VOL. 5, NO. 5, SEPTEMBER 2010 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science


www.arpnjournals.com

ANTIMICROBIAL ACTIVITY OF ETHYL ACETATE EXTRACTS FROM EDIBLE TANZANIAN *Coprinus cinereus* (Schaeff) S. Gray s.lat. CULTIVATED ON GRASSES SUPPLEMENTED WITH COW DUNG MANURE

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ABSTRACT

This study is the first broad investigation of antimicrobial activity of different development stages of *Coprinus cinereus* from Tanzania. The indigenous edible wild *C. cinereus* mushroom was successfully grown in tropical conditions on dried grasses supplemented with different amounts of cow dung manure. Ethyl acetate crude extracts were prepared from different developmental stages of the mushroom, and were screened for antifungal and antibacterial activities using agar well method. Different extracts from black caps, post capping stage and black stem exhibited activity against *Escherichia coli, Candida albicans* and *Aspergillus niger* none of the gram positive tested bacteria was inhibited to grow. Differences in the substrate composition presented differences in the bioactivity of the mushroom extracts with 2: 3 ratio of cow-dung: grasses producing the highest activity. The results obtained clearly indicated that Tanzanian *C. cinereus* extracts contains bioactive components and are potential sources of antimicrobial compounds that could be used for development of new drugs for the treatment and prevention of diseases.

Keywords: *Coprinus cinereus*, antimicrobial activity, crude extracts, mushroom cultivation, grasses, cow dung.

INTRODUCTION

The widespread occurrence of biological activities within the fungal kingdom is now widely accepted. Since ancient times the so called mushrooms meaning Basidiomycota has been used for medicinal purpose. Fungal bioactive metabolites can be obtained from many origins either wild and cultivated fruiting bodies or from mycelial biomass and supernatant of submerged cultured using bioreactors. Tanzania has one of the highest diversity of indigenous wild mushrooms (Buyck *et al*., 2000; Harkonen *et al*., 1995, 2003); however, mushroom eating habit is more in rural areas and relies exclusively on collection from the wild. Species commonly eaten in Tanzania belong to a few genera of Ectomycorrhizal (e.g., *Amanita* and *Cantharellus*,), termite partners (e.g., *Termitomyces*) and saprophytic (e.g., *Auricularia*, *Coprinus*, *Oudemansiella* and *Pleurotus*) (Harkonen *et al*., 1995). The saprophytic mushrooms are cultivated on organic wastes. In Tanzania there has been some development recently in the domestication of the saprophytic mushrooms. *Coprinus*, *Oudemansiella*, *Volvariella* and *Pleurotus* are probably the only wild Tanzanian mushrooms genera known domesticated and cultivated on some agro-industrial residues and wild grasses (Kivaisi *et al*., 2003; Magingo *et al*., 2004; Mshandete and Cuff, 2008). This is good news not only for food security but also for the availability and sustainability of the mushrooms' genetic resource for other uses such as source of medicinal ingredients.

Coprinus cinereus belongs to the genus of edible medicinal mushrooms *Coprinus* which is easy to cultivate and excellent in flavour. The genus *Coprinus* is considered to be with well over 100 species distributed from the Northern Hemisphere to South Africa, and also appears to have been introduced to Australia, New Zealand and Iceland (Crowe 1983; Reid and Eicker 1999; Keirle *et al*., 2004, Uljé 2005). *C. cinereus* is often seen growing on lawns, along gravel roads and waste areas. The young fruiting bodies first appear as white cylinders emerging from the ground, then, the bell-shaped caps open out. The gills beneath the cap are white, then pink, then turn black and secrete a black liquid filled with spores (hence the "ink cap" name). This mushroom is unusual because eventually it turns black and dissolves itself in a matter of hours after being picked or depositing spores. When young it is an excellent edible mushroom provided that it is eaten soon after being collected (it keeps very badly because of the auto digestion of its gills and cap). A study by Hong *et al*. (2004) found that the shaggy ink cap of *Coprinus* kills nematode species *Panagrellus redivivus* and *Meloidogyne arenaria*.

Extracts from several members of the genus *Coprinus* are known in many parts of the world to exhibit among others, antimicrobial, antitumor, hypoglycemic, antinematodes, and antioxidant effects. Examples of the bioactive compounds that have been reported present in *Coprinus* mushrooms extracts are: a broad spectrum bioactive indole compound tryptamine (Worthen *et al*., 1962), micaceol (a sterol) with antibacterial activity against the bacteria *Corynebacterium xerosis* and *Staphylococcus aureus* (Zahid *et al*., 2006), and (Z,Z)-4-oxo-2,5-heptadienedioic acid, which has inhibitory activity against glutathione S-transferase, an enzyme that has been implicated in the resistance of cancer cells against chemotherapeutic agents, especially alkylating drugs (Zahid *et al*., 2006). Although *C. cinereus* is one of worldwide know medicinal mushrooms and grows widely in Tanzania there was no scientific study before the

present on the medicinal potential of the Tanzanian *C. cinereus* variety. The objectives of this study were to cultivate *C. cinereus* and evaluate antibacterial and antifungal activities produced by *C. cinereus* at different developmental stages. Cultivation of this mushroom requires waste organic substrate, which in Tanzania can be found in most areas rotting and polluting the environment, thus the study has potential impact on the control of wastes in the environmental. The results of this study are also significant towards commercial cultivation of *C. cinereus* and in the medicinal industry by broadening the knowledge of discovering new drugs, which can essentially treat various resistant human diseases. Thus with the findings of this study, C. *cinereus* is hereby promoted as a potential source of medicines, income and food particularly for use in the formulation of nutraceuticals and functional foods.

MATERIALS AND METHODS

Coprinus cinereus

Coprinus cinereus (Schaeff) S. Gray s. lat. inoculum was obtained from the authors' collection at the Department of Molecular Biology and Biotechnology of the University of Dar es Salaam, and cultivation was done according to Mshandete and Cuff (2008).

Spawn preparation

Spawn was prepared with intact sorghum grains, which were bought from Kariakoo market in Dar es Salaam. The grains were first soaked in water overnight and thereafter parboiled for 10 min. After draining excess water, 1% (w/w) of calcium carbonate ($CaCO_3$) was added and properly mixed into the grains before spreading them out on a clean plastic sheath. After air-drying for about 20 min, 150 g of the grains were packed in 330 ml wide mouth bottles (Kioo Ltd, Dar es Salaam) and sterilized in an autoclave (Koninklijke AD Linden JR.BN-Zwijinderect, Holland) at 121°C and 1 atm for 1 h. Thereafter, each cooled bottle of sterilized grains was aseptically inoculated with three 1 cm^2 pieces of mycelium malt extract agar (MEA) taken from 4 day-old cultures. Each inoculated bottle, with its cap closed, was shaken thoroughly by hand to distribute the mycelia to the grains. Before use the bottles were incubated with their caps loosely in a ventilated incubator (Memmert GmbH KG, Schwabach FRG, Germany) set at 28°C for 10 days.

Preparation of substrates and cultivation of *Coprinus cinereus*

The substrates used were dry cow dung and dry grasses; the two substrates were prepared and mixed in different ratios (Table-1). Small amount of water was added during mixing to just soak and no a drop of water on squeezing the mixed substrate by hand. The mixture was then put in sterilization container pre-prepared. Each mixture composition was separated from the other by aluminium foil. The container and its content was sterilized at 121°C for 60 minutes after which was left to cool to room temperature. Different mixture composition was then transferred into rectangular plastic containers measuring 23 cm-14 cm-9 cm (length, width and height, respectively) (Cello® Domestoware (Mkate), Dar es Salaam, Tanzania. A total of 136 aeration holes of 0.7 cm in diameter and 3 cm apart were made in all the sides. Spawn-running (mycelia colonizing the substrate) and fructification (fruit body - mushroom) development were done as per Mshandete and Cuff (2008).

Table-1. Mixing ratios of dry grasses and cow dung.

Substrate	Cow dung	Dry grasses
1	10%	90%
2	20%	80%
3	30%	70%
4	40%	60%
5	50%	50%
6	60%	40%

Harvesting of the mushroom

Harvesting of *C. cinereus* was done by picking the mushrooms at different stages of development: early before capping so named as pre-capping stage [PC], capping stage [Y] and after capping or post-capping stage when the fruiting bodies have turned black [K] (Figure-1). Mushrooms were harvested from the substrate, the substrate clinging to the stipe or to the volva was removed and the mushrooms in their entirety were weighed the same day.

Extraction of bioactive compounds

Freshly picked mushrooms were crushed using motor and piston, and were transferred into 250 ml conical flasks for extraction. The crushed mushroom material was soaked two-times in standard grade ethyl acetate for 12 hours and then the ethyl acetate extract was decanted in a clean conical flask. Rotary evaporation was used to concentrate the extracts. Volumes of each extract in different pre-weighed round bottom flask (x) were concentrated using rotary evaporation at constant temperature of 40°C. Each time when the solvent had been evaporated more extract was added until the whole volume was concentrated. Each round bottom flask with concentrated crude extract was weighed (y) to get the weight of the crude extract (y - x). The extracts were kept in the refrigerator for subsequent bioactivity test.

Test microorganisms and culture preparation

Gram positive bacteria *Staphylococcus aureus* (ATCC 25923), Gram negative bacteria *Escherichia coli* (DSM 1103) and *Pseudomonas aeruginosa* (DSM 1117) and the fungi *Cryptococcus sp, Aspergillus niger, Candida albicans* (ATCC 90028) were used to screen for the antimicrobial activity of *C. cinereus* extracts. The test microorganisms were obtained from the Department of

Molecular Biology and Biotechnology, University of Dar es Salaam. Nutrient Agar (LAB MTM, Lancashire, UK) and Malt Extract Agar (PRONADISA®, Conda Ltd. Madrid, Spain) were prepared for bacteria and *Candida* respectively according to the manufacturers' instructions. Immediately after autoclaving, the media was allowed to cool in a 45 to 50^{o}C water bath. The freshly prepared and cooled media was poured into glass, flat-bottomed petri dishes (90mm in diameter) placed on a level, horizontal surface (FASTER® Laminar flow, Cornaredo via Merendi, 22 20010, Italy) to give a uniform depth of approximately 4 mm. The agar media was allowed to cool and solidify at room temperature. About 0.2ml of the test inoculum was evenly spread on the surface of the solidified agar media using a sterile grigalsky spatula and antimicrobial test was done as explained below.

Antimicrobial activity tests

Antimicrobial activity of the crude extracts was determined by Agar well assay methods as previously described by Rojas *et al*. (2006) and Moshi *et al*. (2006). The concentrated crude extracts were re-dissolved in dimethylsulfoxide (DMSO) to make 0.1mg/ml solutions. The prepared agar plates were inoculated with 200 µl bacteria/ fungi culture by spreading evenly over the surface of agar plate using an ethanol flamed glass Drigalsky spatula (spreader). An un inoculated untreated agar plate was incubated at 37^{o}C for 24 hours before use to ensure sterility. Wells of 5 mm in diameter and 4 mm in depth were made on the agar using a sterile cork borer (Figure-2). For each test microorganism, 25 µl of each extract and of control were pipetted into different wells (Rojas *et al.,* 2006). The wells were then labeled to correspond with the code numbers of the test crude extracts and controls. The treated plates were stored in a refrigerator (DAEWOO®, Daewoo Electronics, Europe GmbH, Germany) at 4^{o}C for at least six hours to allow diffusion of the extracts into the agar while arresting the growth of the test microbes. The plates were then incubated for 24 hours at 37^{o}C for bacteria and for 48 hours at 30^{o}C for fungi. The test was carried out in triplicates. Antimicrobial activity was determined by measuring the diameters of zones of inhibition in mm. The means and standard deviations (±SD) of the diameters of zones of growth inhibitions for the treatments are shown in Table-2.

RESULTS

Coprinus cinereus yields in g of fresh mushrooms per kg of wet substrate at pre-capping stage, capping stage and post-capping stage (Figure-1) were 200, 340, and 30 respectively. A total of 7 crude ethyl acetate extracts were prepared from different developmental stages of *C. cinereus* (Figure-1). Preliminary screening revealed that crude extract from negative control samples (DMSO, ethyl acetate, sorghum only) had no growth inhibition activity thus no further antimicrobial tests were done on them.

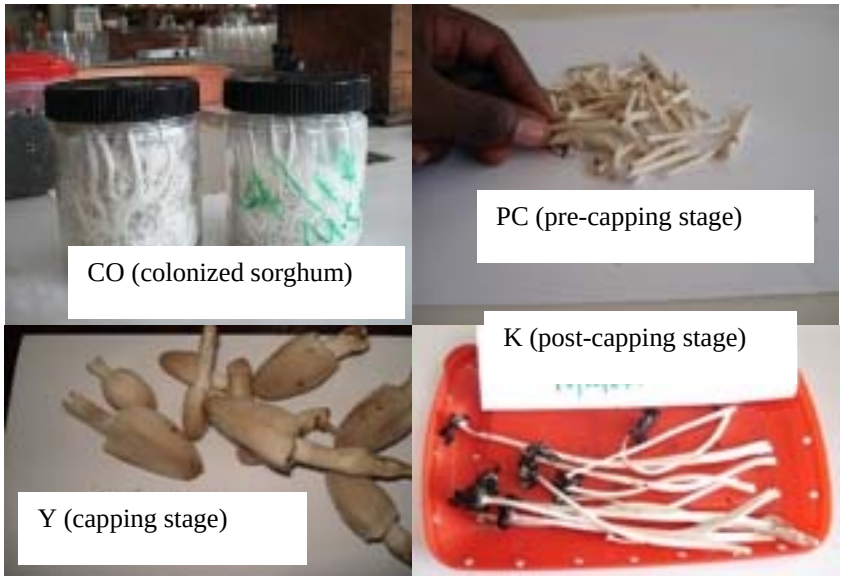

Figure-1. Photographs representing different developmental stages of *Coprinus cenereus*; colonized sorghum (CO), pre-capping stage (PC), capping stage (Y), post capping stage (K).

The test microorganisms' growth inhibition zones (Figure-2) after treatment with the mushroom extracts were measured in mm.

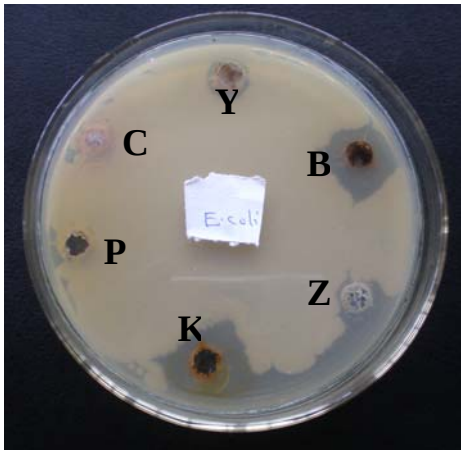

Figure-2. This shows agar plate, in which *Escherichia coli* cells were spread, wells were made and *Coprinus cinereus* colonized sorghum (CO), pre-capping stage (PC), capping stage (Y), post capping stage (K), black caps only (B), black stem only (Z) were introduced and the plate incubated at 37^{o}C for 24 hours.

The presence of anti *E. coli* compound(s) in B, K and Z has created visible zones of growth inhibition of the bacterium.

There was no activity observed for the control extract antimicrobial activity observed from the control S (uncolonized sorghum) extracts, however, some growth inhibition activity was observed from the colonized sorghum (CO) and pre-capping stage (PC) against *E. coli* only. Only extracts from capping stage (Y) and post capping stage (K, B, Z) developmental stages showed broader spectrum antimicrobial activities *E. coli, C. albicans* and *A. niger* (Figure-3). The data showed that *E. coli* was the most sensitive to the extracts from capping and post capping stages especially against the black caps of the post capping (B) with zone of inhibition of 12mm. *C. albicans* and *A. niger* were equally sensitive to the extracts from the post capping stages only with the zones of inhibition ranging from 2 mm to 8 mm. Figure-3 also shows that although the extracts from black caps and that from black stem were active against all three susceptible microbes (*E.coli*, *C.albicans* and *A.niger*), their zones of inhibition varied. The crude extracts from 40% cow dung manure supplementation shown the highest growth inhibitory activity for all the susceptible test microorganisms. No activity was observed at all against the other test miocroorganisms *S. aureus* and *Cryptococcus sp.*

Table-2. The average growth inhibition zones (in cm) formed after treatment of the susceptible microorganisms with the extracts from different developmental stages of *Coprinus cinereus* grown on dry grasses supplemented with different percentages of cow dung manure.

Developmental stages of *Coprinus cinereus*	**Susceptible test microorganism**	**Growth inhibition zones (mm) for different % of cow dung manure supplement of substrate**					
		10%	**20%**	**30%**	**40%**	**50%**	**60%**
Sorghum only (S)	*Escherichia coli*	0	0	0	0	0	0
	Aspergilus niger	0	0	0	0	0	0
	Candida albicans	0	0	0	0	0	0
Colonized sorghum (CO)	*Escherichia coli*	1	1	1	1	1	0
	Aspergilus niger	0	0	0	0	0	0
	Candida albicans	0	0	0	0	0	0
Pre-capping stage (PC)	*Escherichia coli*	1	1	1	2	1	1
	Aspergilus niger	0	0	0	0	0	0
	Candida albicans	0	0	0	0	0	0
Capping stage (Y)	*Escherichia coli*	3	2	3	5	4	2
	Aspergilus niger	0	0	0	0	0	0
	Candida albicans	1	3	2	3	2	2
Post-capping stage (K)	*Escherichia coli*	8	8	9	10	9	8
	Aspergilus niger	5	5	8	8	6	4
	Candida albicans	3	4	6	6	6	3
Black caps (B)	*Escherichia coli*	11	10	10	12	11	9
	Aspergilus niger	4	5	7	6	6	6
	Candida albicans	3	4	7	7	6	5
Black stems (Z)	*Escherichia coli*	7	7	8	8	7	7
	Aspergilus niger	3	3	3	3	2	2
	Candida albicans	2	2	3	4	2	2

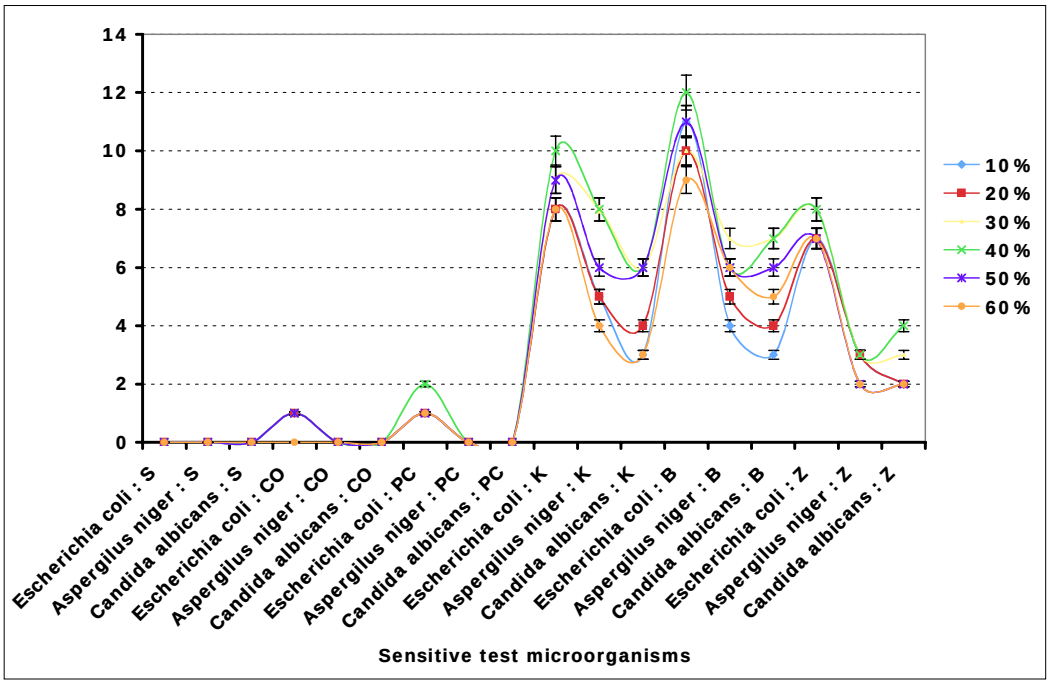

Figure-3. Demonstrating the effect of supplementing substrate with different percentages (10-60%) of cow dung on the antimicrobial activity (inhibition of microbial growth) of the extracts from *C. cinereus*. The extracts were from S (uncolonized sorghum only), CO (colonized sorghum), K (capping whole), B (black cape) and Z (black stem).

DISCUSSIONS

Coprinus cinereus colonization time was 8 days, this result agrees with the findings of Mshandete and Cuff (2008) who reported that the mycelia colonization time on different substrates was 7±1 days. Mycelia of *Coprinus* species seem to colonize various organic materials possibly due to presence of extensive enzyme systems capable of utilizing complex organic compounds, which occurs in organic matter residues (Stamets and Chilton, 1983; Tisdale *et al*., 2006; Atikpo *et al*., 2008). The length of the fruiting period was short about 12 days, which agrees with Mshandete and Cuff (2008) who reported fruiting period for *C. cinereus* to be between 11-12 days.

The results in Figure-3 clearly show that there was no activity from sorghum only, as a control. This implies that the growth inhibition activities observed were due to active substances present in the *C. cinereus* mushroom tissues. In recent years, many new secondary metabolites from higher fungi have been isolated (Zhong J-J, Xiao J-H, 2009) and are more likely to provide lead compounds for new drug discovery, which may include chemo preventive agents possessing the bioactivity of antimicrobial, immunomodulatory, anticancer, etc. *C. cinereus* was observed to produce most bioactivity at post caping stages when the fruiting body has turned black or when growth was restricted (Figure-3). The fact that the ethyl acetate crude extracts from post capping stages presented activity than other stages agrees with some literature (Abraham, 2001), which reported that most active metabolites are produced when normal growth is restricted.

Substrate concentration ratio also has greater effect on the growth and efficacy of mushrooms (Mshandete and Cuff, 2007). In this study a substrate ratio that consisted of 40% cow dung and 60% grasses showed best result (Figure-3). There is no comparable study available in the literature and this is a novel finding which could be useful in use in the formulation of nutraceuticals and functional foods

E. coli was the most susceptible to the *C. cinereus* extracts treatment (Figure-3) and this is a crucial finding because *E. coli* is one of the causes of neonatal meningitis, intestinal and urinary track infections (Ryan, 1984; Madgan *et al.*, 2000). The antibiotic sensitivities of different strains of *E. coli* vary widely. As Gram-negative organisms, *E. coli* are resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotics which, may be used to treat *E. coli* infection include amoxicillin as well as other semi-synthetic penicillins, many cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides. However, antibiotic resistance of *E. coli* is a growing problem due to overuse of antibiotics in humans and the use of antibiotics as growth promoters in food of animals (Johnson *et al*., 2009). A study published in the journal Science in August 2007 found that the rate of adaptative mutations in *E. coli* is "on the order of 10^5 per genome per generation, which is 1,000 times as high as previous estimates," a finding which may have significance for the study and management of bacterial antibiotic resistance (Perfeito *et al*., 2007). Antibiotic-resistant *E. coli* may also pass on the

genes responsible for antibiotic resistance to other species of bacteria, such as *Staphylococcus aureus*. *E. coli* often carry multi drug resistant plasmids and under stress readily transfer those plasmids to other species. Indeed, *E. coli* is a frequent member of biofilms, where many species of bacteria exist in close proximity to each other. This mixing of species allows *E. coli* strains that are piliated to accept and transfer plasmids from and to other bacteria. Thus *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance (Salyers *et al*., 2004). There, finding a new effective agent to inhibit *E. coli* growth is crucial with regard to eradication of the diseases caused by the bacterium infections.

Inhibition of *C. albicans* and *A. niger* suggests that *C. cinereus* produce antifungal secondary metabolites too. The nominal susceptibility of *C. albicans* and *A. niger* to the mushroom extracts could be associated with the presence of supramolecular complexes of their cell wall including chitin, which may be difficult to digest, thus imposing resistance to prospective drugs (Marcilla *et al*., 1991). *C. albicans* is yeast that causes vaginal, oral, or lung infections and, in acquired immunodeficiency (AIDS) patients, systemic tissue damage. There was no activity against the Tanzanian isolated marine yeast *Cryptococcus sp.*, and the mechanism for the resistance is not known.

In the cases where extracts from three different extracts of *C. cinereus* depicted activity against the same microorganism, would possibly mean that the compound responsible for the antimicrobial activity was present in each extract at a different concentration as similarly observed by Rojas *et al*. (2006). However, it is imperative to note that the therapeutics potential of *C. cinereus* could be due to synergistic action of the various compounds present in the mushroom.

The gram-positive bacteria *Staphylococcus aureus* was not sensitive to the mushroom extracts. *S. aureus* is well known to be resistant to many commonly used antibiotics. Since the emergence of penicillinase-producing *S. aureus* in the 1940s, it has proved itself adept at developing or acquiring mechanisms that confer resistance to all clinically available antibacterial classes through the emergency of strains that are methicillin-, glycopeptide-, penicillin-, and vancomycin resistant (Woodford, 2005; Lubowitz and Poehling, 2008). The lack of sensitivity to the *C. cinereus* mushroom extracts by *S. aureus* may also be due to their cell wall composition of peptidoglycan layer,which is much thicker. Imbedded in the Gram positive cell wall are polyalcohols called teichoic acids, some of which are lipid-linked to form lipoteichoic acids. Because lipoteichoic acids are covalently linked to lipids within the cytoplasmic membrane they are responsible for linking the peptidoglycan to the cytoplasmic membrane. Teichoic acids give the Gram positive cell wall an overall negative charge due to the presence of phosphodiester bonds between teichoic acid monomers. All these factors may lead to impermeability of the cell wall against *C. cinereus* extracts.

Extraction of compounds mainly depends on the polarity and solubility of that compound to the solvent "like dissolve like". Ethyl acetate as a solvent extracts none polar compounds; the present study results therefore suggest that the active compounds present in the crude extracts composed mainly of non-polar ones. Studies aiming at purification, isolation and characterization of the active compounds from the *C. cinereus* most active extracts identified in this study are currently under way.

The fact that substrate composition had an effect on potency of *C. cinereus* extracts against some test microbes justifies the rationale for use of mixed substrate for increasing the bioactivity of the extracts for medicinal purpose. To the best of our knowledge, this is the first report on biological assays of *C. cinereus* an indigenous species of Tanzania. The present findings can serve to stimulate further investigation to stimulate mass domestication of this rich source of bioactive secondary metabolites for the use as therapeutic, nutrition and income generation, and thus be incorporated into the MKUKUTA policy. MKUKUTA is a Kiswahili acronym for the National Strategy for Growth and Reduction of Poverty. This strategy is the development framework for the current five-year phase (2005-2010). It forms part of Tanzania's efforts to deliver on its national Vision 2025. The focus is outcome orientated and organized around three clusters: growth and reduction of income poverty, improved quality of life and social well being, and governance and accountability. In conclusion, this study unveils rich resources of bioactive mushroom in Tanzania and serves as a baseline for more research on mushroom natural products

ACKNOWLEDGEMENTS

Thanks to the Department of Molecular Biology and Biotechnology of the University of Dar es Salaam and the Higher Education Student Loan Board (HESLB) for financial support.

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