



ASSESSMENT OF FUNGICIDAL AND FUNGISTATIC PROPERTIES OF SOME NIGERIAN MOSS PLANTS

¹FEMI-ADEPOJU, Abiola G., ²ADEPOJU Adeyinka O. & ³OGUNKUNLE Adepoju T. J.

¹Department of Biosciences and Biotechnology, Kwara State University, Malete, Kwara State, Nigeria;

^{2 & 3} Department of Pure and Applied Biology, Ladoko Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.

Abstract

Extracts from four Nigerian mosses; *Barbula lambarenensis* J. Hedwig, *Octoblepharum albidum* Hedw., *Thuidium gratum* (P. Beauv.) A. Jaeger, and *Calymperes afzelli* Swartz, Jahrv. Gewachsk were prepared using ethanol, petroleum ether, acetone and distilled water with the aid of soxhlet extractor and their antifungal effects tested on *Penicillium chrysogenum* and *Rhizopus stolonifer*. Agar diffusion method was used for *P. chrysogenum* while potato nutrient broth was used to grow *Rhizopus stolonifer*. All the four plant extracts tested had fungistatic effects against the test organisms but none was fungicidal to them. Moreover, ethanolic extracts generally recorded higher retardation of fungal growth than those of petroleum ether, acetone and distilled water.

Keywords: Fungicidal, Fungistatic, Moss extracts, *Penicillium*, *Rhizopus*.

1.0 Introduction

Different plant groups have been recorded in the literature to possess antifungal efficacy against a number of plant and human pathogens (Sibtain *et al.*, 2002; Saadabi, 2006). Investigations have also been conducted on the efficacy of members of lichen (Halama and Van Haluwin, 2004), pteridophyta (Sahayaraj *et al.*, 2009), gymnosperms (Krauze-Baranowska *et al.*, 2002), monocots and dicots (Dey, 2011a,b; Dey and De, 2011) against various pathogenic and some non-pathogenic fungi.

Development of drug resistance in pathogenic fungi due to the use of conventional antibiotics could be dealt with using the natural and novel antibiotics with diverse mechanisms of action from some new and unique sources like bryophytes. In the present study, the antifungal tendencies of extracts of four Nigerian species of mosses in different solvents were tested against *Rhizopus stolonifer*. and *Penicillium chrysogenum*. These organisms were selected for use because they are readily available in our environment and are of notable economic importance to man. *R. stolonifer* is a pathogen that has been implicated in connection with floral and fruit diseases of many plants of economic and medicinal values (Kwon *et al.*, 2001; Echerenwa and Umechuruba, 2004; Shukla *et al.*, 2006). On the other hand *P. chrysogenum* has been acknowledged for its beneficial importance to man, being a source of beta lactam antibiotics and other secondary metabolites. This organism has however, been reported in connection with occasional causes of infection in man, including necrotizing pneumonia (D'Antonio *et al.*, 1997).

2.0 Materials and Methods

2.1 Plant Material and Collection Sites

The moss plants used in this study were collected in Nigeria as follows: *Barbula lambarenensis* J. Hedwig (from a rock surface in Ondo town), *Thuidium gratum* (P. Beauv.) A. Jaeger (from a tree bark in Ondo town), *Octoblepharum albidum* Hedw. and *Calymperes afzelli* Swartz, Jahrv. Gewachsk (both from the campus of University of Ilorin, Ilorin, Kwara State).

2.2 Extraction of Secondary Plant Products

Extraction of the desired active ingredients from the plants was carried out with the aid of the Soxhlet Apparatus at the department of chemistry, University of Ilorin, Ilorin, Nigeria using four different solvents i.e. two polar namely, distilled water and ethanol; and two non-polar i.e., acetone and petroleum ether. Duration for extraction was about 4 hours 30 minutes using 0.75g of the dried plant material and 250 ml of solvent in each case.

2.3 Preparation of Potato Dextrose Agar (PDA)

A 50g portion of peeled Irish potato was sliced into pieces, washed to remove unwanted materials and boiled for 1 hour with distilled water in a conical flask. A muslin cloth was used to obtain a filtrate from the cooked potatoes, to which water was added to make up to 250ml. Then, 5g of dextrose and 3.5g of agar agar was added to the extract in the conical flask. The suspension was dissolved on a burner with occasional shaking, after which the medium was cooled for 2 mins and then sterilized by autoclaving at 121°C for 1hour. Thereafter, 0.25% of Streptomycin sulphate was added to prevent bacterial growth while the laboratory room temperature of about 27 ± 3 °C was maintained. The resulting medium was poured into petri dishes and allowed to solidify.

2.4 Preparation of Potato Nutrient Broth (PNB)

PNB was prepared for the growth of *Rhizopus* by boiling 50g of sliced potatoes in 125ml of distilled water for 1 hour. After cooling, the mixture was sieved with a muslin cloth into another flask and made up to 250ml

with distilled water. Thereafter, 5g of dextrose sugar was added and heated on a burner for the latter to dissolve. The medium was then sterilized by autoclaving at 121°C for 1 hour.

2.5 Fungal Culture Preparation

Pure cultures of two fungi, *Rhizopus stolonifer* and *Penicillium chrysogenum* were obtained from the laboratory of the Department of Biological Sciences, University of Ilorin, Ilorin, Nigeria. These organisms were selected for use because they are readily available in our environment and are of great economic and health values. Sub-culturing was carried out under aseptic conditions by means of a sterilized inoculating loop to transfer some hyphae of *Penicillium chrysogenum* into two petri-dishes containing the prepared PDA as the growth medium; Similarly, two portions of 5mg of the pure culture of *Rhizopus stolonifer* were introduced, each into a 50ml of the broth in a test tube. The mixture was shaken and used immediately to inoculate the experimental broths. The petri-dishes were however incubated for 72 hours at laboratory room temperatures preparatory to the commencement of the experiments.

2.6 Experimental Setup

The experimental setup consisted of five sets of plates or broths. Each of the first four sets was meant to test the effect of the extract of a moss plant on the hyphal growth of a fungus, and it contained four plates/broths, one for each extractant. The fifth set of plates/broths was the control group that was represented by five plates/broths i.e. those of the medium with ethanol, acetone, petroleum ether, and distilled water, and that of the medium only. The total number of plates was 21 and that of the broths was also 21 (Table 1).

Table 1: Experimental setup to test the effects of extracts of some Nigerian moss plants on hyphal growth of *Penicillium chrysogenum* and *Rhizopus stolonifer**.

SET I: Test plates/broths for <i>Barbula lambarenensis</i>		
1.	BL1:	Medium + ethanol extract
2.	BL2:	Medium + Acetone extract
3.	BL3:	Medium + petroleum ether extract
4.	BL4:	Medium + distilled water extract
SET II: Test plates/ broths for <i>Octoblepharum albidum</i>		
5.	OA 1:	Medium + ethanol extract
6.	OA 2:	Medium + Acetone extract
7.	OA 3:	Medium + petroleum ether extract
8.	OA 4:	Medium + distilled water extract
SET III: Test plates/broths for <i>Thuidium gratum</i>		
9.	TG1:	Medium + ethanol extract
10.	TG2:	Medium + Acetone extract
11.	TG3:	Medium + petroleum ether extract
12.	TG4:	Medium + distilled water extract
SET IV: Test plates/broths for <i>Calymperes afzelli</i>		
13.	CA1:	Medium + ethanol extract
14.	CA2:	Medium + Acetone extract
15.	CA3:	Medium + petroleum ether extract
16.	CA4:	Medium + distilled water extract
SET V: Control media plates/broths		
17.	CM1:	Medium + ethanol
18.	CM2:	Medium + acetone
19.	CM3:	Medium + petroleum ether
20.	CM4:	Medium + distilled water
21.	CM5:	Medium only

*Test tubes of potato nutrient broth (PNB) were used for *R. stolonifer* in place of potato dextrose agar plates used for *P. chrysogenum*.

2.7 Experimental Setup for *Penicillium chrysogenum* With PDA

Under aseptic laboratory conditions, the prepared PDA was poured into the sterilized petri dishes in the presence of burning flame and 2ml each of the extracts at 100mg/ml was poured into the agar in their molten form. These were mixed together and allowed to solidify. A 5mm diameter of growth medium together with *P. chrysogenum* hyphae was drilled out by means of a cork borer. A sterilized inoculating loop was then used to pick the hyphae disc on to the centre of the petri dishes containing PDA and the moss extracts. After inoculation, the plastic petri-dishes, properly labeled, were placed in the incubator at 28°C. The experimental set up was then monitored for fungal growth by measuring the diameter of growth at 24 hours intervals for 72 hours.

2.8 Experimental Setup for *Rhizopus stolonifer* With PNB

Following similar aseptic procedures as in the experimental setup for *Penicillium chrysogenum*, the prepared potato nutrient broth (PNB) was poured into the sterilized test-tubes and the extracts were introduced into the broth except for the test-tubes used as the control. By means of a sterilized micro pipette, 1ml of the pure culture of *Rhizopus stolonifer*, containing 0.1mg of the organism was inoculated into the test-tubes which contained PNB incorporated with the moss extracts. Following inoculation, the test-tubes, plugged with sterilized cotton wool, were placed on test-tube racks and were incubated at 28⁰C for 72 hours. The weight of the hyphae at the end of this period was determined as the difference between the final (after incubation) and the initial (before incubation) weights of the setup.

PDA was used for *Penicillium chrysogenum* because the fungus only spreads in spore form, i.e. the hyphae are not pronounced. On the other hand, PNB was used for *Rhizopus stolonifer* due to the fact that the fungus grows with a high rapidity and the hyphae' protruding in all direction to allow maximum spread (Kavanagh, 2005).

3.0 Results

The results of this study are shown in Figures 1 to 8. None of the extracts obtained from *B. lambarenensis*, *O. albidum*, *T. gratum*, and *C. afzelli* was fungicidal to *P. chrysogenum* and *R. stolonifer*.. However, extracts of the four bryophytes appreciably inhibited the growth of *P. chrysogenum* and *R. stolonifer* in comparison with the control. Ethanolic extracts of the bryophytes generally inhibited fungal growth better than did those of petroleum ether, acetone and distilled water (Figures 2 to 6 and 8).

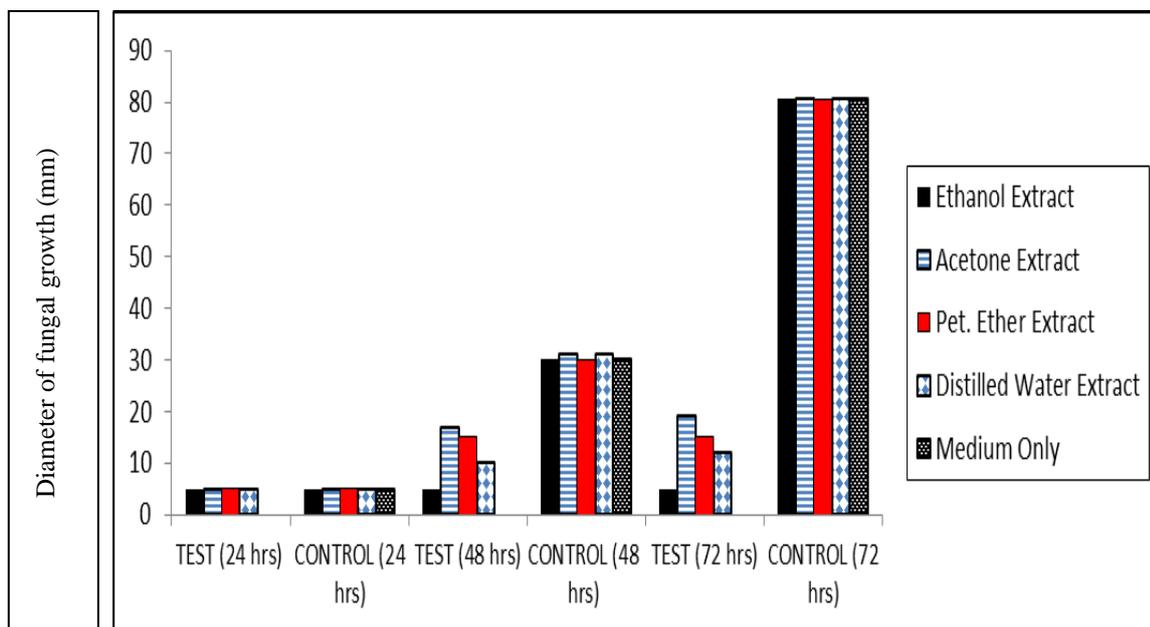


Figure 1: Effects of *Barbula lambarenensis* extracts on the hyphal growth diameter of *Penicillium chrysogenum* over a period of 72 hours.

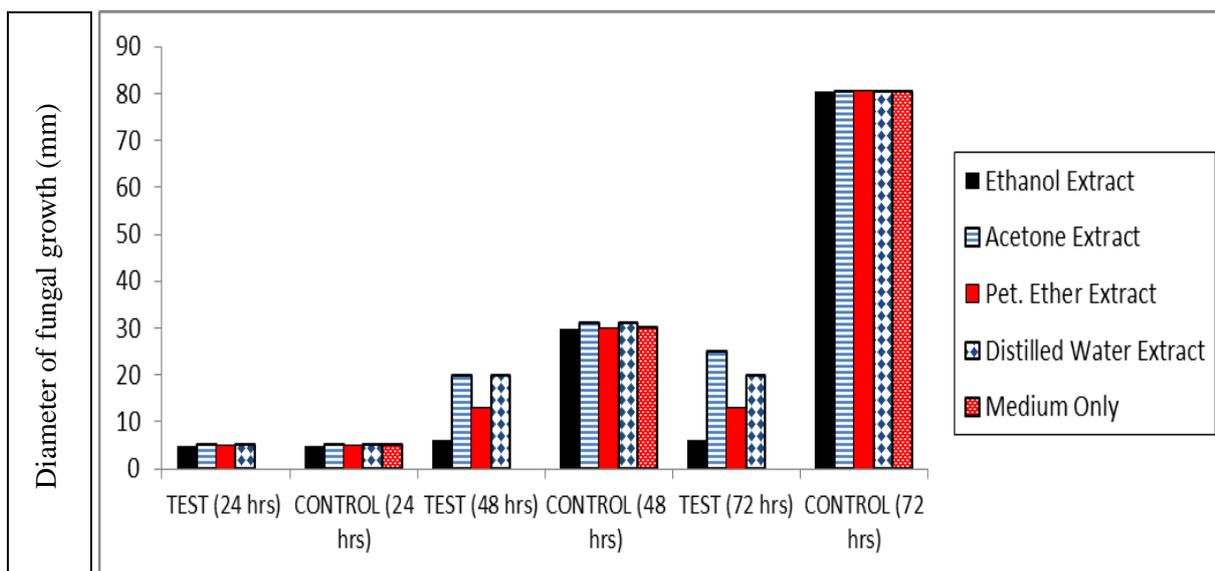


Figure 2: Effects of *Octoblepharum albidum* extracts on the hyphal growth diameter of *Penicillium chrysogenum* over a period of 72 hours.

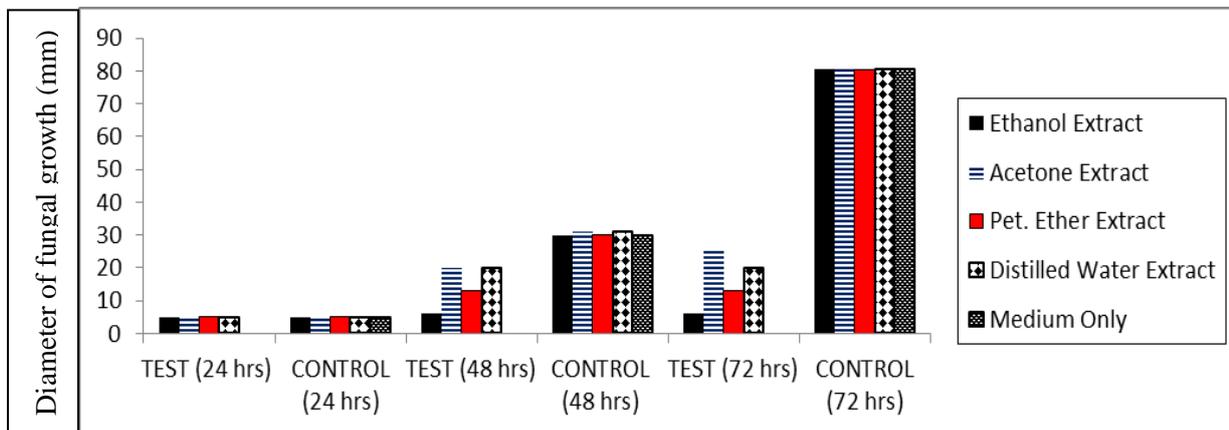


Figure 3: Effects of *Thuidium gratum* extracts on the hyphal growth diameter of *Penicillium chrysogenum* over a period of 72 hours.

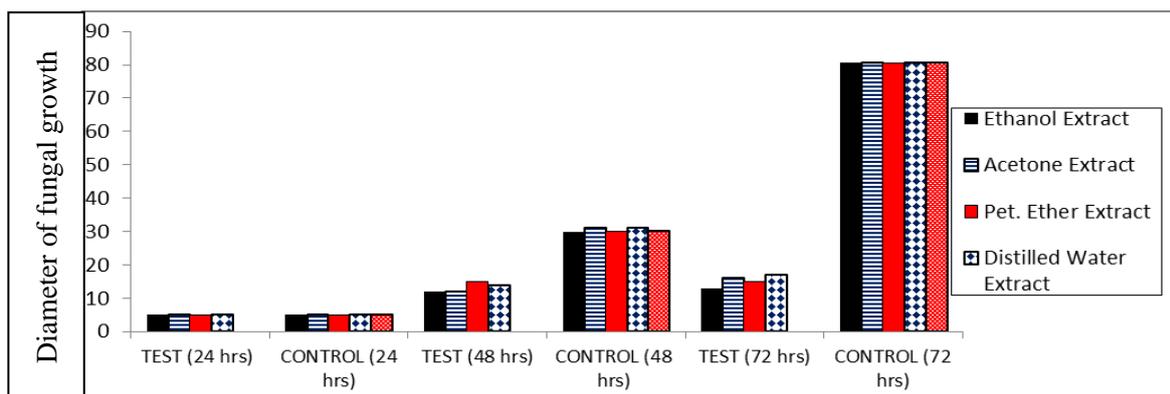


Figure 4: Effects of *Calymperes afzelli* extracts on the hyphal growth diameter of *Penicillium chrysogenum* over a period of 72 hours.

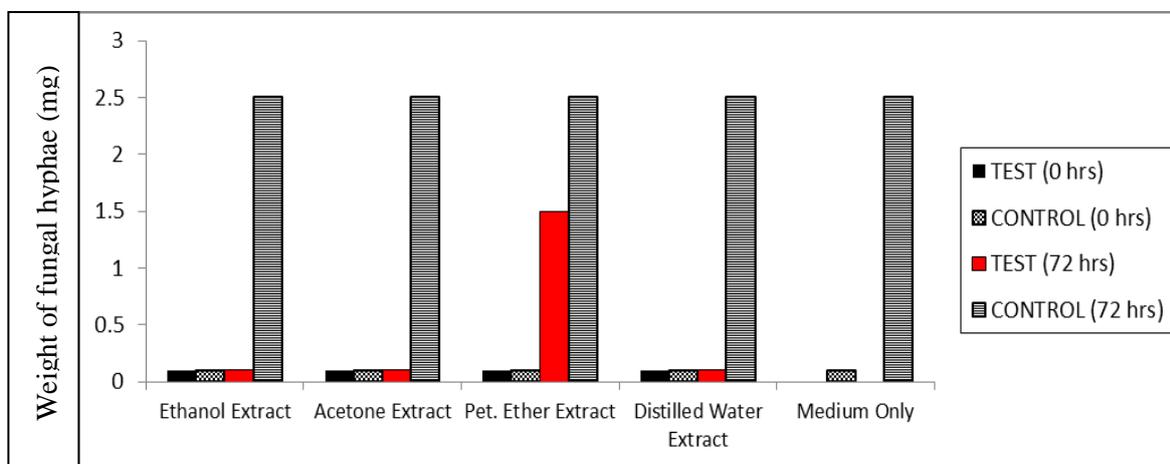


Figure 5: Effects of *Barbula lambarenensis* extracts on the weight of the hyphae of *Rhizopus stolonifer* over a period of 72 hours.

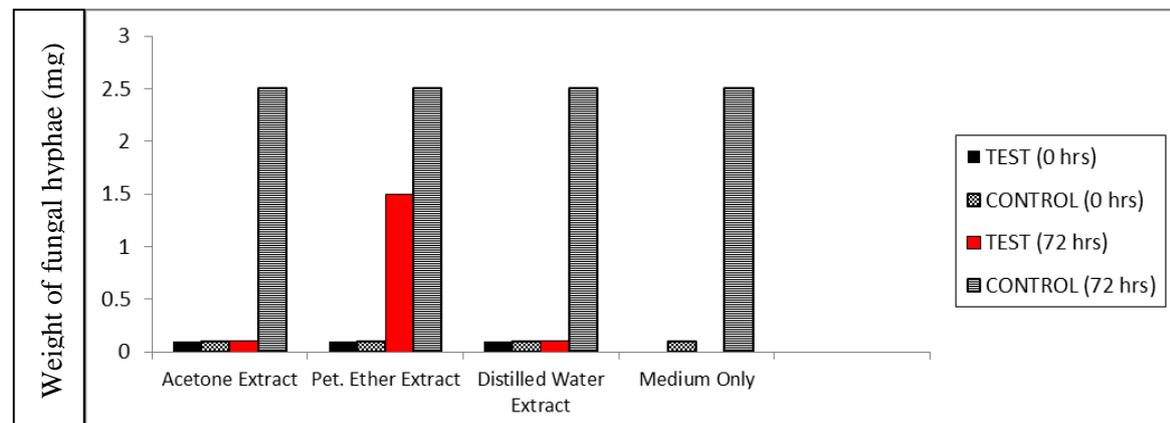


Figure 6: Effects of *Octoblepharum albidum* extracts on the weight of the hyphae of *Rhizopus stolonifer* over a period of 72 hours.

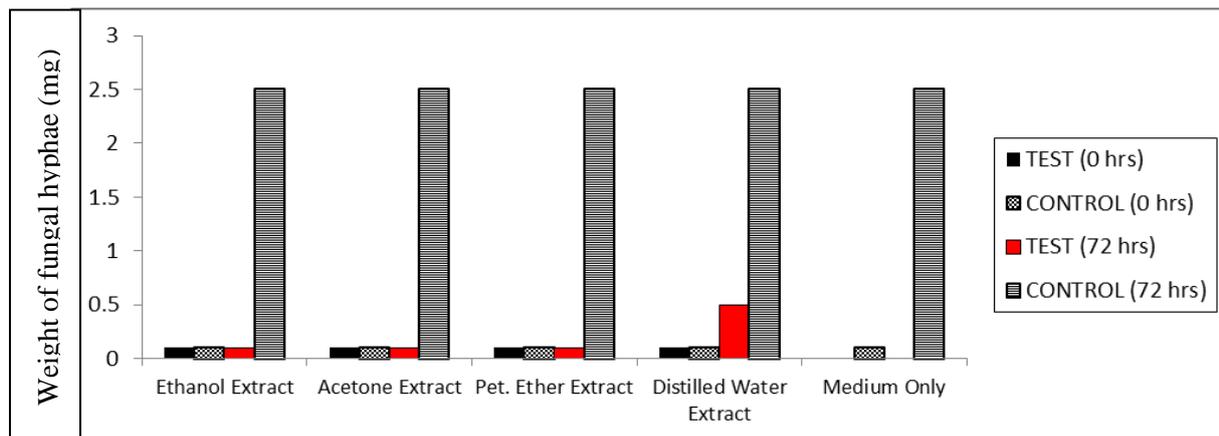


Figure 7: Effects of *Thuidium gratum* extracts on the weight of the hyphae of *Rhizopus stolonifer* over a period of 72 hours.

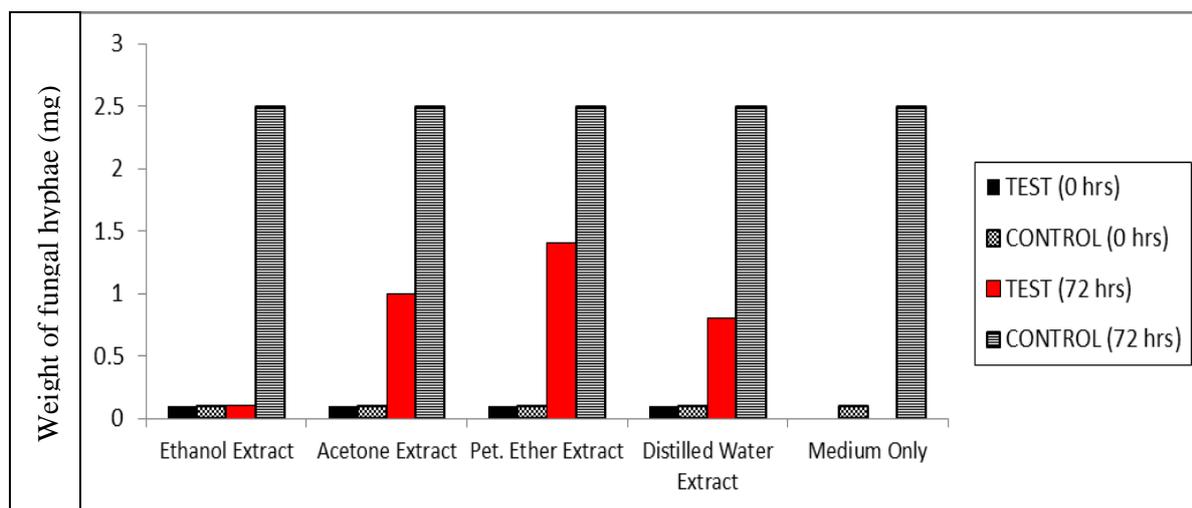


Figure 8: Effects of *Calymperes afzelli* extracts on the weight of the hyphae of *Rhizopus stolonifer* over a period of 72 hours.

4.0 Discussion

Many published reports on the effects of plant extracts on fungal growth involved the use of crude extracts in different solvents. Some of the researchers were able to isolate and purify the active compounds that have been associated with specific mode of fungal growth inhibition. Siddiqui *et al.* (2005) synthesized fungicidal bibenzyls through acetylation of bibenzyl compound which was derived from a bryophyte. In contrary, bibenzyls isolated by Schultz *et al.* (1991) from *Scorzonera humilis* (an angiosperm), did not show fungicidal activity. Apart from the macrocyclic bisbibenzyls, some other constituents of bryophytes that have been found to exhibit fungicidal or fungistatic properties against fungal pathogens include steroids, sesquiterpenoids, acetophenones, stilbenes and essential oils (Abhijit and Jitendra, 2011). Although, the present study did not attempt to isolate the active ingredients in the mosses used, it is a confirmation of the claims by scientists such as Abhijit and Jitendra (2011) that bryophytes could be a rich source of secondary metabolites with diverse pharmacological importance.

With respect to the solvents used for extraction in this study, ethanol performed best, followed in many cases by distilled water, while acetone and petroleum ether were less effective (Figures 1 to 8). Acetone extracts were particularly least effective in extracting the desired fungistatic ingredients against *Penicillium chrysogenum* (Figures 1 to 4) while petroleum ether extract was the least effective against *Rhizopus stolonifer* (Figures 5 to 8). Ethanol extracts of *B. lambarenensis*, *O. albidum*, *T. gratum*, and *C. afzelli* consistently suppressed the growth of *Penicillium chrysogenum* (Figures 1 to 4) and of *Rhizopus stolonifer* (Figures 5 to 8) better than any of the other three extracts. This is an indication that the active principles in these mosses are predominantly polar. These results are in agreement with those of Alam *et al.* (2011) that aqueous extract of the liverwort *Dumortiera hirsuta* was found to inhibit a number of phytopathogenic fungi mediated by different modes of action such as spore germination inhibition, development of anomalies in the hyphae, formation of flaccid cell wall and granulated cytoplasm. The present results also partially agree with those of Basile *et al.* (1998) in which acetone extract of *Lunularia cruciata* (a bryophyte) showed no activity against *Candida albicans* and *Aspergillus niger*. In the tests involving acetone and petroleum ether as solvents, absence of antifungal activity is suspected to be due to the presence of non-polar molecule(s) in the extracts resulting in the inability of the molecules to cross the fungal cell wall (Basile *et al.*, 1998).

Considering the four species of bryophytes employed in this study, only the ethanol extracts of *B. lambarenensis* and *T. gratum* appear to be effective against *Penicillium chrysogenum* (Figures 1 and 3). The extracts of the four mosses, particularly with acetone, petroleum ether and distilled water were less effective (Figures 1 to 4). On the contrary, ethanol, and acetone extracts of three of the four bryophytes tested (*B. lambarenensis*, *O. albidum*, *T. gratum*)

and the distilled water extract of the first two, effectively suppressed the growth of *Rhizopus stolonifer* (Figures 5 to 8). The distilled water extracts of *T. gratum* and *C. afzelli* however were ineffective as they respectively recorded weight increase of fungal growth of 400% and 700% (Figures 7 and 8). Lastly, only the petroleum ether extract of *T. gratum* inhibited the growth of *Rhizopus stolonifer*, while those of *B. lambarenensis*, *O. albidum* and *C. afzelli* did not (Figures 5, 6 and 8). The afore mentioned results of this study appear to be in consonance with those of Wu *et al.*, (2010), from which it was asserted that different bryophytes followed various modes of action to inhibit fungi *in vitro*. According to these authors, the mechanism of activity was not only dependent on the compound but was also pathogen-specific and same compound seemed to follow different modes of action while inhibiting the same or different fungi. The present findings could possibly be justified by this explanation.

5.0 Conclusion

The extracts obtained from *B. lambarenensis*, *O. albidum*, *T. gratum*, and *C. afzelli* did not have fungicidal effects on *P. chrysogenum* and *R. stolonifer*. All the extracts from these four bryophytes however substantially inhibited the growth of *P. chrysogenum* and *R. stolonifer* in comparison with the control. Ethanolic extracts of the bryophytes generally inhibited the growth of the two tested fungi better than did the extracts of petroleum ether, acetone and distilled water.

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