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Free radical scavenging activity of some fungi indigenous to Tanzania

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Abstract: The objective of this study was to evaluate free radical scavenging capacity of crude extracts from forest basidiomycetous fungi, domestic zygomycetous fungi and marine ascomycetous fungi. Lethal concentration values that kill 50% of the brine shrimps (LC_{50}) were determined from 19 fungal extracts using brine shrimp test (BST). The LC_{50} values of fungal extract ranged between 0.28–40μg/ml. The basidiomycetous (*Lactarius volemoides*) was the most toxic fungi with LC_{50} of 0.28μg/ml while ascomycete *Pichia guilliermondii* showed the least toxicity with LC_{50} of 40μg/ml. The concentrations of eleven fungal extracts were further evaluated on their ability to scavenge free radical using 1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl) (DPPH) as a dye reagent for spectrophotometric assay at 517nm. The extract concentrations that decreased the initial DPPH radical by 50% (EC_{50}) were determined. The EC_{50} values ranged from 19–60.4μg/ml ascorbic acid equivalents. Extracts from an edible but undomesticated basidiomycetous fungus isolated from Miombo forest and identified as *Termitomyces microcarpus* showed the highest scavenging effect with EC_{50} at 19μg/ml while that from ascomycete *Candida tropicalis* showed the least EC_{50} at 60.4μg/ml. These results draw attention to wild undomesticated Miombo fungi as potential source of nutritional supplements worth further investigation.

Key words: Fungi**,** *Lactarius volemoides, Pichia guilliermondii, Termitomyces microcarpus, Candida tropicalis,* brine shrimp, Tanzania

Introduction

Antioxidant components are micro constituents present in the diet that can delay or inhibit lipid oxidation by inhibiting the initiation or propagation of oxidizing chain reactions, and are also involved in scavenging free radicals (Othman *et al*., 2007). There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources (Abdalla & Roozen, 1999).

Although almost all humans are well protected against free-radical damage by enzymes such as super-oxide dismutase and catalase or by compounds such as ascorbic acid, tocopherols and glutathione, these systems are insufficient to prevent damage entirely (Asatian *et al*., 2007). Normally, there is a balance between the quantity of free radicals generated in human body and the antioxidants which scavenge these free radicals (Owen *et al*., 2000). But when this balance is shifted towards free radicals production, it leads to oxidative stress which results into aging and diseases such as cancer and diabetes. Therefore, in humans consumption of foods rich in antioxidants or factory made antioxidant supplements is often recommended as part of a healthy diet (Tadesse *et al*., 2007).

Various plants have been investigated as sources of antioxidant activities (Javanmardi *et al*., 2003; Tadesse *et al*., 2007). The work by Masoko & Eloff (2007) has shown that extracts of South African *Terminalia* species contain a wide variety of free radical scavenging molecules such as phenolic acids and alkaloids which are rich in antioxidants

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activity. Epidemiological studies have shown that many of these antioxidants compounds possess antitumor and antiviral activities (Owen *et al*., 2000; Sala *et al*., 2002). Many synthetic chemicals such as phenolic compounds are strong radical scavengers but they usually have side effects. Antioxidant substances obtained from natural sources are of great interest (Masoko & Eloff, 2007). However owing to the environmental concerns associated with harvesting of non fruit plant parts such barks and leaves and whole plants, there is an increasing interest in searching for other microbial sources such as fungi. Already studies done by Jacobson *et al.* (1995) and Lavitschka *et al*. (2007) have investigated antioxidants from black fungi.

Despite the various reports from many parts of the world, there is surprisingly, very little literary information on the production of antioxidants from Tanzanian fungi. This study endeavoured to screen production of antioxidants from indigenous Tanzanian fungi from diverse sources including marine ascomycetes, zygomeycetes and basidiomycetes.

Materials and Methods

Preparation of samples

Marine ascomycetous fungi previously isolated from mangrove sediments were inoculated into separate Erlenmeyer flasks, which contained 1 litre of Malt Extract Broth. Both flasks were then placed on a shaker with 100rpm for 24 hours, at room temperature, allowing optimal equally distributed growth. The cultures were then removed from the shaker and placed on a bench at room temperature for 6 days, totalling a 7 day cultivation process.

Zygomycetous fungi were collected from the Usambara and Pare mountains in north-eastern Tanzania. The fungal strains were found growing on the surface of fermented green bananas (*idundi*). Using a wire loop, inoculation of the fungal sample from the *idundi* banana was done onto a sterile solid malt extract agar media and incubated at 35°C for 72h. Using a wire loop, the fungal species grown on the solid media were transferred aseptically into the conical flasks containing sterile malt extract broth (the liquid media) in the laminar flow. The inoculated culture media were incubated at 30°C on a rotary shaker incubator for 48hr. The cultures were then removed from the shaker and placed on a bench at room temperature for 6 days, totalling a 7 day cultivation process.

Fruiting bodies of basidiomycetious fungi were collected from Miombo woodlands in Tabora, western Tanzania. The collection was done by hand and the fungi were put into small basket and brought to the laboratory for identification by a mushroom taxonomist. Thereafter, specimens were cut into small pieces and sun dried for period of three days.

Isolation and preparation of fungal crude extracts

From seven zygomycetous and six ascomycetous strains, subsequently, the desired secondary metabolites were obtained by liquid-liquid extraction method with ethyl acetate as the organic solvent. Ethyl acetate extracts the non polar metabolites from the cultured broth in a 1:1 ratio. The collected supernatant was then concentrated on a rotary evaporator (Laborota 4001, Heidolph®, Essex Scientific Laboratory Suppliers Ltd) with the bath maintained at 40°C with 90rpm (Muir *et al*., 2002). Once the entire litre of broth completed the above process, the crude extract was dried in desiccators overnight and crude extracts were placed in a refrigerator at 4°C ready for various activity tests. For basidiomycetous, six strains, were cut into small pieces and then sun-dried for period of three days. The dried materials were further pulverized using a national super mixer grinder (MX-119, Emerging

Planet India Ltd., Coimbatore 641011, India). About 200g of the powder materials were extracted by maceration in ethyl acetate. Crude extracts were concentrated *in vacuo* using a rotary evaporator. The extracts obtained were kept in a refrigerator at 4°C until further use.

Brine shrimp test

The brine shrimp test (BST) was used to test the cytotoxicity of the 19 crude extracts from ascomycetous, zygomycetous and basidiomycetous fungi following standard procedures as described by Meyer *et al*. (1982).The seawater was put in a small tank and teaspoon of brine shrimp (*Artemia salina*) eggs added to one side of the divided tank, which was covered. The other side was covered so as to allow light that would attract the hatched shrimps. The tank containing the brine shrimp eggs was left at room temperature for 48h to allow the eggs to hatch. The test tubes used were washed and sterilized in an autoclave machine. Duplicate fungal extracts in dimethyl sulfoxide (DMSO 1 ml) of concentrations 1000, 100, 80, 60, 40, 20μg/ml were prepared in test tubes. Brine shrimp larvae (nauplii, 10) were added to each test tube with fungal extract and sea water. All test tubes were covered at room temperature for 24h. After this period, the numbers of the dead and surviving brine shrimps were recorded.

Antioxidant activities, DPPH assay

1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl) (DPPH)) was from Sigma. The rest of the chemicals used were of the highest grade commercially available. The scavenging activity was estimated according to the method of Molyneux (2004). Eleven fungal extracts were studied on the ability to scavenge DPPH. Test extracts (2ml) in ethanol with concentrations (20, 40, 60, 80, 100, 500 and 1000μg/ml) were mixed with 1ml of 0.1mM DPPH in ethanol. The mixture was then shaken vigorously at room temperature in a dark room. Absorbance was read immediately at 0 minute using a spectrophotometer at 517nm and thereafter after 30min of reaction. Ascorbic acid was used as a standard control. The scavenging effect on of the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \left(1 - \frac{\text{Absorbance of sample at 517nm}}{\text{Absorbance of control at 517nm}}\right) * 100$$

EC_{50} value was determined from plotted graph of scavenging activity against the concentration of extracts, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. Duplicate measurements were carried out and their average scavenging effect was calculated based on the percentage of DPPH scavenged. The antioxidants activity was evaluated in this study by using DPPH assay at wavelength 517nm.

Identification of ascomycetous strains

Overnight cultures of ascomycetous were centrifuged, obtained pellet mixed with equal amount of glass beads then 0.8ml extraction buffer was added and was dismembrated for 1.5min at 2000rpm. This was incubated at 60°C for 1hr, vortex after every 20min and spun down at 14000rpm for 5min the pellet was discarded, equal volume of chloroform was added, vortex for 10sec, spun down for 4min at 14000rpm at 4°C. Upper phase was collected and two times volume of cold isopropanol was added, mixed thoroughly by repeated inversion and then incubated for 15min at -20°C. The mixture was then spun down at 16400

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rpm for 10min and DNA pellet was obtained. The DNA pellet washed with 300µl of 70% ethanol and spun down at 14000rpm for 5min. The pellet was dried out and suspended in 30ml TE and stored at -20°C. For yeast the D1/D2 region at the 5′ end of 26S ribosomal DNA (rDNA) of about 600bp was amplified using NL-1(5′GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′GGTCCGTGTTTCAAGACGG-3′) primers for 30 PCR cycles, denaturation at 94°C 1min, annealing at 60°C for 1min, extension at 72°C for 2min, and final extension at 72°C for 7min.

Data analysis

The obtained data were subjected to Probit analysis (Throne *et al*., 1995), using Statistical Analysis Systems (SAS) computer programme and the lethal concentration values that kill 50% of the shrimps (LC_{50}) was determined for each fungal extract. This was determined by plotting a graph of percentage mortality of brine shrimp larvae against logarithmic concentrations of the fungal extracts tested. The LC_{50} values were determined directly from probit analysis or calculated by substituting 50% for "y" into the curve equation in the graph.

Results

Cytotoxicity of fungal extracts in brine shrimp lethality test

The fungal extracts tested exhibited various levels of toxicity against brine shrimp larvae (Table 1). The LC_{50} values of fungal extract ranged from 0.28–40µg/ml. The basidiomycetous fungus (*Lactarius volemoides*) was the most toxic fungi with LC_{50} 0.28µg/ml while ascomycetous fungus (*Pichia guilliermondii)* was the least toxic with LC_{50} 40µg/ml.

Table 1: Toxicity of fungi extracts to *Artemia salina* and their LC_{50} after 24 h incubation

Type of fungus	Fungal strains	LC_{50} (µg/ml)
Basidiomycetous	*L. volemoides*	0.28
	C. symoensii	2.60
	T. microcarpus	4.00
	L. tanzanicus	8.80
	C. miomboensis	10
	L. medusa	10
Ascomycetous	A6	5.60
	A1	18.32
	A3	20.65
	A5	28.50
	A4	38.94
	A2	40.00
Zygomycetous	Z2	4.00
	Z1	16.00
	Z4	15.00
	Z3	20.65
	Z6	26.95
	Z7	25.05
	Z5	32.95

Key: Z1 – Z7 = Zygomycetous fungi; A1 – A6 = Ascomycetous fungi; Basidiomycetous fungi A2= *Pichia guilliermondii*, A6 = *Candida tropicalis*

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Free radical scavenging activity of Basidiomycetous extracts

The scavenging effects on DPPH radical varied between the strains tested. A basidiomycete *Termitomyces microcarpu*s showed the highest scavenging activity among the strains tested with EC_{50} 19μg/ml while *Lactarius volemoides* showed least scavenging activity with EC_{50} 32 μg/ml (Table 2).

Table 2: Scavenging activity (EC_{50}) of Basidiomycetous crude extracts on DPPH radicals

Sample/crude extracts	EC_{50} (DPPH) μg/ml
C. miomboensis	20 ± 1.0
L. medusa	30 ± 1.4
Termitomyces microcarpus	19 ± 0.1
L. tanzanicus	25 ± 1.4
C. cymoensis	20 ± 0.4
L. volemoides	32 ± 0.4

Free radical scavenging activity Zygomycetous and Ascomycetous scavenging activities

Results showed that scavenging effects on DPPH radical vary from among the strains tested. A zygomycete 1 showed the highest scavenging activity among the strains tested with EC_{50} 30 μg/ml while the ascomycete *Candida tropicalis* showed less scavenging activity with EC_{50} 60 μg/ml (Tables 3).

Table 3: Scavenging activity (EC_{50}) of Zygomycetous and Ascomycetous crude extracts on DPPH radicals

Sample/crude extracts	EC_{50} (DPPH) μg/ml
Zygomycete 1	31 ± 2.0
Zygomycete 2	38 ± 0.4
Pichia guilliermondii	42 ± 0.5
Zygomycete 3	55 ± 0.1
C. tropicalis	60 ± 1.0

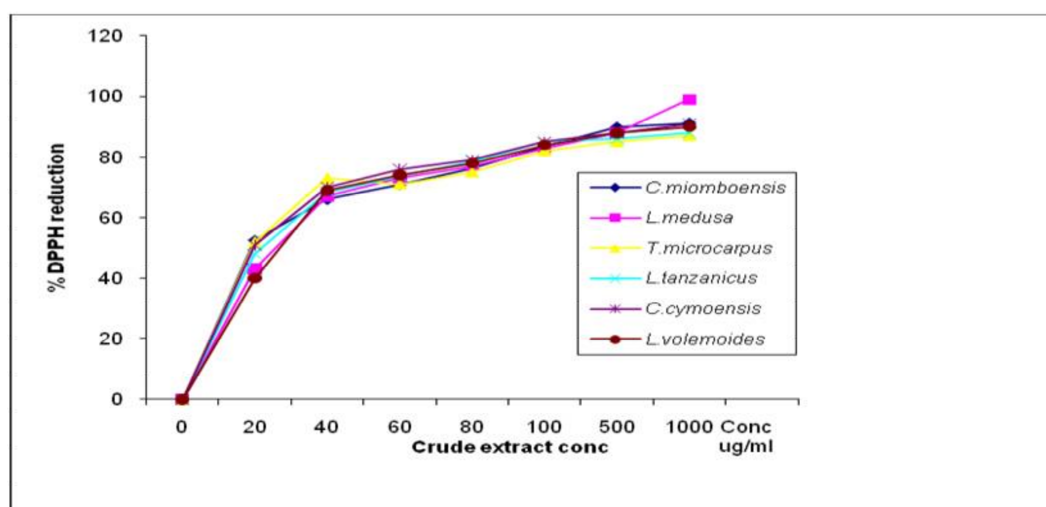

Figure 1: DPPH scavenging capacity of various concentrations of the basidiomycete crude extracts

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It was also observed that scavenging activity increased until a plateau was reached (Figures 1 and 2). From the graph, EC_{50} value (which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%) was determined. The lowest EC_{50} indicates the strongest ability of the extracts to acts as DPPH scavengers.

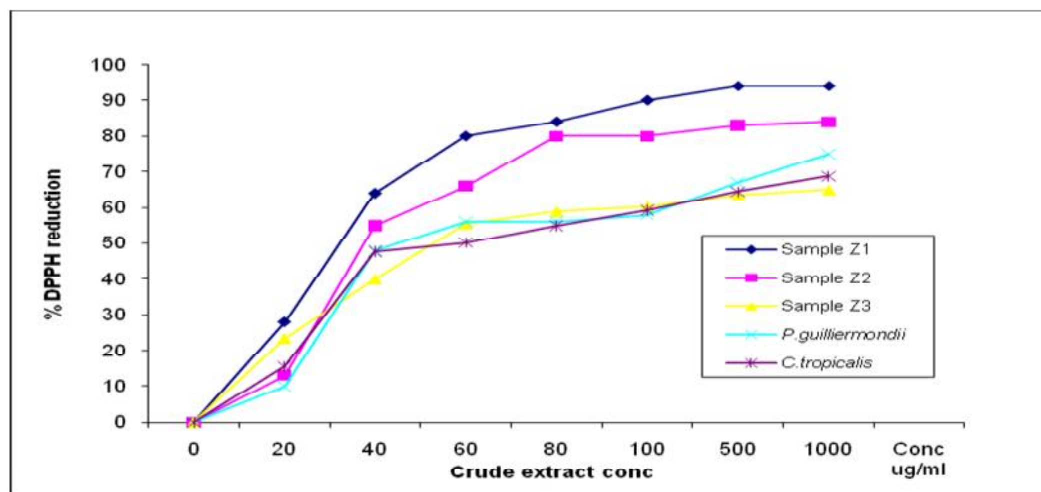

Figure 2: DPPH scavenging activity of various concentrations of zygomycetous (Z) and ascomycetous crude extracts

Discussion

The cytotoxicity activity of 19 fungal extracts was investigated *in vitro* against brine shrimp (*Artemia salina*) using brine shrimp lethality test (BST). The BST assay is considered a useful tool for preliminary assessment of general toxicity and the bioassay has shown cytotoxic activity against some human solid tumours and with pesticidal activity (Meyer *et al*., 1982). In this approach, activities are usually considered significant if the LD_{50} is less than 30µg/ml (Sharififar *et al*., 2009). In this study, a basidiomycetous fungus, *L. volemoides* which was isolated from Miombo woodlands had the highest cytotoxicity (LD_{50} =0.28µg/ml) and *C. symoensii* another wild edible fungus from Miombo was found to posses the least cytotoxicity (LD_{50} = 10µg/ml). Furthermore, in this study, a zygomycetous fungus, named Z2 had the highest cytotoxicity (LD_{50} =4µg/ml) and Z5 had the least cytotoxicity (LD_{50} =32.9µg/ml). This fungus was one of the filamentous fungi isolated from a traditional fermented banana food from north-eastern Tanzania. Among the marine ascomycetous fungi screened, *C. tropicalis* had the highest cytotoxicity (LD_{50} -5.6µg/ml) and *P. guilliermondii* had the least cytotoxicity (LD_{50}=40µg/ml).

Eleven fungal extracts were further analyzed on their ability to scavenge DPPH radical. The selection was based on the highest and lowest toxicity on brine shrimp tests. Results exhibited variables scavenging activity against DPPH radical in a concentration dependent manner. The results are in agreement with study done by Asatian *et al.* (2007) which stated that free radical scavenging activity is species- dependent. The scavenging activity of fungal extracts on DPPH radicals rapidly increased from 20 to 1000 µg/ml although with variations on some extracts, most probably due to light sensitivity of the DPPH radical (Budzianowski & Budzianowska, 2006). The lowest EC_{50} observed in this

study indicates the strongest ability of the extracts to acts as DPPH scavengers as observed in previous studies elsewhere (Lu & Foo, 2000; Molyneux, 2004).

Basidiomycetous mushrooms are widely recognized as a functional food and as a source of various physiologically active compounds. Recently, mushrooms have been found to possess antioxidant activity (Asatian *et al*., 2007). Our results showed that crude extracts from *T. microcarpus* had the highest scavenging ability among eleven fungal extracts. On the other hand, extracts from ascomycete *C. tropicalis* showed very weak scavenging ability towards DPPH. EC_{50} values appeared to be less than 1 mg/ml; thus, the scavenging abilities of these fungi strains are better since EC_{50} values established for these fungi extracts are lower than those from *A. subrufescens*, *F. velutipes*, *A. aegerita*, *C. comatus* fungi as reported by other workers (Asatian *et al*., 2007; Lavitschka *et al*., 2007).

A poor relationship between cytotoxicity test and scavenging capacity of the fungal extracts was found. This could be due to the facts that most of the antioxidant compounds belong to phenolic compounds (Cheung *et al*., 2003; Othman *et al*., 2007). Cytotoxic properties are due to the presence of terpenes and their derivatives (Maridass, 2008). It is also possible that a broad range of structurally diverse compounds contribute to the overall activity of the extracts and synergistic effects between active compounds may exist (Pimentel *et al*., 2002).

In this study only the secondary metabolites contained in the ethyl acetate fraction were evaluated, due to time limitation. However in follow up studies it is proposed that ethanol and water fractions will also be considered. Already, some studies (Mau *et al*., (2005) have reported that water extracts from mushroom (*G. tsugae*) possess higher antioxidants activity than ethanol extracts from edible mushrooms (*G. lucidum*, *F. velutipes*, *D. gibbosa, P. eryngii*, *O. cystidiosus*, *O. olearius*) (Cheung *et al*., 2003)

Generally, the results of this study suggest that fungal extracts tested contain constituents that are good radical scavengers and might serve as a good source of safe natural antioxidants. The high amounts of antioxidants in an edible fungus, *T. microcarpus* reported in this study augment the findings of studies conducted in Israel (Asatian *et al*., 2007). Our findings, therefore, suggest that these edible fungi are potential source for antioxidants supplementary to human diet. However, further studies are needed to determine the physiological mechanisms regulating antioxidant accumulation, to isolate active components and establish the pharmacological efficacy of the promising fungal extracts.

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