IN VITRO TRYPANOCIDAL ACTIVITIES OF *PENICILLIUM RESTICULOSUM* AND ASPERGILLUS FUMIGATUS EXTRACTS ON *TRYPANOSOMA BRUCEI BRUCEI*

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ABSTRACT

Using an *in vitro* experimental model, two Entomopathogenic fungi (*Penicillium resticulosum* and *Aspergillus fumigatus)* was isolated from soil around security office area Bayero University Kano, Old campus. Crude ethyl acetate extracts of the fungus were screened for Trypanocidal activity on *Trypanosoma brucei brucei* parasite. The concentrations of extract used are 0.625, 1.25, 2.5, 5, and 10mg/ml. A dose concentration of 10mg/ml of the ethyl acetate extract of *Penicillium resticulosum* causes mortality of *T.brucei brucei* by 45.6%, 88.6%, and 100% after a period of 1 hour, 2hours and 3hours exposure of the parasite to the extracts respectively, while a dose concentration of 10mg/ml of. *A. fumigatus* extracts causes mortality of *T. b brucei* by 100% after the 1st, 2nd and 3rd hour respectively. These apparently indicate that both fungal extract possess biologically active components which cause the mortality of *T. brucei brucei parasite*. The potent Trypanocidal activity of the extract shown against *T.brucei brucei* requires further work on the isolation identification and purification of the active component(s) for the treatment of *T. brucei brucei*.

Keywords: *Penicillium resticulosum, Aspergillus fumigatus, Trypanosoma brucei brucei,* Trypanocidal, *In vitro activity*

INTRODUCTION

African *trypanosomiasis*, is commonly referred to as sleeping sickness in humans and 'Nagana in cattle, is a disease that is resurgent in Africa (Yusuf *et al.,* 2012). It affects most species of domestic livestock, many types of wild animals, and man (Henry *et al.,* 2012). The disease remains one of the major constraints to ruminant livestock in sub-Saharan Africa (Farougou *et al*., 2012). It has been reported that animal *trypanosomosis* causes not less than 3 million live stock deaths as each year with 20% less in calving, 25% reduction in milk yields with 50% reduction in livestock numbers which leads to the reduction in work efficiency of the animals thereby causing hindrance in crop production (Swallow, 2000).

In Nigeria, animal trypanosomosis constitutes a major obstacle to food security in spite of attempts towards chemotherapeutic treatment and tsetse control. The disease, not only causes millions of livestock deaths, but also reduces calving rates, milk yield and work efficiency of draft animals (Anyaegbunam *et al.,* 2013).

Recent developments in the field of parasitology have led to a renewed interest in the use of different variety of medicinal plants as well as microorganism as alternative sources for the production of antiparasitic drugs (Shaba *et al*., 2011). It is therefore, very important to search for new, safe, effective and affordable alternative sources of anti-parasitic drugs.

Entomopathogenic fungi is fast becoming a vital organism in the development of pesticide, and recent development in the study of Entomopathogenic fungi have highlighted the need for the exploitation of the organism for the development of insecticides as well as antiparasitic drugs (Sehroon *et al.,* 2012). Fungi are recognized as prolific secondary metabolite producers. Fungi have provided several bioactive compounds and chemical models currently used as pharmaceuticals, and soils are traditionally the main source of fungal genetic resources for bioprospection programs (Ali *et al.,* 2011).

Penicillium is a large anamorphic (asexual state) ascomycetous fungal genus with widespread occurrence in most terrestrial environments comprising of more than 200 reported species. Many are common inhabitants of the soil, food borne contaminants and round of food items used in the preparation of cheese and sausages (Frisvad and Samson, 2004). *Penicillium* species produce a much diversified range of active secondary metabolites, including antibacterial (Lucas *et al.,* 2007; Rancic *et al.,* 2006), antifungal substances (Nicolette *et al.,* 2007), immuno-suppressants, cholesterol-lowering agents (Kwon *et al.,* 2002), and also potent mycotoxins (Frisvad and Samson, 2004). These secondary metabolites of *Penicillium* species have been identified as well as proved, their biological activities have been approved (Silva *et al.,* 2004).

Thousands of *Penicillium* isolates have been screened in bioprocessing since the discovery of penicillin. Numerous investigations have reported that various mycotoxins can be produced by *Penicillium* (Faid, 1989). The chance discovery of *Penicillium notatum* by Alexander

Fleming and the production of the revolutionary drug, penicillin, is perhaps the most important finding in the history of therapeutic medicine.

The second Entomopathogenic fungi used in this research was *Aspergillus fumigatus.* These were also reported to produce metabolite due to its broad range of biological activities (Son *et al*., 1996). Fumifungin (Mukhopadhyay *et al*., 1987) and synerazol (Ando *et al*., 1991) were new antibiotic, isolated from a culture broth of *A. fumigatus* which possessed anti fungal activity. Fumagillin is also another antibiotic produce by the fermentation of certain strain of *A. fumigatus* and was reported to have an angiogenesis inhibitory activity. Another report indicates that *Aspergillus* strain have been found to demonstrate a moderate antileshmanial activity (Sergio *et al*., 2014).

MATERIALS AND METHODS

Collection of soil sample

Different soil samples were collected from an area around the security office of Bayero University Kano, Old Campus. The area was under shade and characterized by a lot of leaf liters. Animal mostly found are insects which include flies, dragon flies, Butter flies etc. The soil sample was collected at 5cm, 15cm, and 20cm depth. The soil sample was collected in a white transparent polythene bag and labeled appropriately.

Isolation and identification of Entomopathogenic fungi

20g of Potatoes Dextrose Agar (PDA) was measured and mixed with another 20g of oatmeal agar, supplemented with 0.6g of Cetyl Trimethyle Ammonium Bromide (CTAB) (Sigma Aldrich USA). 0.1g Streptomycin was also weighed, and added to the mixture. The mixture was later transferred into 1000ml conical flask and made up to one liter with distilled water. The mixtures were dissolved by gentle shaking where the opening of the conical flask was wrapped using cotton wool and aluminum foil (Julieta *et al*., 2012). The control medium was prepared similarly without 0.6g CTAB.

0.1g of soil sample was diluted in 10ml of prepared 0.05% of twin 80 in the test tube. One hundred microlitres of soil suspension were then placed on the isolation medium. The suspensions were then spread using an L-shape glass rod in triplicates. The plates were incubated at room temperature until growth of fungi is observed after a period of two weeks.
Colonies of fungi that have grown on oatmeal agar were then sub cultured on Potatoes Dextrose Agar (PDA) in order to obtain a pure growth and then the pure cultured fungi were then taken to the University Central Laboratory for further identification.

Identification of *P. resticulosum and A. fumigatus* species

A sterilized needle was used to collect a small portion of the fungal culture and dropped on a glass slide. A drop of water was added on the glass slide followed by gentle smearing to

enhance visibility of the organism. The slide was covered with a cover slip or spreader and examined under microscope together with oil imation. Observations where made using × 40 objectives (T. Watanabe, 2010).

Preparation of crude extract

A portion of the culture was transferred aseptically into a Potatoes Dextrose Broth and incubated at room temperature for three (3) weeks. The culture was filtered using whatman filter paper no.1 to separate the broth from the mycelia. The mycelia were extracted in 200mls of methanol, while the broth was extracted in an equal volume of ethyl acetate. The methanol extract was concentrated at 40^{o}C. The concentrate of the methanol fraction was further mixed with ethyl acetate fraction washed with brine and finally concentrated at 40^{0}C.

Test organism for *in vitro* assay

Trypanosoma brucei brucei federe strain was the test organism used. It was obtained from vector and Parasitology Section of Nigerian Institute for Trypanosomiasis Research (NITR) Surame road, Unguwan Rimi, Kaduna State. The parasites were maintained in laboratory by serial passage in donor mice. Passaging is necessary when there is rise in parasitaemia i.e. if parasite density is within the range of 15-30 parasite per field, at least 3- 5 days post infection (Atawodi, 2005).

Artificial preparation of carbon dioxide

This was possible by adopting the recent procedure from Bulus *et al*. (2012). Artificial preparation of carbon dioxide was achieved by transferring 2MHCL into a wash bottle. Sodium bicarbonate was placed in another bottle connected with robber pipe linking the two bottles together. Another robber pipe was also linked to 2000ml drip polythene (Plate I). The wash bottle was then pressed to pass the 2MHCL through the pipe to the sodium bicarbonate container where 5% carbon dioxide and 95% atmospheric air was collected to half-fill the 2000 ml drip polythene, i.e. 1000ml, as shown in the equation below:

$$\mathbf{Na_2CO_3 + 2MHCL \longrightarrow NaCl + CO_2 + H_2O}$$

$5500/1000 \times 95 /100 = 5\%\ CO_2$

The 5% carbon dioxide introduced into the desicator with the titer plate containing the test organism and extracts of various concentration in the wells, was used as part of the requirement for the propagation of *T.brucei brucei in vitro* (Bulus *et al*.,2016).

Preparation of Medium for propagation of *Trypanosomes.*

The medium used for the *in vitro* propagation of *Trypanosoma brucei brucei* was RPMI (Royal Pack Memorial Institute) 1640 culture medium. It was prepared by adding16ml RPMI

medium in addition to 25Mm hepes, and 4ml calf serum 20µl micro liter antibiotics (streptomycin) according to manufacturer's instruction and p^H adjusted to 7.4.

In vitro activities of Ethyl acetate crude extracts of *P. resticulosum*

Exactly 10mg of the extract was weighted and dissolved in 1ml of culture medium; this was followed by serial dilution of this stock solution using the culture medium to obtain various concentrations (10mg/ml, 5mg/ml, 2.5mg/ml,1.25mg/ml, and 0.625mg/ml). Assessment of the *in vitro* Trypanocidal activity was performed in duplicate in 24 well microtitre plates (flow laboratories Inc., Mclean, Virginia, USA). Exactly 1ml of the extract was incubated in microtitre wells at 27^0C with 10µl of the parasite suspension (density of about 65.3 x 10^6 parasite/ml). The control well contained 100µl of culture medium with parasites but no extract solution was added to it. Parasite motility was monitored on a glass slide covered with a cover slip under a microscope at ×400 magnification. The number of motile parasites was counted before and 1hour, 2hours and 3hours after the addition of extracts. Drop in the percentage of the motile parasites was taken as an indication of extracts activity against *Trypanosoma brucei brucei* for the period of one hour, two hours and three hours employed.

Results and Discussion

The results of the present study suggest that, both extract (*Penicillium resticulosum* and *Aspergillus fumigates*) shows Trypanocidal activities. These extract were tested on *Trypanosoma brucei brucei* at varying concentrations and time of exposure. Percentage mortality increases with increase in concentration and time of exposure of *T.brucei brucei* to the extract (Figure 1, 2 and 3 respectively). At the lowest concentration (0.625 mg/ml), *P.resticulosum* shows 8.6% mortality were it increases to 45.6% at the highest concentration (10mg/ml) after one hour exposure of *T.b.brucei* to *P.resticulosum* extract (Figure 1). In the other hand, at 0.625mg/ml, *A. fumigatus* extract indicates 20% mortality while mortality increases to 100% at the highest concentration (10mg/ml) after one hour incubation. After the second hour exposure of T.b.brucei to P.resticulosum extract, mortality increases from 51.4% at concentration of 0.625mg/ml to 88.6% at concentration of 10mg/ml after the second hour exposure (Figure 2).

Another indication of increase in Trypanocidal potential of *P.resticulosum* extract with time was recorded after three hours incubation period. This was observed by a record of 65.7% mortality at the lowest concentration (0.625mg/ml) where 100% mortality was observed and recorded at a concentration of 10mg/ml, which is a clear indication of complete cessation of motility after the third hour incubation period (Figure 3). Meanwhile, in A. fumigatus extract, 71.4% was recorded at the lowest concentration (0.625mg/ml) and 100% mortality was also recorded at the highest concentration after three hours exposure (Figure 3).

The results of the current research also indicate that, the reduction in the percentage survival of *T. b. brucei* in relation to increase in concentration and time of exposure by *P. resticulosum,* and *A.fumigatus,* clearly indicate the presence of certain compounds which are

responsible for the Trypanocidal activities on *T. b. brucei* which will be very vital to be isolated and identified in future research. This is in agreement with the findings by Frisvad and Samson (2004), who reported that *P. varrucosum* produces some secondary metabolite, including Griseofulvin and Penitrem A. The study was also in agreement with the findings of Sergio et al. (2014) and David et al. (2014), which reported another isolated compound from fungi Monocerin and 11-hydroxymonocerin which was found to be effective against *P. falciparum*, and *Cladosporium cladosporoid* which produces a secondary metabolite Cladosporine with antiparasitic effect against *Plasmodium* species respectively.

A. fumigatus was also reported to produce metabolite due to its broad range of biological activities (Son *et al*., 1996). Fumifungin (Mukhopadhyay *et al*., 1987) and synerazol (Ando *et al*., 1991) were new antibiotic, isolated from a culture broth of *A. fumigatus* which possessed anti fungal activity. Fumagillin is also another antibiotic produced by the fermentation of certain strain of *A. fumigatus* and was reported to have an angiogenesis inhibitory activity. Another report indicates that *Aspergillus* strain has been found to demonstrate a moderate antileshmanial activity (Sergio *et al*., 2014).

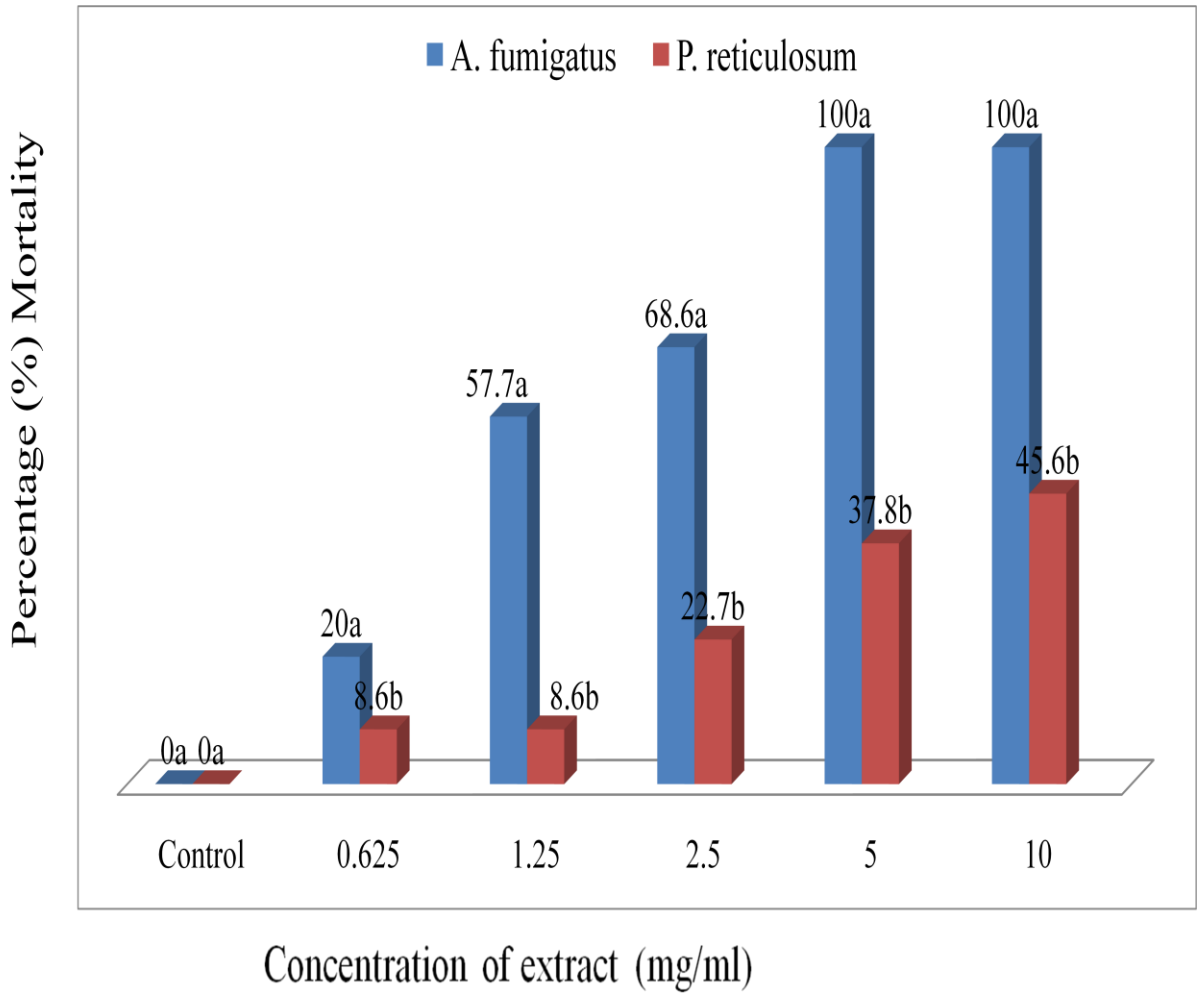

Fig 1: Mean percentage mortality of *T.brucei brucei in* varying concentration of *A.fumigatus* and *P. resticulosum* extract (mg/ml) after 1hour exposure. Mean followed by different alphabet(s) are significant ($p<0.05$) by Pearson correlation test.

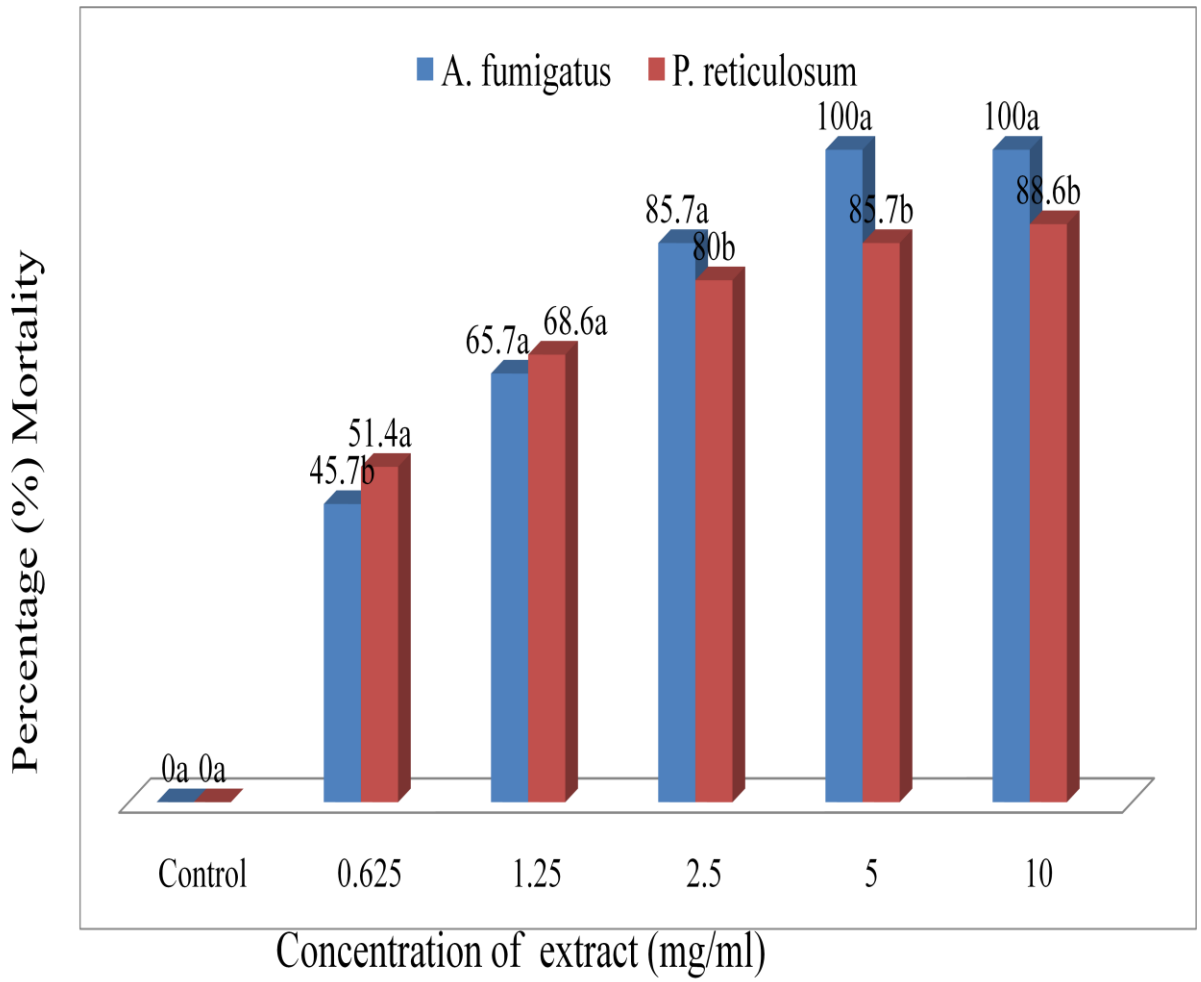

Fig 2: Mean percentage mortality of *T. brucei brucei* in varying concentration of *A.fumigatus* and *P. resticulosum* extract (mg/ml) after 2hours exposure. Mean followed by different alphabet(s) are significant ($p<0.05$) by Pearson correlation test.

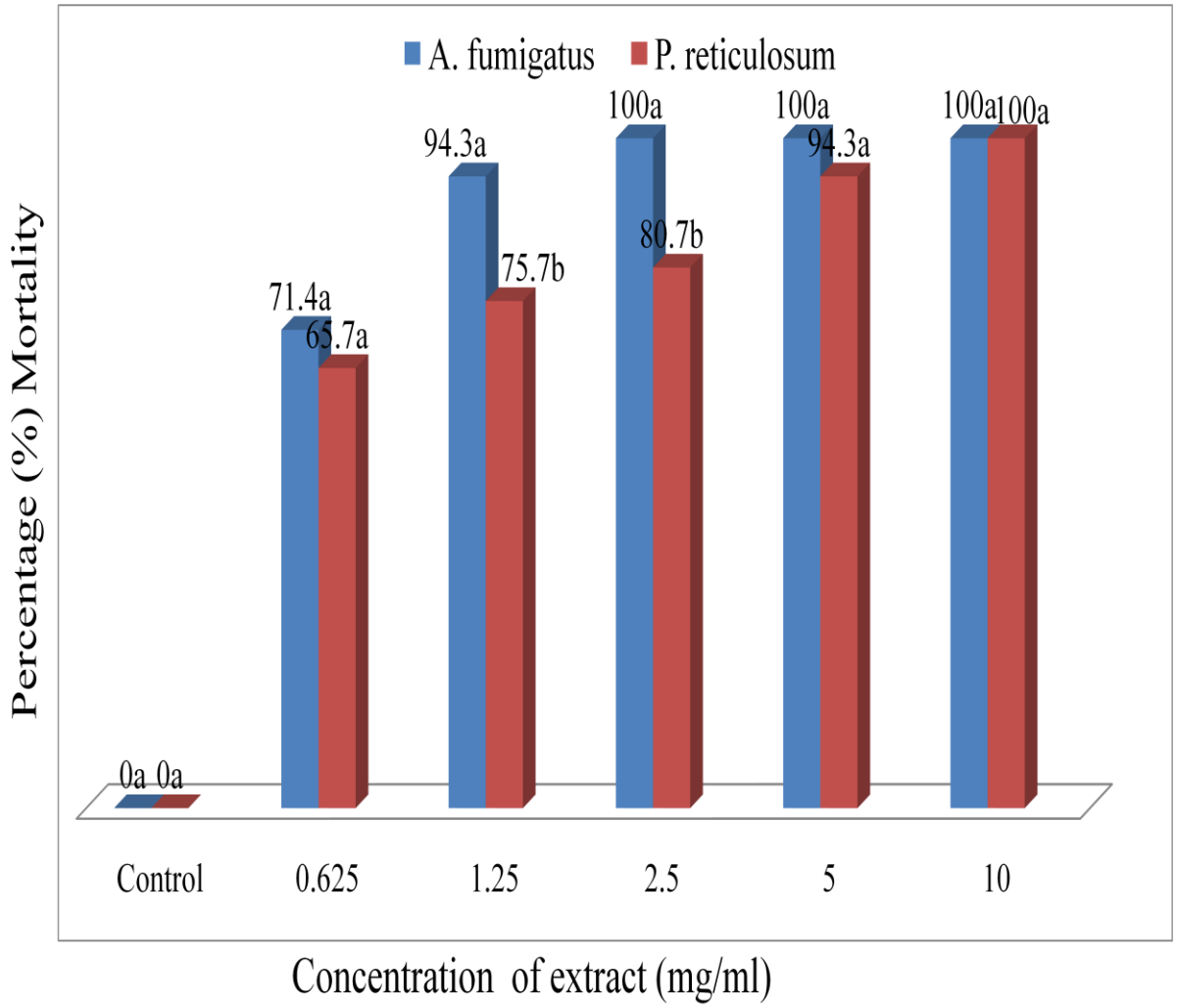

Fig 3: Mean percentage mortality of *T. brucei brucei* in varying concentration of *A. fumigatus* and *P. resticulosum* extract (mg/ml) after 3hours exposure. Mean followed by different alphabet(s) are significant ($p<0.05$) by Pearson correlation test.

Table I: Percentage survival and mortality of *T.b. brucei* in varying concentration of *Penicillium resticulosum* extract after one hour Exposure.

Concentration of extract In mg/ml	% survival	% mortality
10	54	45.6
5	62	37.8
2.5	77	22.7
1.25	91	8.6
0.625	91	8.6
Control	100	0

Table II: Percentage survival and mortality of *T. brucei brucei* in varying concentrations of *Penicillium resticulosum* extract after two hours Exposure.

Concentration of extract In mg/ml	% survival	% mortality
10	11	88.6
5	14	85.7
2.5	20	80
1.25	31	68.6
0.625	48	51.4
Control	100	0

Table III: Percentage survival and mortality of *T.brucei brucei* in varying concentrations of *Penicillium resticulosum* extract after three three hours Exposure.

Concentration of extract In mg/ml	% survival	% mortality
10	0	100
5	5.7	94.3
2.5	19	80.7
1.25	24	75.7
0.625	34	65.7
Control	100	0

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