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In vivo antiviral activity, protease inhibition and brine shrimp lethality of selected Tanzanian wild edible mushrooms

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ABSTRACT

In endeavor to have detailed account of nutritional, medicinal and pesticidal potentials of Tanzanian wild mushrooms, a study was conducted to assess the antiviral and protease activities of five edible species: *Cantharellus platyphyllus* and *C. isabellinus* from genus *Cantharellus* and *Pleurotus djamour, P. sajor-caju* and *P. citrinopileatus* from genus *Pleurotus.* Methanolic extracts were subjected to cytotoxicity lethality tests against *Artemia salina* Leach according to Meyer *et al.*, (1982), *in vivo* antiviral tests using embryonated chicken eggs and protease tests using Screen to Nature methods. *C. platyphyllus* extracts had the highest cytotoxicity activity among *Cantharellus* species (LC_{50} = 7.846 µg/ml) and of all tested species. *P. citrinopileatus* extracts had highest cytotoxicity (LC_{50} = 12.807 µg/ml) among *Pleurotus* species. *C. isabellinus* and *P. djamour* extracts had stronger antiviral activities against both pox virus and infectious bursa disease virus. Other tested mushrooms showed moderate antiviral activities. All tested species showed non protease activities. While the *Cantharellus* species showed no protease inhibition property, *Pleurotus* species inhibited protein degradation similar to protease inhibitor. From these observations, the use of mushrooms as food and therapeutic substances particularly in HIV/AIDS infected persons is highly encouraged. It is recommended that further studies involving detailed biological activities (including antimicrobial, antioxidant and pesticidal activities) and determination of chemical compositions of Tanzanian wild edible mushrooms be done as the knowledge will contribute to the existing knowledge on these useful macro fungi.

Key words*:* wild mushrooms, antiviral activity, protease inhibitor, Tanzania, brine shrimp

INTRODUCTION

A mushroom is a macro fungus with distinctive fruiting body, which can be hypogenous or epigenous, large enough to be seen with the naked eye and to be picked by hand (Härkönen *et al.,* 2003; Lindequist *et al.,* 2005). Wild mushrooms are becoming more and more important in our diet for their nutritional characteristics. The high protein, essential nutrients and low energy contents make them excellent foods comparable to meat, egg and milk (Mdachi *et al.,* 2004; Barros *et al.,* 2007). Beside their use as nutritious food, mushrooms have been used as health-promoting food supplements or nutraceuticals: substances that may be taken as food or part of food but also provide medical and/or health benefits (Barros *et al.,* 2008a; Ogbe, *et al.,* 2008). Edible mushrooms with immuno-modulating activities are used as nutraceuticals since they can stimulate non-specific immune systems and exert anti-tumour activity through stimulation of host's defence mechanism (Lindequist *et al.,* 2005). The commercially grown *Ganoderma frondosa*, *Lentinula edodes and Trametes versicolor as well as the wild occurring Schizophyllum commune*, for example, produce polysaccharides that activate the effector cells to secrete anti-proliferative cytokines and induce apoptosis and differentiation in tumour cells (Ojeda and Skardova, 1997; Lindequist *et al.,* 2005).

Mushrooms can synthesize a dazzling array of biologically active products which have no significant role in their primary physiological processes. Some of the products are merely the end products of aberrant biosynthetic pathways (secondary metabolites) and other excretory products. These bioactive products can affect nerve axons and synapsis (e.g. pyrethrins, nicotine, picrotoxinin), muscles (e.g. ryanodine), respiration (e.g. rotenone, mammein), hormonal balance (e.g. juvenile and molting hormone analogues and antagonists), reproduction (e.g. β-asarone) and behaviour (e.g. attractants, repellents, antifeedants) in insects (Varma & Dubey, 1999; Mugisha-Kamatenesi *et al.,* 2008). Even the toxic constituents present in these organisms represent the secondary metabolites which are biosynthetic products (Tewary *et al.,* 2005).

The bioactive compounds enable mushrooms to survive in their environments which are full of other organisms such as bacteria, viruses and other fungi. Some mushrooms such as those of the *Lepista* and *Cantharellus* genera are never inhabited by insects (Mier *et al.,* 1996; Wang *et al.,* 2002). Research indicates that several compounds of known antimicrobial, anti-viral, anti-tumour, antifungal and immuno-modulating activities, among others, have been isolated and identified from mushrooms (Butcher, 1995; Jackwood, 2005; Lindequist *et al.,* 2005; Barros *et al.,* 2007; Barros *et al.,* 2008b; Selegean *et al.,* 2009). Presence of these compounds enables mushrooms to survive in their natural environments and as such they must have very strong activities to allow them to out-compete their environmental competitors. It is not surprising to find that these compounds can be of great benefit to humans for prevention and cure of diseases and infections caused by pathogens (Nkunya, 2002). Some mushrooms are now being employed in treating chronic stomach diseases. Several compounds of mushroom origin have also been tested for human immuno-deficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and they have shown promising results when tested to HIV/AIDS patients (Lindequist *et al.,* 2005). Triterpenes isolated from *Ganoderma lucidum* have shown to be active against HIV type 1 (Lindequist *et al.,* 2005). Recent studies done in Tanzania have revealed that isolated compounds from wild edible mushrooms possess larvicidal, antimicrobial as well as cytotoxicity activites (Nyigo *et al.,* 2005; Baraza *et al.,* 2007).

While some extracts from this fungal group can cleave proteins into amino acids and peptides, others have the ability to inhibit this process. For example, water soluble lignins extracts from *Inonotus obliquus* (Pers.: Fr) Pilat inhibited HIV protease enzymes at 2.5 µg/ml (Lindequist *et al.,* 2005). Discovery of both new protease and new protease inhibitors in fungi may have medicinal potentials as they are widely used to treat HIV

infections as well as hypertension (GIBEX STN Manual, 2006).

Majority of people particularly in rural areas are known to rely heavily on their own skills, cultural, traditional values and knowledge of the local environment to feed, protect themselves, their crops, livestock as well as stored agricultural products (WHO, 2002; Mugisha-Kamatenesi *et al.,* 2008; Mihale *et al.,* 2009). Recent studies have shown that farmers in Tanzania use indigenous knowledge based pesticides originating from locally available plants to conquer their environments (Mugisha-Kamatenesi *et al.,* 2008; Mihale *et al.,* 2009). They might also be using fungi (or mushrooms) to attain their intended goals. However, studies show that most of the mushroom research is done in the U.S., Europe and Asia and few have been conducted in West Africa. Mushroom studies conducted in East Africa particularly in Tanzania indicate that they were based on either nutritive values (Mshandete, 1998, 2007; Mamiro, 2002; Ndekya, 2002) or amino acid composition (Mdachi *et al.,* 2004). Very few studies have been done on the chemical composition as well as the biological activities of Tanzanian wild mushrooms (Nyigo, *et al.,* 2005; Baraza *et al.,* 2007). As there has been no research to appraise the antiviral and protease potentials of Tanzanian wild edible mushrooms, the research was therefore conducted to assess the *in vivo* activities of mushroom extracts against two viruses: Infectious Bursal Disease Virus (IBDV) and pox virus. In addition, the research was intended to assess the ability of Tanzanian wild edible mushrooms to inhibit or act as proteases.

MATERIALS AND METHODS

Mushroom Materials: Fruiting bodies of *Cantharellus* (*C. platyphyllus* and *C. isabellinus*) and *Pleurotus* (*P. djamour, P. sajor-caju* and *P. citrinopileatus*) species were collected in Muheza District, Tanga, Tanzania in September, 2008. The mushroom samples were identified and authenticated in the Department of Botany, University of Dar es Salaam where voucher specimens were deposited.

Mushroom Extraction: The mushrooms fruiting bodies were dried, grinded to fine powders and later soaked twice in methanol for 48 hours. After that they were filtered and further concentrated using a rotary evaporator. The extracts were kept in refrigerator and later subjected to various chemical tests, bioassays and secondary metabolites screening to detect the presence of different useful phytoconstituents.

Cytotoxicity Lethality Assay (Brine Shrimp Test, BST): Brine shrimp (*Artemia salina* Leach) larvae were used as indicator animals for preliminary cytotoxicity assay of the mushroom extracts (Meyer *et al.,* 1982). Briefly, artificial seawater was prepared by dissolving sea salt (3.8 g) in distilled water to make a concentration of 3.8 g/L and then filtered. The salt solution was filled into a tank that has been divided into two unequal compartments by perforated polythene wall. Shrimp eggs were later sprinkled into the covered part of the tank and a lamp was illuminated on the uncovered part in order to attract the hatched shrimps. The mature nauplii were collected in between 36 and 48 hours of hatching (Baraza *et al.,* 2007). The mushroom extracts were dissolved in Dimethyl sulfoxide (DMSO) in vials in triplicate at an initial concentration 240 µg/ml and decreasing up to 4 µg/ml. In every vial containing the extract in solution, 10 brine shrimp larvae were added. An additional fourth set of vials containing only a solvent (DMSO) in 5 mL of artificial seawater and 10 shrimp larvae acted as control (Meyer, *et al.,* 1982). The number of survived larvae was established after 24 hours and the LC_{50} values - the concentrations required to kill 50% of the shrimp larvae, the concentrations to give 100% mortality rates and confidence intervals were obtained using probit analysis (Finney, *et al.,* 1971).

In vivo Antiviral activities: Embryonated chicken eggs from healthy hens were collected for in *ovo*-testing, arranged in sets of 10 eggs and incubated. On the eighth day of incubation, a set of embryonated eggs were injected with 100 µL of IBDV in 900 µL of phosphate buffer saline (PBS) and 500 µL of the mushroom extract dissolved in DMSO via the allantoic sac. Similar procedures were followed, but this time using pox virus. The concentrations of extracts injected were derived from the brine shrimp 100% mortality rates values (Meyer et *al.,* 1982). In four different sets of embryonated eggs, one set was injected with the virus alone, another set with DMSO alone, another set with virus and DMSO and the last one no treatment was done. These sets of eggs acted as control and

were made available to every set of virus used. All the sets were immediately sealed with hot candle wax, labeled appropriately and placed in an incubator set at 37˚C temperature and 65 -70% humidity. The incubated eggs were later monitored daily by candling process for six (6) days after the treatment day until the final observations.

Protease and Protease Inhibitor tests: The investigations involving protease and protease inhibition were carried out as indicated in the Global Institute for Bioexploration (GIBEX) screens to nature (STN) manual, (2006) with minor modifications. Briefly, trypsin (a protease enzyme) solution was prepared by dissolving 7 mg of trypsin lyophilized powder in 1.5 mL tube into 300 µL of distilled water. The protease inhibitor solution was prepared by weighing 3 mg of the lyophilized inhibitor in a 1.5 mL tube, dissolved in 30 µL of distilled water and flicked to dissolve. Triplicate strips of radiograph film with gelatin coating each was labeled 'P' for protease, 'W' for water, 'I' for inhibitor and numbers according to the extracts to be tested in duplicates with a spacing of 2 cm from each other. Then 10 µL drop of trypsin was placed under the 'P'. Two drops of water (10 µL each) were placed under the 'W' and two drops of inhibitor under the 'I'. Under each labeled number on the strip, two drops of 10 µL of each mushroom extract (with a concentration of 0.5 mg/mL) was added. Later 10 µL of trypsin was added immediately at the bottom row of each of the strips. The strips were then allowed to sit undisturbed for 15 minutes on a flat surface. Thereafter the strips were rinsed gently under running water. Activities of the extracts were judged based on the ability of the extracts to either degrade the protein coated on the strip similar to trypsin or inhibit the trypsin from degrading the protein similar to protease inhibitor.

RESULTS AND DISCUSSION

Cytotoxicity lethality: Mushroom extracts tested showed varied cytotoxicity activities against *Artemia salina* Leach. Extracts from genus *Cantharellus* had higher cytotoxicities compared to those from the genus *Pleurotus*. In addition, there was an inter-species variation within each genus (Table 1). Among the *Cantharellus* species, extracts from *C. platyphyllus* had the highest cytotoxicity (LC_{50} = 7.846 µg/ml) compared to *C. isabellinus* which had LC_{50} = 17.353 µg/ml. On the other hand, *P. citrinopileatus* had the highest cytotoxicity activity (LC_{50} = 12.807 µg/ml) compared to *P. djamour* (LC_{50} = 24.347 µg/ml) and *P. sajor-caju* (LC_{50} = 50.924 µg/ml).

Inter-species cytotoxicity levels in the two study genera indicated that the activities were approximately double from the highest to the lowest LC_{50}. In the *Cantharellus* species values were from 17.353 µg/mL in *C. isabellinus* to 7.846 µg/mL in *C. platyphyllus*, while in the *Pleurotus* species, the activity was doubled from 50.924 µg/mL in *P. sajor-caju* to 24.347 µg/mL in the *P. djamor*. However, the activity was tripled in the *P. citrinopileatus* to LC_{50} = 12.807 µg/mL (Table 1). The observed trend could be due to varied levels of bioactive compounds as well as presence or absence of various chemical constituents that may cause interference with bioactivity in the mushroom species tested.

Table 1: Cytotoxicity Activities of Different Mushroom Extracts

Mushroom	LC_{50} (µg/mL)	100% MR (µg/mL)	95% Fiducial limit (µg/mL) UCL - LCL
Cantharellus platyphyllus	7.846	120	13.593 - 2.680
Cantharellus isabellinus	17.353	260	29.586 - 6.147
Pleurotus djamor	24.347	120	56.527 - 11.286
Pleurotus sajor-caju	50.924	250	67.161 - 35.762
Pleurotus citrinopileatus	12.807	80	33.405 - 5.078

LC_{50} = lethal concentration (concentration to kill 50% of test organisms); MR = mortality rate; UCL = Upper Confidence limit; LCL = Lower Confidence limit

In terms of 100% mortality, *P. citrinopileatus* and *C. platyphyllus* showed the highest potential to kill all the test organisms within their respective genus (Table 1). *C. platyphyllus* and *P. djamor* showed the same concentration to kill 100% of organisms (120 µg/ml) while *C. isabellinus* and *P. sajor-caju* had the lowest mortality rate (Table 1). Inter-species variations exhibited a similar trend in the observed 100% mortality rates as shown in the cytotoxicity activities.. The mortality rates were almost doubling in the *Cantharellus* species and doubling and tripling in the *Pleurotus* species tested (Table 1).

Antiviral activity: Extracts from the wild mushrooms used in this study showed remarkable antiviral activities when tested against pox virus and IBDV. For the whole duration of the candling process, embryos in eggs treated with mushroom extracts from *C. isabellinus* and *P. djamour* were observed to be alive, healthy and with well developed features such as limbs and feathers. This was also observed in eggs treated with extracts from *C. platyphyllus* (Figure 1). The antiviral activities of the extracts from *C. platyphyllus* were observed to be almost the same in both the pox virus and IBDV. Nevertheless, the antiviral activity of *C. platyphyllus* against the viruses were less strong when compared to *C. isabellinus* and *P. djamour* (Table 2).

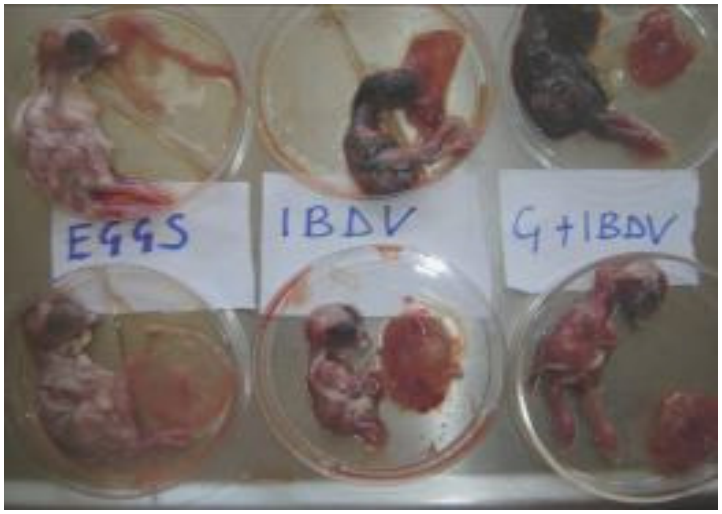

Figure 1: Antiviral activity of *C. platyphyllus* (C_1) towards IBDV as compared to controls (egg alone and the virus alone)

In embryonated eggs treated with extracts from P. sajor-caju and P. citrinopileatus, both sets were observed to have live and health embryos but they were very weak in the pox virus set. The embryos in eggs in the IBDV set, however, were observed to be alive, healthy but moderately weak. In sets where the eggs were treated with the pox virus alone, the embryos were observed to be dead and undeveloped by the 4th day of candling. During this time, about 85% of the treated eggs were observed to have their embryos dead. On the fifth day, all the eggs were observed to have dead embryos. Similar effects were observed on the embryos in the IBDV set. Although they were dead, the embryos in eggs treated with IBDV were observed to be more developed compared to those treated with pox virus. In sets treated with the viruses and DMSO (the solvent), similar effects as those treated with virus alone were observed for both treatments. Eggs incubated without treatment and those treated with DMSO alone resulted in embryos that were observed to be healthy similar to normal growing embryos for the entire experimental period. When compared to those treated with C. isabellinus and P. djamour, there were no clearly observed differences in the embryos as all had well developed features. Table 2 below gives a summary of the antiviral activities of all extracts and the controls.

Table 2: Antiviral Activities of different mushroom extracts toward pox virus and IBDV (The days in brackets indicated the days the eggs were placed in the incubator)

Experiment		Number of Samples		Effect of the virus on embryos after treatment with mushroom extracts											
				1st day (9th)		2nd day (10th)		3rd day (11th		4th day (12th)		5th day (13th)		6th day (14th)	
		Pox	IBDV	Pox	IBDV	Pox	IBDV	Pox	IBDV	Pox	IBDV	Pox	IBDV	Pox	IBDV
Mushroom samples	C. isabellinus extract + Virus	10	10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	C. platyphyllus extract + Virus	10	10	++	+	++	+	++	+	++	+	++	+	++	+
	P. djamour extract + Virus	10	10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	P. sajor-caju extract + Virus	10	10	+	+	+	+	+	+	+	+	+	+	+	+
	P. citrinopileatus extract + Virus	10	10	+	+	+	+	+	+	+	+	+	+	+	+
Controls	Virus alone	7	7	+++	+++	++	++	+	+	--	-	--	-	--	-
	Eggs alone	7	7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	DMSO alone	7	7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Virus and DMSO	7	7	+++	+++	++	++	+	+	-	-	-	-	-	-

+++ = embryo is alive and develops well; ++ = embryo is alive and develops well but weak; + = embryo is alive but very weak; - = embryo is dead with feather development; -- = embryo is dead with no feather development

These results indicate that the extracts from *C. isabellinus* and *P. djamour* had more antiviral activities to both pox virus and IBDV. Extracts from *C. platyphyllus* had less antiviral activity to both viruses compared to *C. isabellinus* and *P. djamour*. *P. sajor-caju* and *P. citrinopileatus* showed similar antiviral activities against both the viruses. The extracts of the two genera had higher antiviral activities against IBDV than pox virus (Table 2). However, their activities were less than the activities of *C. isabellinus*, *P. djamour* and C. *platyphyllus*. The study shows that pox virus was more virulent than the IBDV.

Protease inhibition: Wild edible mushroom extracts showed an interesting trend as regards to the two tested genera. All the five species tested showed no degradation of the protein coating. This indicated that extracts of the tested species have no protease activity when compared to trypsin. On the other hand, while both *Cantharellus* species showed no inhibition of protein coating degradation, the three *Pleurotus* species (*P. djamor, P. sajor-caju and P. citrinopileatus*) showed no clear spots to indicate the absence of degradation of the protein in similar way to the inhibitor. The three species thus blocked the trypsin from degrading the protein and therefore they are protease inhibitors.

CONCLUSION

The findings from this study have shown that wild edible mushroom extracts from the genus *Cantharellus* showed higher cytotoxicity activities against *Artemia salina* Leach than those from the genus *Pleurotus*. Whereas *C. platyphyllus* had the highest cytotoxic activity among the tested *Cantharellus* species, *P. citrinopileatus* had the highest cytotoxic activity among the *Pleurotus* species. Results of the present study also indicate that when tested on embryonated chicken eggs, extracts from *C. isabellinus* and *P. djamour* showed strong antiviral activities against pox virus and IBDV. Extracts from *C. platyphyllus* had relatively weak antiviral activity against both viruses compared to *C. isabellinus* and *P. djamour*. Extracts from *P. sajor-caju* and *P. citrinopileatus*, on the other hand, showed little antiviral activities against both viruses. With regards to protease activity, all the tested species showed no protease activity. While the *Cantharellus* species showed no inhibition, the *Pleurotus* species inhibited the protein degradation in similar way to protease inhibitor.

These interesting findings could lead to the mushrooms being used specifically by HIV /AIDS infected persons as food as well as nutraceuticals. In this regard, the public are highly encouraged to consume these mushrooms due to their multiple advantages as more detailed information continues to be revealed by further research. In addition, further studies involving the determination of chemical structural compositions and the antimicrobial, antioxidant and pesticidal activities of the bioactive constituents present in these wild mushrooms will contribute to the existing knowledge about these macrofungi.

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