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Antiradical and antioxidant activities of methanolic extracts of indigenous termitarian mushroom from Tanzania

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Abstract

Termitomyces mushrooms grow symbiotically with termites. They are abundantly distributed in the country, mostly consumed and liked by people. However, their antiradical and antioxidants activities are not yet established. In this study, both qualitative and quantitative values of antiradical and antioxidant of crude methanolic extracts of six *Termitomyces* species (*T. titanicus*, *T. aurantiacus*, *T. letestui*, *T. clypeatus, T. microcarpus* and *T. eurhizus*) were investigated. The investigation used DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical as a substrate to determine both scavenging abilities and antiradical activities. Antioxidant was further analysed quantitatively for β-carotene, flavonoid content, total phenolic compounds and vitamin C content in the crude methanolic extracts using spectrophotometric assay at 515 ηm.

The result showed that they all exhibited scavenging ability and antiradical activity although the ability differed markedly among the species. The highest antiradical activity unit (EAU_{515}) was from *T. microcarpus* (EAU_{515} 1.48) followed by *T. aurantiacus* (EAU_{515} 1.43) while the lowest was from *T. eurhizus* (EAU_{515} 0.7). The scavenging power was also highest in *T. microcarpus* (EC_{50} < 0.1 mg/ml) followed by *T. letestui* (EC_{50} = 0.14 mg/ml); while *T. eurhizus* showed the least power (EC_{50} = 0.36 mg/ml). In quantitative analysis, *T. microcarpus* was also found having high content of phenols, Flavonoid, and β-carotene except lycopene and Vitamin C content which were high in *T. aurantiacus* and *T. eurhizus* respectively.

Based on these results, all studied termitarian mushroom are good source of antioxidants while *T. microcarpus* could be considered as potential antiradicals of high profile thus thought for selection in preparation of mushroom-based nutraceutics. The results also endorse the continuing harvesting of these wild mushrooms for including them in our daily food for healthy diets.

Keywords: Antioxidant, antiradical, *Termitomyces*, Termitarian, Tanzania

1. Introduction

Normal cellular metabolism and body functions of aerobic organism require a balance between free radical production and antioxidant defenses. While an anti-oxidant refer to a substance that has extra electrons that it can give off to clean up free radicals; Free radical are reactive oxygen species (ROS) in cells, which include hydrogen peroxide (H_2O_2), the superoxide anion, and free radicals, such as the hydroxyl radical (OH^-) which are endogenously constantly produced in the human body. In a situation where excess free radicals occurs, they cause smash up of cells by chain reactions, such as, lipid peroxidation or formation of DNA adducts that could cause cancer-promoting mutations or cell death resulting in abnormal body functions and various diseases (Filipa et al., 2011, Banerjee et al., 2012). Although every organism has natural endogenous defense mechanisms to eliminate free radicals, habitually excess production of ROS overwhelms the system. Thus, taking foods rich in antioxidants such as mushrooms, help the endogenous defense system to reduce oxidative damage (Temple, 2000; Fang et al., 2002; Liu, 2003).

Mushrooms are world wide appreciated for their taste and flavor and are consumed both in fresh and processed Apart from being delicacy and tasty foods, they have special biochemical compositions, with significant contents of antioxidant compounds, proteins, carbohydrates, lipids, enzymes, minerals, vitamins and water which attract more attention as functional health promoters (Ramirez-Anguiano et al., 2007, Chang, 2008, Kim et al., 2008, Lakhanpal et al., 2008, Wong and Chye 2009). Recently mushrooms have also become an attractive source for the development of drugs and nutraceuticals (Lakhanpal and Rana, 2008). Mushroom can be used directly as food, cooked or raw, or can be used as additive nutriceticals (Mau et al., 2002a; Mau et al., 2002b; Wong and Chye 2009; Ramirez et al., 2007). For example, there has been a fast growing trend of exploring different bioactive compounds especially antioxidants for the purposes of selecting and promoting specific types of mushroom with significant amount for

consumption and nutriceutical developments. Among the studies includes evaluation of the presence of tocopherols which act as a free radical scavengers referred to as chain-breaking antioxidants (Ferreira et al., 2009; Heleno et al., 2010).

Termitarian mushroom locally known in swahili as *uyoga mchwa* are among edible and well appreciated mushroom species in many Tanzanian communities. They are easily identified and considered for the dish because of their unique ecology of living symbiotically with termites and usually found growing on termite mounds or in the vicinity of the mounds. Their enormous big size, some being large up to 1 meter (Tibuhwa et al., 2010), and very long pseudorrhiza which is commonly chewed raw, make them food of the sought throughout the rain season. *Termitomyces* mushrooms are only harvested from the wild during the rain season, because they have not yet been successfully developed into fruitibodies since they are mycorrhizal associated with termites of the Termitidae subfamily thus difficult to cultivate. However, *Termitomyces* cultures have been made from mycelium obtained from comb sporodochia (see Petch, 1913; Batra and Batra, 1979; Heim, 1977; Piearce, 1987), while cultures from basidiome context fruit bodies have been done by Heim (1977); Piearce (1987), Botha and Eicker (1991) and Tibuhwa (2012 a). Recently, cultures on the species of the genus have been also done from basidiospore prints of *T. microcarpus* of the Lake Victoria basin by Olila et al. (2007) and successfully grown into cultures and spawn. Though *Termitomyces* species grow extensively and are widely consumed in different part of Tanzania, their scavenging ability and antioxidant capacity has not yet been fully determined. This study therefore, focuses on determining their antioxidant status from their methanolic crude extracts both qualitatively and quantitatively.

*2. **Materials and Methods***

*2.1 **Sample collection***

Mushroom samples were collected from different field sites in Tanzania (Figure 1). They were then identified using colored field guide books, monographs (Arora, 1986; Härkönen et al., 1995, 2003; Kirk et al., 2001; Lodge et al., 2004, De greef, 2010) and internet facility. After freshly observation mushroom were sundried and deposited at the University of Dar es Salaam herbarium (UDSM) and a duplicate of *T. titanicus* is also available at herbarium of Belgium (BR). All details of the studied taxa are summarized in Table 1 and accompanying field photo in Figure 2.

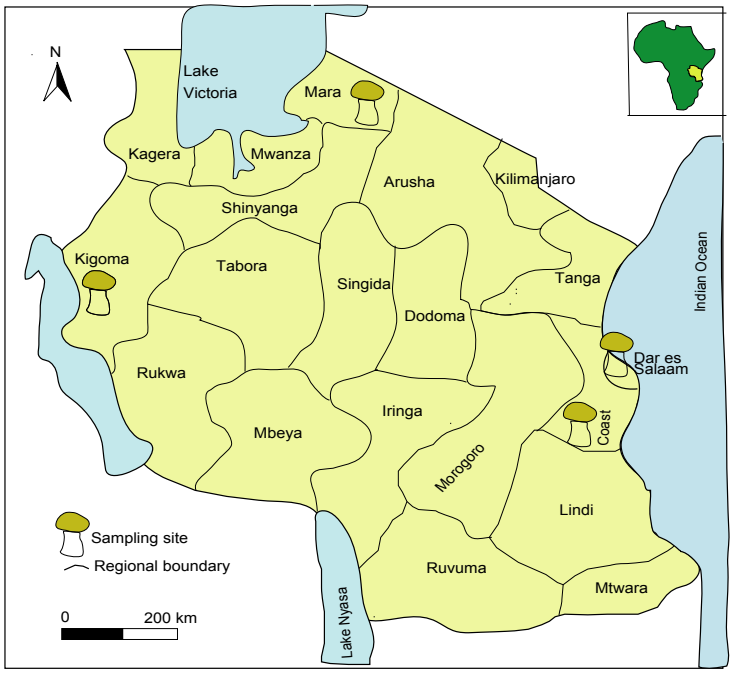

Figure 1: Tanzania map showing sites where samples were collected

2.2. Mushroom crude extracts preparation and yield determination

Methanolic extractions were carried out using 25gm of the whole mushroom fruitbody weighed using analytical balance at room temperature. The mushroom were powdered in motor using pestles and soaked in 250 ml of methanol and extraction proceeded as explained in Jaita et al. (2010). It involved constant stirring of the material for 48 hrs then filtered using whatman filter paper. The filtrates were evaporated to dryness at 40ºC in a rotary evaporator with 90 rpm (Labrota 4001, Heidolph® Essex Scientific Laboratory Supplies LTD) under reduced pressure. The obtained concentrated extracts were stored in dark at 4ºC until further analysis. The yields of evaporated dried extracts were obtained by gravimetric method. The percentage yield extracts were calculated based on dry weight as:

$$\text{Yield (\%)} = \frac{(W_1 \text{ X } 100)}{W_2}$$

Where W_1= weight of extract after methanol evaporation; W_2 = Weight of the grinded mushroom powder

2.3 Qualitative Antioxidant assay

2.3.1 DPPH free radical scavenging activity

The qualitative assays were performed according to the method of Masuda et al. (2000), Jaita et al. (2010). A series of methanol crude extracts to (1:10 − $1:10^7$) were prepared. 1ml of the extract was mixed with 1ml of 0.4 $mmol^{-1}$ methanolic solution containing a very stable radical, 1:1− diphenyl−2picryhydrazyl (DPPH). At maximum concentration (1mg/ml) each sample was duplicated and standard deviation for the two readings was statistically determined. The mixture was left in the dark for 30 min. and the absorbance measured at 515 ηm. The percentage of DPPH radical scavenging activity of each extract was determined at these five concentrations within the range of dose response and was calculated as:

$$\text{DPPH radical scavenging activity} = \left[A_0 - \frac{(A_1 - A_s)}{A_0} \right] * 100$$

Where A_0 = Absorbance of the control solution containing only DPPH

A_1= absorbance in the presence of mushroom extract in DPPH solution and

As = the absorbance of the sample extract solution without DPPH

The EC_{50} value (total antioxidant necessary to decrease the initial DPPH radical concentration by 50%) was determined from plotted graph of scavenging activity against the concentration of extracts.

Table 1: Details of the studied mushrooms

No	Species	Voucher	Locality-TANZANIA	Collection/herbarium number	GPS
1.	*Termitomyces microcarpus*	DDT11-153	Dar es Salaam, JKN Mlimani -campus	DDT 1053.2011 (UDSM)	04°52'57" S, 29°48'41" E
2.	*Termitomyces titanicus*	DDT11-36	Kigoma, Kidawe forest	DDT 1053.2011 (UDSM, BR)	04°52'57" S, 29°48'41" E
3.	*Termitomyces letestui*	DDT10-68	Serengeti National Park	Tibuhwa 1068.2007 (UDSM)	06°52'34" S, 37°67'29" E
4.	*Termitomyces aurantiacus*	DDT-03	Kisarawe near Kazimzumbwi forest	Tibuhwa 03.2003 (UDSM)	06°52'34" S, 37°67'29" E
5.	*Termitomyces striatus*	DDT10-49	Serengeti National Park	Tibuhwa 049.2010 (UDSM)	04°59'22" S, 32°40'20" E
6.	*Termitomyces clypeatus*	DDT12-70	Dar es Salaam, JKN Mlimani-campus	Tibuhwa 1070.2007 (UDSM)	06°55'11" S, 37°34'20" E

2.3.2 Determination of antiradical activity

Determination of antiradical activity was based on the principal behind that DPPH (di phenyl-2picryhydrazil) radical in its radical form has a characteristic absorbance at 515 ηm which disappear after its reduction by an antiradical

compound. The reduction of DPPH thus can be monitored by measuring the decrease in its absorbance at 515 ηm during the reaction. In this study, 40µl of extract in methanol was added to 1460 µl of 0.0037% DPPH in methanol W/V. Sample control was prepared by adding 40 µl of Methanol to 1460 µl of 0.0037% DPPH according to Sroka (2003). Absorbance was measured at 515 ηm at time 0, and after 1 min. The antiradical activity of each extract was calculated as:

$$AU_{515} = (A_0-A_1) - (A_{0C}-A_{1C})$$

Where AU_{515} = antiradical activity of the extract

A_0= the absorbance of the sample at the beginning of the reaction; A_1= the absorbance of the sample after one minute of the reaction; A_{0C}= the absorbance of the control sample at the beginning of the reaction; A_{1c}= the absorbance of the control sample after one minute of the reaction.

Figure 2: Field photo of the studied *Termitomyces* species (All photo taken by the author in their respective study sites except *T. titanicus* taken by Dr. Deo Baribwegure in Kigoma, as the author hold the mushroom.

The number of antiradical activity (EAU_{515}) was calculated per 1mg of each extract according to the equation:

$$EAU_{515} = \frac{AU_{515}}{Ie}$$

Where EAU_{515} = number of antiradical activity; Ie = amount of extract in the sample (mg) and
AU_{515} = antiradical activity of the extract

The total number of antiradical activity units in the extract was calculated according to the equation

$$PAU_{515} = \frac{cle \times AU_{515}}{Ie}$$

Where cle = total amount of extract (mg) and Ie = amount of extract in the measured sample.

2.4 Quantitative Antioxidant assay

2.4.1 Determination of total flavonoid

The total flavonoid was determined with Aluminium chloride according to Jaita et al. (2010), Pitchaon et al. (2005) using quercetin as standard. 1 ml of each extract was diluted with 4.3 ml of 80 % aqueous ethanol containing 0.1 ml of 10% Aluminium nitrate and 0.1ml of 1M aqueous Potassium acetate. After 40 min. incubation at room temperature the absorbance was determined calorimetrically at 415 ηm using the SAME spectrophotometer Uv-vis model 6305 Jenway UK. Total flavonoid concentration was calculated using quercetin standard calibration curve. Data were expressed as Rutin equivalent/100g of mushroom extracted.

2.4.2 β-carotene and Lycopene contents

The assay was carried out according to the method of Nagata and Yamashita (1992). The mushroom extract (100 mg) was shaken with 10 ml of Acetone-hexane mixture 92:3) for 1 min. and filtered through Whatman number 4 filter paper. The absorbance of the filtrate was measured at 453, 505 and 663 ηm. The β-carotene and Lycopene content were calculated as:

Lycopene mg/100mg = $0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$

β-carotene mg/100mg = $0.216\ A_{663} - 0.304 A_{505} + 0.452 A_{453}$

2.4.3 Total phenolic content determination

The total phenolic content in each mushroom species crude methanolic extracts was determined using the Folin-Ciocalteu colorimetric method (Singleton et al., 1999). Each 0.1 gm of concentrated extract was diluted with 5 ml of methanol. 200 µl of the mushroom extract was transferred into a test tube then mixed thoroughly with 1ml of Folin-Ciocalteau reagent. After 3 min, 0.8 ml of 7.5% (w/v) Sodium carbonate was added to the mixture. The mixture was agitated for further 30 min. in the dark and centrifuged at 3300 g for 5 min. The absorbance of mushroom extract and prepared blank were measured at 765ηm using spectrophotometer (Uv-vis model 6305 Jenway UK). The total phenolic content in the mushroom extract was expressed as milligram of gallic acid equivalent per 100g weight of mushroom using the linear equation obtained from standard gallic acid calibration curve.

2.4.4 Determination of Vitamin C

The vitamin C content was determined titrametrically using 2, 6 Dichloropheno Indophenol methods. Known weight of grounded sample was mixed with 25 ml of 5% metaphosphoric acid solution and shaken for 30 min. The mixture was then filtered through Whtaman no 42 filter paper using sunction pump. 10 ml was pippeted from the extract in 250 ml conical flask and titrated against 0.025% of 2.6 Dichlorophenol Indophenol reagents. The amount of vitamin C in each extract was calculated from the equation:

$$\text{mg of ascorbic acid per 100g} = \frac{\text{Ax Ix V x 100}}{V_2 x W}$$

Whereas A = quantity of ascorbic acid (mg) reacting with 1ml 0f 2, 6 Indophenol
I = volume of indophenol (ml) required for the completion of extract titration;
V1 = Total volume of extract and W = Weight of the grinded mushroom

3. Results and Discussion

3.1Crude extract % yield

In extraction, 25 gm of grinded powder of mushroom were soaked in 250 ml of extraction solvent (Methanol). Although the same procedures were done for each studied mushroom species, the extraction yield differed considerably among the studied species (Table 2). While *Termitomyces aurantiacus* had the highest yield of (21.7%) followed by *Termitomyces microcarpus* (20.12%), *Termitomyces clypeatus* had the least yield (13.98%).

3.2 Antioxidant activities

3.2.1 Free radical scavenging activities

The effect of the concentration of antioxidant compounds on the DPPH radical was observed as a function of reducing power. The antioxidant properties of all the studied mushrooms were evaluated through scavenging activity on DPPH radicals (examined by the capacity to decrease the absorbance at 515 ηm of DPPH solution). The result showed that, the free radical scavenging activity increased with increasing concentration of extract in all studied species indicating the concentration dose dependency of antioxidative activities (Figure 3). This observation concur

with that of Banerjee (2012) who also noted a similar trend of antioxidative activities dose dependency and associated it with the presence of reductones that are reported to be the terminators of free radical chain reactions.

Table 2: % Crude extract yield of the studied mushrooms and Antiradical activity values

Extracted sample	% yield in 25gm/250ml	Antiradical activity (AU_{515})	Number of antiradical activity units in 1 mg of extract (EAU_{515})	Number of antiradical activity units in the Extract (PAU_{515})
Termitomyces aurantiacus	21.7	0.879 ± 0.05	1.43 ± 1.25	657.08 ± 10.35
Termitomyces eurhizus	18.3	1.139 ± 0.12	0.7 ± 1.50	631.68 ± 15.55
Termitomyces titanicus	19.2	1.599 ± 0.5	1.245 ± 0.55	829.56 ± 20.23
Termitomyces letestui	15.8	1.12 ± 0.35	1.36 ± 1.75	705.37 ± 15.75
Termitomyces clypeatus	13.98	0.959 ± 0.55	1.24 ± 0.55	681.94 ± 10.15
Termitomyces microcarpus	20.12	1.852 ± 0.72	1.48 ± 0.45	892.78 ± 20.25

In this study, the maximum scavenging activity values were at dilution of 1mg/ml. The highest percentage scavenging power was found in species *Termitomyces microcarpus* (98.65%) followed by *Termitomyces letestui* (95.95%) while *Termitomyces titanicus* showed the lowest value (62.14%). However, the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%, determined from plotted graph of scavenging activity against different concentration of the extracts, showed *Termitomyces microcarpus* to have the highest ability (EC_{50} < 0.1mg/ml) followed by *Termitomyces letestui* (EC_{50} = 0.14mg/ml) while the least ability was from *Termitomyces eurhizus* (EC_{50} = 0.36 mg/ml). This result shows that the termitarian mushroom studied have super scavenging ability compared to other mushrooms. Although in this study *Termitomyces eurhizus* was the one with least ability (EC_{50} = 0.36 mg/ml), this value is still better compared to other well appreciated antiradical mushrooms. For example, the established EC_{50} values in *Paxillus involutus* and *Pisolithus arhizus* by Filipa et al. (2011) are (EC_{50} = 0.61 and EC_{50} = 0.56 mg/ml) respectively which show them having relatively low free radical scavenging ability compared to *Termitomyces eurhizus* with least ability in this study.

3.2.2 Antiradical activity

Antioxidative properties are associated with the development of the reducing power whereby antioxidant activity of reductones break the radical chains by donation of a hydrogen atom (Gordon, 1990).This property is often used as an indicator of its electron donating ability, thus used in testing antioxidative, and reducing action of a given compound. In this study, the numbers of antiradical activity units per 1 mg of extract (EAU_{515}) were calculated for all studied species and are presented in Tables 2. They all showed high antiradical activity although the highest antiradical activity unit (EAU_{515}) was from *Termitomyces microcarpus* (EAU_{515} 1.48) followed by *Termitomyces aurantiacus* (EAU_{515} 1.43) while the lowest were from *Termitomyces eurhizus* (EAU_{515} 0.7). Antiradicals are whole food anti-oxidant which helps the body to neutralize excess free radicals by providing them with extra electrons Barnard (2004). The presented result shows the studied mushrooms having high antiradical activity comparable to some medicinal plant extracts (see Sroka 2006), this support them to be potential Antiradicals of high profile.

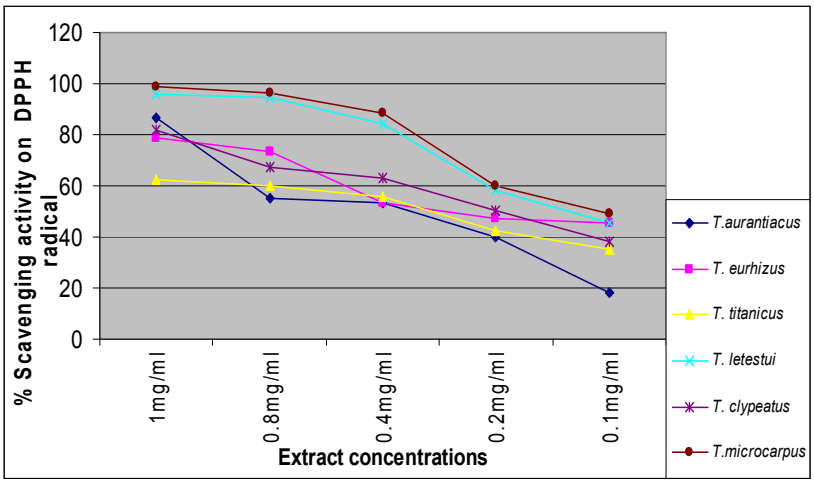

Figure 3: DPPH scavenging ability of the studied mushrooms

3.3 Antioxidant contents

3.3.1 Flavonoid and ß-carotene

The amounts of flavonoid established in the studied mushrooms were very low compared to other observed compounds (Figure 4a). Low values of flavonoid in mushroom have been also observed by Peralta et al. (2012) in *Agaricus brasiliensis* although they used HPLC analysis method. This study thus concur with the suggestion by Peralta et al. (2012) that study of flavonoids needs more sensitive and specific methods. The reducing power of a compound indicates its potential antioxidant activity. Test for β-carotene measure the ability of a compound or mixture to inhibit the oxidation of β-carotene. Carotenoids have linoleic acid free radical which attack highly unsaturated β-carotene models thus making it a strong antioxidant. However, Lee et al. (2011) found that the antioxidative components in the mushroom extracts can reduce the extent of β-carotene destruction by neutralizing other free radicals formed in the system (Jayaprakasha et al., 2001). Despite of all these possibilities of interferences, the studied mushrooms showed high β-carotene content (Figure 4b), with *Termitomyces microcarpus* having highest content while *Termitomyces titanicus* had the least content. These results suggest that, the consumption of these mushrooms can be beneficial for health, since they presumably offer antioxidant protection against oxidative damage. In order to improve their shelf life for wider distribution they should be processed into nutriceuticals and be used as food supplement or in the pharmaceutical industry.

3.3.2 Vitamin C content and lycopene

Vitamin C, one of the simplest vitamins, and lycopene were found in all studied mushroom species using the spectrophotometer procedure based on the reaction with 2, 6-dichlorophenolindophenol. Average concentration of

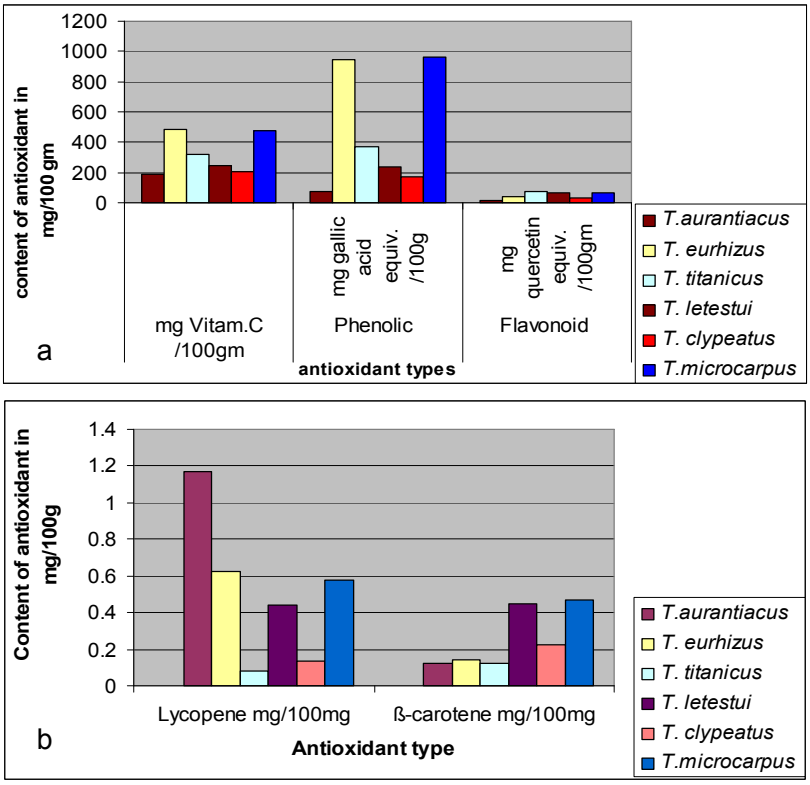

Figure 4: Different antioxidant types and contents in the studied mushrooms

vitamin C content ranged from 191. 88 to 486.19 mg/100g, while lycopene ranged from 0.081 to 1.167 mg/100g among all studied mushrooms (Figure 4).
Specifically the amount of Vitamin C content was found higher in *Termitomyces eurhizus* followed by *Termitomyces microcarpus*. However, the concentration of lycopene was found maximum in *Termitomyces aurantiacus* (486.19 mg/100g) and minimum in *Termitomyces titanicus* (0.081 mg/100g). Vitamin C is well known for its effectiveness against superoxide, hydroxyl radical, hydrogen peroxide, peroxyl radical and singlet oxygen (Sies et al., 1992). Vitamin C as an antioxidant blocks free radical damage, potentially warding off illnesses such as arthritis, heart disease and cancer.

3.3.3 Phenolic content

Phenolic compounds are well known as secondary metabolites commonly found in plants and mushrooms and reported to have vital biological functions including antioxidant activity (Dimitrios, 2006). Knowing the amount of total phenolic compounds in mushrooms is of great importance in their nutritional and functional characterization. Normally, the profile of the phenolics is species-specific Banerjee (2012); this was true for the mushroom studied, the profiles of the phenolics in the extract also differed among the species (Figure 4). This might be attributed to the polarities of the phenolics present in the mushrooms. It is known that Phenolics extraction from any of the natural material depends on their solubility in the used solvent (Naczk and Shahidi, 2006). This study used methanol which is a polar solvent thus generating high extraction yield as it has been also found in Banerjee (2012). Methanol a polar solvents use the electron transfer mechanism which forms strong hydrogen bonds with the phenol molecules. Antioxidant activity of fungal extracts is not solely given by phenolics (Kayashima and Katayama, 2002; Valentão et al., 2005); this study as well, found several types of other antioxidants compounds such as Flavonoid, Vitamin C, Lycopene and β-carotene (Figure 4) in the studied mushrooms (fungi). This suggests that the antioxidant activity of the mushroom studied is due to the entirety of different types of antioxidants they contains, and not a one specific type.
Comparatively, *Termitomyces microcarpus* was found having high antioxidant compounds studied (phenolic content,

Flavonoid, and β-carotene) than other studied taxa except lycopene which was found high in *Termitomyces aurantiacus* and Vitamin C which was relatively higher in *Termitomyces erhizus* (Figure 4). The observed high power of scavenging and antioxidant activities in *Termitomyces microcarpus* correspond well with the locally myth in its medicinal applications. *Termitomyces microcarpus* is well appreciated in different communities in boosting immunity of long ill people and is usually given to lactating mothers of newly born babies (Tibuhwa 2012 b). This finding strengthens further its medicinal application hence this study, propose it as a potential source of natural antioxidant of high profile.

4. Conclusion

To the best knowledge of this study, this is the first report to demonstrate the antioxidant activity of the Tanzanian termitarian mushrooms which are highly appreciated and widely consumed. These results suggest that the consumption of these mushrooms is beneficial for health, since they presumably offer antioxidant protection against oxidative damage. There is thus a need to promote their use not only as food but also as food supplements or in the pharmaceutical industry.

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