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Chelela, Baraka

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Baraka Luca Chelela, Musa Chacha and Athanasia Matemu*

The School of Life Science and Bio-Engineering,
Nelson Mandela African Institution of Science and Technology (NM-AIST),
P. o. Box 447, Arusha-Tanzania
* Corresponding author: athyone@yahoo.com

ABSTRACT

The crude extracts from six wild mushroom species (*Russula cellulata, Afrocantharellussymoensis, Lactarius sp, Lactarius denigricans, Russula kivuensis, Amanita muscaria and Amanita phalloides*) collected from the Southern Highlands of Tanzania were evaluated for cytotoxicity effects against Brine shrimp (*Artemia salina)* nauplii. The lethal concentration which kills 50% of the larva, LC_{50} (µg/mL) of the wild mushroom extracts was evaluated. The results showed that, *A. muscaria* ethanol (MS1E) and petroleum ether (MS1PE) extracts exhibited highest cytotoxicity activity with LC_{50} of 11.00 and 13.72 µg/mL respectively. *Lactarius. denigricans* ethanol extract (BM10E) also possessed highest cytotoxicity activity with LC_{50} of 12.77 µg/mL as compared to its petroleum ether extract (BM10PE) with LC_{50} of 18.96 µg/mL, followed by chloroform (BM10C) and acetone (BM10A) extracts with LC_{50} of 32.56 µg/mL and 47.79 µg/mL respectively. In contrast, petroleum ether extract of *A. symoensis* (BM6PE) showed non-toxic effect on Brine shrimp larvae with LC_{50} of 202.96 µg/mL. The study on cytotoxicity effect of wild mushrooms will give an insight into possibility of isolating stronger anticancer agents.

Key words: Wild mushrooms, Brine shrimp, cytotoxicity

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INTRODUCTION

Mushrooms have been used as food for a long time since ancient times due to their pleasant flavor and appealing texture (Ferreira *et al*, 2010). Their therapeutic property makes them a good health promoting food as they contain minerals, vitamins and nutritive compounds, proteins, polysaccharide and have a low fat content (Giri*et al*, 2012). Also, mushrooms have been found to have potential biological activities such as anti-bacteria, anti-fungi, antitumor, anti-inflammatory, anti-hepatotoxic, cardio-tonic activity, cholesterol lowering, antiviral and immune-modulatory (Miles and Chang, 1997; Nkunya, 2002; Quang*et al*., 2006; Halpern, 2007; Petrova*et al.,* 2007; Mshandete and Cuff, 2008; Salahddin, 2008; Nyigo*et al*., 2009; Ferreira *et al.,* 2010 and Saral, 2013). Mushrooms have been also tested with achievement in therapy of cancer, viral diseases, diabetes, hypercholesterolemia, impaired blood platelet aggregation and hypertension, (Wang et al, 1996; Waiser and Weis, 1999). Moreover, mushrooms have been reported to cause ill health and fatalities in both human and animals in the whole world, due to the increasing cases of mushroom poisoning (Amber *et al*, 2008).

The growing drug resistance against pathogenic microorganisms and tumor cells has drawn much concern for researchers to look for alternative drugs. Toxicological concerns related to the use of synthetic drugs and growing consciousness on natural foods as nutraceuticals has increased interest in the study of natural materials as nutraceuticals and pharmaceuticals. Wild mushroom are potential sources of these natural materials since they synthesize the secondary metabolites which can demonstrate a broad spectrum of bioactivity (Bendahou*et al*, 2008).

In Tanzania, many tribes include mushrooms in their diet, especially during rainy season as they are collected in abundance (Härkönen *et al*, 1995). Furthermore, wild mushrooms may be a potential source of bioactive compounds for different physiological activities. Considering the geographic differences and edibility of some wild mushrooms (Ambali *et al*, 2008), the need for toxicological screening turns out to be potential. Therefore, studies to exploit wild mushrooms as a source of biologically active compounds is relevant. Proper screening of toxic wild mushrooms may further reduce wastage of some edible, nutritious mushrooms, which are regarded by some of the communities as inedible besides being abundant in the wild. Therefore, the objective of the study was to evaluate cytotoxicity effect of some wild mushroom species against Brine shrimps.

MATERIALS AND METHODS

Materials

The edible (*Russula cellulata, Afrocantharellus symoensis* and *Lactarius densifolius)* and inedible *(Lactarius sp, Lactarius denigricans, Russula kivuensis, Amanita muscaria, Amanita phalloides* and *Lactarius gymnocarpoides*) wild mushroom species were collected in the Southern Highlands of Tanzania in January, 2014. Mushroom samples were identified by a taxonomist at the University of Dar Es Salaam where voucher specimens were deposited. Brine Shrimp eggs were purchased from Aquaculture Innovations (Grahamstown, South Africa) and sea salt was prepared by evaporating the unpolluted water collected from the Indian Ocean, along Tanga coast line. All other chemicals and reagents used in this study were of analytical grade.

Wild Mushroom Extracts Preparation

Fresh mushrooms (whole) 25 g were ground (Singsung, Singapore) and extracted sequentially by soaking twice in 250 mL of ethanol and chloroform for 48 h. The extracts were then filtered and further concentrated in a vacuum evaporator (Heildolph, Germany). The collected wild mushroom extracts were stored at 4 °C for further bioassays.

Cytotoxicity on Brine Shrimps

Brine shrimp (*Artemia salina* Leach) larvae were used as indicator animals for preliminary cytotoxicity assay of the mushroom extracts according to (Meyer *et al.*, 1982). In summary, artificial seawater was prepared by dissolving sea salt (3.8 g) in distilled water to make a concentration of 3.8 g/L (Ramachandran *et al.*, 2011). The salt solution was filled into a tank and the shrimp eggs were spread and a lamp was illuminated from one side in order to attract hatched shrimps. The hatched shrimps (mature nauplii) were collected after 36 and 48 h of hatching (Golla *et al.*, 2011). The test was done in triplicate at an initial concentration 240 µg/mL and declining up to 4 µg/mL. Using a glass capillary, ten brine shrimp larvae (nauplii) were added in a vial containing mushroom extracts. Control was done in a solvent (50% DMSO) in 5 mL of artificial seawater (Meyer *et al.*, 1982, Krishnaraju *et al.*, 2005). The number of surviving larvae was established after 24 h of exposure and the percentage mortality was determined by comparing the mean surviving larvae of the test and the control. Mortality (%) = (Number of dead Nauplii/ Initial number of live Nauplii) x 100 (Peteros & Uy, 2010).

Statistical Analysis

The brine shrimp lethality test was done in triplicates and data were analyzed using Microsoft Excel (2013) where the fit line was obtained after plotting the mortality rate against the log of concentration. The regression line was then used to calculate the LC_{50}, LC_{16}, LC_{84}, 95% confidence interval and R^2 (Krishnaraju *et al*., 2005).

RESULTS

The result of cytotoxicity activities of the crude extracts of wild mushroom species (*Lactarius densifolius, Afrocantharellus symoensis, Russula cellulata, Lactarius sp, Lactarius gymnocarpoides, Russula kivuensis, Amanita muscaria, Lactarius denigricans* and *Amanita phalloides*) against Brine shrimps (*Artemia salina* leach) are presented in **Table 1**. *Amanita muscaria* ethanol (MS1E) and petroleum ether extracts (MS1PE) exhibited higher cytotoxicity activity with LC_{50} of 11.00 µg/mL and 13.72 µg/mL respectively. The *L. denigricans* ethanol extract (BM10E) also exhibited higher cytotoxicity activity with LC_{50} of 12.77 µg/mL whereas its petroleum ether extract (BM10PE) showed LC_{50} of 18.96 µg/mL, followed by chloroform (BM10C) and acetone extracts (BM10A) with LC_{50} of 32.56 µg/mL and 47.79 µg/mL respectively. The cytotoxicity effect of *Lactarius spp*. ethanol extract (BM1E) was 20.04 µg/mL and 22.09 µg/mL for the petroleum ether extract (BM1PE) respectively. The ethanol extracts of *R. kivuensis* (MS4E) and *A. phalloides* (MS2E) showed cytotoxicity level of 22.50 and 22.60 µg/mL compared to their petroleum ether extracts (MS4PE and MS2PE) with LC_{50} of 31.51 and 68.99 µg/mL while its chloroform extracts (MS4C) had LC_{50} of 41.05 µg/mL **(Table 1)**. Conversely, petroleum ether extract of *A. symoensis* (BM6PE) was non-toxic activity against brine shrimp larvae (LC_{50} = 202.96 µg/mL). This confirms that *A. symoensis* is nontoxic since it is an edible specie in most parts of Tanzania. Generally, ethanol extracts of wild mushrooms exhibited substantially higher lethality on Brine shrimps followed by petroleum ether extracts. Generally, almost all mushroom species tested showed the range of activity from high to moderate cytotoxicity except for BM6PE which was non-toxic.

Table1: Cytotoxicity of crude extracts from wild mushrooms

Mushroom Extracts	LC_{50} (µg/mL)	95%Confidence interval (µg/mL)	Regression equation	Regression coefficient (R^2)
MS1PE	13.72	8.5966-21.8921	Y=42.35x+1.8337	0.94
MS1E	11.00	6.6007-18.3316	Y=38.76x+9.6374	0.92
BM10AC	47.79	34.03-67.11	Y=58.30x-47.91	0.93
BM10C	32.59	22.83-46.53	Y=55.1x-34.141	0.96
BM10E	12.77	9.30-17.54	Y=88.19x-47.551	0.94
BM10PE	18.96	14.07-25.55	Y=81.27x-53.853	0.86
BM1E	20.04	13.41-29.95	Y=49.26x-14.139	0.94
BM1PE	22.09	14.35-34.03	Y=45.83x-11.614	0.95
MS4E	22.50	15.05-33.65	Y=49.20x-16.536	0.98
MS4C	41.05	19.41-86.83	Y=26.43x+7.3685	0.94
MS4PE	31.51	23.08-43.00	Y=63.64x-45.351	0.97
MS2PE	68.99	63.09-75.43	Y=70.071x-78.844	0.90
MS2E	22.60	12.90-39.58	Y=35.31x+2.1973	0.90
BM8E	63.51	46.03-87.62	Y=61.50x-60.873	0.83
BM8C	65.67	42.58-101.29	Y=45.68x-33.014	0.92
BM4PE	25.59	19.89-32.92	Y=96.204x-85.462	0.97
BM6PE	202.96	112.15-367.29	Y=33.37x-26.998	0.97
BM2C	37.58	27.40-51.54	Y=62.68x-48.713	0.86
Cyclophosphamide	16.39	12.01-22.31	Y=69.97x-34.936	0.98

Key: BM4PE = *Russula cellulata* petroleum ether extract, BM1PE = *Lactarius sp.* petroleum ether extract, BM10C = *Lactarius denigricans* chloroform extract, MS4PE = *Russula kivuensis* petroleum ether extract, MS1PE=*Amanita muscaria* petroleum ether extract, BM10PE = *Lactarius denigricans* petroleum ether extract, MS2PE = *Amanita phalloides* petroleum ether extract, BM6PE = *Afrocantharellus symoensis* petroleum ether extract, MS4E = *Russula kivuensis* ethanol extract, MS2E = *Amanita phalloides* ethanol extract, MS1E = *Amanita muscaria* ethanol extract, MS4C = *Russula kivuensis* chloroform extract, BM1E = *Lactarius sp*. ethanol extract, BM2C = *Lactarius gymnocarpoides* chloroform extract, BM10A = *Lactarius*

denigricans acetone extract, BM10C = *Lactarius denigricans* chloroform extract, BM8E = *Lactarius densifolius* ethanol extract and BM8C = *Lactarius densifolius* chloroform extract.

DISCUSSION

Brine shrimp bioassay is considered as a quick preliminary screening for the presence or absence of bioactivity and also is used to determine the cytotoxicity of crude extracts (Syahmi *et al.*, 2010). Evaluation of toxic properties of a material is vital when considering for community health safety for the reason that exposure to chemicals can be perilous or hazardous, resulting in adverse effects on human being (Assessment, 2005). Toxicity is a state of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells (Syahmi et al., 2010). According to Meyer et al. (1982) and Nondo *et al.* (2011), extracts from natural products with LC_{50} of less than 100 µg/mL are considered to hold toxic effects, also extract is generally regarded as non-toxic if its LC_{50} value is greater than 100 µg/mL in the brine shrimp lethality assay.

From the study, all wild mushrooms crude extracts except BM6PE exhibited cytotoxicity effect against brine shrimp since the LC_{50} values were below 100 µg/mL. These findings concur with Baraza *et al.* (2007) where *Lactarius edulis* petroleum ether, dichloromethane and methanol extracts, and *T. letestui* ethanol extract exhibited mild cytotoxic activity (brine shrimp test; LC_{50} = 88, 69.6, 26.7 and 69.7 µg/mL respectively) while *Agaricus sp*. aff. *arvensis* ethanol extract had the highest cytotoxic activity (LC = 19.90 µg/mL). Also, Nyigo *et al.* (2009) observed that the crude extracts of *Agaricus* and *Termitomyces* species exhibited significant activity with brine shrimp bioassays. The LC_{50} values of the brine shrimp larvae were 19.54 µg/mL, and 59.93 µg/mL for ethanol extracts of *Agaricus* sp. and *Termitomyces letestui* respectively. This also suggests why *Agaricus* specie is not edible by most ethnics of Tanzania. On the other hand, Kidukuli *et al.* (2010) reported varied cytotoxicity activities of mushroom extracts against *Artemia salina* larvae. Extracts from genus *Cantharellus* had higher cytotoxicity than *Pleurotus* genus and also interspecies variation was observed where *Cantarellus platyphyllus* exhibited higher cytotoxicity (LC_{50} = 7.85 µg/mL) than *Cantharellus isabellinus* with LC_{50} = 17.35 µg/mL also *Pleurotus citrinopileatus* had higher cytotoxicity activity (LC_{50} = 12.81 µg/mL) compared to

Pleurotus djamour (LC_{50} = 24.35µg/mL) and *Pleurotus sajorcaju* (LC_{50} = 50.92 µg/mL). Variation in cytotoxicity effect among the extracts was also observed in this study, this may be due to variation in levels of bioactive compounds as well as the presence or absence of several chemical compounds which interfere with bioactivity of the mushroom species. In both tests of the Brine shrimps, the LC_{50} showed dose dependency, this means that mortality rate increased as the concentration of extracts increased.

Ethanol extracts were observed to have higher cytotoxicity activity than chloroform extracts petroleum ether and acetone. However, the LC_{50} of *A. muscaria*, *A. phalloides* and *L. denigricans* ethanol extracts were smaller than that of cyclophosphamide which was 16.39 µg/mL. This suggests that ethanol extracts can be a potential solvent for isolation of antitumor compounds. The brine shrimp lethality shows the bioactivity of the extract which in most cases correlates reasonably well with cytotoxic and anti-tumor properties (Krishnaraju et al. 2005). In the present study, inedible *A. muscaria, A. phalloides* and *L. denigricans* mushrooms were observed to have higher cytotoxicity than other mushroom species. The high cytotoxicity of these species proposes the reason why these mushroom species are not edible in most parts of Tanzania. However, edible *R. cellulata* and *L. densifolius* mushrooms were observed to have a certain degree of toxicity higher than the regarded by the community as inedible species. The same case was observed by Ambali *et al*. (2008) where edible *Macrolepiota procera* mushrooms were toxic against mice, the situation which demonstrates some toxic principles which may be detrimental to health. Conversely, this toxicity may not be seen to human possibly because the toxin is heat labile and must have been damaged during cooking or may be shattered by gastric juices and proteolytic enzymes in the gastrointestinal tract or may not even be absorbed when ingested possibly due to its size (Ambali *et al*, 2008). Moreover, further study on the threshold of toxicity is needed during screen of bioactive compounds wild mushrooms. Therefore, significant lethality of these wild mushroom crude extracts to brine shrimp is an indication of the presence of potent and compelling cytotoxic components with merits further investigation as anticancer agents.

CONCLUSION

All screened wild mushrooms crude extracts demonstrated cytotoxicity effect, although the degree of their toxicity was different among species. The observed results give an insight for

further exploitation of indigenous wild mushroom species found in Tanzania as potential source of bioactive compounds against cancer cells. Also, poisonous wild mushroom species may be easily identified based on their cytotoxicity threshold, hence extreme caution should be taken during wild mushrooms collection.

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