-1} and 22.00±0.76 mm at 31.25 mg mL^{-1}. Zones of inhibitions were observed to be concentrations dependent and also dependent on the duration of treatment. Results obtained are shown in Table 2.

Susceptibility of *Aspergillus* isolates to the standard antifungal drug: The positive control, Itraconazole showed zones of inhibition ranging from 8.0-17.0 mm against *A. fumigatus* and 15.0-27.0 mm against *A. flavus* at various concentrations. However, *A. fumigatus* showed lesser susceptibility compared to *A. flavus*. The negative controls of distilled water and 30% DMSO showed no inhibition against the *Aspergillus* species. Results are shown in Table 2.

Table 1: Phytochemical screening of the root extracts of *T. glaucescens*

	Extracts		
Phytochemicals	Pet ether	Dichloromethane	Ethyl acetate
Alkaloids	-	+	+
Anthraquinones	-	-	+
Carbohydrates	+	+	+
Cardiac glycosides	-	-	+
Flavonoids	-	-	+
Reducing sugar	+	+	+
Saponins	-	-	+
Steroids	+	+	+
Tannins	-	+	+
Triterpenes	+	+	+

+: Presence, -: Absence

Table 2: Antifungal activity of the ethyl acetate root extract of *T. glaucescens* and fluconazole against *Aspergillus* species

	Fungal pathogens	
Antifungal agents (mg mL^{-1})	*A. flavus*	*A. fumigatus*
Ethyl acetate root extract (EARE)		
500	24.83±0.29	28.83±0.29
250	23.83±0.29	28.00±0.00
125	22.50±0.50	27.33±0.29
62.5	21.00±0.29	26.17±0.29
31.25	20.00±0.00	22.00±0.76
Itraconazole		
200	27.00±0.71	17.00±0.36
100	22.00±0.19	12.00±0.00
50	19.33±0.36	10.00±0.00
25	15.33±0.00	8.00±0.00
12.5	12.00±0.00	6.83±0.29
Distilled water	-	-
30% DMSO	-	-

Values are zones of inhibition (mm), -: No inhibition

Table 3: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *T. glaucescens* root extracts and fluconazole

	A. flavus (mg mL^{-1})		*A. fumigatus* (mg mL^{-1})	
Parameters	MIC	MFC	MIC	MFC
Ethyl acetate	125.0	+	62.5	250
Itraconazole	12.5	50	12.5	100

+: Fungal growth in tubes/plates

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC): The ethyl acetate root extract had MIC values of 125 and 62.5 mg mL^{-1} against *A. flavus* and *A. fumigatus* respectively while the control drug, Itraconazole, had MIC of 12.5 mg mL^{-1} against both test fungi. The extract was only fungicidal at 250 mg mL^{-1} against *A. fumigatus* while Itraconazole had MFC values of 50 and 100 mg mL^{-1} against *A. flavus* and *A. fumigatus*, respectively. The MFC values were greater than those of the MIC but showed similar pattern with the MIC values (Table 3).

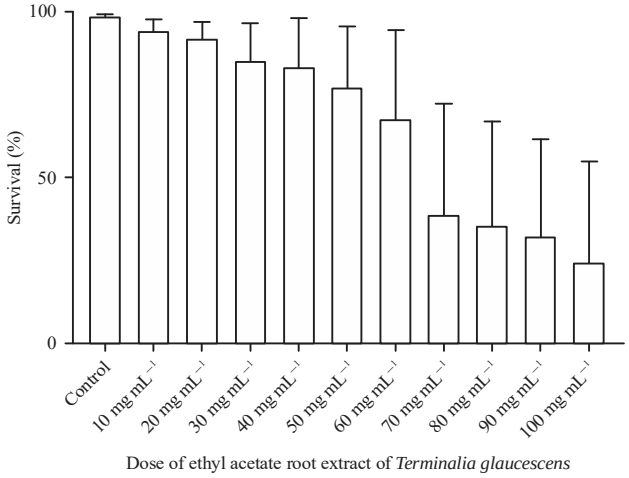

Fig. 1: Acute toxicity of ethyl acetate root extract of *T. glaucescens* on *Drosophila melanogaster* (Drs-LacZ)

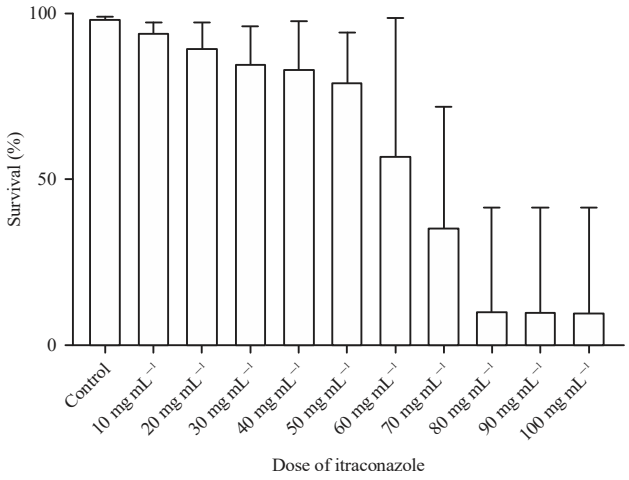

Fig. 2: Acute toxicity of itraconazole on *Drosophila melanogaster* (Drs-LacZ)

Acute toxicity studies of ethyl acetate root extract of *T. glaucescens* and itraconazole in *D. melanogaster*: At the end of 7 days of acute toxicity studies, fly mortality rate increased with increasing concentration. An LD_{50} of 70 mg mL^{-1} was recorded at which point mortality was recorded in half of the population. LD_{50} for Itraconazole was 50 mg mL^{-1}. These results are presented in Fig. 1 and 2.

Effects of *T. glaucescens* ethyl acetate root extract and Itraconazole on survival rate of *D. melanogaster*: The infectious studies showed a dose dependent increase in survival rate of the flies treated with ethyl acetate root extract of *T. glaucescens* at 20-100 mg mL^{-1} compared to the

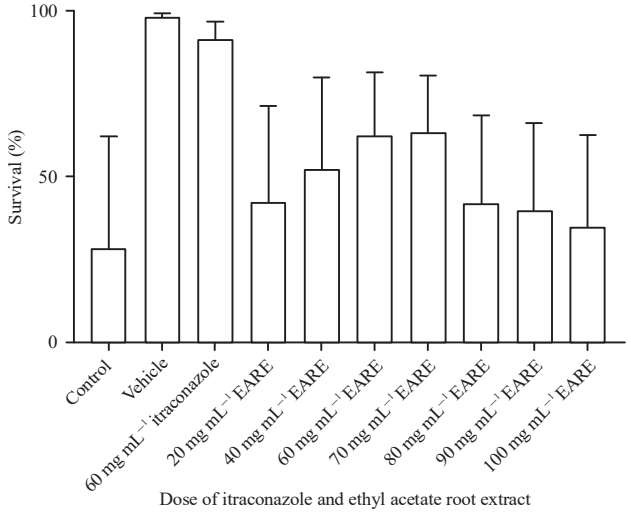

Fig. 3: Survival of *Drosophila melanogaster* (Drs-LacZ) infected with *Aspergillus flavus* and treated with ethyl acetate root extract (EARE) from *Terminalia glaucescens* and itraconazole

negative control of *A. terreus* infected flies that were not treated There was increased in survival rates in infected flies treated with the standard drug as shown in Fig. 3, Itraconazole compared to the plant extract which improved survival at relatively lower and safer doses of 30 and 40 mg mL^{-1}.

DISCUSSION

The presence of the following phytochemicals like alkaloids, saponins, tannins, cardiac glycosides, flavonoids, triterpenes, steroids and anthraquinones which have been reportedly detected in *T. glaucescens*[18-23] are hereby confirmed. Success in isolating compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Ethyl acetate was quantitatively the best extractant, with a greater quantity of plant material extracted than any of the solvents used.

In vitro antifungal activity of *T. glaucescens* has been reported against fungi[24]. In this study, zones of inhibitions were observed to be dose dependent as well as increasing with increasing duration of treatment. Although antifungal effects of ethyl acetate root extract was lower than Itraconazole against *A. flavus*, its activity against *A. fumigatus* was considerably higher but not more than Itraconazole. The observed antifungal effects of the plant extract *in vitro* varied with the data obtained in the *in vivo* study. This could be as a result of variations in the quantity of active principle in the extract administered and its availability, possible antagonism

from other phytochemicals present, physiological interactions, pharmacokinetics, extract toxicity, mode of infection, fungal virulence or activity of antimicrobial peptides. However, the antifungal effects of ethyl acetate extract of the root *in vivo* were comparable to Itraconazole and at significantly lower doses.

The ethyl acetate root extract of *T. glaucescens* showed good inhibitory potential against *Aspergillus* species *in vitro*, against *A. fumigatus in vivo* at lower and less toxic concentrations than Itraconazole. This validates the medicinal traditional use of *T. glaucescens* in fungal infections and calls for the isolation and elucidation of the mechanism of action of the active metabolite. The ethyl acetate extract of *T. glaucescens* inhibited the growth *in vitro* of *Aspergillus* species as well as *in vivo* in this study, indicating that ethyl acetate extract from root of *T. glaucescens* is a potential source of antifungal compounds and therefore, supports the traditional uses in treatment of various fungal infections of plants and animals. That the survival rate of *D. melanogaster*, increases to 50% and above could be traced to phytochemicals in ethyl acetate fraction in the plant extract. These phytochemical constituents aid *D. melanogaster* to combat the fungal infection through the Toll pathway, activated mainly by gram-negative bacteria and fungal pathogens making *D. melanogaster* exhibit a potent host defense. This study is limited to the use of one plant *T. glaucescens* and only one part, the roots. It also uses *Aspergillus* species and the only fungal pathogens.

CONCLUSION

Ethyl acetate root extract from *T. glaucescens* inhibited significantly, at lower to higher concentrations virulent Aspergillus species used in this study. Ethyl acetate root extract based on the LC_{50} values significantly protected *Drosophila melanogaster*, when compared with standard antifungal drug, Itraconazole. It is not evident what mechanism underlies this protective ability of the ethyl acetate root extract, but more than 50% of *D. melanogaster* infected with virulent *Aspergillus* species exposed to varying dosed survived at doses ranging from 30-70 mg mL^{-1}.

SIGNIFICANCE STATEMENT

This study discovered that ethyl acetate root extract of *T. glaucescens* inhibit virulent *Aspergillus* species *in vitro* and protects *D. melanogaster* against virulent *Aspegillus fumigates*, causing more than 50% survival rate when treated with 60 mg mL^{-1} of ethyl acetate root extract. This study will help researchers to uncover the critical areas of how phytochemicals regulate the molecular mechanisms underlying the fly defense reactions and how each of these molecular mechanisms contributes to defense during an infection.

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