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Full Length Research Paper

Improved antimicrobial activity of the Tanzanian edible mushroom *Coprinus cinereus* (Schaeff) Gray by chicken manure supplemented solid sisal wastes substrates

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The Tanzanian edible mushroom species *Coprinus cinereus* was grown on sisal waste substrates supplemented with chicken manure with the aim to evaluate the effects of the chicken manure supplement on the antimicrobial activity of the mushroom's extracts. Crude ethyl acetate extracts were prepared from the mushroom's fruiting bodies harvested at pre-capping, capping and post capping stages, and the extracts were tested for antimicrobial activity, using the agar well method. The antimicrobial activity was observed only in capping and post capping stages of the mushrooms and the activity generally increased with increased percentage of manure supplementation. These findings show that Tanzanian edible *C. cinereus* mushroom contains antimicrobial compounds and chicken manure could be used in the cultivation of the mushroom to increase the production of active secondary metabolites, which could be used as lead compounds for discovery of new and more effective drugs against microbial infections.

Key words: *Coprinus cinereus*, antimicrobial, chicken manure.

INTRODUCTION AND LITERATURE REVIEW

Coprinus cinereus belongs to a small genus of mushrooms, Coprinus, in a black spored family Coprinaceae. All species go through an auto digestion at maturity in which the cap forms black spores that become a soupy black glob. *C. cinereus* has several close relatives, which are *Coprinus sterquilinus*, *Coprinus calyptratus*, *Coprinus spadiceisporus* and *Coprinus comatus* (Philips, 1981). However, the classification of the Coprinus species is still unclear because it was only based on morphological characteristics without molecular analyses. As a result, *C. cinereus* has been given different names by different scientists such as *Coprinosis cinerea* or *Coprinus macrorhizus* (Ohtsuka et al., 1973). Some Tanzanian indigenous strains of *Coprinus* were reported for the first time by Härkönen et al. (2003), also based on the morphological caharacteristics. Mshandete and Cuff (2008) reported for the first time, the successful domestication of wild edible mushroom beleaved to be *C. cinereus* indigenous to Tanzanian. Recently, we showed that the Tanzanian *C. cinereus* can also grow on dried grasses supplemented with cow dung manure (Ndyetabura et al., 2010).

Mushrooms are important sources of medicines and nutritive proteins and minerals (Bahl, 1994). Many cultures worldwide recognize that extracts from certain mushrooms could have profound health promoting benefits and consequently, became essential components in many traditional medicines. There are at least 270 species of mushrooms that are known to possess various therapeutic properties (Ying et al., 1987) and hence the term "medicinal mushroom". Examples of edible mushroom genera mentioned by Ying et al. (1987), which demonstrate medicinal or functional properties are Lentinula, Hericium, Grifola, Flammulina, Coprinus and

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Pleurotus.

Whereas the majority of fungi produce very similar primary metabolites, secondary metabolites are more species–specific and products are often unique to a particular species (Isaac, 1997). For this reason it is likely that, although a very large number of fungal secondary metabolites have now been identified, many more will be described in future as the activities of more fungal species are investigated. By manipulation of the growth conditions, it is possible for the production of a secondary metabolite to be prevented or a fungus be induced to over-produce some of these compounds.

Different Coprinus species have been found to have medicinal and bioactive compounds by several researches. Polysaccharides extracted from the mycelia culture of *C. cinerea* (as *C. macrorhizus*) have been shown to contain antitumor effects (Ohtsuka et al., 1973). Polysaccharide solutions extracted from *C. comatus* and given to mice had the ability to increase serum lysozyme activity, which is used as a general indicator of immune system fitness. In addition to breaking down polysaccharides found in bacterial cell walls, lysozyme can also bind to the surface of some invading bacteria and make it easier for white blood cells to engulf them (Li et al., 2001). Water extract of *C. comatus* has been identified as containing potent antitumor compounds for breast cancer (Gu and Leonard, 2006). In the more recent study, it was found that the Tanzanian *C. cinereus* grown on dried grasses supplemented with cow dung manure exhibits activity against *Escherichia coli*, *Aspergilus niger* and *Candida albicans*, with the highest activity observed at 40% of cow dung manure supplementation (Ndyetabura et al., 2010). The potential of the Tanzanian *C. cinereus* as a possible source of bioactive compounds which could be used as lead compounds for drug discovery needs to be researched further. Varying the growth environmental conditions, such as supplementing the mushroom growing substrates with chicken manure, could lead the mushroom to produce secondary metabolites of medicinal or commercial significance.

According to 2002 census, Tanzania had 47 million chickens out of them 27 million were free-range chicken (*Gallus gallus* domesticus). Average manure production per chicken is 139 g per day with dry matter content of 22% (Thomsen, 2004) . Based on 27 million free-range chicken populations in Tanzania, about 3,753 tons (826 tons dry matter) chicken manure can be generated per day equivalent to 1,369,845 tons (301,366 tons dry matter) per annum. Chicken manure has a high nitrogen and phosphorus contents; fresh chicken manure has 0.9% while on dry weight basis is 4.5% (Thomsen, 2004).

In the present study, crude ethyl acetate extract of *C. cinereus* grown on sisal wastes supplemented with different amounts of chicken manure was prepared and then antimicrobial activity was determined on the crude extracts by agar well method.

MATERIALS AND METHODS

C. cinereus

C. cinereus inoculum was obtained from the 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 of *C. cinereus* mushroom were 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 (300 ml jar, 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 1 cm^2 pieces of mycelium MEA taken from 4 to 7- day-old cultures of *C. cinereus*. The inoculated bottles, with caps closed, were 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 *C. cinerius*

Diferent types of sisal wastes namely: sisal leaf decortication waste (also referred to as sisal leaves) and sisal fibre dust (also called sisal dust) were used each supplemented with chicken manure at 5, 10, 15, 20 and 25%. *C. cinereus* fruit bodies were harvested at capping and post capping developmental stages. 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 1 h after which it was left to cool to room temperature. Different mixture composition was then transferred into different plastic dishes with small hole on their sides and covers. Spawn-running (mycelia colonizing the substrate) and fructification (fruit body - mushroom development) were done as per Mshandete and Cuff (2008).

Harvesting of the mushroom

C. cinereus fruit bodies were harvested at young or pre-capping stage, firm and fleshy (immature/juvenile stage) or capping stage and when the mushroom caps turned into an inky mass considered matured or post capping stage. 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

Extraction of bioactive compounds was done according to Ndyetabura et al. (2010) . Freshly picked mushrooms were crushed using motor and piston, and transferred into 250-ml conical flasks for extraction. The crushed mushroom material was soaked twice

in standard grade ethyl acetate for 12 h and then the ethyl acetate extract was decanted in a clean conical flask. 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 immediately tested for bioactivity or kept in the refrigerator for subsequent bioactivity test.

Test microorganisms and culture preparation

The test microorganisms used were bacteria *Pseudomonas aeuroginosa* and *E. coli* (Gram negative), *Staphylococcus aureus* (Gram positive) and one fungi *Candida albicans*. 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°C water bath. The freshly prepared and cooled media was poured into glass, flat-bottomed Petri dishes (90 mm 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 0.2 ml 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 shortly.

Antimicrobial activity tests

Antimicrobial activity of the crude extracts was tested according to Ndyetabura et al. (2010) by agar well assay methods as previously described by Rojas et al. (2006), Moshi et al. (2006). The concentrated crude extracts were re-dissolved in dimethylsulfoxide (DMSO) to make 0.1 mg/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) . Un inoculated untreated agar plate was incubated at 37°C for 24 h 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.

For each test microoganism, 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°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 h at 37°C for bacteria and for 48 h at 30°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 of the diameters of zones of growth inhibitions for the treatments are shown in Table 1.

RESULTS

Antimicrobial activity was observed only in capping and post capping stages of *C. cinereus* mushrooms extracts and the mocrobial growth inhibition zones (in mm) are presented in Table 1. Figure 1 shows that the sisal leaves and sisal dusts without chicken manure supplementation produced *C. cinereus* mushroms whose extracts exhibited activity only against *E. coli* (Figure 1a to d) and *C. albicans* (Figure 1b). *P. aeuroginosa* and *S. aureus* growth was inhibited only after supplemention with chicken manure *P. aeuroginosa* being sensitive to broader levels of the chicken manure. The activity generally was increased with increased percent of supplementation (Figure 1a to d).

Figure 2 shows that the capping stage of *C. cinereus* mushrooms grown on sisal dust exhibited the highest antibacterial activity 17 mm for *E. coli* at 20% chicken manure supplimentation (Figure 2a), 16 mm for *S. aureus* (Figure 2c) and 17 mm for *P. aeuroginosa* at 25% chicken manure (Figure 2d). For *C*. *albicans* (fungi) the highest growth inhibition zone was 19 mm observed from the post capping stage of the mushrooms grown on sisal leaves (Figure 2b) . E. coli was the most sensitive inhibited to grow by mushroom extracts from all levels of chicken manure supplements (Figure 2a) whereas *S. aureus* was the least sensitive inhibited to grow only by mushroom extracts from higher levels of chicken manure supplements (Figure 2c). At 10% chicken manure supplementation a reduced activity for *E. coli* (Figure 2a) or no activity for all other test microbes was observed (Figure 2b to d). Both Figures 1 and 2 show that, the mushrooms grown on sisal dust had broader spectra and higher antimicrobial activity than those grown on sisal leaves.

DISCUSSION AND CONCLUSION

Crude ethyl acetate extracts from mature stages of *C. cinereus* exhibited antimicrobial activity (Table 1) whereas younger stages did not. This observation agrees with the previous study by Ndyetabura et al. (2010), which showed that only extracts from capping stage and post capping stage of *C. cinereus* development exhibited antimicrobial activities against *E. coli*, *C. albicans* and *A. niger*. This observation also agrees with some older literature (Isaac, 1997; Abraham, 2001) which reported that most active secondary metabolites produced at the end of active growth are derived from primary products which were synthesized earlirer, and their formation may accompany differentiation and sporulation in the fungus. It is also true that when growth becomes restricted by some factors, products and intermediate compounds will then accumulate in their bodies or culture medium. This can be brought about by manipulation of the particular nutrients provided for growth or the provision of specific environmental conditions (Isaac, 1997). It is this attribute which is exploited for the commercial production of useful primary and secondary fungal products.

Table 1. The growth inhibition zones (in mm) formed after treatment of the test micro-organisms with the extracts from *C. cinereus* grown on sisal wastes supplemented with different percentages of chicken manure.

Sisal waste/developmental stages of *C. cinereus*	Test micro-organism	Mean of growth inhibition zone (mm)						
		Control	Chicken manure supplements (%)					
			0	5	10	15	20	25
Dust / Capping	*S. aureus*	0	0	0	0	0	0	16
	P. aeuroginosa	0	0	12	0	9	1	17
	E. coli	0	4	12	11	1	17	16
	C. albicans	0	0	0	0	0	0	9
Dust/ Post capping	*S. aureus*	0	0	0	0	7	12	0
	P. aeuroginosa	0	0	14	0	15	12	11
	E. coli	0	5	9	3	10	9	7
	C. albicans	0	8	3	0	8	13	0
Leaves/ Capping	*S. aureus*	0	0	0	0	0	13	0
	P. aeuroginosa	0	0	0	0	5	15	0
	E. coli	0	5	1	0	10	10	0
	C. albicans	0	0	0	0	0	5	19
Leaves/ Post capping	*S. aureus*	0	0	0	0	0	0	0
	P. aeuroginosa	0	0	8	0	0	16	0
	E. coli	0	5	9	4	11	10	9
	C. albicans	0	0	0	0	0	5	0

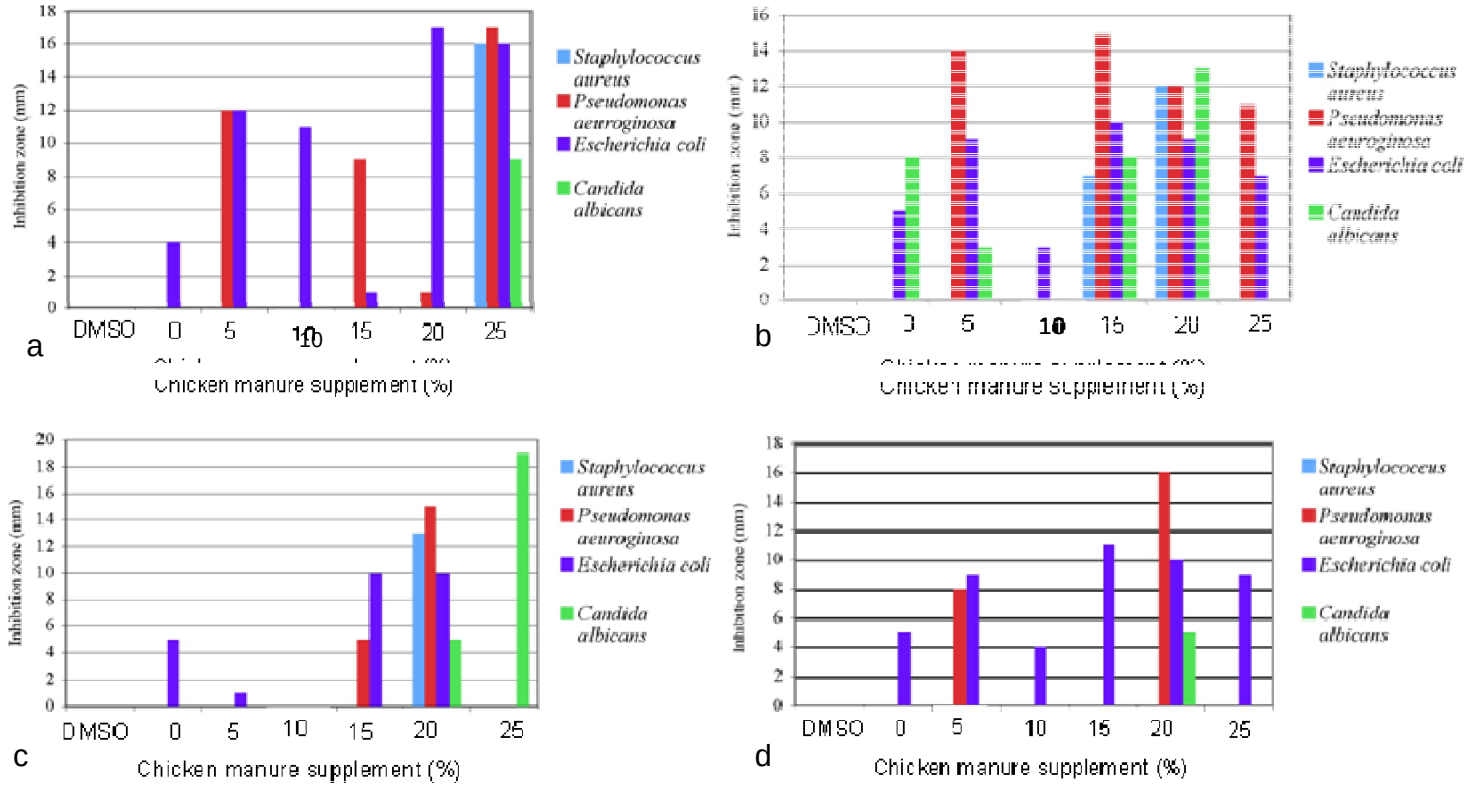

Figure 1. Effects of substrate types and developmental stages of *C. cinereus* on the antimicrobial activity: (a) Activity of *C. cinereus* capping stage grown on sisal dust, (b) activity of *C. cinereus* postcapping stage grown on sisal dust, (c) activity of *C. cinereus* capping stage grown on sisal leaves and (d) activity of *C. cinereus* postcapping stage grown on sisal leaves.

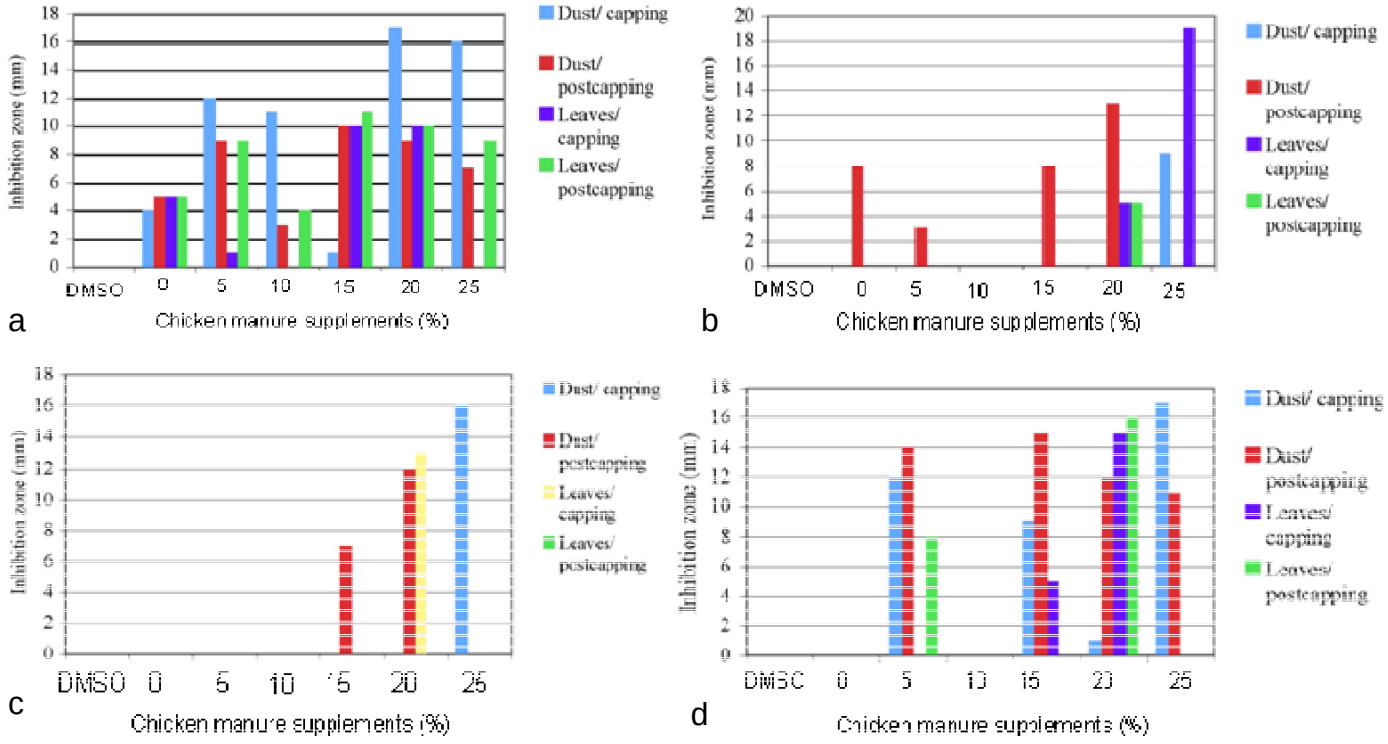

Figure 2. Comparing sensitivity of different microorganisms to *C. cinereus* extracts: (a) sensitivity of *E. coli* to *C. cinereus* extracts, (b) sensitivity of *C. albicans* to *C. cinereus* extracts, (c) sensitivity of *S. aureus* to *C. cinereus* extracts, and (d) sensitivity of *P.aeuroginosa* to *C. cinereus* extracts.

The antimicrobial activity of extracts from *C. cinereus* grown on sisal waste without manure supplements against *E. coli* and *C. albicans*, strongly suggest that the Tanzanian *C. cinereus* produces bioactive secondary metabolites naturally. This observation agrees with the study by Ndyetabura et al. (2010) again which showed that *C. cinereus* exhibited antimicrobial activity against all Gram-negative bacteria and all fungi species tested, including *E. coli* and *C. albicans*. The observation also agrees with previous studies elsewhere which showed that Coprinus representatives are a possible source of antibiotics (Zenkova et al., 2003). Zenkova et al. (2003) study showed that, the Coprinus representatives were able to inhibit growth of all, common Gram- negative bacteria, Gram-positive bacteria and fungal strains contrary to Ndyetabura et al. (2010). The environmental growth conditions of the Tanzanian *C. cinereus* is different from where the study by Zenkova et al, took place, leading to different types and rate of biochemical processes (Sher et al., 2010) and so different types and/or quantities secondary metabolites were produced. The present study therefore shows that, supplementation of chicken manure had positive effects on the antimicrobial activity of the Tanzanian *C. cinereus* regardless of whether Gram-pisitive or Gram-negative bacteria or *C. albicans* was used and generally, the inhibition zones increased with increase in percentage of chicken manure supplementation. These observation could be due to the fact that, increasing amount of chicken manure supplementation raises amounts of nutrients in the substrates which enhance the metabolic reactions that are taking place in the mushroom and therefore increase the production of secondary metabolites. The present study shows that, addition of free-range chicken droppings manure to the sisal waste substrate led to the production of active metabolites against Gram-positive bacteria *S. aureus* and *P. aeuroginosa* which were previously not sensitive to the mushroom extracts at all. The availability of high nitrogen and phosphorus contents 4.5 to 7.5% on dry weight basis of chicken manure (Nahm, 2003; Thomsen, 2004; Chen et al., 2009), Increased biochemical processes in the growing mushrooms, leading to the production of new types of or higher quantities of the existing active secondary metabolites. In addition, free range chickens eat a whole range of complex combination of different floral, fauna and innorganic material from their environments and so the manure may contain a variety of new other molecules which eventually led to the activation of other different pathways, leading to the production of active secondary metabolites. This study provides a baseline for scientist to unraval more facts on the subject. Based on the 301,366 tons of dry matter chicken manure per annum produced by free-range

chicken populations in rural Tanzania (Thomsen, 2004), it is obvious that this novel use lead to income generation and improved health standards in communities.

More studies are underway to shed light on the mechanisms of the observed activities and will be published in the next article. It may be difficult to explain the differences in the sizes of the microbial growth inhibition zones among the different extracts because concentrations of active components in the crude extracts are not known yet, and compounds present in some of the crude extracts are unknown yet. However, the observed difference in the activities could also be due to differences in diffusion of an antimicrobial through the solidified medium, which could be the result of molecular size or chemical nature of the active compounds. Small molecules diffuse more easily than large molecules (Hewitt, 1977). The reason to why at 10% chicken manure supplementation, a reduced activity for *E. coli* (Figure 2a) or no activity for all other test microbes (Figure 2b to d) is a subject of further investigation. Mshandete and Cuff (2008) reported for the first time, the suitability of sisal organic waste as substrate for cultivation of local edible *C. cinereus*, *P. flabellatus* and *V. volvacea* mushrooms. The present study shows for the first time, the increased antimicrobial effects of *C. cinereus* by chicken manure supplements on non-composted sisal leaves decortications residue and sisal dust. However, the reasons for the observed broader spectra and higher activity of the mushrooms grown on sisal fiber dust than those grown on the sisal leaves remain to be investigated.

In conclusion, *C. cinereus* grown on sisal wastes without chicken manure supplementation exhibited activity against *E. coli* only, whereas for *S. aureus* and *P. aeuroginosa* inhibiton zones were observed after supplementation with chicken manure. The activity increased with increase in percentage of manure supplementation. These findings show that the Tanzanian *C. cinereus* mushrooms contain antimicrobial compounds and that chicken manure could be used in the mushroom cultivation to increase the production of active secondary metabolites, which could be used as lead compounds for discovery of new and more effective drugs against microbial infections. By manipulation of the growth conditions, it is possible for the production of a secondary metabolite to be prevented or a fungus may be induced to over-produce some of these compounds.

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