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Full Length Research Paper

Cytotoxicity and Antioxidant Activities of *Ganoderma tsugae* - A Basidiomycetes Mushroom Indigenous from Tanzania

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Abstract

Ganoderma species non-edible medicinal mushroom is amongst Basidiomycetes utilized in traditional medicine in the form of an extract, tea or powder, since are very bitter and/or hard to eat in Tanzania. The methanolic extracts of Ganoderma tsugae fruit body indigenous from Tanzania widely used in folk medicine system in the country were tested for cytotoxic activity by brine shrimp toxicity (BST) test. The methanolic extracts were also investigated for their antioxidant activities using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method to determine radical scavenging abilities. Moreover, antioxidant properties (phytochemicals content) such as ß-carotene, Lycopene, total phenolic compounds and vitamin C content were also determined using methanolic extracts by spectrophotometric assay at 515 nm. The result showed relatively mild cytotoxic activity against brine shrimp larvae (113.75–4356.76 µg/ml) while the phenolic compounds were high up to 197.55 GAE mg/g, ß-carotene 16.8 mg/g and lycopene 25.81 mg/g, Vitamin C content 6.09 mg/100g. Moreover, the radical scavenging ability was also high and it was concentration dependent. The maximum ability was at 1ml/100g with EC_{50} value of 0.04 mg/ml. This shows that the studied mushroom is less toxic to cells, rich in antioxidants with high scavenging abilities thus can be considered as good sources of antioxidants as shown by the values obtained for antioxidant activity, phenolic, β-carotene and lycopene contents. In conclusion, the findings obtained in this study support Ganoderma tsugae traditional medicinal use. Also provide baseline data for further research focusing antimicrobial activities, isolation of bioactive compounds, pharmacological efficacy, up scaling and developments into valuable medicines.

Keywords: *Cytotocity, DPPH Radical Scavenging, Phenolics, ß-carotene, Lycopene*

Introduction

In the 21st century the alarming spread of drug resistance around the world, high cost of treatment and the threats from emerging diseases that as of yet have no cure all are amongst public health challenges, which underscore the need for novel therapeutics to tackle diseases and the searching for new drugs in pharmacological industries to reduce the problems. For a long period plants and mushrooms have been a valuable source of natural products for maintaining human health, especially in the last decade, with more scientific explanation to show useful biologically active agents derived from mushrooms function in humans are increasingly being established (Fagbohun et al., 2012; Sofowora, 2008; Imtiaj and Lee, 2007). About 80% of individuals from developing countries use traditional medicine, which has compounds derived from medicinal plants and mushrooms, which should be investigated to better understand their properties, safety and efficiency (Ellof, 1998). Mushrooms are well known not only as the source of essential nutritive, medicinal values and essential amino acids (Cheung and Cheung 2005; Agrahar-Murugkar and Subbulakshmi, 2005; Bonatti et al, 2004) but also are well document in preventing diseases such as hypertension, hypercholesterolemia, and cancer (Chang, 1991; Breene, 1990). Recent findings also show that mushrooms play other great medicinal roles including immunomodulatory, liver protective, anti-fibrotic, anti inflammatory, antidiabetic, antiviral and antimicrobial activities (Russell and Paterson, 2006). These findings affirm the fact that mushrooms are vital sources of medicinal compounds that may be used in curing different body ailment and prevent pathogenic organism. *Ganoderma* is a cosmopolitan genus belonging to basidiomycetes well known in a combination of the mythical, pseudo-science and science for producing novel myco-chemicals (Stanley et al., 2005;Mau et al., 2002). The mushrooms in the genus are mainly white rot fungi, which are involved, in the fundamental process of lignocellulose biodegradation in nature of economic importance. The taxonomic situation within *Ganoderma* is unclear as the species and genus concepts are confused. For example, similar fungi are found in Fomes, Polyporus and Tomophagus (Russell and Paterson, 2006). Laccate *Ganoderma* species often refer to *G. lucidum* species complex which includes *G. tsugae* Murr., *G. valesiacum* Boud., *G. oregonense* Murr., *G. resinaceum* Boud., *G. pfeifferi* Bres., *G. oerstedii* (Fr.) Torr., *G. ahmadii* Stey, and several other taxa that are restricted to tropical areas (Moncalvo et al., 1995). A field photo of *Ganoderma tsugae* used in this study, identified based on morphological character is presented in Figure 1. *Ganoderma* species are nowadays artificially cultivated due to their medicinal uses in more than 10 countries, lead by China where it is nicknamed as 'the mushroom of immortality' and is the number one medicinal mushroom and the leader in terms of world medicinal mushrooms production. Korea is the second largest producer followed by Taiwan, Japan and USA and other Asian counties. Its cultivation has recently begun in some Latin American countries like Colombia and Brazil as well as African countries including

Tanzania. Although *Ganoderma* species have been long used in folk medicine since time memorial, the species in the genus may have different bioactive compounds that may be influenced by the compounds produced by the host (Russell and Paterson, 2006), leading to different medical applications. This study thus evaluated the *Ganoderma tsugae* extracts indigenous from Tanzania, growing on two different hosts (*Ficus benjamina and Dalbrgia melanoxylon* among others, by determining cytotoxicity using brine shrimp toxicity (BTS) test and its antioxidant activities, in order to provide a scientific proof for its wide application in folk medicine system Tanzania.

Materials and Methods

Collection of mushroom

Ganoderma tsugae fruit bodies (Figure 1) were collected at the University of Dar es Salaam, Mwalimu J.K. Nyerere Mlimani campus in Tanzania. Dr. D.D. Tibuhwa (PhD) mushroom taxonomist identified the mushrooms and the voucher specimen specimens DT 137.2012, DT 128.2012 are kept in the mycological herbarium of the University of Dar es Salaam (DSM). The mushroom is among the dominant species fruiting out in clusters on several dead logs including the tree species of *Ficus benjamina*, *Albizia petersiana*, *Pteleopsis myrtifolia*, and *Dalbrgia melanoxylon*.

Standards and reagents standards

Quercetin, Folin-ciocalteu's Phenol, DPPH (2,2-diphenyl-1-picrylhydrazyl), dimethylsulfoxide (DMSO), 2,6 Dichloropheno Indophenol, Gallic acid, Acetone, Hexane, Metaphosphoric acid, Ascorbic acid were all purchased from Sigma Aldrich Co. (St Louis, MO, USA). Sodium carbonate and Methanol were purchased from BDH Poole, England. All the chemicals used were of analytical grade. Deionized-Distilled water was used throughout the experiment. Jenway 6305 UV-Visible Spectrophotometer, Jenway UK was used for analysis. Brine shrimp eggs were purchased from Great Salt Lake, USA.

Brine Shrimp Test Lethality Test (BST)

Extracts preparation

Powdering of air-dried fruit bodies of *G. tsugae* was done by cutting the mushroom into smaller pieces using a coping saw and then grinding it with a mortar and pestle to pass through 1 mm sieve (Model 4,Thomas Wiley; Arthur K Thomas, Philadelphia, PA, USA). It was then **s**ubjected to extraction using analytical grade methanol (BDH Poole, England) for 48 hours. After incubation period the mixture was filtered using cheesecloth and separating funnel to obtain the filtrate. The filtrate was then allowed to evaporate by aid of vaporizer to obtain the crude extract, the crude extract was weighed using weighing balance (Adventurer TM balance, Ohaus Corp, Pine Brook, NJ, USA) and found to be 0.5 gram.

Serial dilution

0.3g of crude extract was dissolved in 10 ml of dimethylsulfoxide (DMSO) to obtain the stock solution of 0.03 g/ml which is equivalent to 30 mg/ml. Serial dilution were then made from a stock solution to obtain a concentration of 15 mg/ml, 7.5 mg/ml, 3.75 mg/ml, 1.875 mg/ml and 0.9375 mg/ml by taking 1ml from the sock solution and diluted in DMSO of equivalent volume deduced from the dilution formula, CiVi=CfVf.

Brine Shrimp Test Assay

The BST was carried out to screen the cytotoxic activity in the crude extract of the *G. tsugae*. The practical procedures are as detailed in Sosovele et al. (2012), which included dissolving the crude extracts in DMSO to make a concentration of 4 mg/ml (stock solution) for while illuminated using an electric bulb, which helped attracting the hatched shrimps. Using 100 µl pipette, 10 hatched shrimp larvae were selected and transferred into different sample wells, which contained 1m of each dilution of extract and one control with only DMSO. The mixture were mixed gently to allow good mixing and incubated at room temperature of 28-30ºC for 24 hours. Using the microscope the number of survivor and dead nauplii were counted and recorded. Then the percentage mortality was then calculated logarithmically. Based on the concentration in relation to the number of dead nauplii the graph was plotted and the regression equation was obtained which was used to calculate the LC_{50}.
The percentage mortality were obtained by using the following formula

$$\% \text{ Mortality} = \frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}} \times 100\%$$

Antioxidant activity and Antioxidant properties (Phytochemical content) assay

The antioxidant ability was analyzed using DPPH radical and antioxidant properties (phytochemical content) were analysed by determining the polyphenols (Total phenolic compounds, vitamin C, and Carotenoids (ß -carotene, lycopene).

Mushroom extracts preparation and yield

Methanolic extractions were carried out using 25 g of the whole mushroom fruit body weighed using balance (Adventurer TM balance, Ohaus Corp, Pine Brook, NJ, USA) at room temperature range of 28-30ºC. The mushrooms were powdered in a motor using

pestles and soaked in 250 ml of methanol and extraction proceeded as explained in (Tibuhwa, 2012 adopted from Jaita et al., 2010). It involved constant stirring of the material for 48 hours then filtered using Whatman filter paper (Whatman, GmbH, Dassel, Germany). The filtrates were evaporated to dryness at 40ºC in a rotary evaporator 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:

$$Yield(\%) = \frac{W_1 x100}{W_2}$$

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

Determination of Total Phenolic Contents

The total phenolic content was determined using the Folin-Ciocalteu colorimetric method (Tibuhwa 2012 adopted in Singleton et al., 1999). Each 0.1 gm of extract was diluted with 5 ml of methanol. 200 µl of the mushroom extract was transferred into a test tube then mixed thoroughly with 1 ml 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 minutes in the dark and centrifuged at 3300 g for 5 minutes. The absorbance of mushroom extract and prepared blank were measured at 515 nm 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 100 g weight of mushroom using the linear equation obtained from standard gallic acid calibration curve.

β-Carotene and Lycopene antioxidant activity assays

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 minute and filtered through Whatman number 4 filter paper (Whatman, GmbH, Dassel, Germany). The absorbance of the filtrate was measured at 453, 505 and 663 nm using spectrophotometer (Uv-vis model 6305 Jenway UK). The ß-carotene and Lycopene content were calculated as:

Lycopene mg/100 mg = $0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$
ß-carotene mg/100 mg = $0.216\ A_{663} - 0.304A_{505} + 0.452A_{453}$

Determination of Vitamin C

The vitamin C content was determined titrametrically using 2,6 Dichloropheno Indophenol methods following the method detailed in Tibuhwa et al. (2011). 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 Whatman no 42 filter paper (Whatman, GmbH, Dassel, Germany) using suction pump (Handy Aspirator, Model WP-25, Yamato Scientific Co. Ltd., Tokyo, Japan). 10 ml was pipetted 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:

$$\text{mg of ascorbic acid per } 100g = \frac{AxIxV_1 x100}{V_2 XW}$$

Where; A = quantity of ascorbic acid (mg) reacting with 1 ml of 2,6 Dichlorophenol Indophenol
I = volume of 2,6 Dichlorophenol Indophenol (in ml) required for the completion for the titration with extract
V_1 = Total volume of extract
W = Weight of the mushroom sample extracted
V_2= volume of aliquot

DPPH free radical scavenging activity assays

The qualitative assays were performed according to the method of Masuda et al. (2000), Jaita et al. (2010). A series of extracts to methanol (1:10-1:10^7) were prepared. 1ml of the extract was mixed with 1ml of 0.4 $mmol^{-1}$ methanolic solutions containing 1:1-diphenyl-2picryhydrazyl (DPPH) radical that are very stable. Each free radical scavenging activity assay was done three times from the same extract in order to determine their reproducibility and standard deviation for the three readings were statistically determined. The mixture was left in the dark for 30 min. and the absorbance measured at 515 nm. 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:

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

Where; A_0=Absorbance of the control solution containing only DPPH
A_1=absorbance in the presence of mushroom extract in DPPH solution and
A_s = 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.

Data analysis

Data analysis was carried out using Analysis Systems (SAS) computer programme for Probit analysis according to Throne et al. (1995). The lethal concentration values that kill 50% of the shrimps (LC_{50}) were determined for each fungal extract by graphical method where by a graph of percentage mortality of brine shrimp larvae against logarithmic Concentrations was used. The LC_{50} values were directly determined from probability analysis or calculated by substituting 50% for "y" into the curve equation in the graph.

Results and Discussion

Morphological identification of Ganoderma tsugae

A number medicinal mushroom species belonging to the Polyporaceae family are now being explored as candidates for production valuable medicines. Fungal fruiting bodies, fungal mycelium or the culture fluid in which the mycelium has been cultivated could all be explored for biological activity. In this study results revealed a Tanzania indigenous mushroom *Ganoderma tsugae* (Figure 1) with cytotocity and antioxidant activities.

The mushroom was distinctively found growing on tree logs and seldom on trunks of live trees. It was identified based on the following morphological characters: Basidiocarp large, fan to kidney-shaped, very showy with whitish tan to orange when young then becoming deep red then bright red colors that darkens as it matures appearing, shiny and varnished. Margin smooth, distinctive white to light yellow contrasting the rest of the basidiocarp. Hymenium well developed, whitish to light tan pores on the outside but often brown on the inside with brown basidiospores. Stipe accentric, if present and very short. Flesh whitish turning brown on bruising or cut.

Figure 1. *Ganoderma tsugae* (a&b) young stages, (c) growing on a tree log, (d) old fruit body with varnished look surface.

Cytotocity

Nowadays, many bio-compounds with potential biomedical application are being explored and utilized, although some could be potentially toxic when ingested at high doses or in combination with other medications Phan et al. (2013). The results of this study however, indicated that no cytotoxicity was detected for the concentrations tested. The overall observed cytotoxicity was very low (113.75–4356.76 µg/ml) the results which second the observation by Phan et al. (2013) who also noted the ethanol extracts of *Ganoderma* species carried out against *Artemia salina* (brine shrimp) after 24 h to possess no significant toxicity.

Antioxidants activities

Results from this study showed that *Ganoderma tsugae* portrayed good antioxidant activities depicted by high phenolic compounds up to 197.55 GAE mg/g, ß- carotene, 16.8 mg/g and Lycopene 25.81 mg/g, Vitamin C content 6.09 mg/100g and high radical scavenging ability with EC50 value of 0.04 mg/ml. Many *Ganoderma* species has been widely investigated for their potential therapeutic benefits and longevity. For example *Ganoderma lucidum* (also known as Lingzhi in Chinese or Reishi in Japanese) has been widely investigated (Phan et al., 2013). *Ganoderma tsugae* locally in Swahili known as *'uyoga mfupa'* in Tanzania meaning bone like mushroom is traditionally used to cure different diseases including tumors, gastric ulcers, hypercholesterolemia, hepatitis, hypertension (Tibuhwa 2013;Tibuhwa 2011). It is commonly boiled and taken, as tea in believes that it promotes health and longevity, as well as improve the immune system (Tibuhwa 2013;Tibuhwa 2011). This study found it to have non-toxic and with strong antioxidant activities. These unique properties support some of its traditional uses and believe. For example, the high antioxidant activities are well known to portray anti cancer properties, since they presumably offer antioxidant protection against oxidative damage (Russell and Paterson 2006). Nevertheless, the high antioxidant activities which suppress excess free radical species, are in line with other scientifically proven study, which shows that excess free radicals 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 (Banerjee et al., 2012; Filipa et al., 2011). The *G. tsugae* studied portrayed high antioxidant activities depicted by high phenolic compounds of 197.55 GAE mg/g. This result is inline with the finding by Mau et al. (2002) who also noted the very species to have high antioxidant properties also associated with total phenols as the major naturally occurring anti-oxidant components 24.0–35.6 mg/ g. It should be noted that the well-known and widely explored species *Gadomerma lucidum* species is morphologically hard to distinguish from *G. tsugae*, the diagnostic method used in this study. The former is well documented in its ability to induces apoptosis, inhibits cell proliferation, and suppresses cell migration of highly invasive human prostate cancer cells PC-3 Stanley et al. (2005) which all these are essential in medical fields. Lycopene and β- Carotene possess the ability to scavenge singlet oxygen. Consumption lycopene and β- Carotene have been reported to be inverse with incidence of cancer (Sarada et al., 2002). The consumption of *G. tsugae* could be considered as good source of natural antioxidant with health benefits since the extracts were found to possess high amount ß- carotene and Lycopene in range between 16.8 mg/g - 25.81 mg/g.

Conclusion

In this study *G. tsugae* was found to have mild cytotoxic activity against brine shrimp larvae and outstanding antioxidant activities with strong radical scavenging ability. The new findings in the present investigation thus offer a scientific support to the ethno medicinal uses of this mushroom. These finding thus, support its traditional medicinal use and provide baseline data for purposeful thoroughly study focusing antimicrobial activities, isolation of bioactive compounds, up scaling and possible developments into valuable medicines.

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