Research Journal of Medicinal Plant

ISSN 1819-3455

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Medicinal Plants

ISSN 1819-3455
DOI: 10.3923/rjmp.2019.64.73

Research Article
Antifungal Activity of *Terminalia avicennioides* in *Drosophila melanogaster* as an Infectious Model

[1]Ziriyi Winifred Achi, [2]Satkat Longchi Zacchaeus, [1]Dafam Dalen Gwatau, [2,3]Kakjing Dadul Falang and [2]Ponchang Apollos Wuyep

[1]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, University of Jos, Nigeria
[2]Department of Plant Science and Biotechnology, Faculty of Natural Sciences, University of Jos, Nigeria
[3]Department of Pharmacology, Faculty of Pharmaceutical Sciences, University of Jos, Nigeria

Abstract

Background and Objective: Conventional antifungal agents have become less effective against fungal pathogens especially, *Aspergillus fumigatus* and *Aspergillus flavus* because of the toxicity and resistance. This has prompted the search for novel prototype antifungals from higher plants. The present study determined the antifungal activity of leave, stem bark and root extracts of *Terminalia avicennioides in vitro* and *in vivo* against *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus terreus in Drosophila melanogaster*. **Materials and Methods:** In the *in vitro* and *in vivo* evaluation of antifungal potentials of the extracts was measured using the microdilution method in a 96-well diffusion method. Survival of *Drosophila melanogaster* (Drs-LacZ III) challenged with *A. fumigatus*, *A. flavus* and *A. terreus* 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. *A. fumigatus*, *A. flavus* and *A. terreus* was more susceptible to dichloromethane followed by ethyl acetate extract of the leave and roots of *T. avicennioide.* MIC of 0.32-0.18 mg mL^{-1} was recorded against test organisms after 24 and 48 h with Amphotericin B as standard antifungal drug and with the petroleum ether, ethyl acetate, dichloromethane and ethanol leave, stem bark and root extracts. Survival rate increased by more than 50% in flies treated with dichloromethane and ethyl acetate leave and root extract of *T. avicennioides*. Drosomycin, an AMP increased as fungal infection progressed. **Conclusion:** The antifungal results *in vitro* and *in vivo* validated 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: *Terminalia avicennioides*, *Drosophila melanogaster*, drosomycin, antimicrobial peptides, amphotericin B, *Aspergillus* species

Citation: Ziriyi Winifred Achi, Satkat Longchi Zacchaeus, Dafam Dalen Gwatau, Kakjing Dadul Falang and Ponchang Apollos Wuyep, 2019. Antifungal activity of *Terminalia avicennioides* in *Drosophila melanogaster* as an infectious model. Res. J. Med. Plants, 13: 64-73.

Corresponding Author: Ponchang Apollos Wuyep, Department of Plant Science and Biotechnology, Faculty of Natural Sciences, University of Jos, Nigeria

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 search for new antifungal remedies that would be efficient and less cytotoxic against the host is a paradigm shift fungal disease treatment therapies for both plants and animals. On the whole, the discovery of novel antimicrobial and antifungal agents representing new chemical classes working through distinctive modes of action than obtainable antifungal and antimicrobial agents[1]. Higher plants can be a potential source for these kinds of compounds, because secondary metabolites are widely distributed among higher plants. However, only a few have been evaluated for their activity against human, animal and plant pathogenic fungi. Moreover, fungal diseases of human, animal and plant poses a threat to these organisms[2,3]. The World Health Organization (WHO) estimates that 80% of the population of developing countries presently use herbal medicine for some aspect of Primary Health Care[2,3]. Medicinal properties of plants are normally dependent on the presence of certain phytochemical principles such as alkaloid, anthraquinones cardiac glycosides, saponins, tannins and polyphenols which are the bioactive bases responsible for the antimicrobial property[4,5]. Traditional Medicines continues to provide health coverage for over 80% of the world population. During the past decade, the interest in finding new plant-derived medicines and therapies for various diseases ailments has increased. Terminalia species are important medicinal plants in rural Africa, with assorted pharmacological spectrum. Diverse phytochemicals are present in these plants such as gallic acid, ellagic acid, corilagin and unidentified tannins which are responsible for many of the pharmacological activities. Due to the presence of number of phytoconstituents, the different extracts have exhibited antimicrobial, antioxidant, antibacterial, antidiabetic[6,7]. Fungal pathogens are becoming prospective agents of bioterrorism, there is widespread emergence of fungi other than *Aspergillus species* being implicated in invasive diseases, leading to increased use of antifungal prophylaxis[8] and with resistance to standard antifungal drugs[9]. 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[10,11]. There are many methodological problems associated with evaluation of antifungal activity *in vitro*, resulting in discrepancies with clinical outcomes[12]. Thus *in vivo* studies carried out in animal models are preferred only that those models are costly, labour intensive and not conducive for rapid testing of antifungal potency[13].

This study investigated natural substances in *Terminalia avicennioides*, which may have potential activities against virulent Aspergillus species, focusing on the protective role of the potential bioactive compounds in *Drosophila melanogaster* against virulent Aspergillus species. Considering the antifungal properties shown by these plants, this study emphasized the protective role of these antifungal agents in *D. melanogaster* infected with virulent *Aspergillus flavus, A. fumigatus* and *A. terreus*.

MATERIALS AND METHODS

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

Materials: Fresh and disease free parts of the plant, *Terminalia avicennioides* was collected between March and April, 2018 to ensure high concentration of bioactive constituents. The leave, stem bark and roots were collected and the specimen was deposited in the Department of Plant Science and Biotechnology, Herbarium Unit for proper identification. The parts, leaves, stem bark and roots were dried at room temperature. The dried plant parts were milled to a fine powder and stored at room temperature in tightly closed containers in the dark until required.

Extraction of plant parts: Plant extracts were prepared using aqueous (distilled water) and solvent method (Petroleum ether, ethyl acetate, dichloromethane and ethanol). The extraction was done using the solvent partitioning. The 200 g of the powdered plant sample transferred into a large Erlenmeyer flask. The content was soaked with 1500 mL of petroleum ether and allowed to stand for 72 h, with continuous shaking using a mechanical shaker and stirring. The plant residue was subjected 3 times rinsing for exhaustive extraction to ensure that no metabolite was left in the residue. The solution of the extract was evaporated to dryness using a rotary evaporator at 40°C, then dispensed into a beaker and finally dried to powder in the drying cabinet and the weight of the extracts was determined. The final residue was air dried and repacked for extraction with ethyl acetate, dichloromethane and ethanol. The steps above were repeated

for stem bark and root powder of *Terminalia avicennioides* to get the fractions. In all, a total of 9 fractions were obtained and tested for their phytochemical constituents and their *in vitro* and *in vivo* antifungal potentials in *Drosophila melanogaster* as the animal model

Test fungi: Standard Isolates of *A. flavus, A. fumigatus* and *A. terreus* were obtained from National Veterinary Research Institute (NVRI), Vom. The isolates were sub cultured twice on Sabouraud Dextrose Agar SDA from stock before use.

Establishment of virulence and re-identification of the standard isolates: Stock culture of *A. fumigatus* was streaked onto formulated Yeast Agar Glucose YAG plates and while *A. flavus* and *A. terreus* was streaked unto Potato Dextrose Agar PDA and incubated at 29°C for 3-7 days. Subcultures were produced and colonies identified based on macroscopic colony morphology, micro morphological characteristics and the ability to grow at 37°C (for *A. fumigatus*), thereby establishing their virulence[14].

Preparation of fungal inoculum: The spores from the surface of the agar plates were collected with inoculating needle and suspended in 3-4 mL of sterile distilled water. The mixture was homogenized and heavy particles were allowed to settle. 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[15] ($0.4\text{-}5\times10^6$) CFU mL^{-1}.

Preparation of standard antifungal stock solutions: The 0.08 g pellet of standard antifungal Amphotericin B was dissolved in 3.2 mL of sterile distilled water to give a concentration of 200 μg mL^{-1}, after which a double fold serial dilution was made to obtain[16] 100, 50 and 25 μg mL^{-1}.

Preparation and re-constitution of plant extracts: For the preparation of dilutions of crude extracts for antifungal assay, the extracts were reconstituted by dissolving in 10% DMSO (Dimethyl sulfoxide) solvent according to modified method described by Elumalai *et al.*[16]. The 1 g of the solid plant extract was dissolved in 2 mL of 30% DMSO in distilled water to make a stock of 250 mg mL^{-1} and further double dilutions were made to obtain 125, 62.5, 31.25 and 15.625 mg mL^{-1}, 7.81 mg m^{-1}, 3.9 and 1.95 mg mL^{-1}. The reconstituted extracts were maintained at a temperature between 2-8°C, under refrigerated condition until they were used for the experiment[16].

***In vitro* antifungal assay**

Antifungal susceptibility testing of the isolates to the plant extracts: Once the medium (PDA) had solidified, a sterile 6 mm cork-borer was used to bore 4 equidistant wells of 2.5 mm deep on the agar plates. The wells were drilled far from each other to avoid overlap of zone of inhibition, at least 24 mm apart, allowing about 10 mm distance to the edge of the plate[17]. The plant extracts (50 μL) were placed in wells. The plates were allowed to stand on a level laboratory bench for one hour to allow for proper pre-diffusion of the extract solution into the medium under strict aseptic conditions. The culture was incubated for 24 h for bacteria and 24-72 h for fungi at 37°C. The evaluation of antifungal activity or sensitivities of the fungal species to the plant extracts was determined by measuring the average sizes of inhibitory zones (including the diameter of wells) on the agar surface around the wells with a meter rule. To account for the inhibitory effect of the solvent (30% DMSO), negative and positive controls were included for all pathogens, 30% DMSO in sterile distilled water served as negative control while the antifungal drug, Amphotericin B served as positive control[18].

Determination of minimum fungicidal concentration (MFC): The MFC was determined for each of the extracts by dispensing 3 mL of potato dextrose broth in vial, 100 μL of extract and a drop of fungal inoculum and plant extracts at different concentrations into each vial, swirled to mix and incubated at 37°C for 24 h, it was observed for odour, turbidity and visible growth. The contents of the vials were sub-cultured from each vial showing no visible growth in media plates. The plates were incubated at 29°C until growth was seen in the control plates. The MFC is defined as the concentrations required killing 99.9% of the cells[17].

***In vivo* antifungal assay**

***Drosophila melanogaster* fly stock selection:** The fly *Drosophila melanogaster* (Drs-Lac Z III) was obtained from UPLEM-Lemaitre Laboratory-EPFL (Switzer Land). The flies were maintained and reared on cornmeal medium at temperature of 26°C and 60% relative humidity under 12 h dark/light cycle conditions. All the experiments were carried out with the same *D. melanogaster.*

Sexing and sorting of the flies: While being anesthetized on ice, male and female flies were distinguished based on their genitalia, size, mark and shape of abdomen, stripes or bands, bristle on forelegs. Virgin female flies were identified according to the dark mark on the ventral abdomen, which is

an embryonic residue that is excreted from their gastrointestinal tract upon maturation 8-12 h after eclusion[18,19]. Two to 4 day old female flies were consistently used because they have significantly lesser mortality rates after *Aspergillus* infection than 10-15 day old. In addition, they are larger than male flies and relatively more resistant to injection stress and also to minimize potential sex-dependent effects on infection susceptibility.

Preparation of *Aspergillus* inoculum for fly infection: The spores from the surface of frozen glycerol stock of *Aspergillus fumigatus A. flavus* and *A. terreus* was streaked unto YAG plates and incubated at 37°C for 24 h. Then, a single colony was inoculated using a sterile loop onto a YAG agar pate and incubated at 37°C for 72 h. A uniform lawn of *Aspergillus* conidia forms on the agar surface which was collected from the Agar plate by adding 0.5 mL of autoclaved water using a glass spreader which was adjusted to the desired concentration (range 10^7-10^{10} conidia mL^{-1})[19,20].

Infection of adult female flies by ingestion technique: A fresh lawn of *Aspergillus* conidia was grown in YAG-containing fly vials by adding 100 µL of 10^8 conidia mL^{-1} solutions to the surface of the agar and incubated at 37°C for 72 h. Ice-anesthetize 50 (age 2-4 days) female flies were transferred into the vials and allowed to feed on the *Aspergillus* conidia for 6-8 h. As starvation control 50 (age 2-4 days) female flies were placed into the vials that contain YAG medium only for 6-8 h (starvation control). The flies were closely observed over the first 3 h post -ingestion. Flies that died within the 3 h period post infection (typically 1%) have not died from aspergillosis but because of starvation and/or stress related to the procedure which was excluded from the survival analysis. The infected flies were maintained at 29°C, the temperature at which susceptibility to infection is maximal. The infected flies were transferred to fresh food vials every 2 days and mortality monitored[12,13,17,21,22] every 3-6 h.

Preparation of Amphotericin B and extract-containing food vials and fly fasting: The spatula was flame sterilized and used to make superficial abrasions on the fly food surface. Amphotericin B (at concentrations of 40 µg mL^{-1}) was added on the surface of the fly food, 1 mL dry yeast was slowly sprinkled on the damp food surface and allowed to sit for 24-48 h at room temperature to dry prior to use, otherwise the flies will stick to the damp surface and will die. The vials were now ready to use for the antifungal protection experiment. This procedure was also repeated for preparing the plant extracts leave petroleum extract (PEL), Stem bark petroleum extract (PES), root petroleum extract (PER), leave ethyl acetate extract (EAL), stem bark ethyl acetate extract (EAS), root ethyl acetate extract (EAR), leave dichloromethane extract (DCML), stem bark dichloromethane extract (DCMS), root dichloromethane extract (DCMR), leave ethanol extract (EtL), stem bark ethanol extract (EtS) and root ethanol extract (EtR) at concentrations[17] of 60 mg mL^{-1}).

Treatment of *drosophila* with Amphotericin B and extract-containing food vials and assessment by fly survival rate Experimental design

A = Normal flies on normal diet
B = Normal flies on diet containing extract
C = Normal flies on diet containing control drug
D = Infected flies on diet containing control drug
E = Infected flies on normal diet
F = Infected flies on diet containing extract

The 45 female flies (age, 2-4 days) in empty vials for 6-8 h to starve, after the starvation period, the flies were transferred into the antifungal (Amphotericin B at concentration of 40 µg mL^{-1} and extract (at concentration of 60 mg mL^{-1} containing vial and in triplicates. The set up was maintained at 28°C, the temperature at which susceptibility to infection is maximal. For the controls, 45 females flies (age 2-4 days) were infected with *A. fumigatus, A. flavus* and *A. terreus* placed in vials with fly food that does not contain the antifungal agent. The infected flies were transferred to fresh antifungal-containing food vials every 24 h and the mortality monitored for 3-6 h[17].

Beta-galactosidase titration: Three sets of 5 adult flies exposed to plant extract were collected and stored in eppendorf tubes at -20–C to freeze the flies. When required, samples on ice. After which 250 µL of buffer Z was added to each tube and homogenized for 30 sec. Another 250 µL of buffer Z was added to each tube and the samples quickly vortexed. Then samples were centrifuged at 6000×g for 5 min. The supernatant was collected and the protein concentration in the samples was estimated with the Bradford assay using bovine serum albumin (BSA) as a protein standard. Furthermore, a 96-well plate was used for the titration assay. 30 µL Aliquot of (Dpt) or 10 µL (Drs) of the samples was placed into 96-well plates. Then 250 µL of Buffer Z+ONPG ([ONPG] final = 0.35 mg mL^{-1}) was added to each well rapidly and plate incubated at 37°C. The β-galactosidase activity was monitored

at regular time intervals (2-30 min) by measuring the OD at 420 nm using UV-spectrophotometer. β-galactosidase activity was estimated as earlier described by Lemaitre and Coen[21]:

$$\beta\text{-galactosidase activity} = \frac{((OD)/T\ min)V}{(Protein\ concentration\ (v))/0.0045}$$

Data collection and statistical analysis: Data were obtained from various stages of evaluation with time and subjected to statistical analysis. The survival rates for the infected flies at different stages were calculated and survival curves plotted using Kaplan-Meier analysis. Differences in survival rates between treatment groups were analyzed and survival curves were compared using the log-rank test (Graphpad Prism software, version 8.0.2 and graphpad Software). All survival data are presented as the mean survival time (days) ±standard error. The results obtained were tested for significant difference at 5% level.

RESULTS

Percentage yield of *Terminalia avicennioides* extracts: All plant parts were extracted with petroleum ether for 72 h and subsequently by partitioning between ethyl acetate-Dichloromethane and ethanol-water, to yield concentrated extracts. The yield percentage per solvent is shown in Table 1.

***In vitro* antifungal assay:** Minimum inhibition concentration (MIC) values were determined by after 24 and 48 h to note the end point. The MIC values for most of the extracts were in order of 0.32 mg mL^{-1} with extracts having values as low as 0.18 mg mL^{-1}, especially against *Aspergillus fumigatus, A. flavus* and *A. terreus*, respectively. Results are shown in Table 2. It was observed that all extracts of *T. avicennoides* were active against the fungal pathogens used in this study. Ethyl acetate and Dichloromethane fractions of leave and root extracts were the most effective against *Aspergillus fumigatus* and *Aspergillus flavus* as shown in Table 3. Moreover, *Aspergillus fumigatus* was more susceptible to all fractions by solvents and plant parts of *T. avicennioides* as seen in Table 3. It was also observed that petroleum ether fractions of plant parts of *T. avicennioides* had the least total activity on all fungal pathogens used in this study followed by those of ethanol.

Table 1: Percentage yield by solvent of extracts from *Terminalia avicennioides*

Solvent used for extraction	Yield leaves (%)	Yield stem bark (%)	Yield roots (%)
Petroleum ether	1.84	0.17	0.16
Dichloromethane	3.60	0.72	0.74
Ethyl acetate	1.66	0.48	0.95
Ethanol	13.20	18.60	18.50
Water	7.00	14.60	7.40

Table 2: MIC values (mg mL^{-1}) of *Terminalia avicennoides* after 24 and 48 h incubation

		MIC values (mg mL^{-1}) of *Terminalia avicennoides*					
Organism	Time (h)	Petroleum ether	Ethyl acetate	Dichloromethane	Ethanol	Average	Amphotericin B
Aspergillus flavus	24	0.18	0.48	0.42	0.32	0.35	0.5
	48	0.18	0.96	0.42	0.32	0.47	0.5
Aspergillus fumigatus	24	0.28	0.38	0.62	0.64	1.44	0.4
	48	0.56	0.76	2.20	0.64	3.68	0.4
Aspergillus terreus	24	0.32	0.22	0.12	0.16	0.21	0.6
	48	0.32	0.44	0.24	0.32	0.33	0.5
Average		0.31	0.54	0.67	0.40		

Table 3: Total activity (mL g^{-1}) of *Terminalia avicennoides* extracted after 24 and 48 h incubation

		Total activity (mL g^{-1})				
Organism	Time (h)	Petroleum ether	Ethyl acetate	Dichloromethane	Ethanol	Average
Aspergillus flavus	24	125	167	275	145	178
	48	125	163	275	145	177
Aspergillus fumigatus	24	205	268	325	165	241
	48	205	285	345	163	250
Aspergillus terreus	24	195	145	285	132	189
	48	193	138	285	132	187
Average		175	194	298	147	

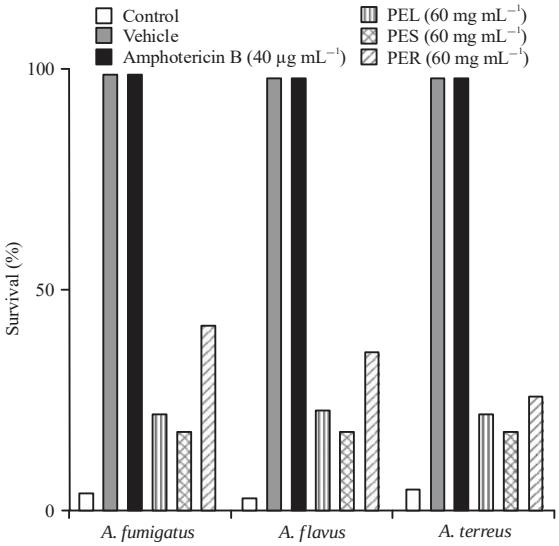

Fig. 1: Effect of petroleum ether fraction of leave, stem and root of *Terminalia avicennioides* on the survival of *D. melanogaster* infected with fungal pathogens after 14 days

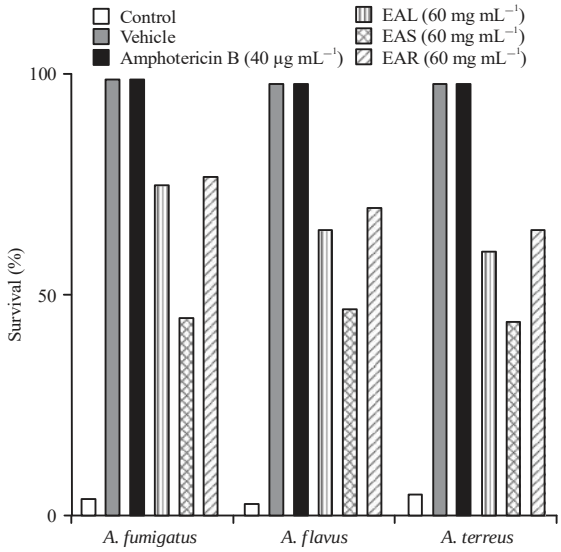

Fig. 2: Effect of ethylacetate fraction of leave, stem and root of *Terminalia avicennioides* on the survival of *D. melanogaster* infected with fungal pathogens after 14 days

Acute toxicity of fractions of *terminalia avicennioides* on *drosophila melanogaster*. The acute toxicity of the fractions of *Terminalia avicennioides* and Amphotericin B (10-100 µg mL^{-1}) was determined after 4 and 8 days when *D. melanogaster* (15 flies per vial with 3 replicates) per concentration (10-100 mg mL^{-1}) were exposed each fraction. The experiment was conducted for 10 days at 29°C. The acute toxicity profile of the fractions showed that ethanol was more toxic to *Drosophila melanogaster*, recording 45.25% population of *D. melanogaster* alive after 4 days and reduced to 26.75% after 8 days, as shown in Table 4. The fractions of Dichloromethane and ethyl acetate were less toxic to *D. melanogaster* with survival rate of 76.12 and 62.10%, respectively after 8 days of exposure. It was also observed that toxicity was dose dependent. Higher doses of each fraction became toxic to *D. melanogaster*. Moreover, the dose concentration seen to favour survival of *D. melanogaster* was observed to be 40-60 mg mL^{-1}.

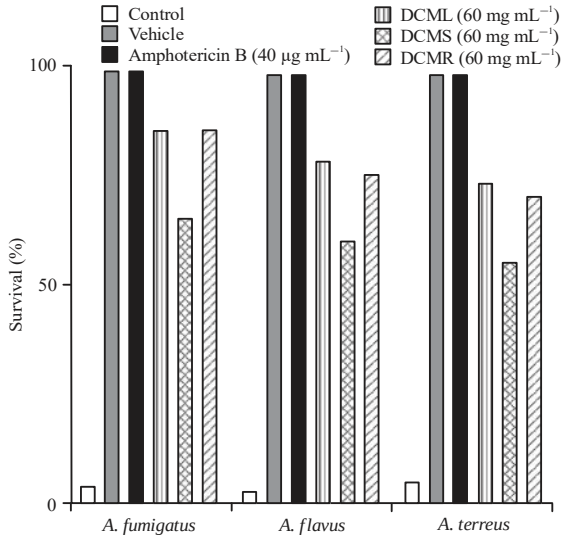

Fig. 3: Effect of dichloromethane fraction of leave, stem and root of *Terminalia avicennioides* on the survival of *D. melanogaster* infected with fungal pathogens after 14 days

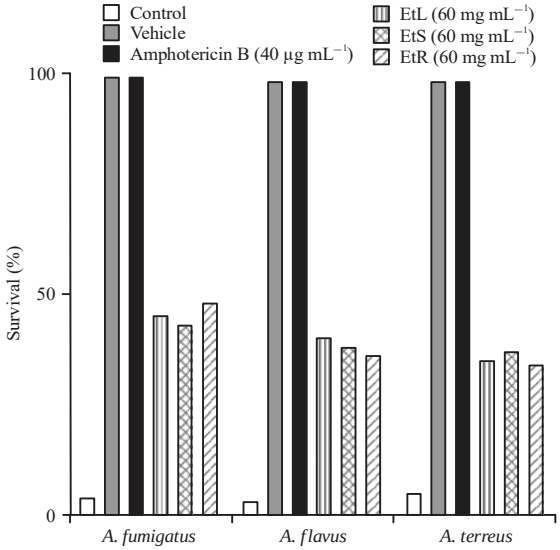

Fig. 4: Effect of ethanolic fraction of leave, stem and root of *Terminalia avicennioides* on the survival of *D. melanogaster* infected with fungal pathogens after 14 days

***In vivo* antifungal assay:** The survival rate of *D. melanogaster* infected with three fungal pathogens used in this study was monitored with a treatment dose of 60 mg mL^{-1}, of petroleum ether extracts (PER) of leave, stem bark and root. It was observed comparatively that extracts from roots (PER), had more antifungal activity against the three fungal pathogens used in the study followed that extracts from leave and stem bark, as shown in Fig. 1. However, the survival rate for all the extracts from plant parts of *T. avicennioides* was less than 50% for the fungal pathogens tested, when compared with standard antifungal drug, 40 µg mL^{-1} of Amphotericin B, which recorded about 95% survival in *D. melanogaster* infected with the test fungal pathogens as shown in Fig. 1. Figure 2 shows the result for *D. melanogaster* survival infected with test fungal pathogens and treated with 60 mg mL^{-1} of ethyl acetate of leave, stem bark and root. It was observed that more 50% of *D. melanogaster* survived when treated with ethyl acetate leave and root extracts from *T. avicennoides*. However, stem bark extracts did not protect *D. melanogaster* infected with the test fungal pathogens as survival was less than 50%.

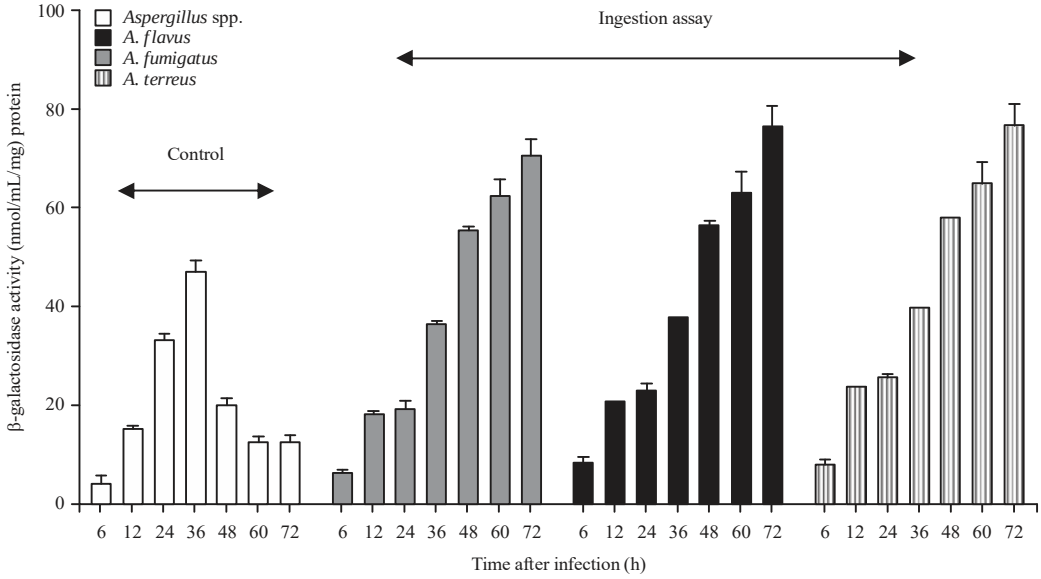

Fig. 5: Time-course analysis of AMPs (Drosomycin activity after infection of adult *D. melanogaster* (Drs-Lac Z III) by ingestion assay of pathogenic fungal spores
β-galactosidase activity was determined by the method of Romeo and Lemaitre (2008)

Table.4: Acute toxicity of *Terminalia avicennioides* on *Drosophila melanogaster*

	Survival (%)		
Parameters	Flies number	4 days	8 days
Petroleum ether	450	35.15	18.20
Ethyl acetate	450	83.27	62/10
Dichloromethane	450	88.41	76.12
Ethanol	450	45.25	26.75
Amphotericin B	450	98.75	98.55

The Dichloromethane extracts of plant parts of *T. avicennioides* protected *D. melanogaster* remarkably, with each extract enabling infected *D. melanogaster* achieve more than 50% survival rate as shown in Fig. 3. The survival rate for fractions from leave (DCML) and root (DCMR) which was used to treat infected *D. melanogaster*, was above 85% as shown in Fig. 3. Extracts from ethanol of leave, stem bark and root of *T. avicennioides* like those of petroleum ether, did not improve the survival of *D. melanogaster* infected with all three fungal pathogens. Less than 50% *D. melanogaster* infected with test fungal pathogens and treated with ethanol extracts of leave, in Fig. 4.

Beta-galactosidase activity: The antimicrobial peptide (AMP), drosomycin level was seen to be high at 48 h post infection as shown in Fig. 5. The drosomycin level peaked steadily even at 72 h post infection for all fungal pathogens.

DISCUSSION

The presence of tannins, flavonoids, saponins, terpenoids and triterpenoids in *Terminalia avicennioides*, is obviously the source of antifungal potential of this plant. This study has confirmed the antifungal potentials of *Terminalia avicennioides* as earlier reported by Azeez *et al.*[4], Eloff *et al.*[5], Mann *et al.*[7] and Lionakis and Kontoyiannis[13], which occurs in diverse components in Terminalia species[22, 23]. The potential antifungal agents have been used to inhibit the growth of fungal agents like *Aspergillus fumigatus, Cryptococcus neoformans* and *Candida albicans* which fungal pathogens associated with immunocompromised individuals[24,25]. The MIC of leave and root extracts of *Terminalia avicennioides* showed significant antifungal activity against *Aspergillus fumigatus, A. flavus* and *A. terreus*. The antifungal activity ranged from 0.18-0.32 mg mL^{-1} higher than the previous report by Baba-Moussa *et al.*[26], which was 0.08-0.04 µg mL^{-1} against *Aspergillus flavus* and *Aspergillus niger*. The leave and root had antifungal activities with significant difference of $p<0.05$ compared to those from stem bark.

The acute toxicity revealed that LD_{50} for *D. melanogaster* was 40-60 mg mL^{-1} for leave extract, 50-60 mg mL^{-1} for stem bark and 40-70 mg mL^{-1} for root extract for all solvents used in the study. It was that the concentration was dose dependent with *D. melanogaster* population reducing

drastically at higher dose/concentration of each extract, regardless the solvent. 60 mg mL^{-1} for all the extracts was used in this study since it enables *D. melanogaster* population to tower above 50% suggesting that 60 mg mL^{-1} of the extracts is the acceptable threshold for *D. melanogaster*. 40 µg mL^{-1} of the standard antifungal drug Amphotericin B was used due to that fact being a pure compound containing all the active ingredients in exact quantity.

In the in vivo antifungal assay, concentration of the extract was essential in the treatment of *D. melanogaster* infected with fungal pathogens. Results obtained in this assay revealed that *D. melanogaster* survival was minimal at the LD_{50} threshold and when *D. melanogaster* were exposed to treatment regimes to plant extracts across all concentrations, the survival rate increased as the concentration increased from 50-70 mg mL^{-1}. However, at 80-100 mg mL^{-1}, the population of *D. melanogaster* in the experiment crashed significantly. This could be due to fungal infection burden and already toxic nature of the extracts. At 60 mg mL^{-1}, the extracts protect *D. melanogaster* from the fungal infection burden and no drastic reduction in population was observed. It was not clear if 60 mg mL^{-1} is the optimum dose for exposure of *D. melanogater* infected with *Aspergillus* species. It will appear that *D. melanogaster* tolerated this concentration couple with the fly innate immune ability; switch on the necessary pathway to combat the fungal infection. The observed increase in levels of Drosomycin as seen in the β-galactosidae titration data might be a possible explanation. Drosomycin is an antimicrobial peptide (AMP), which is involved in the Toll and pathway. Antimicrobial peptides (AMPs) are an important group of immune effectors that play a role in combating microbial infections in invertebrates. Increasing levels of Drosomycin in *D. melanogaster* treated with plant extracts and Amphotericin B suggested that Drosomycin is inevitably helping the infected *D. melanogaster* to comb at the *Aspergillus* infections. It was not clear about the role of the plant extracts, however *D. melanogaster* survived the infectious *Aspergillus* species. Drosomycin levels were seen to increase till the experiment in which *D. melanogaster* was infected with fungal pathogens and exposed to plant extracts, was terminated after 72 h. However, the levels in control experiments, where *D. melanogaster* was infected with fungal pathogens and not exposed to plant extracts reduced leading to massive death of *D. melanogaster.* There is dearth of evidence to support this observation/finding in literature, particularly where the effect of plant based antifungal agents caused any significant change in the amount of antimicrobial peptides products being quantified. This is the first report in which this observation may have been made. Moreover, the role of pure compounds as antifungal agents on the Toll and Imd pathway, especially as their impact on immune respond has been elucidated[8,13,18,21]. Present observation, in which factions of plant extract have impact antimicrobial peptide, appears to be a 1st report. However, the molecular mechanism which supports this observation is not yet clear.

CONCLUSION

All extracts of *Terminalia avicennioides* protected *D. melanogaster* against fungal infections. Worthy of note were the extracts from leave and root at 60 mg mL^{-1} enabled *D. melanogaster* exhibited a survival rate above 50%. The antifungal potent phyto-constituents present in the extracts of *T. avicennioides* aided *D. melanogaster* to combat fungal infection through the Toll pathway. *D. melanogaster* innate immunity served as the first line defense against microbial invaders.

SIGNIFICANCE STATEMENT

The infection model used in this study was vital to the understanding of how plant extracts may have mediated in the survival of *D. melanogaster* infected with fungal pathogens. *D. melanogaster* immune response consists of multiple cellular and humoral response mechanisms, including activation of phagocytosis by specialized blood cells, melanization, coagulation and synthesis of antimicrobial peptides (AMPs). Based on the β-galactosidase data, the presence of Drosomycin, one of the AMPs in high levels is an indication that the Toll pathway mediated *D. melanogaster* immune response significantly and that plant extracts played a role too, though not clear which molecular mechanism was employed, especially when the Drosomycin levels was seen to be depleting in control experiments of *D. melanogaster*.

REFERENCES

1. Anyanwu, M.U. and R.C. Okoye, 2017. Antimicrobial activity of Nigerian medicinal plants. J. Intercult. Ethnopharmacol., 6: 240-259.
2. Suleiman, M.M., L.I. McGaw, V. Naidoo and J. Eloff, 2010. Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. Afr. J. Tradit. Complement. Altern. Med., 7: 64-78.

3. Elujoba, A.A., O.M. Odeleye and C.M. Ogunyemi, 2005. Review-Traditional medicine development for medical and dental primary health care delivery system in Africa. Afr. J. Tradit. Complement. Altern. Med., 2: 46-61.
4. Azeez, M.A., T.A. Yekeen, D.A. Animasaun, F.A. Durodola and O.B. Bello, 2015. *Terminalia avicennioides* as a potential candidate for pharmaceutical industry: A review. Res. J. Pharmaceut. Biol. Chem. Sci., 6: 748-754.
5. Eloff, J.N., D.R. Katerere and L.J. McGaw, 2008. The biological activity and chemistry of the Southern African Combretaceae. J. Ethnopharmacol., 119: 686-699.
6. Shuaibu, M.N., P.T.A. Wuyep, T. Yanagi, K. Hirayama, A. Ichinose, T. Tanaka and I. Kouno, 2008. Trypanocidal activity of extracts and compounds from the stem bark of *Anogeissus leiocarpus* and *Terminalia avicennoides*. Parasitol. Res., 102: 697-703.
7. Mann, A., Y. Yahaya, A. Banso and F. John, 2008. Phytochemical and antimicrobial activity of *Terminalia avicennioides* extracts against some bacteria pathogens associated with patients suffering from complicated respiratory tract diseases. J. Med. Plants Res., 2: 94-97.
8. Lewis, R.E., R.A. Prince, J. Chi and D.P. Kontoyiannis, 2002. Itraconazole preexposure attenuates the efficacy of subsequent amphotericin B therapy in a murine model of acute invasive pulmonary aspergillosis. Antimicrobial Agents Chemother., 46: 3208-3214.
9. Abd El-Baky, R.M., 2016. The future challenges facing antimicrobial therapy: Resistance and persistence. Am. J. Microbiol. Res., 4: 1-15.
10. Cos, P., A.J. Vlietinck, D.V. Berghe and L. Maes, 2006. Anti-infective potential of natural products: How to develop a stronger *in vitro* proof-of-concept. J. Ethnopharmacol., 19: 290-302.
11. Bognan, A.J.A.A., Y.Y. Guillaume, Y.H. Felix, K.A.K. Mathieu and D.A. Joseph, 2013. Antifungal activity of *Terminalia mantaly* on the *in vitro* growth of *Cryptococcus neoformans*. Int. J. Biochem. Res. Rev., 3: 63-73.
12. Mylonakis, E., A. Casadevall and F.M. Ausubel, 2007. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog., Vol. 3, No. 7. 10.1371/journal.ppat.0030101.
13. Lionakis, M.S. and D.P. Kontoyiannis, 2010. The growing promise of Toll-deficient *Drosophila melanogaster* as a model for studying Aspergillus pathogenesis and treatment. Virulence, 1: 488-499.
14. Campbell, C.K., E.M. Johnson, C.M. Philpot and D.W. Warnock, 1996. Identification of Pathogenic Fungi. Public Health Laboratory Service, London, UK., Pages: 298.
15. CLSI., 2010. Method for antifungal disk diffusion susceptibility testing of non dermatophyte filamentous fungi; approved guideline. CLSI Document M51-A, Clinical and Laboratory Standards Institute, Wayne, PA., USA.
16. Elumalai, E.K., P. Sivamani, T. Thirumalai, P. Vinothkumar, A. Sivaraj and E. David, 2009. *In vitro* antifungal activities of the aqueous and methanol extract of *Abrus precatorius* Linn (*Fabaceae*) seeds. Pharmacologyonline, 2: 536-543.
17. Lionakis, M.S. and D.P. Kontoyiannis, 2005. Fruit flies as a minihost model for studying drug activity and virulence in *Aspergillus*. Med. Mycol., 43: 111-114.
18. Lionakis, M.S., R.E. Lewis, G. Chamilos and D.P. Kontoyiannis, 2005. *Aspergillus* susceptibility testing in patients with cancer and invasive aspergillosis: Difficulties in establishing correlation between *in vitro* susceptibility data and the outcome of initial amphotericin B therapy. Pharmacotherapy: J. Hum. Pharmacol. Drug Ther., 25: 1174-1180.
19. Romeo, Y. and B. Lemaitre, 2008. *Drosophila* Immunity: Methods for monitoring the activity of Toll and Imd signaling pathways. Methods Mol. Biol., 415: 379-394.
20. Eloff, J.N., P. Masoko and J. Picard, 2007. Resistance of animal fungal pathogens to solvents used in bioassays. South Afr. J. Bot., 73: 667-669.
21. Lemaitre, B. and D. Coen, 1991. P regulatory products repress *in vivo* the P promoter activity in P-lacZ fusion genes. Proc. Natl. Acad. Sci. USA., 88: 4419-4423.
22. Hamilos, G., G. Samonis and D.P. Kontoyiannis, 2012. Recent advances in the use of *Drosophila melanogaster* as a model to study immunopathogenesis of medically important filamentous fungi. Int. J. Microbiol., Vol. 2012. 10.1155/2012/583792.
23. Apulu, N., J.O. Dada, L.E. Odama and M. Galadima, 1994. Antibacterial activities of aqueous extracts of some Nigerian medicinal plants. Niger. J. Bot., 7: 45-48.
24. Nair, R. and S. Chanda, 2008. Antimicrobial activity of *Terminalia catappa*, *Manilkara zapota* and *Piper betel* leaf extract. Indian J. Pharmaceut. Sci., 70: 390-393.
25. Masoko, P., J. Picard and J.N. Eloff, 2005. Antifungal activities of six south African *Terminalia* species (Combretaceae). J. Ethnopharmacol., 99: 301-308.
26. Baba-Moussa, F., K. Akpagana and P. Bouchet, 1999. Antifungal activities of seven West African Combretaceae used in traditional medicine. J. Ethnopharmacol., 66: 335-338.