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Antifungal Potentials of *Terminalia cattapa* Linn on *Aspergillus terreus* and *Trichoderma mantas* using *Drosophila melanogaster* (Iso) as a Model

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ABSTRACT

One of the contemporary threats facing man is antimicrobial resistance (AMR). Available antimicrobials have been distorted and are almost unproductive, with some of these drugs associated with dangerous side effects in some individuals. The development of new, effective, and safe antimicrobials is one of the ways of tackling the problem of AMR. Medicinal plants are potential sources of new antimicrobial molecules. *Terminalia catappa* is one of these medicinal plants in the family of combreteceae that has been used traditionally for treatment of various diseases in different parts of the world, Nigeria inclusive. The research is designed to determine the antifungal potentials of *T. cattapa* on *Aspergillus terreus* and *Trichoderma mantas* using *Drosophila melanogaster* (Iso) as a Model. Leaves and stem of *T. catappa* were collected and extraction was carried out using cold maceration method with methanol. Phytochemical screening was carried out using standard methods, antifungal susceptibility was done using the agar well diffusion techniques and minimum inhibitory concentration and minimum fungicidal concentration were also determined using broth dilution methods. Ingestion methods were used to infect flies with different fungal pathogens and treated on a diet containing different concentrations of plant extracts. Survival rate was taken for seven days and mortality was recorded. Phytochemical screening of the extracts revealed the presence of the following bioactive constituents; Saponins, Flavonoids, Tannins, Steroids and Carbohydrates. The antifungal activities were concentration dependent, with the highest activity against *Aspergillus terreus* with a zone of inhibition of 10±0.82 to 16±0.41mm and 11±0.41 to 20±0.45mm at the different concentrations for leaves and stem extract respectively. The results of the infectious studies showed that flies infected with *A. terrues* and treated with 60 mg/ml of leaf extracts had the highest survival rate of 58.33% and 61.44% of 50mg/ml of stem extract as compared with the standard drug itraconazole with a survival rate of 65.11% at 60 mg/ml respectively. The survival rate of the flies decreased at higher concentrations. Both leaves and stems of *T. cattapa* at various concentrations manifested antifungal activities *in vitro* and *in vivo* on both *Aspergillus terreus* and *Trichoderma mantas.* These support the use of *T. catappa* in the treatment of fungal infections.

Keywords: *Aspergillus terreus, Trichoderma mantas, Drosophila melanogaster*, phytochemistry, antifungal activities, survival rate.

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INTRODUCTION

Traditional medicine has been used for centuries in many parts of the world, including Nigeria mostly in rural areas due to availability and low cost. Nature has provided a source of medicinal agents for thousands of years, and an impressive number of modern drugs are isolated from natural sources, many based on their use in traditional

medicine (Cragg and Newman, 2002). Most of the plant materials used for traditional medicines are readily available in rural areas and have made traditional medicine relatively cheaper than modern medicine (Mann et al., 2008; Gilani et al., 2010; Hussain et al., 2012).

There has been an increasing incidence of multiple resistances in human pathogenic micro-organism, mainly due to the indiscriminate use of commercial antimicrobial drug commonly explored in treating infectious diseases (Hussain et al., 2012). The development of fungal resistance to presently available antibiotics has necessitated the search for new antimicrobial agents. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds (Chanda et al., 2007; Hediat and Marraiki, 2010).

Using mammalian models to study fungal pathogenicity has logistical limitations because they are expensive and labor-intensive. When large-scale screening of fungal mutants is warranted, the costs can be prohibitive. Therefore, several invertebrate models, including *Caenorhabditis elegans*, *D. melanogaster*, and *Galleria mellonella* have been developed as promising alternatives to study the pathogenesis of medically important fungi (Chamilos et al., 2007; Binder et al., 2016). Consequently, non-vertebrate mini hosts such as *D. melanogaster* and *Caenorhabditis elegans* are used to study the pathogenesis of *Aspergillus* spp. and other fungi as well as Gram-positive bacteria and yeast (Wuyep et al., 2020). The edge with *D. melanogaster* is because it has a short life cycle and can be studied in large numbers with relative ease and at a lower cost. *Drosophila melanogaster* was used in this study as a model host to evaluate the antifungal potentials of *Terminalia cattapa* methanol leaves and stem extracts against virulent *Aspergillus terreus and Trichoderma mantas*. The immune deficiency (Imd) pathway and the Toll pathway are the two conserved signaling pathways activated during the immune response to pathogen challenge. The Toll pathway confers protection in *D. melanogaster* against fungi, and with its mutations, the fly becomes easily infected by *Aspergillus* species and other fungi (Wuyep et al., 2020).

Terminalia catappa L. belongs to the family Combretaceae. *T. Catappa* is used primarily as an ornamental, shade, and salt-tolerant stress-free, but the leaves provide food for the Tasar Silkworm, and the seeds are edible like almonds with similar oils. *T. catappa* plant has been investigated in various pharmaceutical studies as it contains various chemical components (Yeh et al., 2014; Triantafillidis et al., 2016; Zacchaeus et al., 2024). *T. catappa* L. leaf extracts exhibit biological activities, including antioxidant (punicalagin, punicalin, terfluvina A and B, chebulic acid, benzoic acid, cumaric, and its derivatives), antidiabetic (β-carotene), anticancer (punicalagin), antiviral (ellagic acid), anti-inflammatory (triterpenic acids, especially ursolic acid and its derivatives), antimicrobial (flavones and flavanols), and hepato-protective activities (punicalagin, punicalin), (Naitik et al., 2012; Anand et al., 2015; Zacchaeus et al., 2024). Therefore, this research seeks to evaluate the antifungal activities of the leaves and stem methanol extracts of *T. Cattapa,* both in-vito and in-vivo, using *D. melanogaster* as a model.

MATERIALS AND METHODS

Study area

The study was carried out between January 2021 and September 2022 at the Drosophila Laboratory: Fungal Pathogens and Plant Bioactive Compounds, Department of Plant Science and Biotechnology, University of Jos, Nigeria.

Collection of plant samples/parts and their identification

Leaves and stems of the plant species tested in this study were collected from Naraguta village, Jos North Local government of Plateau State Nigeria, between March and April 2021 to ensure a high concentration of bioactive constituents. All plants were identified with a number (UJH000297), and Voucher specimens was prepared and stored at the Department of Plant Science and Biotechnology, University of Jos. All plant materials were air-dried in the shade and pulverized into a fine powder.

Extraction of plants materials

Extraction was carried out using cold maceration by taking 50g each of leaves and stem separately and macerating in 500ml of methanol for 72 hours. The extracts were filtered through filter paper (Whatman No.1). The extracts were subsequently allowed to dry at room temperature (Parekh and Chanda, 2007).

Purity test of the extract

Purity tests of the different extracts were carried out by inoculating 0.1g of the dried extracts on a poured plate of Sabronuad dextrose agar and incubated for any growth. The absence of growth in the media signifies that the extracts are pure.

Phytochemical determination

The plant fractions were screened for their phytochemical

constituents to determine the presence of Alkaloids, Saponins, Tannins, Flavonoids, Carbohydrates, Steroids, Anthraquinones, Cardiac glycosides and Terpernoids using standard phytochemical screening procedures as described by Sofowora (2008).

Determination of Percentage Yield

The percentage yield of the crude extract was determined (Mahmood, 2009; Parekh and Chanda, 2007). The percentage yield of the methanolic extracts was calculated as

$$\text{Percentage Yield} = \frac{\text{Weight of plant extract before extraction} \times 100}{\text{Weight of plant extract after extraction}}$$

Re-constitution of plant extracts for antifungal activities testing

Leaves and stem extracts were reconstituted by dissolving in 30% DMSO (Dimethyl Sulphuroxide) solvent according to the modified method described by Elumelai et al. (2009). One gram (1g) each of leaves and stem extracts was dissolved in 2 mL of 30% DMSO in distilled water to make a stock of 500mg/ml, and further double dilutions were made to obtain 250 mg/ml, 125 mg/ml, and 62.5 mg/ml respectively. The reconstituted extracts were maintained at a temperature between 2 - 8°C in a refrigerator until they were used for the experiment (Eloff et al., 2008).

Source of micro-organisms

Standard isolates of the fungi *Aspergillus terreus* and *Trichoderma mantas* were obtained from the Veterinary Research Institute Vom. The organisms were collected in a suspension of sabronuad broth (SB). The organisms were subcultured on sabronuad broth agar (SDA) where pure cultures were obtained and viewed microscopically for re-identification.

Antimicrobial susceptibility testing

Agar well diffusion techniques

The antimicrobial susceptibility test was performed with the clinical isolate of *Aspergillus terrues* and *Trichoderma mantas* using the agar well diffusion technique as described by Nair and Chanta (2005). The fungal inoculum was prepared from subculture as follows; the spores of three day old fungi were suspended in broth and turbidity was adjusted to 0.5 McFarland standard. The pour plate method was used to inoculate the organism on SDA. 1 ml of broth containing spores was poured into a petri dish and 20 ml of SDA was added and shaken for even distribution and was allowed to solidify. Wells of about 6mm in diameter were aseptically punched with a sterile cork borer (5 holes per plate) and the wells were filled with 100 microliters of the different concentrations of the plant extracts. The plates were left for 30 minutes before incubation for the extracts to diffuse into the agar. The plates were incubated at 37^{0}c for 24 hours and the zones of inhibition were measured to the nearest millimeter (mm).

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentration which is the concentration with the least inhibitory activity was determined using the broth dilution method. A standardized suspension of 1 ml of broth containing the organism was introduced into a test tube containing 5 mls of sterile broth, 200 µmL of the re-constituted extract at various concentrations was introduced into the test tubes and incubated at 37°c for 24 to 48 hours and observed for growth in the form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection was considered as the MFC value (Elumelai et al., 2009).

Drosophila melanogaster Fly Stock Selection

The fly *Drosophila melanogaster* (ISO*)* was obtained from the National Species Stock Center (Switzerland). The flies were maintained and reared on cornmeal medium at a temperature of 23±1°C and 60% relative humidity under 12 h dark/light cycle conditions. *D. melanogaster* (ISO) was used in all the experiments.

Preparation of fly food and plant extracts for acute toxicity testing and infectious studies

The fly food was prepared using the ratio of grams per diet to obtain food with concentrations of 100 mg/ml, 90 mg/ml, 80 mg/ml, 70 mg/ml, 60 mg/ml, 50 mg/ml, 40 mg/ml, 30 mg/ml, 20 mg/ml and 10 mg/ml. The acute toxicity was carried out by putting 15 unsex flies per vial in triplicates to have a total number of 45 flies per concentration. The survival of the flies was recorded for seven days, and mortality was taken.

Establishment of virulence and re-identification of the standard isolates

Stock culture of *Aspergillus niger was* streaked onto formulated Yeast Agar Glucose (YAG) plates and incubated at 37°C for 3-7 days. Subcultures were

produced, and colonies were identified based on macroscopic colony morphology, micro morphological characteristics, and the ability to grow thereby establishing their virulence (Wuyep et al., 2020).

Preparation of fungal inoculum

The spores from the surface of the agar plates were collected with an 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 (0.4–5 × 10^6) CFU/ml.

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, and 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 eclosion (Lionakis and Kontoyiannis, 2010). Two to 4-day-old female flies were consistently used because they have significantly lesser mortality rates after infection than 10- to 15-day-old.

Establishment of infections in the flies

Flies were harvested and starved for between 8 to 10 hours, and then they were introduced into Yeast Agar Glucose media containing 3-day-old culture of *A. terreus* to feed for 6 hours. After which they were taken for various treatments, and survival rates were observed.

Experimental groups

A = uninfected flies fed with normal diet (Vehicle).
B = infected flies that were not treated (control)
C = flies infected with *A. terrues* and treated on different concentrations of the methanol leaves extracts of *T. cattapa*.
D = flies infected with *A. terrues* and treated on different concentrations of the methanol stem extracts of *T. cattapa*.
E = flies infected with *A. terrues* and treated on different concentrations of the standard drug itraconazole.
F = uninfected flies placed on different concentration of leaves, stem and standard drug (acute toxicity).

Data collection and statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and results were presented as means ± standard error of means using Graph Pad Prism version 8 software. The results obtained were tested for significant differences at a 5% level.

RESULTS

Extract yield, characteristics and phytochemical screening

The results of the extraction revealed that the methanol leaves extract had the highest percentage yield of 10.5% whereas the stem extract had the percentage yield of 7.3%. The phytochemical investigation of the methanol extracts of leaves and stem showed the presence of Saponins, Tannins, Flavonoids, Carbohydrates and Steriods. Alkaloids, Anthraquinones, Cardial glycosides and Terpenoids were absent in all the extracts (Table 1).

Table 1. Biochemical constituents of the methanol leaves and stem extracts of *T. Catappa.*

Constituents	Leaves	Stem
Alkaloids	-	-
Saponins	+	++
Tannins	+++	++
Flavonoids	+++	+++
Carbohydrates	++	++
Steroids	+	+
Anthraquinones	-	-
Cardial glycosides	-	-
Terpenoids	-	-
Percentage yield	10.5%	7.3%

- = absent, + = present, ++ = More Present and +++ = Highly Present.

Susceptibility of *Aspergillus Terreus* and *Trichophyton Mantas* to methanol leaves and stem extract

Evaluation of the antifungal activity of the methanol leaves and stem extract of *T. catappa* was measured by the zones of inhibition of mycelia growth of fungal species observed around the wells containing varying concentrations of the extracts (ranging from 62.5-500 mg/mL) as well as the positive and negative controls. *Aspergillus terreus* was the most susceptible to the leaves and stem extract with zone of inhibition of between 10±0.82mm to 16±0.41mm and 11±0.41mm to 20±0.54mm for leaves and stem extract respectively. While *T. mantas* had the least inhibition of between 9±0.53mm to 15±0.89mm and 11±0.71mm to 20±0.82mm for leaves and stem extract respectively (Table 2).

Table 2. Antifungal activity of the methanol Leaves and stem extracts of *T. Catappa* and itraconazole on *Aspergillus* species.

EXTRACTS	*Aspergillus Terreus*	*Trichophyton Mantas*
Methanol leaves extract		
62.5	10± 0.82	9 ± 0.53
125	12 ± 0.83	10 ± 0.83
250	14 ± 0.58	12 ± 0.82
500	16 ± 0.41	15 ± 0.89
Methanol stem extract		
62.5	11 ± 0.41	11 ± 0.71
125	14 ± 0.96	13 ± 0.71
250	17 ± 0.5	16 ± 0.58
500	20 ± 0.54	20 ± 0.82
Itraconazole		
25	15± 0.41	13 ± 0.36
50	19± 0.19	17 ± 0.58
100	22± 0.36	21 ± 0.41
200	27± 0.71	25 ± 0.85
Distilled water	-	-

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

Similarly the standard drug also had a broad spectrum of activities on the test fungi with *A. terrues* the most susceptible with zone of inhibition of between 15±0.41mm to 27±0.71mm and *T. mantas* had the least inhibition of between 13±0.36mm to 25±0.85mm respectively. No inhibition was observed with the negative control distil water (Table 2).

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The methanol leaves extract had MIC of 250 mg/ml and MFC 500mg/ml on *A. terrues* and MIC 125 mg/ml and MFC 250mg/ml on *T. mantas* respectively. Similarly the stem extract had MIC of 62.5 mg/ml and MFC 125 mg/ml on *A. terrues* and MIC 250mg/ml and MFC 500 mg/ml on *T. mantas* as compared to the standard drug with MIC of 25mg/ml and MFC 50mg/ml on *A. terrues* and MIC 50mg/ml and MFC 100 mg/ml on *T. mantas* (Table 3).

Acute toxicity studies of methanol leaves and stem extract of *T. catappa* and itraconazole in *D. melanogaster*

At the end of 7 days of acute toxicity studies, fly mortality rate increased with increasing concentration. A Lethal concentration (LC_{50}) of 60 mg/mL was recorded for both leave and stem extract at which point mortality was recorded in half of the population. LC_{50} for Itraconazole was 40 mg/ml. These results are presented in Figure 1, 2 and 3 respectively.

Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *T. Catappa* and itraconazole on *Aspergillus* species.

EXTRACTS	***A. terreus***(mgml^{-1})		***T. mantas***(mgml^{-1})	
	MIC	MFC	MIC	MFC
Methanol leave extract	250	500	125	250
Methanol stem extract	62.5	125	250	500
Itraconazole	25	50	50	100

Effects of *T. catappa* methanol leaves and stem 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 methanol leaves and stem extract of *T. catappa* at 10-100 mg/ml compared to the negative control of *A. terreus* infected flies that were not treated. The results of the infectious studies showed that flies infected with *A. terrues* and treated on 60 mg/ml of leaves extracts had the highest survival rate of 58.33% and 61.44% of 50mg/ml of stem extract as compared with the standard drug itraconazole with survival rate of 65.11% at 60 mg/ml respectively. But at very extreme concentration the population of the flies crashes to zero (Figure 4, 5 and 6).

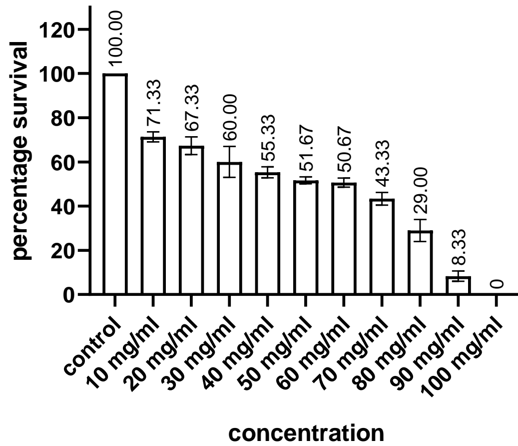

Figure 1. Percentage survival of the acute toxicity of different concentrations of methanolic stem extract of *T. catappa* on *D. melanogaster* (ISO).

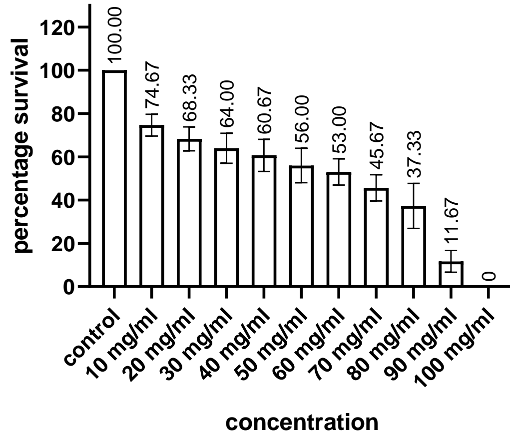

Figure 2. Percentage survival of the acute toxicity of different concentration of methanol leaves extract of *T. catappa* on *D. melanogaster* (ISO).

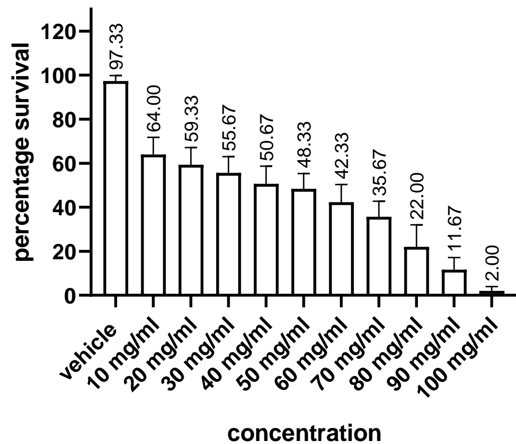

Figure 3. Percentage survival on the acute toxicity of the different concentrations of Itraconazole on *D. melanogaster* (ISO).

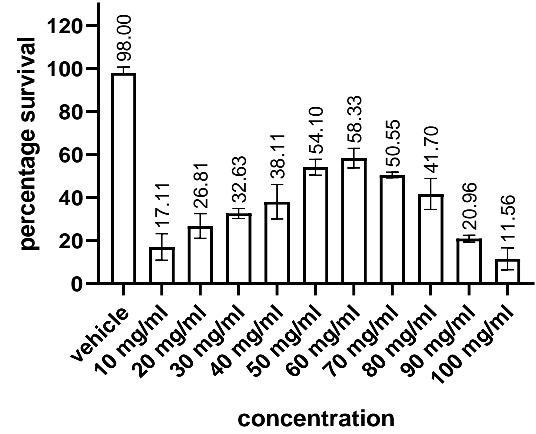

Figure 4. Percentage survival of *Drosophila melanogaster* (ISO) infected with *A. terrues* and treated with the different concentrations of methanolic leaves extract of *T. catappa.*

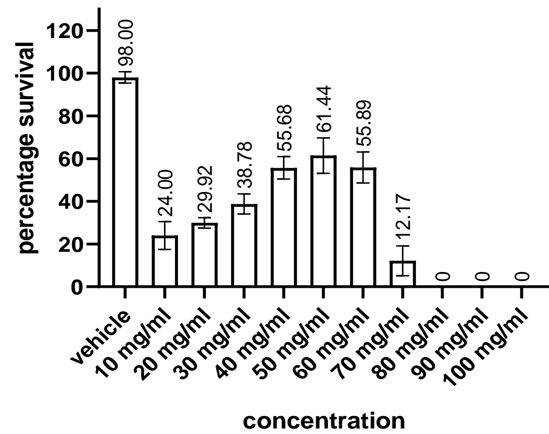

Figure 5. Percentage survival of *Drosophila melanogaster* (ISO) infected with *A. terrues* and treated with the different concentrations of methanol stem extract of *T. catappa.*

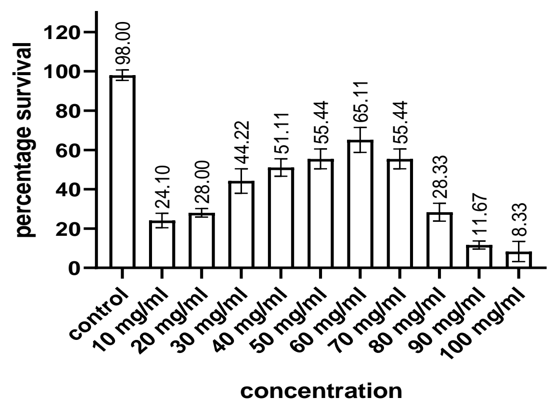

Figure 6. Percentage survival of *Drosophila melanogaster* (ISO) flies infected and treated with different concentrations of Itraconazole.

DISCUSSION

Herbal medicine in developing countries is commonly used for the traditional treatment of health problems. In recent years, multiple drug resistance in human pathogenic microorganisms has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host, including hypersensitivity, immune suppression and allergic reaction (Limem et al., 2011). Therefore, there is a need to develop alternative antimicrobial drugs from various medicinal plants for the treatment of infections (Penduka et al., 2011; Hagg et al., 2011).

In the present study, the leaf and stem extracts of *T. Cattapa* in methanol were investigated for antimicrobial potentiality, both in vitro and in vivo, using *Drosophila melanogaster* as a research model. The results of the extraction reveal that leaves extract had the highest percentage yield of 10.5% and stem extracts had a yield of 7.3% respectively. These agree with the findings of Hediat and Marraiki (2010), which reported that methanol has the highest percentage yield of the three solvents used to extract the powders of the leaf of *T. Cattapa* using cold maceration methods. The high yield with methanol is due its volatility and ability to dissolve several secondary metabolites of plants.

The Phytochemical screening of the extracts revealed the presence of the following bioactive constituents; Saponins, Flavonoids, Tannins, Steroids and Carbohydrates but Alkaloids, Anthraquinone, Cardiac glycosides and Terpenoids were absent in all the extracts. The results of the finding concur with the work of Wuyep et al. (2020) and Zacchaeus et al. (2024) who also reported the presence of these metabolites in different fractions of plants from the same family (Table 1). The antifungal activities were also concentration-dependent with the highest activity against *Aspergillus terreus* with a zone of inhibition of 10±0.82 to 16±0.41mm and 11±0.41 to 20±0.45mm at the different concentrations for leaves and stem extract respectively (Table 2). The results of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) showed that *T. mantas* was the most susceptible with MIC of 125 mg/ml and MFC 250 mg/ml, whereas *A. terrues* was the most susceptible to the stem extracts with MIC of 62.5 mg/ml and MFC 125 mg/ml as compared to the standard drug with MIC of 25 mg/ml and MFC 50 mg/ml (Table 3). The presence of secondary metabolites in plants, produce some biological activity in man and animals and it is responsible for use as herbs. The presence of antifungal activity in alcoholic fraction of *Terminallia* extracts finds agreement with the work of other scientists (Parekh and Chanda 2007; Shinde et al., 2011). Zirihi et al., 2012), who reported that the hydroalcoholic extracts of *T. catappa* and *T. mantaly* inhibit the in vitro growth of *Aspergillus* species.

The results of the acute toxicity testing reveal that the survival of the flies is concentration-dependent in a negative progression. The lower the concentration the higher the survival, and the higher concentration the lower the survival. The lethal dose (LD_{50}) for the methanol leaves and stem extract is 60 mg/ml while the control drug Itraconazole is 40 mg/mg. This concentration is essential for the treatment of the flies in the infection studies. Similar results have been reported by Wuyep et al. (2020) stating that the ethyl acetate roots extracts of *T. glaucescens* had a toxicity activity in *D. melanogaster* with LD_{50} of 50 mg/ml. The results of the infectious studies showed that flies infected with *A. terrues* and treated with 60 mg/ml of leaf extracts had the highest survival rate of 58.33% and 61.44% of 50mg/ml of stem extract as compared with the standard drug intraconazole with survival rate of 65.11% at 60 mg/ml respectively. But at very extreme concentrations the population of the flies crashes to zero. There was no significant difference at P≤0.05 between the survival of the flies on the plant extracts and the control drug Itraconazole. The result is in agreement with the antifungal activity of the extracts *in vitro*. Zacchaeus et al. (2024) also reported that the methanol extracts of *T. cattapa* improved the survival rate of *D. melanogaster* infected with different species of *Aspergillus* and treated on 60 mg/ml of five different species of Terminalia plants. 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 (Wuyep et al., 2020). However, the antifungal effects of the methanol leaves and stem extract in vivo were comparable to Itraconazole and at significantly lower doses.

CONCLUSION

The methanol extract of *T. catappa* leaves and stem manifested varying degrees of antifungal activity *in vitro* against *Aspergillus terreus* and *Trichoderma mantas* and *in vivo* on *D. melanogaster* infected with virulent *A. terreus* in the fly survival rate assay. The presence of secondary metabolites must be responsible for the antifungal activity both *in vitro* and in vivo against *A. terreus* and *T. mantas.* Therefore, the antifungal potency of *T. catappa* leaves and stem fractions manifested in this research is the reason for its use in African traditional herbal medicine for the treatment of animal and plant diseases caused by these pathogenic fungi.

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