Research Article

FUNGAL INFESTATION OF TERMITE MOUNDS IN KOPONG, BOTSWANA AND THEIR EFFECTS IN MICE RED BLOOD CELLS

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ABSTRACT

This paper aims to determine the fungal infestation, pH and moisture contents of termite mound soil from five locations in Kopong, Botswana and *in vitro,* effect of fungal extracts of species recovered from termite mound on hemolysis in rats' red blood cells. The moisture contents of the five termite mounds ranged from 20.5 to 37.9% whereas pH ranged from 7.8 to 8.07. The CFU/g of termite mound soil varied from 3.0×10^3 to 1.9×10^4. No correlation could be established among moisture contents, pH and the fungal count. The termite mounds contained species belonging to three genera, *Aspergillus, Penicillium and Fusarium. Aspergillus* species were found to be the most frequent and recovered from almost all soil samples. The fungal species recovered from soil samples of five termite mounds include; *Aspergillus niger, A. fumigatus, A. candidus, A. ochraceus, A. niveus, A. flavus, A. parasiticus, A. terreus, Penicillium sclerotiorum, P. thomii, P. digitatum and Fusarium culmorum*. Out of twelve species, fungal extracts of seven species were assayed for hemolysis of mice red blood cells, and all of them caused red blood cell hemolysis (92.7% to 96.5% hemolysis with 2 ml of fungal extract). The highest percentage hemolysis was obtained for *P. digitatum* (97.5%). There was no significant difference in percentage hemolysis between fungal isolates at p>0.05 but differed significantly with different volumes of fungal filtrate. The percentage hemolysis increased with the increase in fungal titrate volume for all fungal isolates except for *A. ochraceus* and *P. sclerotiorum.* Thus, geophagy of termite mounds may result in hemolysis by fungal toxins contained in them.

KEYWORDS: Fungal infestation, termite mounds, hemolysis, mice red blood cells.

INTRODUCTION

Termites, in the order Isoptera, also known as white ants have the ability to break down dead plant matter and return nutrients to the soil, hence they are economically and ecologically important.[1] They tend to inhabit wood in hollow trees; some make nests underground while others make mounds. Inside termite mounds, there are fungi which are consumed by termites.[2,3] Most termites require moist environment in order to become established and also for maintaining the fungal combs in the mounds. The fungus growing - termites are known as *Macrotermitinae*. The fungi mutually associated with termites in the termite mound belong to the genus *Termitomyces*. Species of *Termitomyces* are edible mushrooms that contain high levels of amino acids such as methionine and cysteine.[4] These Fungi belong to Basiodiomycetes and help to break down ligno-cellulotic materials found in the combs into substances that are easier for termites to assimilate. Without this association, termites would continuously eat but die of starvation due to lack of conversion.

Termite mounds have been used widely by indigenous people in the tropical world for medicinal purposes. Many people such as the Aborigines have directly eaten this soil, smoked or made it into slurry which is then drunk or used as poultice.[5] In many African countries, including Botswana, termite mound soil craving is a very common phenomenon among expectant mothers. As much as these soils, presents a nutritive potential as they have been found to contain some important elements such as potassium and iron, the long term effects of such practices outweigh the benefits.[6] Soils in general are a haven for potentially pathogenic microorganisms such as *Penicillium, Aspergillus* and *Fusarium*. The species of these genera are potential producers of mycotoxins, which are secondary metabolites capable of causing disease and death in humans and other animals.[7,8]

Different species of *Aspergillus* are known to produce some of the economically important mycotoxins and these include aflatoxin, ochratoxins, Fumonisins and Patulins.[9,10] The mycotoxins of greatest significance in foods and feeds are aflatoxins which are produced mainly by *A. flavus, A. parasiticus* and *A. nominus*.[11]

Mycotoxins produced by *Fusarium* species include trichothecenes, zearalenone, moniliformin and the fumonisins. Some of the *Fusarium* species known to produce mycotoxins include *F. equiseti*, *F. graminearum*, *F. proliferatum, F. moniliforme* and *F. sporotrichioides*. *Penicillium* species produce mycotoxins known as OTA, patulin and citrinin. Mycotoxin producing *Penicillium* species include *P. verrucosum*, *P. expansum,* and *P. citrinum*.[11]

Trichothecines, zearelenones and fumonisins produced by *Fusarium* sp. can cause clinical symptoms such as subcutaneous hemorrhaging, alimentary toxic aleukia, exhaustion of bone marrow abortion of fetuses and frequent death.[12] Ochratoxin A produced by *Penicillium* and *Aspergillus* species causes kidney disease in pigs. This fat soluble toxin accumulates in the depot fat of affected animals and can be passed on to human when the pork is ingested by humans when eating pork.[13] With view of the above, there is a need to ascertain if these commonly eaten termite mound soils do not pose a health risks to individuals who consume them, through production of potentially human pathogenic toxins. This study hence aims at identifying fungi associated with the termite mounds and their effect on mice red blood cells.

MATERIALS AND METHODS
Study area and sample collection
Soil samples were collected from five different termite mounds located in different sites in Kopong in the Southern part of Botswana (Latitude 24.4771° S, longitude 25.8906° E) from January, 2014 – April, 2014. Samples were collected at a depth of 20 cm then placed in polyethylene bags, closed tightly, labeled and brought to the microbiology laboratory, Department of Biological Sciences. Samples were stored in a refrigerator for further use.

Physiochemical properties
Soil parameters measured included the soil pH and moisture content. The soil pH was measured using the pH meter. The dry samples were sieved, 1g suspended in 10 ml distilled water and allowed to stand for 1 hour before taking the pH measurements. Soil moisture content was determined using the difference between the wet and the dry weights. To get dry weight the samples were put in an oven set at 105^0C for 24 hours. The following formula was used to calculate the percentage moisture content.

$$W\% = [(W_2 - W_3) / (W_3 - W_1)]*100$$

Whereby
W = weight of the empty container
W2 = weight of the wet sample and the container
W3 = the final constant weigh of the sample

Isolation of fungi
To get fungal isolates, samples were first serial diluted in distilled water to 10^{-4}. For each dilution six replicates were made and 1ml from each was cultured in Sabouraud dextrose agar (SDA) with streptomycin sulphate and incubated at 25^0C for 2 days. Isolated colonies were then sub cultured in Potato dextrose agar (PDA). These were then identified to species level using morphological characteristics.

Culture filtrate preparation
To obtain culture filtrates, 2 mm mycelia discs were cut from the each fungal colony and grown in three replicates of 100 ml enrichments broths. Different enrichments broths were used for collection of different fungal isolates. Yeast extract sucrose was used for collection of *Aspergillus* species, malt extract broth for *Fusarium* species while yeast extract sucrose was used for *Penicillium* species. These were then incubated at 25^0C for 14 days under constant shaking at 100 rpm. Cell free fungal filtrates were obtained by filtering the cultures through sterile Whatman No. 1 filter paper.

Preparation of red blood cells
2 ml of mice whole blood sample was mixed with 9 ml of isotonic buffer and centrifuged at 1000 rpm for 10 minutes in order to separate the red blood cell pellet from plasma proteins. Aliquot of supernatant was removed and the pellet was washed twice with saline by centrifugation.

Effects of fungal filtrates on mice red blood cells
Four test tubes containing of 0.5 ml red blood cell suspension were prepared. In three test tubes the following volumes, 1 ml, 1.5 ml and 2 ml, of the different fungal isolates (*Aspergillus, Penicillium* and *Fusarium*) were added. The fourth test tube was treated as the control and to it 3.5 ml of distilled water was added. The tubes were then incubated at 37^0C for 16 hours with constant shaking at 100 rpm. The tubes were then centrifuged at 1000 rpm for 10 minutes and absorbance measured at wavelength of 540 nm. The absorbance was converted to transmittance and percentage transmittance using the formula below.[14]

% Transmittance = antilog (2- Absorbance)
The following formula was then used to calculate percentage hemolysis of red blood cells as a result of addition of fungal filtrate;
%Hemolysis= [Transmittance (control – extract) / Transmittance of control] x100

RESULTS AND DISCUSSION
The moisture content of the five termite mounds sampled ranged from 20.5% to 37.9% (Table 1). This is much lower than the ideal soil moisture content of 50%. The low soil moisture content of the termite mounds is mainly attributed to the semi-arid climatic conditions of the Botswana whereby the average rainfall is not only low (600 mm/year) but the rains are also sporadic. The difference can also be due to the specific part of the mound where the soil was obtained, that is, in case of top soil, the moisture content will be very low while the bottom soil is very moist. In dehydrated soil, few species

of fungi may survive.[15] In this case, soil samples were obtained from the top hence low moisture content.

The soil pH on the other hand was slightly alkaline and ranged from 7.8 to 8.07. Studies done in Nigeria, indicated the normal mound pH to be around 7.67.[16]

However, soil pH is influenced by different inherent factors such as parent material, time, relief or topography, climate, and activities of microbial populations in the soil hence likely to differ from place to place.

Table 1: Average moisture content, pH and the population count of the five termite mound soil samples obtained from Kopong Termite mounds in Botswana.

Sample	Moisture content (%)	pH	CFU/g of soil
1	30.3	8.04	1.9×10^4
2	37.9	8.06	5.0×10^3
3	23.5	8.07	1.6×10^4
4	20.5	8.05	1.1×10^4
5	29.8	7.80	3.0×10^3

The CFU/g of five samples of termite mounds ranged from 3.0×10^3 to 1.9×10^4. However,[19] reported that under optimal growing conditions the CFU/g of fungi range from 4.5×10^4 to 2.03×10^5. The low CFU/g obtained may be due to influence of alkaline pH and very low moisture content. There was no correlation between the moisture content and microbial count. The termite mound that had the highest moisture content (Sample 2) had quite low microbial count (Table 1). The slightly alkaline conditions of the mounds are probably having an inhibitory effect since fungi are found to grow better under acidic conditions as compared to alkaline conditions. A fivefold increase in fungal growth with decrease in soil pH was observed.[17] Again the termite mound structure influence growth of fungi at different parts of the mound. That is, because the fungal combs are located deep down in the mound, fungi will be more concentrated at the combs than the top soil.[18]

Table 2 shows that a total of 13 species belonging to three genera. *Aspergillus*, *Penicillium* and *Fusarium* could be isolated from the five samples of termite mound soils. *Aspergillus* species were found to be the most frequent and abundant in almost all soil samples. *Aspergillus niveus* however had the highest population. *Penicillium* and *Fusarium* species were not abundant as compared to *Aspergillus*. Fungal species occurrence differed with the termite mounds. Two of the mounds were dominated by *Aspergillus* species only, while in the other three, *Aspergillus* was isolated with either *Penicillium* or *Fusarium* species. These species were part of the eighteen genera isolated from termite hill.[19] *Aspergillus, Penicillium* and *Fusarium* are generally saprophytic and ubiquitous in the soil. However, their presence in the termite mounds also suggests their possible role in cellulose decomposition in this environment.[19]

Table 2: Frequency incidence of fungi isolated from five termite mounds and grown on Sabouraud dextrose agar plates at 25° C.

Fungal species	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Aspergillus niger	10	4	-	-	-
Aspergillus fumigatus	7	1	5	-	1
Aspergillus candidus	10	-	-	-	-
Aspergillus ochraceus	7	1	6	-	-
Aspergillus niveus	-	1	1	33	3
Aspergillus flavus	-	-	2	-	-
Aspergillus parasiticus	-	1	-	-	-
Aspergillus terreus	-	1	-	-	-
Penicillium sclerotiorum	-	1	-	-	-
Penicillium thomii	-	2	-	-	-
Penicillium digitatum	-	-	1	-	-
Fusarium culmorum	-	-	-	1	-

Species assayed were found to cause red blood cell hemolysis (Fig. 1). The highest percentage hemolysis was 96.7% obtained for *P. digitatum*. However, there was no significant difference in the percentage hemolysis between the different fungal isolates at p>0.05. These observed trends may be due to production of mycotoxins which have the ability to cause hemolysis.[8] However, even though different genera may contain different

mycotoxins, there are a few species from different genera which produce similar mycotoxins such as *Aspergillus niger* and other *Penicillium* species which contain Ochratoxin A. This type of mycotoxin tends to have a similar effect on cells regardless of the species they are produced from.
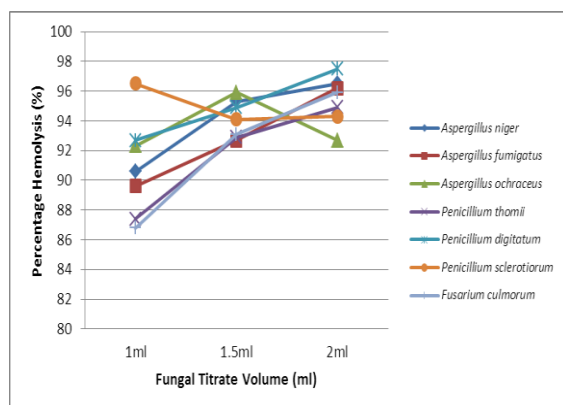
Fig. 1: Percentage Hemolysis against different volumes of the fungal filtrates added.

Percentage hemolysis increased with the increase in fungal titrate volume for all fungal isolates except for *A. ochraceus* and *P. sclerotiorum*. *Penicillium sclerotiorum* had the highest haemolysis percentage (96.2%) at the lowest fungal isolate volume, and remained stable between 1.5 ml and 2 ml. Percentage hemolysis for *A. ochraceus* increased between the first two volumes and dropped in the highest fungal filtrate volume. Percentage hemolysis was however, found to differ significantly with different volumes of fungal filtrate used at $p > 0.05$. *Fusarium culmorum* produce mycotoxins known as Trichothecines which contains T-2 toxin that generates free radical which have the ability to cause hemolysis. Due to these toxins, red blood cell membrane is perforated leading to excessive entry of water in the red blood cells hence hemolysis. At wavelength of 540 nm, hemoglobin is the major source of protein in the red blood cell sample, so when hemolysis occurs, red blood cells burst open and the hemoglobin is released to the outside of the cell and settles at the bottom of the tube.[20] At this wavelength, hemoglobin does not absorb light but the red blood cells scatters the light giving a high transmittance.

Some species such as *P. thomii* are not known to produce any mycotoxins.[22] However, penicillic acid is produced as a form toxin.[21] This may have been another factor that contributed to hemolysis. *P. digitatum* does not produce any mycotoxins but have been found to be toxic to chicken embryo.[22] This toxicity might also explain hemolysis caused by *P. digitatum*. Several fungi are also known to produce volatile metabolites such as aldehydes, aromatic compounds and alcohols.[23] These volatile substances may play a role in toxicity.

The flactuating results of *A. ochraceus* and *P. sclerotiorum* may be due to error in the spectrophotometer while taking the absorbances. The control also which was suppose to have 100% hemolysis due to 100% transmittance however, it gave a percentage transmittance of 95.9%. This may also be due to instrumental error or may be the water was not high enough to cause total hemolysis.

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