Bioaccumulation of heavy metals together with medicinal properties of *Pleurotus* spp cultivated on agro-industrial substrates supplemented with wheat bran and maize flour

by

Senzosenkosi Surprise Mkhize

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PREFACE

The research was conducted and completed within the Discipline of Biochemistry, School of Life Sciences, College of Agriculture, Engineering, and Science, at the University of KwaZulu-Natal, Westville, South Africa. The candidate conducted the research with assistance and guidance fromthe supervisors (Dr. O.J Pooe and Prof. M.B.C Simelane). Financial support was received fromthe National Research Foundation (NRF), FoodBev SETA, and the UKZN research office.

This work has not been submitted or presented in any form for any degree or diploma to any other University. Where the work involved the use of work from others, it was duly acknowledged within the text in the form of references.

[redacted] …

Candidate: Senzosenkosi Surprise Mkhize

[redacted] …

Supervisor: Dr. O.J. Pooe

[redacted] …

Co-supervisor: Prof. M.B.C Simelane

DECLARATION 1: PLAGIARISM

I, Senzosenkosi Surprise Mkhize, declare that:

1. The research reported in this thesis, except where otherwise indicated or acknowledged,is my original work.
2. This thesis has not been submitted in full or in part for any degree or examination to anyother university.
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5. This thesis is primarily a collection of material, prepared by me, submitted for publication or presented at conferences. In some cases, additional material has been included.
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DECLARATION 2- PUBLICATIONS

This thesis consists of various manuscripts or research paper articles published in numerous journals. Each chapter was prepared according to all journal's specifications and guidelines, hence the formatting differed for every chapter.

All the work within this thesis was conducted by the candidate with assistance from the supervisors, hence the candidate was the first author of all the publications and conference proceedings.

1. **Mkhize, S. S**., Machaba, K. E., Simelane, M. B. C., & Pooe, O. J. (2022). Mushroom Derived Products as an Alternative Antimalarial Therapeutics: A Review. In: Kendrekar, P. (ed.). Drug Development for Malaria: Novel Approaches for Prevention and Treatment. *Wiley*. pp. 235-249. https://doi.org/10.1002/9783527830589.ch10.

2. **Mkhize, S.S**., Simelane, M. B.C., Gasa, N.L., & Pooe, O.J. (2021). Evaluating the antioxidant and heavy metal content of *Pleurotus ostreatus* mushrooms cultivated using sugar cane agro-waste. *Pharmacognosy Journal*, 13(4), 844–852. https://doi.org/10.5530/pj.2021.13.108. (**Chapter 2**).

3. **Mkhize, S. S**., Cedric Simelane, M. B., Mongalo, I. N., & Pooe, O. J. (2022). The Effect of Supplementing Mushroom Growing Substrates on the Bioactive Compounds, Antimicrobial Activity, and Antioxidant Activity of *Pleurotus ostreatus*. *Biochemistry Research International*, 2022, 1-10. https://doi.org/10.1155/2022/9436614 (**Chapter 3**).

4. **Mkhize, S. S**., Simelane, M. B. C., Mongalo, N. I., & Pooe, O. J. (2023). Bioprospecting the Biological Effects of Cultivating *Pleurotus ostreatus* Mushrooms from Selected Agro-Wastes and Maize Flour Supplements. *Journal of Food Biochemistry*, 2023, 1-16. https://doi.org/10.1155/2023/2762972. (**Chapter 4**).

5. **Mkhize, S. S**., Pooe, O. J., Khoza, S., Mongalo, I. N., Khan, R., & Simelane, M. B. C. (2022). Characterization and biological evaluation of zinc oxide nanoparticles synthesized from *Pleurotus ostreatus* Mushroom. *Applied Sciences*, 12(17), 8563. https://doi.org/10.3390/app12178563. (**Chapter 5**).

CONFERENCE PRESENTATIONS

Mkhize, S.S., Pooe, O.J., Bio-absorption of heavy metals by *Pleurotus ostreatus* grown from sugarcane waste product supplemented with wheat bran. College of Agriculture, Engineering and Science. *Postgraduate Research & Innovation Symposium 2019*, University of KwaZulu-Natal, Westville, South Africa. *Theme: Big Data and the 4th Industrial Revolution*.

Mkhize, S.S., Pooe, O.J., The effect of supplementing mushroom growing substrates on the bioactive compounds, antimicrobial activity, and antioxidant activity of *Pleurotus ostreatus*. College of Agriculture, Engineering and Science. *Postgraduate Research & Innovation Symposium 2023*, University of KwaZulu-Natal, Coastland Musgrave Hotel, South Africa. Theme: *Water for sustainability into the 21st Century*.

ABSTRACT

Over the years, mushrooms have been used as a source of food and as medicinal therapeutics, with numerous biological properties such as; antimicrobial, anticancer, hepatoprotective, and antidiabetic. Identifying optimum mushroom growing conditions and substrates may improve mushroom productivity, quality, safety, and subsequent biological properties of *P. ostreatus* mushrooms. Therefore, the current study sought to investigate the effects of supplementing the mushroom-growing substrates on the biological properties of mushrooms. The study also evaluated the ability of *Pleurotus ostreatus* to accumulate heavy metals from locally available mushroom-growing substrates. Our observations indicated that the *P. ostreatus* mushroom potentially absorbed heavy metals from all the growing substrates, indicating its potential for bioremediation. The absorption of heavy metal by *P. ostreatus* was not influenced by the type of substrates used to cultivate the mushroom. The addition of supplements significantly improved the mushroom yield, and biological properties of *P. ostreatus*. The *P. ostreatus* mushroom extracts showed significant radical scavenging activity against DPPH and ABTS. Significant antimicrobial activities against *Staphylococcus aureus* and *Escherichia coli* were observed.

Finally, the study investigated the potential of biosynthesis of zinc oxide nanoparticles (ZnONps) using *Pleurotus ostreatus* mushroom as the capping and reducing agent. The synthesized ZnONps were stable and proved to have antioxidant and antimicrobial activity against *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumonia*, and *Enterococcus faecalis*. Finally, the findings suggest that edible *P. ostreatus* mushrooms grown from supplemented substrates can potentially be used for green synthesis of ZnONPs, and also as an alternative source for antioxidant and antimicrobial products.

Keywords: *P. ostreatus*, Substrates, Nanoparticles, antioxidant, antimicrobial, supplements

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
µg	Microgram
µl	Microlitre
AAS	Atomic Absorption Spectrometry
ABTS	2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid
Ac	Absorbance of the control
Ag	Argon
Al	Aluminium
ANOVA	Analysis of Variance
APX	Ascorbate peroxidase
As	Absorbance of the sample
As	Arsenic
AsA	Ascorbate
AsA-GSH	Ascorbate-glutahione
BHT	Butylated hydroxytoluene
C	Carbon
$CaCO_3$	Calcium carbonate
$CaSO.\ 4\cdot_2H_2O$	Calcium sulfate dihydrate
CAT	Catalase
Cd	Cadmium
Cr	Chromium
Cu	Copper
DARD	Department of Agriculture and Rural Development
DHAR	dehydroascorbate reductase
DLS	dynamic light scattering
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribose Nucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDS	Energy Dispersive Spectrometry

EDX	Energy Dispersive X-ray
eV	Electron voltage
FAO	Food and Agriculture Organization
Fe	Iron
FTIR	Fourier transform infrared
g	Grams
GCMS	Gas column mass spectrophotometry
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
Hg	Mercury
INT	p-Iodo-nitrotetrazolium viol
KZN	KwaZulu-Natal
MDHAR	Monodehydro ascorbate reductase
MF	Maize Flour
mg	Milligrams
MIC	Minimum Inhibitory Concentration
Min	Minutes
ml	Millimetres
MY	Mushroom yield
N	Nitrogen
NC	Negative control
Ni	Nickel
NIST	National Institute of Standard Technology
Pb	Lead
PC	Positive control
PDA	Potato dextrose agar
pH	Potential of hydrogen
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAED	Selected area electron diffraction

Sb	Antimony
Se	Selenium
SEM	Scanning electron microscope
SOD	superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TEM	Transmission Electron Microscopy
TF	Transfer Factor
UV	Ultraviolet
WHO	World Health Organization
XRD	X-Ray diffraction analysis
Zn	Zinc
$ZnNO_3$	Zinc nitrates
ZnO NPs	Zinc oxide nanoparticles

CHAPTER 1

Literature Review

1.1 The nature and type of mushrooms

Mushrooms have been defined in different ways, however, they are usually defined as macro-fungus with a fruiting body that is either below (hypogeous) or above (epigeous) the soil and can be large enough to be observed by the naked eye (Usha & Suguna, 2014). Mushrooms are different from bacteria, plants, and animals hence they belong to the kingdom of fungi (Holkar & Chandra, 2016). Various factors, such as cell wall composition, heterotrophic nature, and their osmotrophic absorptive nature make the mushrooms distinct from plants, animals, and microbes (Okan et al., 2014).

Mushrooms obtain nutrients by secreting acids and extracellular enzymes, that breaks down lignin, hemicellulose, and cellulose from the dead organic matter (Semwal et al., 2014). In nature, mushrooms comprise structures such as cap/pileus, stem/stipe, and gills or lamellae underneath the cap (Figure 1) (Kamalakannan et al., 2020), Their special structural feature of being parasol or umbrella-shaped, protects them from the environmental stress such as rains and sun heat (Chang, 2011). In terms of classification, they are said to belong to the higher phyla Ascomycota under the sub-division of Basidiomycota (Wu et al., 2016).

There are close to 12,000 species of mushrooms recorded worldwide, of which 2000 are regarded as being edible and or medicinal, however, only about 35 are cultivated commercially (Das et al., 2021), with only 25 species accepted as food (Valverde et al., 2015). These mushrooms are categorized into three main groups, whereby 54% belong to the edible group, 38% belong to the medicinal group and 8% belong to the wild type (Grimm & Wösten, 2018). Mushrooms such as *Agaricus bisporus*, *Agaricus blazei*, *Agrocybe aegenta*, *Flammulina velutipes*, *Ganoderma lucidum*, *Grifola frodosa*, *Lentinula edodes*, *Pleurotus ostreatus*, *Pleurotus eryngii,* are the mostly grown mushrooms due to their medicinal and nutritional values (Anusiya et al., 2021).

Among the edible mushrooms, *Pleurotus ostreatus* is recognized as one of the most cultivated and consumed mushrooms globally because of its taste, flavor, nutritional content, and mostly its medicinal attributes (Törős et al., 2022; Fufa et al., 2021). The oyster (*Pleurotus*) mushroom species have the advantages of being easily cultivated, having bioremediation potential, produce extracellular enzymes and has neutraceutical properties (Rashad et al., 2009). Furthermore, the *Pleurotus ostreatus* mushrooms have organoleptic and medicinal properties with fast growth character (Gregori et al., 2007). It is worth noting that the organoleptic and medicinal properties of *P. ostreatus* can be influenced by various conditions. Hence, Li et al., (2022) mentioned that the metabolite profiles of mushrooms are influenced by environmental conditions, however, this phenomenon needs to be further investigated.

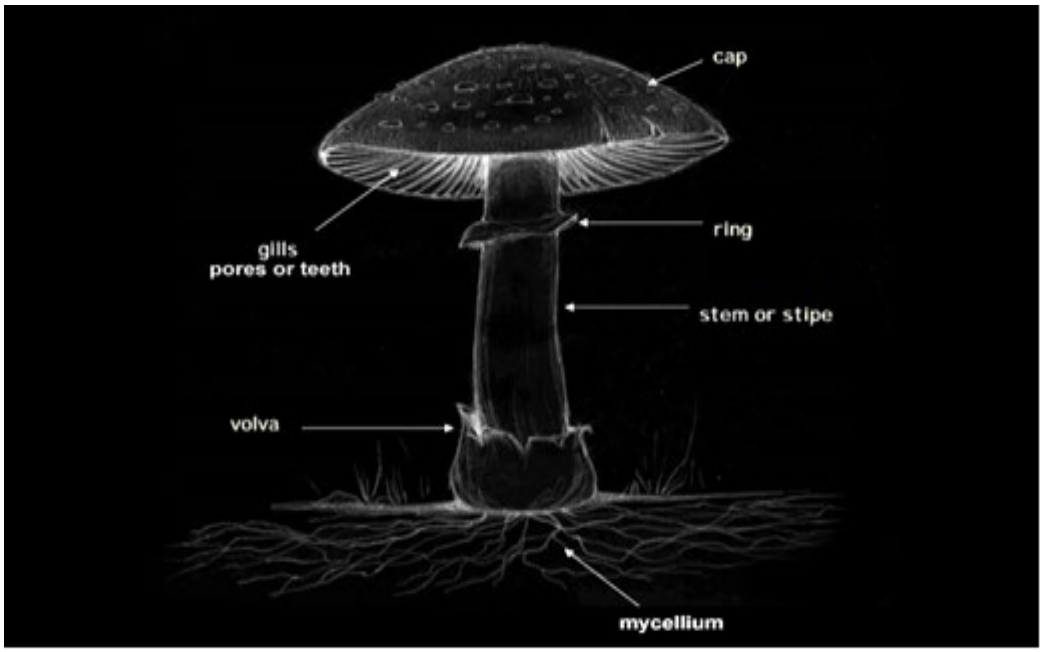

Figure 1: The general structure of mushroom. (Source: Wille & da Costa Bento, 2021).

1.2 Techno-economic value of *P. ostreatus* mushrooms

The recent technology of mushroom cultivation is a new revolution that can improve the economic and social status of poor/developing farmers by adding this nonconventional crop to their existing agricultural systems (Niazi & Ghafoor, 2021). This technology of mushroom cultivation has the advantage of requiring low capital, and low technical knowledge and produces high returns using low investments, hence it can be used to empower rural communities especially (Easin et al., 2017). Therefore, poverty could be eliminated from the grassroots level (Ferdousi et al., 2020). Furthermore, mushroom fungi add value to the bioremediation of the environment since they secrete strong extracellular enzymes that transform or degrade numerous numbers of hazardous and toxic environmental pollutants/chemicals (Adenipekun, 2012). This technology of mushroom cultivation is regarded as green technology especially since it involves the use of neglected organic biomass that practically ends up as waste/garbage if they are not converted into valuable products (Lopez et al., 2021). This process of growing *P. ostreatus* mushrooms using agro-waste has attracted great interest since after cultivation, the spent mushroom substrates can be collected and applied in the production of enzymes, biomass, feed ingredients, bioethanol, and functional foods (xylo-oligosaccharides) (Seekram et al., 2021).

1.2.1 Biomass Conversion by P. ostreatus

The disposal of large quantities of agro-industrial has been one of the major problems worldwide (Garg et al., 2012), especially since the agro-industrial wastes are not properly managed (Sadh et al., 2018), which endangers the environment and the health of the public. Hence, *Pleurotus ostreatus* mushroom could combat the above-mentioned problem since it is popularly known as the fungi that efficiently bio-convert waste from agriculture, forests, industries, and municipalities into a highly nutritious food (mushroom) (Hu et al., 2019). Thus, the cultivation of *P. ostreatus* has been expanding throughout the world (Raman et al., 2021), since *P. ostreatus* efficiently converts substrate biomass into mushroom biomass, with minor adjustments in the environmental growth conditions (Sánchez, 2010). This wood-decaying fungus only manages to

convert these lignocellulosic residues through the secretion of extracellular enzymes (Inácio et al., 2015), such as β-amylase, cellulase, xylanase, manganese peroxidase (MnP), and laccase (Massadeh et al., 2010). Therefore, components such as lignin, cellulose, and hemicellulose are degraded into low molecular weight compounds, which the mushroom utilizes for its growth and production (Inácio et al., 2015). It is worth noting that the composition and the type of lignocellulosic substrates employed during mushroom cultivation have a significant influence on the types of enzymes secreted by the mushroom (Xie et al., 2016). The composition and the type of substrates also have a significant impact on the antioxidant activities of mushrooms (Atíla, 2022), since it is associated with the phenolic compounds extracted by mushrooms from substrates (Elmastas et al., 2007; Cheung et al., 2003;). Hence, former studies have indicated that enzymes such as laccase promoted phenolic oxidation, which was in accordance with low levels of total phenolic compounds (Inácio et al., 2015). Other enzymes such as oxidoreductases that are associated with the conversion of lignocellulose are very useful in the process of converting lignocellulosic biomass into soluble sugars that are required by mushrooms to grow and produce optimally (An et al., 2019).

For the *Pleurotus ostreatus* mushroom to successfully bio-convert biomass into a mushroom, several factors should be considered, such as oxygen, moisture, carbon, and nitrogen ratio, and temperature together with the pH of the growing substrates should be monitored (Suwannarach et al., 2022). Furthermore, nutrient deficiency within some lignocellulosic biomass is another factor that should be considered when cultivating mushrooms, hence supplementation of lignocellulosic biomass is recommended before the addition of spawn to enhance mushroom yield (Naraian et al., 2016). Therefore, the major purpose of adding supplements is to bring an optimum level of carbon to nitrogen (C/N) ratio for mushroom growth and yield, (Subedi et al., 2023). Supplementation of substrates not only improves mushroom yield but can also increase the production of secondary metabolites, hence, supplementation could potentially have beneficial

effects on the antimicrobial and antioxidant activity of mushrooms without having negative cytotoxic effects (Cardoso et al., 2021).

1.2.2 Re-purposing potential of P. ostreatus mushroom

Besides malnutrition and poverty alleviation, mushroom cultivation has multiple purposes, one of them being the synthesis of organic manures from the substrates after the full harvesting of mushrooms (Ferdousi et al., 2020). They have also proven to be very useful in bioremediation, hence they were previously reported to filter pollutants since they are effective bio-absorbers of toxic heavy metals from radioactive nuclear fallout that pollutes the soil and water (Das, 2005). Thus, it is worth noting that this process of environmental myco-remediation is through various mechanisms, that involve biotransformation, bioconversion, bioaccumulation, precipitation, and surface sequestration (Manimaran et al., 2021). An extracellular enzyme found within the mushrooms greatly contributes to the above-mentioned mycoremediation processes or pollutant degradation within the environment (Adenipekun, 2012). Hence, ligninolytic enzymes from the white rot fungi have been previously applied in the biotransformation of numerous organic pollutants from wastewater contaminated by pesticides (Deshmukh et al., 2016).

Furthermore, extracellular ligninolytic enzymes produced by white rot fungi, have proven to have the capacity to adsorb dyes, thus these mushrooms have dominated the niche of dye degradation (Deshmukh et al., 2016). The white rot fungi have become more useful since industries release basic and acidic dyes that are toxic to aquatic (fishes, algae, etc.) and human life via the food chain (Watharkar et al., 2015). Therefore, these fungi manage to degrade toxic dyes by producing extracellular ligninolytic enzymes (laccase, lignin peroxidase, and manganese peroxidase) that degrade complex organic compounds via an enzyme catalysis system (Dewi et al., 2019). Hence enzymes such as laccases have been observed to be one of the most used enzymes in the biodegradation or treatment of dyes (Robinson et al., 2001).

1.3 Yield Optimisation of Mushrooms

Mushrooms are said to obtain nutrition from substrates such as lignocellulose substrates that play a role in the growth, development, and fruiting of the mushroom (Chang & Miles, 2004). The mushroom mycelium secretes enzyme complexes that degrade the lignocellulose into nutrients for the growth and fruiting of the mushroom (Angelova et al., 2021). As a result, the substrates that mushrooms grow on determine their yield, growth, and composition (Khare et al., 2010). This means that the mushroom farmer must choose the most appropriate substrate for producing mushrooms (da Luz et al., 2012). However, it should be taken into consideration that an ideal substrate for growing mushrooms must meet the following criteria, (a) be free from contaminants or sterile, (b) be rich in important nutrients such as phosphorus, magnesium, nitrogen, potassium, and iron and (c) available locally at low cost (Masevhe et al., 2016).

It has been noted by Jafarpour et al., (2010) that utilization of substrate with high amounts of nitrogen and protein results in added advantages such as a short growing period and at the same time increases yield and biological efficiency of mushrooms. This therefore necessitates the supplementation of mushroom substrate with additional nitrogen, especially for substrates with lower protein content (Oseni et al., 2012). The technique of supplementing the mushroom substrate is of benefit to the mushroom since it may increase the mushroom yield (Carrasco et al., 2018). The organic supplements which are major sources of nitrogen (wheat bran) are significantly beneficial in increasing the yield and biological efficiency of oyster mushrooms (Vieira & de Andrade, 2016) since they are metabolized more efficiently by oyster mushrooms (Rizki & Tamai, 2011). Although substrate supplementation contributes to the yield improvement of mushrooms, however, it poses the risk of perpetuating contamination by competitor microorganisms (Doroški et al., 2022). This is because the supplements provide nutrients not only to mushrooms but also to other microorganisms within the growing substrates (Tesfaw et al., 2015). Thus, precautions should be taken when supplementing the growing substrates. Besides supplements, many parameters can affect the yield of the mushroom (Rai et

al., 2015), including light, humidity, and temperature because if these factors are controlled properly then the yield gets higher (Turković, 2015).

1.4 Bioaccumulation of heavy metals by mushrooms

Heavy metals are said to be metallic elements that have high density and are known to be toxic even at lower concentrations (Ogidi et al., 2021). Heavy metals such as Al, Fe, Cr, Sb, As, Be, Cd, Cu, Pb, Hg, Ni, Se, Ag, and Zn are recognized as primary contaminants by the United States Environmental Protection Agency (US EPA) (Patnaik, 2010). They are the major environmental pollutants that are widely distributed within the environment since they originate either from natural or anthropogenic sources (Figure 2) (Jan et al., 2015). The heavy metals disturb the intracellular balances which include damage to proteins, lipids enzymes, and even DNA (Jan et al., 2015). Mushrooms have a high capacity to accumulate heavy metals that are toxic for consumption (Orywal et al., 2021), hence they have the potential to enter the food chain through various sources as observed in Figure 2. Thus, mushrooms that have been exposed to toxic heavy metals may pose health hazards to humans who consume them (Nowakowski et al., 2021). Hence the contaminated mushrooms cause disorders such as the ones of the central nervous system, dementia in adults, kidney diseases, insomnia, and depression to name a few (Kumar Yadav et al., 2018). Mushrooms can accumulate heavy metals because they consist of spacious mycelium that is responsible for the uptake of heavy metals from the substrates or environment (Uddin et al., 2020; Quarcoo & Adotey, 2013). Out of all the edible mushrooms known, *Pleurotus* species are known to have the ability to bio-accumulate metal contaminants from the environment (Onifade et al., 2016). They can even accumulate/uptake toxic metals such as Pb, Cd, and Hg from the polluted substrate into their fruiting bodies (Adenipekun et al., 2013). This process by which mushrooms uptake such heavy metals is through various pathways such as (i) active uptake and (ii) passive uptake (Choma et al., 2018). The active uptake or bioaccumulation or intracellular uptake depends on cell metabolism to transfer heavy metals into the cell via cell

membranes, whereas, in passive uptake or biosorption, metal ions bind to the cell wall surface and into binding sites, hence it does not depend on the cell metabolism (Banerjee et al., 2018).

In brief, the accumulation of heavy metals by mushrooms can be summarized as follows: heavy metals get intercepted by the cell wall (Vimala and Das, 2011), and get transported via carriers (Severoglu et al., 2013), or they get chelated in the plasma membrane by bioactive compounds (Bellion et al., 2007). It has been observed previously that the type of uptake mechanism is influenced by factors such as environmental conditions and also the toxicity of the metal, hence the absorbed metal ions are unequally distributed within the mushroom fruiting body (Choma et al., 2018). However, generally, the accumulation of heavy metals by mushrooms is governed by three factors that include the types of species of mushroom, pH, age of the mushroom, source, and distance of heavy metal pollution, type and the number of enzymes and proteins produced (Yamaç et al., 2007; Falandysz et al., 2013).

During the process of heavy metal bioaccumulation, the mushroom cap/fruiting body usually releases stress-related factors such as metallothionein, glutathione, and plastocyanin that govern the metal ion uptake by the fungi (Damodaran et al., 2013). The cysteine-rich proteins called metallothioneins have a high affinity for binding metals and xenobiotics (Khullar & Sudhakara Reddy, 2016), due to their thiol group that binds to metals and results in metalloprotein complex that accumulates into the vacuole, which later gets released as a metallic complex (Choma et al., 2018). Metals such as Zn, Cu, Cd, and Hg are said to be the major inducers of metallothioneins (Khullar & Reddy, 2016), which usually bind to these metals depending on the type of metal and host species (Ediriweera et al., 2022).

Besides the metallothioneins, fungi also produce a tripeptide (L-γ-glutamyl-L-cysteinyl glycine) that has dual protection such as metal scavenging and antioxidant during oxidative stress (Khullar & Reddy, 2016). When the heavy metals enter the cytosol of hyphae, they stimulate the synthesis of GSH (Pawlik-Skowronska et al. 2002). Thereafter the GSH binds to metal and forms the GSHX complex through the catalysis by Glutathione-S-transferase enzyme. These conjugates

are then transported to vacuoles via the ABC (ATP-binding cassette) transporters (Schlunk et al., 2015). It is through the above mechanisms by which mushrooms accumulate heavy metals (Yu, 2020) such as mercury (Hg), lead (Pb), cadmium (Cd), arsenic (As), Cadmium (Cd) (Orywal et al., 2021). These heavy metals cause harmful effects on human health, for example, Cd has been shown to affect the kidneys and cardiovascular system (Khani et al., 2017).

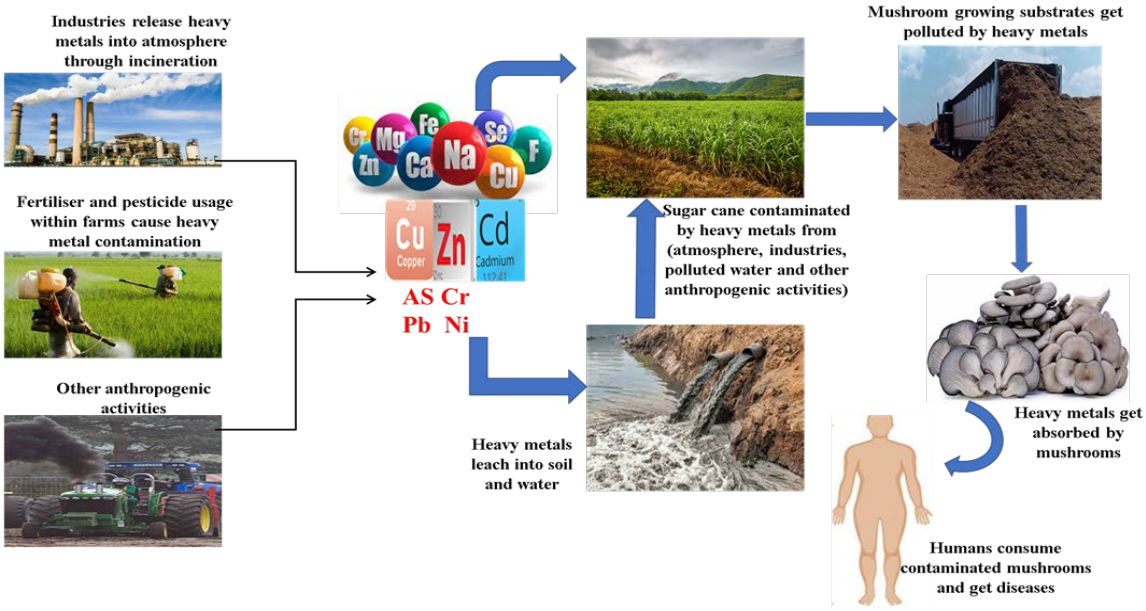

Figure 2: Bioaccumulation of heavy metals by mushrooms from growing substrates via a different route, which end up in food chain (Source: (Ab Rhaman et al., 2022).

1.4.1 Mercury (Hg)

Mercury is one of the heavy metals that is toxic and dangerous and can efficiently bioaccumulate in numerous mushrooms (Širić et al., 2017). This type of heavy metal poses some threat to animals and human health in different ways, such as dermal contact, ingestion, and via soil-to-plant pathways (Li et al., 2012). Mercury (Hg) is found to be in different forms such as organic mercury with compounds, whereby Hg is attached to carbon-containing structures (ethyl, methyl, phenyl, etc..) and inorganic mercury that consists of mercury vapor (Hg^{o}), mercuric salts (Hg^{++}) or (Hg^{2++}), and metallic mercury (Bernhoft, 2012).

The existence of mercury is either caused by man-made contamination or occurs naturally (Rice et al., 2014). Mercury concentration within the soil can be influenced by various factors such as

soil pH, fertilizers, pesticide application in agriculture, sewage irrigation, atmospheric deposition, and organic matter content of the soil and other man-made activities (Wang et al., 2016). However, the soil that has been contaminated by mercury or has been tainted by water with mercury can potentially distribute the mercury into plants and livestock or the food chain (Rice et al., 2014). Mercury has various toxic effects on humans who get exposed to this heavy metal. Such toxicological effects of mercury include cardiovascular, cellular, pulmonary, renal, hematological, embryonic, endocrine, neurological, and reproductive toxicological effects (Bernhoft, 2012; Rice et al., 2014). Therefore, health effects such as impaired vision, tremors, insomnia, hearing paralysis, instability of emotions, fetal development deficits, and delayed childhood development are experienced by individuals exposed to mercury (World Health Organization & Water Sanitation, 2005).

1.4.2 Lead (Pb)

Lead (Pb) is said to be a bluish-gray heavy metal that is found in nature but rarely exists in the earth's crust (Handan Dökmeci, 2021). The exposure of plants and animals to Pb has proven to pose a health hazard since it causes continuous toxicity, especially to humans who have been exposed to lead (Kumar et al., 2020). This type of heavy metal is usually transmitted via atmospheric dust, automobile exhaust, paint, polluted food, industrial areas, and water (Kumar et al., 2020). Once humans get exposed to this heavy metal, they turn to develop some pathological changes in the organs of the body and also cause damage to the central nervous system (Fang et al., 2014).

1.4.3 Cadmium (Cd)

Cadmium is one of the naturally occurring heavy metals that is usually found on the earth's crust. The content of cadmium on the earth's crust may also be due to anthropogenic activities such as agriculture (agrochemicals), sewerage disposal, metallurgy, and energy production (Nkwunonwo et al., 2020). This type of heavy metal is highly toxic to the human body since it can destroy many organ systems within the human body (Bernhoft, 2013). It can particularly cause an

increased risk of cancer mortality (Watanabe et al., 2020), such as those related to prostate, kidney, lung, pancreas, breast, urinary system, and bladder cancer (Peana et al., 2022). Some researchers have indicated that cadmium (Cd) is also toxic to bones, kidneys, and cardiovascular system (Fang et al., 2014). This heavy metal negatively affects the human body because it causes oxidative stress, resulting in tissue injury (Das & Al-Naemi, 2019). Cadmium also causes the inhibition or upregulation of transport pathways (Wan & Zhang, 2012), especially in the kidney (proximal SI segment) (Vesey, 2010). Hence target cells get damaged by Cd^{2+} if they consist of receptors or transport pathways with an affinity for this toxic metal, therefore this process is through free ions or complexes with a carrier (Thévenod, 2010). Thus, diseases such as pulmonary emphysema, diabetic and renal complications, deregulated blood pressure, immunosuppression, and bone disorders are associated with Cd exposure (Järup & Akesson, 2009). There are many sources of cadmium as it can be found near metal mining and refining, disposal, waste incineration, and lastly in the areas where phosphate fertilizer is produced and applied (Keil et al., 2011). This rare dangerous element can be released by human activities into air, water, and land, whereby an increase of Cd within the soil causes the cadmium to be taken up by plants (Maihara et al., 2012) and crops (Keil et al., 2011). Therefore, mushrooms can be found to contain a high amount of Cd if they have been grown within substrates (plant material or soil) which has been exposed to cadmium because factors such as substrate and the place where the mushroom is cultivated contribute to the presence of toxins within the mushroom (Maihara et al., 2012).

1.5 The relationship between mushrooms' therapeutic capabilities and heavy metal bioaccumulation

The majority of researchers have been interested in the prospect that edible mushrooms could become enriched with heavy metals from the environment they are growing in (Sevindik, 2020; Yang et al., 2011). This came after it was established that fungi tolerate heavy metal accumulation through extracellular (chelation and cell wall binding) and intracellular (heavy

metal binds to proteins) sequestration (Fawzy et al., 2017). Sithole and colleagues, (2022) highlighted that there is insufficient research data concerning health hazards from consuming *P. ostreatus* grown in a polluted environment (Sithole et al., 2022). Previous studies noted that the reducing power and chelating capacity of mushrooms increases as the concentration of heavy metals increases (Sarikurkcu et al., 2010). In contrast, Soceanu and colleagues (2024) argued that the presence of toxic metals in edible mushrooms poses minimal health risks (Soceanu et al., 2024). Nonetheless, additional studies are required to fully elucidate if the therapeutic benefits and nutritional benefits obtained from mushrooms grown in heavy metal-rich environments may outweigh the safety concerns.

1.6 Medicinal properties and type of compounds found in mushrooms

Infectious diseases have become a worldwide health concern (Adenipekun et al., 2013), especially in developing countries (Modi et al., 2014) due to the increase in resistance to traditional antibiotics, which have since elevated mortality and morbidity (Murugaiyan et al., 2022). Thus, infectious diseases are observed to be on the rise because of the pathogens that evolve and develop resistance over time to some of the currently prescribed antibiotics (Chokshi et al*.,* 2019). Previous findings indicate that pathogens such as the methicillin-resistant *Staphylococcus aureus* (MRSA) have resulted in mortality of about 50,000 people per year in Europe and the United States, and even more deaths in other countries (O'Neill et al., 2014). Whereas the World Health Organization (WHO) has indicated that non-communicable diseases are the leading cause of mortality worldwide, with cardiovascular diseases (17.9 million deaths per year), diabetes (1.6 million), cancer (9.0 million), and respiratory disease (3.9 million) recognized as a major cause of deaths globally (Budreviciute et al., 2020). In general, the main challenge of finding the cure to treat communicable and non-communicable is finding a drug with little or no side effects (Dzobo, 2022). Hence innovative and novel strategies for the discovery of novel drugs are a necessity currently, thus nature could potentially be an option for the discovery of numerous compounds that are novel (Dzobo, 2022). Therefore, natural products

could be used as an alternative source of therapeutics since they consist of novel biologically active compounds that could be used as new therapeutics (Rodrigues, et al., 2016).

Mushrooms have previously been identified in *in-vitro* studies to have numerous medicinal properties (Wasser, 2014), such as antitumor (Reis et al., 2015), antioxidant (Geng et al., 2016), anti-inflammatory, antimicrobial (Shigesue et al., 2000), immunomodulating (Gao et al., 2013), hepatoprotective (Zhang et al., 2002), antidiabetic (Kim et al., 2010), prebiotic properties (Singdevsachan et al., 2016), antiviral, and hypocholesterolemic properties (Cohen et al., 2002).

Therefore, mushrooms are regarded as the natural source of antibiotics because they contain compounds such as oxalic acids, peptides, benzoic acids, terpenes, sesquiterpenes, anthraquinone, oxalic acids, proteins, and steroids (Valverde et al., 2015). The extracts from mushrooms, allow the mushrooms to be used more medicinally since this approach is more natural, cost-effective, and consists of minimal side effects in the fight against diseases (Poucheret et al., 2006). The medicinal use of mushrooms comes from the fact that the mushrooms usually produce organic compounds called secondary metabolites which protect the mushrooms against predators and pathogens (Künzler, 2018). Thus, the medicinal properties of mushrooms are due to these metabolites which are of course beneficial to the human immune system (Mocan et al., 2018). The bioactive compounds found in oyster mushrooms such as *Pleurotus ostreatus* mushrooms have been responsible for therapies such as antitumor, antiviral, antibacterial, hematological, and immunomodulating treatments (Mohamed & Farghaly, 2014). The bioactive compounds are however divided into three major categories namely secondary metabolites (terpenoids, sterols, alkaloids, acids, sesquiterpenes, polyphenols, lactones, metals, chelating agents, vitamins, and nucleotide analogs), glycoproteins and polysaccharides (Pleuran, pyran, and β-glucans as a major polysaccharide) (Kumar, 2015). Bioactive compounds such as phenolics, terpenoids, polysaccharides, glucans, and lectins, have been previously reported to have more than 126 beneficial health effects, which include anti-microbial antioxidant, antiviral, immune-modulating, and hypo-cholesterolemic activities (Badalyan, 2014). It is worth noting

that these compounds are affected by various factors such as the type of substrates used during cultivation and the type of supplements employed during cultivation (Magdziak et al., 2021). Thus, previous authors have proven that the addition of wheat bran and thymus post-extraction waste as supplements influenced the production of low-molecular-weight organic acids (quinic, malic, and citric acids) within the *P. citrinopileatus*. Therefore, small changes in the composition of growth media alter the amounts of specific compounds and also result in the production of new molecules (Bode et al., 2002). This is the most effective approach in the discovery of novel compounds and optimization of antibiotic production (Ochi et al., 2016).

1.6.1 Bioactive compounds found in mushrooms

1.6.1.1 Polysaccharides

Polysaccharides may be defined as biopolymers that consist of monosaccharides that are joined by glycosidic bonds, they can be either branched or linear (Jorge & Figueiredo, 2012). Mushroom polysaccharides can be divided into homopolysaccharides (with one kind of monosaccharide) and heteropolysaccharides (with two or more kinds of monosaccharides), based on the number of monomers present (Ren, 2014). The basidiomycetes mushrooms are known to have polysaccharides in their fruiting bodies, culture broth, and in their cultured mycelium (Zidan & Alneameh, 2014). However, the mushroom polysaccharides are majorly found within the mushroom cell wall which is composed of two major types of polysaccharides namely chitin or cellulose (rigid fibrillary) and a matrix-like (β-glucan, α-glucan, and glycoproteins) (Choong et al., 2019). These mushroom polysaccharides have gained a lot of interest since they contain health benefits such as anticancer, immunomodulation, antiviral effects, and antimicrobial effects and are responsible for the prevention and treatment of cardiovascular diseases (Villares et al., 2012). The *P. ostreatus* mushrooms have been observed to consist of numerous polysaccharides such as pleuran (high molecular weight β-(1→3) (1→6)-glucan) which have immunomodulating, antioxidant, antiproliferative, and prebiotic activity (Radzki et al., 2016).

Another class of polysaccharides produced by *P. ostreatus*, namely selenium-enriched polysaccharides (Se-POP-21 and Se-POP) has demonstrated potent scavenging activity for DPPH, hydroxyl, and ABTS radicals. Furthermore, the Se-POP has been shown to have an inhibitory effect against oxidative damage caused by H_2O_2 in C_2 $C1_2$ cells (Zhang et al., 2021).

Numerous studies have indicated that polysaccharides can be either natural or semi-synthetic depending on their source or origin (Jorge & Figueiredo, 2012). Polysaccharides can be either natural or semi-synthetic depending on their source or origin (Jorge & Figueiredo, 2012). Those that are natural are obtained from various organisms including algae, microorganisms, and plants, whereas those that are semi-synthetic are obtained through chemical and enzymatic modification of parent macromolecules (Jorge & Figueiredo, 2012). Within the mushroom polysaccharides, the polysaccharides such as hemicellulose, chitin, β- and α-glucans, xylans, galactans, and mannans are known to be found in abundance within the mushroom (He et al., 2017). However, the major polysaccharides in mushrooms are glucans that consist of different types of glycosidic linkages, such as (1→3)-, (1→6)-β-glucans and (1→3)-α-glucans, however, heteroglycans are also found in mushrooms (Ren et al., 2012). The homopolysaccharides (β-glucans) are usually perceived as the major bioactive polysaccharides even though there are some polysaccharides such as heteropolysaccharides which also have biological properties (Friedman, 2016). This may be due to the fact that the β-glucans have a wide range of activities, and they are versatile in nature (Kumar & Shankar, 2017). This enables the mushrooms with β-glucans to affect numerous pathways of both immune and non-immune systems by causing the macrophages to produce cytokines and nitric oxide and further promote the adhesion of monocytes (Carbonero et al., 2012). The neutrophils and natural killer cells are also activated by this property of β-glucans to fight against cancer cells (Yoon et al., 2008). Such mechanism of β-glucans makes them biological response modifiers (BRM) (Novak & Vetvicka, 2009), since they indirectly fight viruses, bacteria, and cancer cells (Zidan & Alneameh, 2014). However, the biological activities

of β-glucans are dependent on factors such as the structure, solubility, size, and the branching degree of polysaccharides (Han et al., 2020).

1.6.1.2 Phenols

Phenolic compounds are said to be aromatic hydroxylated compounds that consist of one or more aromatic rings and one or more hydroxyl groups (Palacios et al., 2011; Sánchez, 2017a). The phenolic compounds include various subclasses, namely flavonoids, phenolic acids, hydroxycinnamic acids, hydroxybenzoic acids, tannins, lignans, oxidized polyphenols, and stilbenes (Sánchez, 2017b). They are distinguished according to the number of phenol rings and the elemental structures that bind the rings together (Stalikas, 2007). However, phenols can be classified as either simple or polyphenols (Table 1), depending on the number of phenol units contained in a molecule as shown in Figure 3 (Khoddami et al., 2013; Santana-Gálvez & Jacobo-Velázquez, 2019).

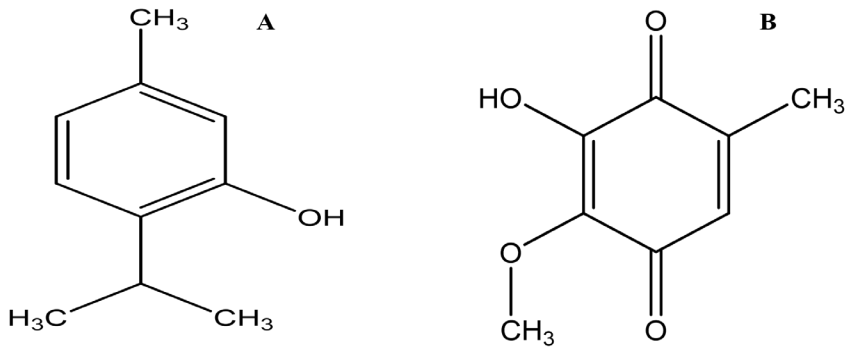

Figure 3: Different structures of phenols: (A) Thymol, a monoterpene, (B) Fumigatin, a quinone. Source: (Ueitele, 2016).

Table 1: Classification of Phenolic compounds

Simple Phenols	**Polyphenols**		
Phenolic Acids: • Benzoic acid • Cinnamic acid	**Flavonoids:** • Anthoxanthins a) *Flavones* b) *Flavonols* c) *Flavanols* d) *Isoflavones* • Anthocyanins	**Tannins:** • Hydrolisable • Non-hidrolisable	**Stilbenes & lignans**

Previous studies have shown that phenolic compounds are the major naturally occurring antioxidants found in medicinal mushrooms (Mau et al., 2002; Ribeiro et al., 2007). These compounds are considered antioxidants because of their ability to neutralize ROS when they are in excess within the cells (Hussein et al., 2015). Such characteristics of phenolic compounds allow them to be regarded as peroxide decomposers, free radical inhibitors (chain breakers), oxygen scavengers, and metal-in activators (Palacios et al., 2011).

Liu et al., (2012) have reported that phenolic compounds contribute to anti-inflammatory, antibacterial, and antihyperglycemic activities of mushrooms. Hence, *P. ostreatus* mushroom has attracted attention as an ideal candidate for the production of functional foods, pharmaceuticals, and cosmetic formulations due to its wide spectrum of phenolic compounds with pharmacological properties (Ianni et al., 2021). Furthermore, phenolic compounds found in mushrooms have been reported to counteract the oxidative damage caused by malaria, hence they are deemed to be a significant source of bioactive compounds with anti-malarial activity (Abugri et al., 2019). However, out of the several phenolic compounds, the main phenolic compound found in mushrooms is phenolic acid (Karaman et al., 2010), which contributes greatly to mushrooms' antioxidant activity and protection of crucial cellular structures, such as enzymes, structural proteins, nucleic acids, and membrane lipids (Froufe et al., 2009). Even though the

phenolic compounds are considered to be major antioxidants in mushrooms, however, there are other antioxidants (β-carotene, vitamin C, and γ-tocopherols) that are also present in mushrooms in minimal quantities (Mau et al., 2002).

The structure of phenolic compounds contributes to their antioxidant activity, specifically the number and the positioning of hydroxyl groups together with the type of substitutions of the aromatic rings (Kumar & Goel, 2019). For example, Ching-yu, (2013) reported that the phenol structures with the following three features can be effective in scavenging radicals: (a) with the O- dihydroxy structure in the B ring, (b) with the 2,3-double bond in conjugation with 4-oxo function in the C ring, (3) with the 3- and 5-OH group with 4-oxo function at A and C ring. Even though the structure of phenolic compound influences antioxidant activity, also the quantity of phenols in mushrooms influences antioxidant activities since Hussein et al., (2015) have indicated that, the higher the phenol content within the mushroom extract, the stronger the antioxidant activities. However, it is noteworthy that the levels of polyphenols are influenced by factors such as the substrate where the mushroom is cultivated, species, maturity stage, and a portion of the analyzed mushroom (Oboh & Shodehinde, 2009). Furthermore, researchers such as Angelini et al., (2015), have also testified that besides genetic factors, the mushroom cultivation conditions such as pH and substrate composition may influence the metabolic pathway, which then influences the phenol content of mushrooms

1.6.1.2.1 Phenolic Acids

Phenolic acids are phenolic compounds that have an aromatic ring that is substituted by at least one carboxyl and hydroxyl group (Balik et al., 2020). They are usually found in different foods such as mushrooms (Çayan et al., 2020), and plant-based foods (skins of fruits, seeds, and vegetable leaves (Kumar & Goel, 2019). Within the mushrooms, phenolic acids are said to be the major phenolic compound (Çayan et al., 2020), that greatly contributes to the medicinal properties of mushrooms (Heleno et al., 2015). Hence, phenolic acids have been documented to have health effects such as anticancer, antimicrobial, anti-mutagenic, and anti-inflammatory

(Kumar & Goel, 2019). The above-mentioned activities of phenolic acids are due to the presence or the number of hydroxycarboxylic groups and their positioning on the aromatic ring (Balik et al., 2020). Hence, they manage to act as antioxidants by neutralizing the free radicals and also chelating metal ions (Balik et al., 2020). They are reported to have various mechanisms which they use for antioxidant activity, one of them being through the reactivity of phenol moiety and hydroxyl substituent on the aromatic ring. They also scavenge radicals via hydrogen donation, which is the major method they usually utilize for antioxidant activity (Kumar & Goel, 2019). Different phenolic acids are reported to have different antioxidant activities since substituents on the aromatic ring affect the stabilization of the phenolic acid structure, thus influencing the radical-quenching ability of the phenolic acid (Chalas et al., 2001).

These compounds are said to be grouped into two classes such as hydroxybenzoic and hydroxycinnamic, which are derivatives of non-phenolic molecules such as benzoic and cinnamic acid (Figure 4) (Heleno et al., 2015). The hydroxybenzoic acids (gallic, vanillic, p-hydroxybenzoic, protocatechuic, and syringic acids) have a C6-C1 structure whereas hydroxycinnamic acids (ferulic, caffeic, p-coumaric, and sinapic acids) are aromatic consisting of three carbon side chain (C6-C3) (Ozcan et al., 2014). The phenolic acids such as p–hydroxybenzoic, protocatechuic, vanillic acid, gallic acid, p–coumaric acid, gentisic acid, and ferulic acid, are recognized as the most prominently present phenolic acids within the fruiting bodies of higher fungi (mushrooms) (Balik et al., 2020). A study by Gąsecka et al., (2016) confirmed that *P. ostreatus* mushroom consists of six phenolic acids such as 4-hydroxybenzoic, ferulic, p-coumaric, protocatechuic, t-cinnamic and vanillic acids, which contributes to the medicinal attributes of the *P. ostreatus*.

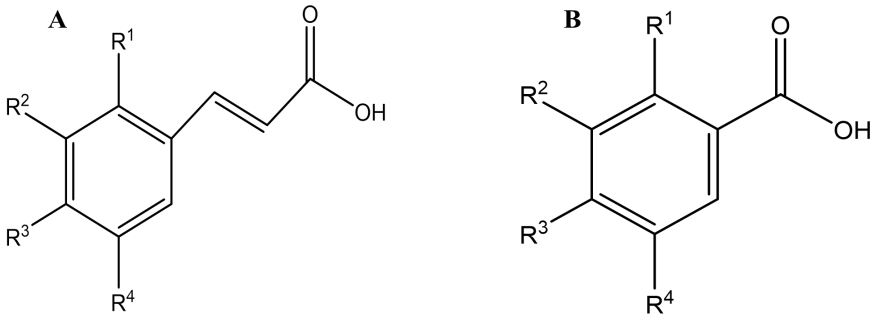

Figure 4: (A) cinnamic acid; (B) benzoic acid derivative

1.6.1.2.2 Flavanoids

The polyphenols also consist of a class of phenols named flavonoids which play an important role in the reduction of oxidative stress and result in the cell death of cancer cells together with the inhibition of the growth in cancer cells (Flora, 2009). The flavonoids are said to be low molecular weight compounds (Ozcan et al., 2014) that have C6-C3-C6 skeletal structures that consist of numerous groups namely flavones, flavonols, anthocyanins, flavanones, and isoflavonoids (Hollman & Arts, 2000; Nijveldt et al., 2001). Flavonoids consist of a structure with two aromatic rings A and B that are fused by a 3-carbon bridge, usually called heterocyclic C (figure 5) (Balasundram et al., 2006). The flavonoids can however be categorized into two classes, namely, (i) anthoxanthins (flavonols, flavans, flavones, isoflavones and their glycosides) and (ii) anthocyanins (glycosylated derivatives of anthocyanidin) (Balasundram et al., 2006; Lotito & Frei, 2006).

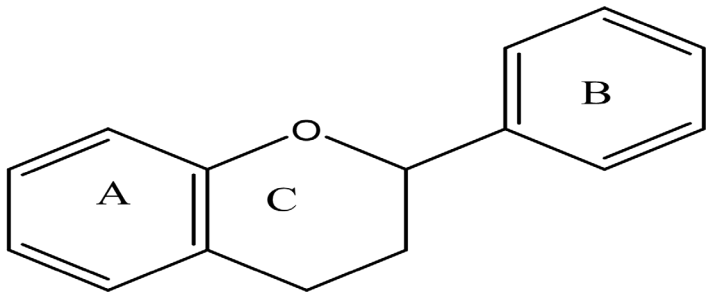

Figure 5: The general structure of flavonoids. (Source: (Kumar & Pandey, 2013)).

The flavonoids are known to have numerous health effects, including anticancer (Zhao et al., 2019), antiviral, antimicrobial, antiangiogenic (Zhao et al., 2018), antioxidant, antimalarial, neuroprotective, antitumor, and anti-proliferative (Patel et al., 2018). Thus, the above-mentioned health properties of flavonoid compounds have linked them to the reduction of major chronic diseases (Rahaman & Mondal, 2020). The flavonoids manage to perform antioxidant activities in various ways which include, scavenging reactive species (RS) directly, chelating metal ions responsible for RS production, inhibiting enzymes such as xanthine oxidase (XO), lipoxygenases (LOX'S), protein kinase C, cyclooxygenase, microsomal monooxygenase, mitochondrial succinoxidase, and NADPH oxidase enzymes that are involved in RS formation (Procházková et al., 2011). They further regenerate antioxidants (α-tocopherol) which are bound to the membrane (Renaud & de Lorgeril, 1992), for example, quercetin and (+)-catechin each can regenerate alpha-tocopherol, which results in a co-antioxidant effect (Pedrielli & Skibsted, 2002). The radicals are easily inactivated because of the presence of highly reactive hydroxyl group within flavonoids (Nijveldt et al., 2001), whereby the scavenging of radicals by flavonoids is through the donation of hydrogen atoms (Zheng et al., 2022) according to the following equation and thus radicals are made to be inactive (Korkina & Afanas'Ev, 1996):

flavonoid (OH) +R* > flavonoid (O*) + RH

R* is the free radical, whereas O* is said to be an oxygen-free radical. Different studies have indicated that different edible mushrooms contain flavonoids (Ferreira et al., 2009) and their concentration differs depending on the mushroom species. Jeena et al., (2014) observed that *P. sojarcaju* had higher flavonoid content when compared to *P. ostreatus* and *P. sapidus*. However, there is some controversy about mushrooms having flavonoids since Gil-Ramírez et al., (2016) have speculated that mushrooms cannot produce flavonoids because they do not have enzymes responsible for flavonoid production within their metabolic pathways.

1.6.2 Antioxidant Activity of Mushrooms

The human body undergoes the oxidation process whereby nutrients like fats, proteins, and carbohydrates are transformed into energy and maintenance of the immune system (Sánchez, 2017a). During this process, the free radicals in the form of reactive oxygen species (ROS) are produced usually at low concentrations, which is normal in living systems (Sánchez, 2017a). However, during the normal metabolism of aerobic cells, most free radicals are neutralized by both enzymatic and nonenzymatic cellular antioxidants (Muthangya et al., 2014). The enzymatic antioxidant defence includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydro ascorbate reductase (MDHAR), and glutathione reductase (GR) (Sharma et al., 2012). The nonenzymatic antioxidants include ascorbate (AsA), glutathione (GSH), carotenoids, phenolics, and tocopherols (Sharma et al., 2012). These antioxidants play an important role in maintaining equilibrium between free radicals and antioxidant defences, so to keep the organism functioning normally (Hollman & Arts, 2000). This is because excessive production of free radicals within the system results in a condition known as oxidative stress (Shankar & Mehendale, 2014).

During oxidative stress numerous cellular functions are affected since nucleic acids get denatured, proteins get oxidized and lipid peroxidation is formed due to excessive ROS production (Sánchez, 2017a). Such damage that is caused by free radicals, has been reported to play a role in various diseases such as heart disease, cancer, liver damage, impairment of immune function, Parkinson`s and Alzheimer`s diseases, cataracts, and muscular degeneration in elderly individuals (Hu et al., 2010; Kozarski et al., 2011).

The free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) when they are in excess, cause oxidative stress leading to increased diseases (Phaniendra et al., 2015). This is because the free radicals (ROS/RNS), turn to randomly oxidize all molecules in biological membranes and tissues, leading to damage or injuries since they break DNA strands,

and they also start lipid peroxidation (Li et al., 2015). However, a study by Ramkumar et al., (2010) showed that some antioxidants can scavenge free radicals, which therefore play a huge role in prevention of the free radical-induced diseases.

The antioxidants manage to play such a role by delaying the rate of oxidation and inhibiting the initiation or interrupting the proliferation of the oxidation reaction of lipids (Olajire & Azeez, 2011). The antioxidants manage to scavenge free radicals by donating an electron to a rampaging free radical and therefore the free radical gets neutralized, and its damaging capacity is minimized (Lobo et al., 2010). For example, Figure 6 indicates the antioxidant defense mechanism used by endogenous antioxidants under normal physiological conditions to neutralize free radicals and produce non-toxic molecules (Mosa, 2014). The enzyme superoxide dismutase (SOD) converts the radical ($O^{2\bullet-}$) into O_2 and H_2O_2 which is still a reactive ROS, therefore enzyme catalase (CAT) converts the H_2O_2 into water and O_2. The H_2O_2 can also be broken down into water and a dimer of glutathione (GS-SG) by glutathione peroxidase (GPx) through the action of glutathione (GSH) (Figure 6) (Ching-yu, 2013).

According to Mosa, (2014), the increased activity of these enzymes is very important under pathophysiological conditions to prevent cellular damage caused by oxidative stress. However, some studies have shown that mushrooms consist of numerous secondary phytochemical metabolites (such as polyphenolic compounds, terpenes, saponins, tannins, and terpenoids) which have high radical scavenging activity (Doughari, 2012) since they may consist of the above enzymes.

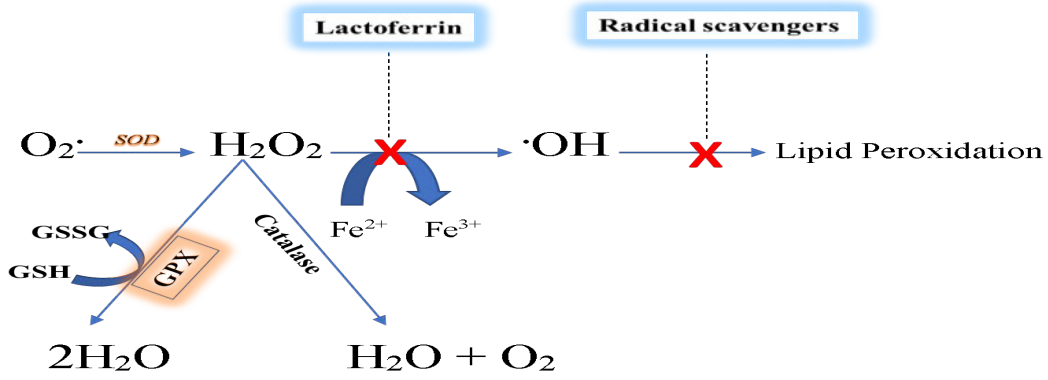

Figure 6: Antioxidant defense mechanism used by antioxidants (enzymes) neutralize free radicals (ROS). (Source: Mosa, 2014).

Mushrooms are known to consist of two types of antioxidants namely primary (chain breakers and scavengers of free radicals) and secondary antioxidants (Kozarski et al., 2015). The primary antioxidants act on oxidative stress by donating hydrogen and generating stable radicals which therefore breaks the oxidation reaction chain (Shah & Shafi, 2019), whereas the secondary antioxidants act by slowing the rate of oxidation, by mechanisms such as metal chelation, primary antioxidants regeneration, oxygen scavenging and hydrogen peroxide decomposition, to name a few (Shahidi et al., 2020). The in vitro study by Boonsong et al., (2016) indicated that the ethanolic extract of *P. ostreatus* potentially induced antioxidant activity by trapping hydroxyl radicals and superoxide, reducing ferric ions, inhibiting lipid peroxidation, chelating ferrous ions, and quenching 2,3-diazabicyclo [2,2,2]oct-2-ene (ene DBO). Furthermore, the in-vivo study also highlighted that the *P. ostreatus* extract induced antioxidant activity by reducing lipid peroxidation and increasing enzymatic and non-enzymatic antioxidant activity.

In terms of the quality and the contents of antioxidants in mushrooms, it has been documented that antioxidants may vary from different strains of mushrooms, which also depend on culture conditions (Kanagasabapathy et al., 2011). The antioxidant activities in different strains may also vary because the content of bioactive compounds (responsible for properties of antioxidants)

vary from strain to strain due to factors such as cultivation, stage of development, age of fresh mushroom, extraction method, and conditions of storage (Mishra et al., 2013).

1.6.3 Antimicrobial activity of oyster mushroom

Mushrooms are known to contain compounds that have numerous antimicrobial activities within their fruiting bodies and their mycelium (Onuegbu et al., 2017). During the life cycle, mushrooms produce secondary metabolites which have been reported to have antioxidant, antimicrobial, and anti-inflammatory activities (Alves et al., 2012). The *Pleurotus* mushroom has been reported to possess an important antibacterial effect on Gram-positive and Gram-negative bacteria (Pauliuc et al., 2013) and therefore it has been regarded as a major promising source of antimicrobial drugs (Bawadekji et al., 2017).

This is because *Pleurotus* fungus contains bioactive compounds such as α and β linkages of glucan compound, phenol (*p*-benzoic acid, *p*- phenylacetic acid, O- coumaric acid, ferulic acid, and chrysin), alkaloids, flavonoids, polyphenols, citric acid, pigments, cinnabarinc acid and enzymes which gives the mushroom antimicrobial activity (Adebayo et al., 2012). For example, *Pleurotus ostreatus* mushrooms have been reported to be effective in fights against both simple and multiple drug-resistant microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis* (Akyuz et al., 2010), *Streptococcus*, *Enterococcus* and *Candida* species (Olatubosun et al., 2019).

Since mushrooms produce a wide range of bioactive metabolites that have important antimicrobial activities (Mukul Jana et al., 2014), they could be used as a good source of natural antimicrobials (Alves et al., 2014). They can be an alternative source of medicine, particularly given the uncertainty surrounding the screening of natural products in search of alternative antimicrobials as a result of the emergence of several drug-resistant microbes (Appiah et al., 2017). However, the efficiency of the mushroom as an antimicrobial is influenced by factors such as the type of mushroom species, concentration of the active compounds, and the type of the selected or tested microorganisms (Kosanic and Dasic, 2012). For example, Bach et al., 2019

have observed that Gram-negative bacteria are more resistant to mushroom phenolic extracts when compared to Gram-positive bacteria. The resistance of Gram-negative is due to the lipopolysaccharide barrier found in the Gram-negative which limits the breakthrough of various compounds into the inside of bacteria while permitting nutrients into the cell (Oliveira et al., 2016). In general, the bioactive compounds from mushrooms, exhibit antibacterial activity via different mechanisms, for example, phenolic and tannin produced by *P. ostreatus* mushrooms have been observed to cause anti-bacterial activity via cell mem-brane lysis, microbial adhesion, proteolytic enzyme inhibition, and also via inhibition of protein synthesis (Lesa et al., 2022). The lipophilic character of phenolic acids causes them to be able to cross the bacterial cell membrane through passive diffusion, thus disrupting membrane structure, and possibly causing acidification of the cytoplasm which leads to protein denaturation within the bacteria (Campos et al., 2009).

Other studies have indicated that compounds such as flavonoids elicit antibacterial activity through interacting with critical enzymes, inhibition of nucleic acid synthesis, and inhibition of the functions of cytoplasmic membrane by disturbing the formation of porins, biofilms, thus disturbing bacterial permeability (Farhadi et al., 2019). Gallic acid inhibits efflux pumps on the *Pseudomonas* strains, which is the most important mechanism the bacteria use to produce antimicrobial resistance (Abreu et al., 2012). The gallic acid compound has been observed to cause irreversible changes in the bacterial membrane through changes in the hydrophobicity of bacteria, hence the negative surface charge decreases and results in rupture or pore formation in cell membranes which causes the leakage of essential intracellular constituents (Borges et al., 2013). It should however be noted that the metabolite profiles of fungi strongly depend on the cultivation conditions of the fungi, thus changing the composition or the content of the growing substrates, the type and the content of certain compounds within the fungi could be altered (Brakhage et al., 2011). Therefore, new compounds that have unknown and known mechanisms in the fight against various pathogenes could be developed by altering the substrate's

composition. Thus, fungi are said to be an ideal source of a large number of novel compounds that have been rarely analyzed and summarized (Zhang et al., 2022).

1.6.4 Antimalarial Activity of Mushroom Extracts

To date, there is still ambiguity about the mechanism of action of most antimalarials and their stage-specific targets in the malaria life cycle. Furthermore, most antimalarials were primarily designed to alleviate symptoms of the disease malaria in the blood stage. However, some drugs such as artemisinin (Piyaphanee et al., 2006), artemisinin combination therapies (ACTs) (Stepniewska et al., 2008), and primaquine (Bousema et al., 2010) have been shown to effectively reduce gametocyte carriage. Antimalarial drugs, such as pyronaridine and atovaquone, have been shown to target liver and sexual stages along with the asexual blood stages in the *P. falciparum* life cycle (Delves et al., 2012). The continual discovery of nontoxic therapeutics with reliable multistage-targeting capabilities is pivotal to continued and effective malaria control. It should be noted that major attention has focused on fungi for the discovery of natural products with antimalarial agents (Katsayal et al., 2009), hence Figure 7 indicates some of the mushrooms that have been previously studied for antimalarial activity. Animal studies demonstrated that treatment with *Agaricus blazei* water extracts (100 mg/kg) displayed antioxidant activity, reduced weight loss, and significant malaria control on *Plasmodium berghei*-infected mice (Val et al., 2015). Val et al. (2015) directly demonstrated that treatment with *A. blazei* reduced parasitaemia by disrupting the erythrocytic stage of malaria parasites (Val et al., 2015). Previous studies by Adam et al. (2010), have indicated that the ethyl acetate extract of *Ganoderma lucidum* mushroom (Figure 7) demonstrated the antiplasmodial activity as it showed 79% inhibition of plasmodium at a minimum concentration of 4.9 µg/ml. Oluba et al. (2012) demonstrated the antimalarial and liver-protective effects of crude ethanolic extract, *G. lucidum* mushrooms, in *P. berghei*-infected mice (Oluba et al., 2012). Although mushrooms are promising in the fight against malaria, however, several mushroom species have not been evaluated as possible treatments for malaria. To date, at least 126 medicinal activities, including

antimicrobial, anti-fungal, antiviral, and mostly antiparasitic activities, have been reported from isolates derived from mushrooms and fungi (Oluba et al., 2012); some of these are highlighted in Table 2. Previous studies on anti-malarial mushrooms and plants have mainly focused on their antiparasitic activity but rarely investigated other mechanisms that may play a role in the killing of the parasite. Several studies have reported a synergistic parasite-killing action when crude extracts are administered with anti-malaria drugs (Mallard et al., 2019).

Table 2: Biological properties of different mushrooms found in nature.

Mushroom species	Biological activity	Type of crude extract	References
P. linteus	Antimalarial (*P. falciparum*): IC_{50} of 3.15 µg/ml	Methanol (MEOH)	(Samchai et al., 2009)
L. piperatus	Antimicrobial activity (*Enterococcus faecalis*): MIC of 1.25-5 mg/ml	Acetone extract	(Kadian et al., 2017)
P. ostreatus	Antimicrobial activity (*C. albicans, P. aeruginosa*, and *S. aureus*)	Water (H_2O)	(Urban et al., 2005)
P. ostreatus & P. var florida	Antifungal (*Trichoderma harzianum*): 16 mm inhibition at 2 mg/disc	Water (H_2O)	(Stevenson, & Riley, 2004)
P. ostreatus	Anti-parasitic/antimalarial (*Plasmodium berghei*)	Ethanol and water	(Malaguarnera, & Musumeci, 2002)
P. ostreatus	Anti-parasitic/antimalarial (*P. falciparum*)	n-Hexane extract	(Nsiah et al., 2019)
Agaricus bisporus	Antifungal activity (*Candida* spp.)	MEOH	(Kosanic et al., 2013]
G. lucidium	Antifungal activity (*Trichoderma viride, A. fumigatus*, *A. versicolor*, and *A. Niger*): MIC of 0.005¬1.5 mg/ml	MEOH	(Lin et al., 2014)
G. lucidum	Anti-parasitic/antimalarial (*P. berghei*)	Ethanol extract (EtOH)	(Adams et al., 2010)
G. lucidum	Anti-parasitic/antimalarial (*P. falciparum*): IC_{50} of 6.0¬20 µM	MEOH and EtOAc	(Owaid et al., 2017]
Terfezia pfeilii	Antimalarial (*P. falciparum* 3D7 (chloroquinesensitive strain): 5, 10, and 50 µg/ml	Water (H_2O)	(Basnet et al., 2017)

Plant crude extracts are likely to contain phenolics, and terpenoids amongst other components (Ayeni et al., 2019). Some researchers have speculated that phenolics from plant extracts such as *Artemisia annua* may also inhibit enzymes involved in metabolism, thus, elevating the action of artemisinin, in vivo (Ashraf et al., 2017). Little research has been conducted to evaluate mushroom extract-to-drug interactions. However, it is plausible that other components in the extract may influence the bioavailability and metabolism of the antimalarial drugs. On the other

hand, some researchers have reported clinical evidence indicating that some herbs may have side effects and adverse events may occur due to herb-to-drug interactions (Izzo, 2012). Thus, more research and assays are required to adequately investigate potential interactions between herbal medicines, mushroom extracts, and prescribed drugs.

Figure 7: Some mushrooms with known antimalarial activity. (a) *G. lucidum*. Source: Photo Courtesy of University of Namibia Zeri project. (b) *A. blazei*. Source: Photo Courtesy of Andrej Gregori, Marija Gregori, Andrej Piltaver, Tamara Korošec. (c) *P. ostreatus*. Source: Photo Courtesy of Andrej Gregori, Marija Gregori, Andrej Piltaver, Tamara Korošec. (d*) Lentinula edodes*. Source: Photo Courtesy of Hyde Kevin. (e*) Phellinus linteus*. Source: Photo Courtesy of Wenhua Chen.

1.7 Green Synthesis of Nanoparticles using Mushrooms

Nanoparticles are defined as tiny materials that have a size that can range from 1 to 100 nm. Depending on their sizes, shapes, and other properties (physical and chemical), they can be classified into distinct categories such as metal, ceramic, fullerenes, and polymeric nanoparticles (Khan et al., 2019). The nanoparticles that are synthesized from biomaterials have recently gained much interest due to their unique properties, which includes, (i) low cost, (ii) highly soluble in water, (iii) eco-friendly, and easy method of synthesis (Bhardwaj et al., 2020). This approach of synthesizing nanoparticles via biological materials is called green nano-biotechnology (Patra & Baek, 2014), hence biological materials such as plants, fungi, actinomycetes, bacteria, corncob, mushrooms, *citrus limetta* peel, *aloe vera*, and *concarpus lancifoliu* are used for the synthesis of nanoparticles (Elsakhawy et al., 2022). Thus, mushrooms

such as *Agaricus bisporus, Pleurotus* spp., *Lentinus* spp., and *Ganoderma* spp. are known to produce nanoparticles that have great nutritional, antimicrobial (antibacterial, antifungal, and antiviral), immune-modulatory, anticancerous, and antioxidant properties (Bhardwaj et al., 2020). Components such as enzymes, phenolics, flavonoids, sugars, and proteins from these mushrooms act as stabilizing and reducing agents during the green synthesis of nanoparticles (Nair et al., 2022). The advantage of this green synthesis method is that the production of toxic or harmful products is avoided using natural resources (organic system) together with an ideal solvent, hence this procedure is deemed reliable, sustainable, and eco-friendly (Singh et al., 2018). Hence, Figure 8 represents a summarised key benefit of green synthesis of nanoparticles.

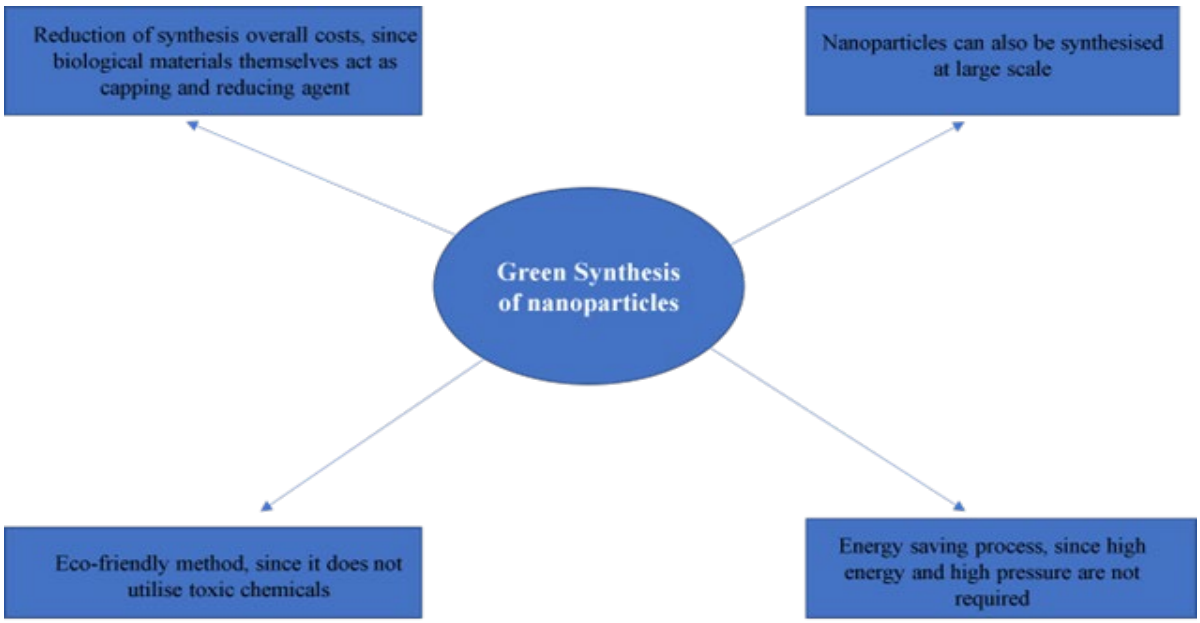

Figure 8: Key Benefits of green synthesis method of nanoparticles (Source: Singh et al., 2018).

The green synthesis of nanoparticles became an alternative method after it was observed that other methods of synthesis such as sol-gel, solvothermal, hydrothermal, micro-emulsion, and solution combustion methods use high-energy and toxic solvents (Thunugunta et al., 2015). Hence, methods such as chemical synthesis cause traces of toxic chemicals to be adsorbed by nanoparticles,

might have bad effects on the biomedical field, thus incorporating green synthesis methods could minimize such adverse effects (Parashar et al., 2009).

There are various nanoparticles, that have been synthesized using *Pleurotus* mushrooms as shown in Table 3, however, the synthesis of zinc oxide using *Pleurotus* mushrooms has been scarcely explored since the zinc oxide NPs have been majorly synthesized using costly and environmentally unfriendly methods such as sol-gel, solvothermal, hydrothermal, micro-emulsion methods (Mahamuni et al., 2019), and solution combustion synthesis (Khaliullin et al., 2019). Thus, green synthesis of zinc oxide nanoparticles using *P. ostreatus* could potentially be useful in many aspects such as biomedical applications.

Table 3: Various types of nanoparticles which have been biosynthesised by *Pleurotus* spp

***Pleurotus* spp**	**Nanoparticles**	**Activity**	**References**
P. florda	Au-NPs (Gold nanoparticles)	Anticancer (A-549 Human lung carcinoma; K-562 Human chronic myelogenous leukemia	(Bhat et al., 2013)
P. djamor	ZnO-NPs (Zinc oxide nanoparticles)	Mosquito, Larvicidal, Antibacterial (*S. aureus, P. fluorescens, C. diptheriae*), Antioxidant (DPPH, H_2O_2 and ABTS), A549 lung cancer	(Manimaran et al., 2021a)
P. djamor	ZnO-NPs (Zinc oxide nanoparticles)	Antibacterial (*S. aureus*, *C. diptheriae*), Anticancer (A549 lung cancer), Antioxidant (DPPH, H_2O_2, ABTS)	(Manimaran et al., 2021a)
P. sajor-caju	Ag-NPs (Silver nanoparticles)	Antifungal (Candida Albicans)	(Musa et al., 2018)
P. sajor-caju	Au-PtNPs (Gold platinum nanoparticles)	Cancer cell inhibition (human colon cancer)	(Chaturvedi et al., 2021)
P. sajor-caju	TiO_2-NPs (Titanium dioxide)	Antilarval (*Aedes aegypti*)	(Manimaran et al., 2022)
P. tuber-regium	Se-NPs (Selenium nanoparticles)	Anticancer	(Wong, 2012)
P. giganteus	AgNPs (Silver nanoparticles)	Antimicrobial (*E. coli*, *P. Aeruginosa*, *S. Aureus, B. Subtilis)*	(Debnath et al., 2019)
P. ostreatus	AgNPs (Silver nanoparticles)	Antimicrobial	(Irshad et al., 2020)
P. ostreatus	Ag-NPs (Silver nanoparticles)	Antioxidant (ABTS and DPPH)	(Martínez-Flores et al., 2021)
P. ostreatus	ZnS-NPs (Zinc Sulfide nanoparticles)	Biomedical, food packaging	(Senapati & Sarkar, 2014)

The majority of the green synthesized nanoparticles have numerous applications in fields such as biomedicine (Tran et al., 2022; Velsankar et al., 2022), cosmetics (Ong & Nyam, 2022),

agriculture (Sharma et al., 2022), textiles (Zayed et al., 2022), electronics (El-Desouky et al., 2022), and in the environmental remediation (Khan et al., 2022). Most of the metallic nanoparticles have been previously tested for biocidal activity on bacteria (Patra & Baek, 2017), viruses (Narasimha, 2013), and fungi (Arciniegas-Grijalba et al., 2017), however, little information has been reported on their antiplasmodial activity (Kojom Foko et al., 2019).

1.7.1 Antimicrobial activity of mushroom synthesized nanoparticles

Nanotechnology has recently played a major role in drug delivery, biotherapeutics, nano fertilizers, cosmetics, pharmaceuticals, electronics, biotechnology, ointments, creams, and water treatment (Prasannaraj & Venkatachalam, 2017). The useful applications of nanoparticles in the above-mentioned sectors are due to their unique properties, which include shape, size, high reactivity, charge, and large surface area to volume ratio (Kim et al., 2007). For example, it has been previously noted that the nanoparticle's large surface area to volume ratio, gave them major attention as an antimicrobial agent with a unique delivery system (Ingle et al., 2014). Thus, nanoparticles are deemed as the promising solution for combating the emergence of multidrug-resistant bacteria, especially since nanoparticles act as carriers for antibiotics and natural compounds (Wang et al., 2017). Hence nanoparticles such as Zinc oxide (ZnO), titanium dioxide, (Firouzabadi et al., 2014), AgNPs, CuONPs, TiONPs, AuNPs, and Fe_3O_2NPs (Hemeg, 2017), have been demonstrated to have antimicrobial activity against various multidrug-resistant bacteria such as *P. aeriginosa*, *K. Pneumonia*, *S. aureus*, *E. coli* (Varaprasad et al., 2016).

Out of all these nanoparticles, ZnO nanoparticles have proven to have greater selectivity, heat resistance, and good durability, hence it can be utilized to fight various types of microorganisms, such as *S. aureus* (Manoharan et al., 2015), *E. Coli* (Liu et al., 2009), and *C. albicans* (Janaki et al., 2015). Furthermore, the ZnO nanoparticle is regarded to be more advantageous as it is a low-cost material and its synthesis is very simple (Babayevska et al., 2022). Therefore, the synthesis of ZnO nanoparticles has recently been performed using biological materials such as mushrooms,

even though it is still a scarce technology (Manimaran et al., 2021b). Researchers such as Preethi et al., (2019) have successfully synthesized zinc oxide nanoparticles using *Agaricus bisporus* mushrooms, hence great antimicrobial activity was observed. This proved that mushrooms can be used as an alternative green method for synthesizing useful zinc oxide nanoparticles. A study conducted by Velsankar et al., (2022) provided the advantages of green synthesized ZnO nanoparticles compared to chemically synthesized ones. It was observed that the green synthesized ZnO nanoparticles have strong bactericidal activity compared to the chemically synthesized ZnO nanoparticles. The actual mechanism by which ZnO nanoparticles, induce bactericidal activity in bacteria is not clear as some queries need to be deeply explained (Sirelkhatim et al., 2015), however, several scientists have suggested the following possible mechanism (Figure 9):

(i) Direct contact of ZO nanoparticles with bacterial cell wall

The direct interaction of ZnO nanoparticles and the cell surfaces usually affects the permeability of bacterial cell membranes, which results in a loss of membrane integrity due to the disruption of the bacterial phospholipid bilayer. Hence, this mechanism is considered as one of the important mechanisms utilized by ZnO in fighting against bacteria (Agarwal et al., 2018). The attachment of ZnO nanoparticles to the membrane of bacteria and their transportation towards the inner part of the cell differs for Gram-negative and Gram-positive bacteria due to their difference in membrane structure (Agarwal et al., 2018). For example, Gram-positive bacteria have thick peptidoglycan layer with teichoic acid and lipoteichoic acid which chelate Zn^{2+} ions from the ZnO nanoparticles complex and thereafter transport them inside the bacterial cell (Hood & Skaar, 2012). The Gram-negative bacteria have a thin peptidoglycan layer with ion channels called porin on their outer layer, which facilitates the diffusion of nanoparticles toward the inside of the cell (Agarwal et al., 2018). Hence, the thinner peptidoglycan layer of Gram-negative bacteria causes an easier rupture of the

bacterial cell membrane compared to the rupture of the Gram-positive bacterial cell membrane (Shinde et al., 2014).

(ii) Release of antimicrobial ions such as Zn^{2+}

The partial dissolution of ZnO nanoparticles within the solution causes the release of Zn^{2+} ions, which usually damages the bacterial cell membrane and further disrupts its metabolic pathways (Burman et al., 2013). These ions contribute towards antimicrobial activity via amino acid metabolism and interruption of the enzymatic system. For example, once the Zn^{2+} ions are inside the cell, they interact with the functional groups of proteins and nucleic acids and hinder the normal physiological activities of bacteria together with their enzymatic activities (Yu et al., 2015). The Zn^{2+} reaches the bacterial DNA and causes nuclear damage, which therefore results in irreversible damage to chromosomes, hence inducing cell death (Shoeb et al., 2013).

Several reports indicate that the antimicrobial activity of Zn^{2+} is affected by the duration of exposure and the concentration of Zn^{2+} (Pasquet et al., 2014). However, the antimicrobial activity of ZnO through Zn^{2+} is still debated since the dissolution of Zn^{2+} is affected by numerous parameters as suggested by Pasquet et al., (2014). It has been stipulated that parameters such as the pH of the media (Han et al., 2010), duration of exposure (Peng et al., 2011), UV irradiation (Han et al., 2010), and the physicochemical properties of the nanoparticles affect the dissolution of Zn^{2+} (Bai et al., 2010; Peng et al., 2011; Yebra et al., 2006).

(iii) Formation of reactive oxygen species (ROS)

The generations of radicals such as superoxide anions (O_2^{2-}), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2), cause damage to the cellular components (proteins, DNA, and lipids) of the bacteria (Lallo da Silva et al., 2019). The actual mechanism involves the

activation of ZnO by UV and visible light such that the electron-hole pairs ($e^{-}h^{+}$) are generated, hence H_2O from ZnO nanofluid splits into OH^- and H^+ through the action of electron-hole (Jalal et al., 2010). Furthermore, the dissolved oxygen gets to be transformed into $^{\bullet}O^{-2}$, which thereafter reacts with H^+ and therefore generates $HO_2^{\bullet}$ radicals, that collide with electrons and result in the production of hydrogen peroxide anions (HO_2^{-}) (Jalal et al., 2010). The hydrogen peroxide anions, thereafter, react with hydrogen ions and produce an H_2O_2 molecule which manages to penetrate the cell membrane and get internalized within the bacteria and eventually leads to the loss of cellular integrity as observed in Figure 8 (Lallo da Silva et al., 2019).

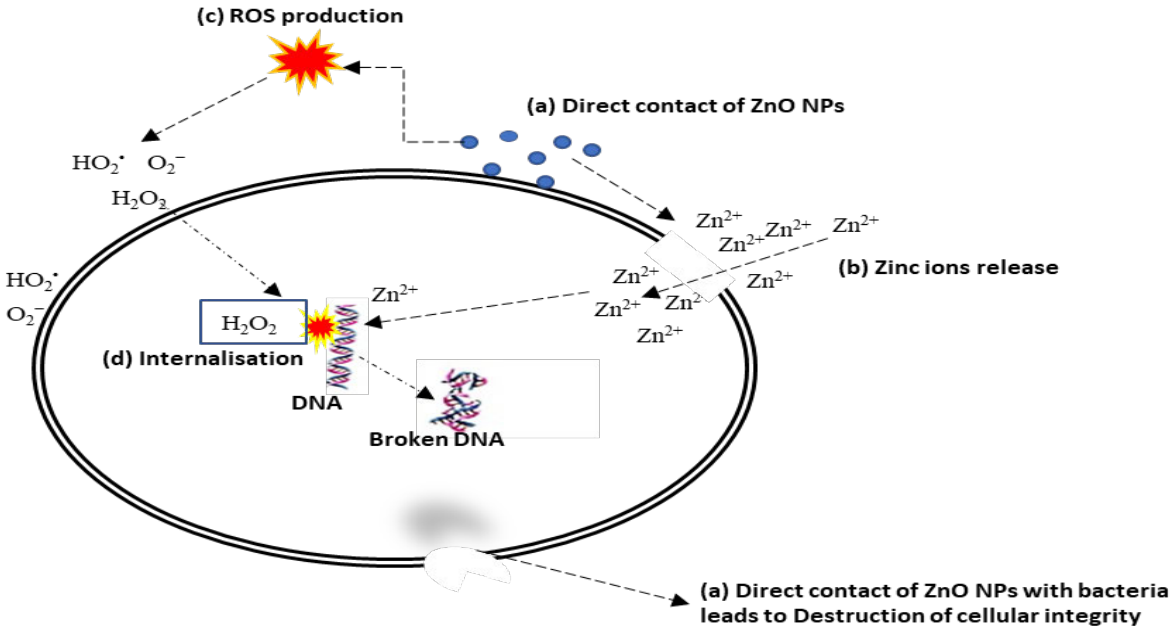

Figure 9: Possible mechanism by which ZnO NPs induce bactericidal activity on bacteria. (Source: Lallo da Silva et al., 2019).

It should however be noted that the mechanism may vary depending on the media since the species of dissolved Zn changes depending on the components of the medium (Li et al., 2011). Generally, the ZnO nanoparticles manage to exhibit significant antimicrobial activity when the particle size is minimized into nanometre ranges (Sirelkhatim et al., 2015). It has been observed by numerous researchers that a decrease in the particle size of nanoparticles enhances the

antibacterial activity of ZnO nanoparticles against *S. aureus* and *E. coli* bacteria (Zhang et al., 2010). During the synthesis of ZnO nanoparticles, mushrooms act as reducing and oxidizing agents which could potentially contribute to the nanoscale of nanoparticles, especially since mushrooms contain bioactive compounds such as proteins, flavonoids, tocopherols, and carotenoids (Eskandari-Nojehdehi et al., 2016). Furthermore, a polysaccharide such as glucans that are found in mushrooms endows the mushroom with great reducing (Kozarski et al., 2015; Mahakham et al., 2016) and capping properties which decrease particle size of the nanoparticles into nanoscale (Guilger-Casagrande & Lima, 2019), hence improves the antimicrobial activity of the nanoparticle.

1.7.2 Antioxidant activity of mushroom synthesized nanoparticles

The human body generates reactive oxygen species (ROS) as the by-products of cellular metabolic reactions; however, cells require low levels of ROS to be able to conduct cellular processes (cell progression, cell defence, and intracellular signaling). Therefore, the production of high levels of ROS due to the antioxidant system's failure leads to oxidative stress within the human body (Madhanraj et al., 2017). The oxidative stress potentially causes various diseases within the human body, due to the imbalance of ROS and antioxidant defenses (Ge et al., 2022). Chronic diseases such as diabetes, cancer, cardiovascular, and neurogenerative diseases have been linked to oxidative stress (Sharifi-Rad et al., 2020). There are various causes of oxidative stress, namely the modern lifestyle of consuming processed food, exposure to toxic chemicals, and the lack of physical exercise (Sharifi-Rad et al., 2020). To minimize such effects, the antioxidant defense systems must minimize the ROS while still permitting enough ROS to remain for useful purposes within the cell such as signaling and redox regulation (Poljsak et al., 2013). According to Poljsak et al., (2013), supplementary antioxidants may help to reduce excessive levels of oxidative stress that were improperly managed by endogenous antioxidants.

It is worth noting that out of all supplementary antioxidants, the synthetic ones such as butylated hydroxyanisole, butylated hydroxytoluene, tertbutylhydroquinone, and propyl gallate turn to

have carcinogenic and toxic effects (Hassanpour & Doroudi, 2023). Therefore, the utilization and the development of natural effective antioxidants such as antioxidants from plants (phenolic compounds, vitamins, and carotenoids) and green synthesized nanoparticles are becoming more desirable (Sowmya et al., 2020). The metal nanoparticles have been observed to have significant antioxidant activity within the intracellular and extracellular environment (Bhardwaj et al., 2020). Previous studies indicate that functional substances such as natural fruit extract which assist in the formation of inorganic nanoparticles, potentially get absorbed on the surface of nanoparticles to scavenge the free radicals, hence promoting the antioxidant effect of nanoparticles (Ge et al., 2022). Furthermore, nanoparticles such as ZnO have been shown to inhibit oxidation through the transfer of electron density situated at the oxygen atom to the nitrogen atom within the DPPH free radicals (Murali et al., 2021). Some studies indicate that Zinc from the nanoparticle potentially acts as an antioxidant by maintaining oxidative stress, maintaining membrane damage, and maintaining integrity within the cell, thus protecting the cellular defence against oxidative stress (Manimaran et al., 2021).

1.7.3 Anticancer potential of mushroom synthesized nanoparticles

Cancer disease is one of the major causes of high mortality worldwide, since chemotherapeutic agents and chemopreventives cause unwanted side effects within the body such as anemia, cellular resistance (Gurunathan et al., 2013), suppression of bone marrow, gastrointestinal and skin disorders (Gavas et al., 2021). Thus, finding alternative drugs or therapies to overcome these drawbacks is of paramount importance (Franco-Molina et al., 2010). Nanoparticles can be used as anticancer since they have advantages over chemotherapeutic agents as they have proven to be stable, have reduced toxicity, are biocompatible, have enhanced permeability and retention effect, and also have precise targeting ability (Gavas et al., 2021). Nanoparticles such as ZnO have gained a lot of interest due to their anticancer, and antibacterial activity, and their affordability and availability within nature (González et al., 2021). The main mechanisms that ZnO nanoparticles use to fight against cancer are ROS generation, apoptosis, and necrosis cell

death (Hussain et al., 2019). Furthermore, cell membrane rupture is also regarded as one of the possible dominant mechanisms of cell death, used by ZnO nanoparticles against cancer cells (Hussain et al., 2019). During apoptosis, the cancer cells commit programmed death due to internal or external stimuli, whereas, in necrosis, the cancer cells are destroyed by external injury (Hussain et al., 2019). However, out of these mechanisms, apoptosis has been reported to be the mechanism used by most of the nanoparticles (Bendale et al., 2017).

1.7.4 Synthesis and Characterization of mushroom synthesized Zinc oxide nanoparticles

Fungi are well known to have high metal tolerance and can bioaccumulate metals since they have high intracellular metal uptake capacity and maximum wall binding capacity (Bhardwaj et al., 2020). Thus, fungi utilize materials such as mycelia, proteins, fruiting bodies, and polysaccharides for the synthesis of metal nanoparticles (Owaid, 2019). This method of synthesis of nanoparticles using fungi is deemed a bottom-up strategy, whereby the reaction is based on oxidation or reduction of the substrate. Previous studies indicate that enzymes and metabolites produced by fungi are responsible for the reduction of metallic compounds in nanoparticles (Kashyap et al., 2013). During the synthesis of nanoparticles, fungal extracts act as capping and reducing agents, however, when the fungal mycelium is exposed to metal precursor, the fungi liberate enzymes and metabolites that the fungi use for survival (Mirunalini et al., 2012). This means both the fruiting body and the mycelium of mushrooms can be used for the synthesis of nanoparticles (Bhardwaj et al., 2020). Hence, the mechanism of synthesis of nanoparticles using mushrooms seems to be simple, however, various factors influence the stability and the biocompatibility of the synthesized nanoparticles (Elsakhawy et al., 2022). Such factors are temperature, pH during the reaction (preferably use of higher pH), the quantity of the used biomass, and lastly the composition of the medium (Srivastava & Bhargava, 2022).

The process of myco-synthesis of nanoparticles using mushrooms can be either intracellular or extracellular, whereby the intracellular method involves the synthesis of nanoparticles inside the cells through the transportation of ions during the exposure of enzymes (Chan & Mashitah,

2012). The intracellular synthesis incorporates metal precursors into mycelia cultures and results in nanoparticles of small size when compared to those synthesized extracellularly. The disadvantage of the intracellular method is that it is slower compared to the extracellular (Narayanan & Sakthivel, 2010), and its downstream is complicated resulting in increased cost of synthesis (Dhillon et al., 2012). Therefore, the extracellular synthesis method is the most used method for nanoparticle synthesis (Devi & Joshi, 2015) due to its advantages such as being simple and cost-effective. The method involves adding metal precursors to an aqueous mushroom filtrate, allowing metal ions to be adsorbed onto the surface of fungal cells (Guilger-Casagrande & Lima, 2019). Amongst the *Pleurotus* spp, the *Pleurotus ostreatus* is capable of synthesizing nanoparticles using both intracellular and extracellular approaches, while other spp such as *P. florida*, *P. cornucopiae var*. *citrinopileatus, P. platypus*, *P. ostreatus*, *P. sajor-caju*, *P. eous*, and *P. djamor var* could only synthesize nanoparticles using extracellular approach (Yehia & Al-Sheikh, 2014). Little information is available on the synthesis of ZnO nanoparticles using edible mushrooms such as *Pleurotus ostreatus* (Mkhize et al., 2022), *P. florida* (Bhat et al., 2013), *P. sapidus*, and *P. sajor caju* (Chan & Mat Don, 2013). Therefore, more studies are needed to explore the synthesis of ZnO nanoparticles using *Pleurotus* mushrooms. After the synthesis of ZnO nanoparticles, X-ray diffraction (XRD) is used to characterize the nanoparticles with good peaks and standard diffraction peaks indicating successful synthesis of the nanoparticles (Dimapilis et al., 2018). The diffraction peaks also give information about the crystallinity of the nanoparticles, thus narrower and intensive diffraction peaks symbolize a good crystalline nature of synthesized ZnO, whereas a peak that is broadening at the bottom symbolizes a small crystalline size (El Saeed et al., 2015). Besides the above-mentioned characterization techniques, there are various techniques such as Scanning electron microscopy (SEM) and Transmission Electron Microscopy (TEM) which usually determine the morphology, size, and structure of the synthesized nanoparticles (Dimapilis et al., 2018).

1.8 Problem Statement

The production and cultivation of mushrooms such as *Lentinula edodes* and *Pleurotus ostreatus* is growing globally (Mleczek et al., 2021). However, their cultivation using agricultural waste raises concerns since global environments are continuously exposed to toxic heavy metals due to human and natural activities (Kaur & Sharma, 2021). For example, Sugar cane production involves the use of pesticides and fertilizers, potentially affecting the food chain, as some mushroom growers use sugar cane by-products as substrates. Therefore, there is a critical need to assess the bioaccumulation of these toxicants on edible mushrooms, particularly given that many agricultural sectors produce edible mushrooms from agricultural waste.

Antimicrobial resistance (AMR) is an issue that is still evolving and spreading in both developed and developing nations (Dhingra et al., 2020). Additionally, non-communicable diseases like diabetes, cancer, cardiovascular disease, and chronic respiratory conditions are also becoming more prevalent (Babel et al., 2021). Finding emergency solutions to the above-mentioned challenges is of paramount interest to most researchers. Hence several researchers have focused on natural compounds obtained from microbes, plants, and animals as a strategy to fight the above-mentioned challenges (Gyawali and Ibrahim 2014). The focus has shifted to natural compounds mainly because they have the potential to combat drug tolerant infections, which conventional antibiotics are failing to eliminate (Rossiter et al., 2017). Therefore, the focus of the research was based on optimizing the mushroom-growing substrates through the addition of supplements such as wheat bran and maize flour in order to promote the mushroom's secondary metabolite with therapeutic properties. It has been observed previously that the metabolite profiles of fungi are strongly influenced by varying cultivation conditions of fungi, hence even small changes in the composition of growth media can alter the amount of specific compounds and can also result in different types of compounds (Brakhage et al., 2011).

Therefore, by varying the levels and type of supplements added to the mushroom growing substrates, probably certain health and biological activities of *P. ostreatus* mushroom would be

enhanced or otherwise reduced. These supplements are usually added to provide nutrients such as carbon and nitrogen which are required by the mushroom to grow optimally, hence potentially influencing the mushroom yield as well.

Another alternative method that could be used in combating the challenges of antimicrobial resistance (AMR) and non-communicable diseases such as cancer, is through the synthesis of nanoparticles (Himanshu et al., 2023). However, the major challenge in the synthesis of nanoparticles is that most of the methods used are toxic, costly, and time-consuming (Pal et al., 2022). Hence finding a reliable and better production method that is cheap, safe, and environmentally friendly is of utmost importance (Bhardwaj et al., 2020). Thus, our study green synthesized Zinc NPs using *P. ostreatus* mushroom as a capping and reducing agent. To the best of our knowledge, this would be the first time the *P. ostreatus* mushroom is being used to synthesize Zinc nanoparticles.

1.9 Research Aims and Objectives

1.9.1 Aim of the study

The study aimed to investigate heavy metal bioaccumulation by *P. ostreatus* mushrooms grown in agro-waste, and further aimed to ascertain the impact of adding supplements on agro-waste towards mushrooms' medicinal properties. Additionally, the study sought to biosynthesize nanoparticles using *P. ostreatus* as a capping and reducing agent to enhance its therapeutic benefits.

1.9.2 Objectives of the Study

To achieve the above-mentioned aims, the following objectives were undertaken:

(i) Evaluating the concentration or presence of heavy metals within both the substrates/agro-waste (sugar cane bagasse and sugar cane tops/leaves) and within the *P. ostreatus* mushrooms cultivated on the above-mentioned agro-waste products.

(ii) Investigating heavy metal transfer or absorption from the agro-waste into *P. ostreatus* mushrooms.

(iii) Evaluating the type of heavy metals that have high affinity to be absorbed by *P. ostreatus* mushroom and further confirming if they are above or below the recommended daily intake which is recommended by WHO or FAO.

(iv) Investigating the effect of supplementing the sugar cane substrates with wheat bran (WB) supplements on biological activities (DPPH and DNA cleavage) and yield of *P. ostreatus* mushroom.

(v) Evaluating the influence of supplementing mushroom growing substrates (sugar cane bagasse and sugar cane tops/leaves) with wheat bran towards parameters such as carbon to nitrogen (C/N) of substrates, mushroom yield, content/type of bioactive compounds, antimicrobial activity (multidrug-resistant strains) and antioxidant activity of *P. ostreatus* mushroom.

(vi) Evaluating the biological activities and profile bioactive compounds found in *Pleurotus ostreatus* cultivated from agro-waste (sugar cane bagasse and sugar cane tops/leaves) supplemented with maize flour. Thus, the influence of supplementing mushroom growing substrates with maize flour supplements was investigated towards parameters such as, the carbon to nitrogen(C/N) ratio of substrates, mushroom yield, bioactive compounds, and antimicrobial, antioxidant, and antimalarial potential of *P. ostreatus* was investigated.

(vii) Evaluated the biological usefulness of *P. ostreatus* mushroom extracts as capping and reducing agents in the biosynthesis of ZnONPs, hence an alternative cheap and eco-friendly method of synthesizing a stable and biocompatible ZnO NPs with biological properties (antimicrobial, DNA cleavage, cytotoxicity on Hek293 and HepG2 cells) was evaluated.

1.10 Outline of the thesis

This thesis is presented in six chapters, that are formatted into research paper format. The first chapter outlines the rationale and the literature of the research study, together with the aims and objectives of the research. Since the current thesis is presented in manuscript format, it then entails the following chapters that are in paper format:

Chapter two, Outlines the bio-absorption of heavy metals by *P. ostreatus* mushrooms cultivated on agro-bio-waste products. This chapter also outlines the influence of adding wheat bran supplements to the free radical scavenging properties of *P. ostreatus* mushrooms. This chapter was published by the Pharmacognosy Journal; hence it was formatted according to the journal's standards.

Chapter three mainly focuses on the influence of wheat bran supplements on mushroom productivity, the content of bioactive compounds, and the biological activity of oyster mushrooms. Thus, new information on the benefits of adding wheat bran supplements to improve quality, content of bioactive compounds, productivity, antimicrobial activity, and antioxidant properties of *Pleurotus ostreatus* mushrooms will be stipulated in this chapter.

In **Chapter four**, the emphasis was based on growing *Pleurotus ostreatus* mushrooms on sugarcane waste substrates that were majorly supplemented with maize four, which has been rarely used when compared to other supplements such as wheat bran. Therefore, this chapter focuses on the effect of maize four supplements on the mushroom's bioactive compounds, antioxidant properties, antimicrobial properties, and antimalarial properties of the mushroom. Thus, the medicinal benefits of adding maize four to the mushroom-growing substrates could be established.

Chapter five involves the synthesis of stable and biocompatible ZnO nanoparticles using the green synthesis method by employing mushroom extracts as the capping and reducing agent. This chapter also involves the characterization of the synthesized ZnO nanoparticles and further

evaluates the antimicrobial, antioxidant, and anticancer potential of the synthesized ZnO nanoparticles, using a fast, safe, cost-effective, and eco-friendly green method.

Chapter six represents the general discussion of the study's overall findings and provides conclusions together with potential recommendations for future studies that would address various challenges facing the world.

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CHAPTER 2

Evaluating the Antioxidant and Heavy Metal Content of *Pleurotus ostreatus* Mushrooms Cultivated using Sugar Cane Agro-Waste

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This published article investigated the absorption of heavy by *P. ostreatus* mushrooms cultivated on agro-waste, that was supplemented with wheat bran. It further elucidated the influence of wheat bran supplementation on the free radical scavenging properties of mushrooms. This chapter has been published in *Pharmacognosy Journal* with the tittle: Evaluating the Antioxidant and Heavy Metal Content of *Pleurotus ostreatus* Mushrooms Cultivated using Sugar Cane Agro-Waste.

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Original Article

Evaluating the Antioxidant and Heavy Metal Content of *Pleurotus ostreatus* Mushrooms Cultivated using Sugar Cane Agro-Waste

Senzosenkosi Surprise Mkhize[1], Mthokozisi Blessing Cedric Simelane[2], Nothando Lovedale Gasa[1], Ofentse Jacob Pooe[1,*]

ABSTRACT

Background: *Pleurotus ostreatus,* is one of the most cultivated mushrooms with great economic and medicinal value that can be easily grown on various bio-waste substrates. However, biosafety evaluations on these mushrooms are rarely conducted. Thus, we sought to evaluate the concentration or presence of Heavy metals in *P. ostreatus* mushrooms cultivated on agro-bio-waste products. Furthermore, the effect of adding agro wastes on wheat bran (WB) cultivated mushrooms was evaluated. **Methods:** Mushrooms grown in sugar cane tops and bagasse were supplemented with varying levels of WB. Atomic absorption spectrophotometer was applied to evaluate the concentration of heavy metals in the substrates and within mushrooms. Furthermore, DPPH free radical scavenging activity was used to determine antioxidant activity of mushroom extracts. **Results:** The transfer factor analysis (TF) showed that mushrooms have an affinity to absorb Zn, Cd, Cu and Cr from all tested substrates during cultivation (TF>1). The addition of WB supplement into substrates resulted into significant increase in mushroom yield. However, the increased addition of WB, inversely affected the DPPH scavenging activity of the *P.ostreatus* methanolic extracts. **Conclusion:** The bio-absorption of heavy metals by *P. ostreatus* is depended on the metal type. Based on these findings, mushrooms grown on these agro-waste appear to be safe and potent scavenging ability against free radicals.

Key words: Heavy metals, Mushrooms, *Pleurotus ostreatus*, DPPH, Antioxidant.

Senzosenkosi Surprise Mkhize[1], Mthokozisi Blessing Cedric Simelane[2], Nothando Lovedale Gasa[1], Ofentse Jacob Pooe[1,*]

[1]Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, SOUTH AFRICA.
[2]Department of Biochemistry, University of Johannesburg, P.O. Box 524, Auckland Park, 2006, SOUTH AFRICA.

Correspondence

Ofentse Jacob Pooe

Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, SOUTH AFRICA

E-mail: PooeO@ukzn.ac.za

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INTRODUCTION

The oyster mushroom, namely *Pleurotus* spp., can be classified as one of the white-rot fungi under the class of basidiomycetes, which belongs to the family of Tricholomataceae. Mushrooms have been reported as potent bio-absorber of heavy metals in bioremediation studies, accumulating both essential and non-essential (toxic) heavy metals from the growth substrate.[1,2] Mushrooms have previously been shown to absorb toxic heavy metals such as mercury (Hg), lead (Pb), cadmium (Cd) and aluminum (Al), which are detrimental to human health.[3,4] These heavy metals may originate from pesticides, organic and inorganic fertilizers, livestock and poultry manure.[5] Hence, it is imperative to adequately analyze the chemical and elemental composition of growing substrates since some toxic metals could be transferred from the substrates into mushrooms during cultivation.[6] During mushroom cultivation, the growing media is usually supplemented with nitrogen sources which increase both biomass and productivity of mushrooms.[7,8] Furthermore, it has also been noted that supplementation of substrates with nitrogen cause mushrooms to become a great source of protein and minerals.[9] Hence supplements such as wheat bran have been reported to be a great source of minerals such as magnesium (Mg), zinc (Zn), manganese (Mn), iron (Fe) and phosphorus (P) for the mushrooms.[10] Recently, mushroom farmers across developing countries have resorted to using low cost agro-waste products such as sugar cane waste residues as base substrates in mushroom cultivation. However, the potential transfer of heavy metals from agro-waste products into mushrooms during cultivation is yet to be elucidated. Some of these agro-wastes may contain heavy metal elements.[11] Thus, the main goal of the present work was to investigate heavy metal absorption by mushrooms cultivated on agro-waste, namely, sugar cane tops (leaves) and bagasse in the presence of WB. Furthermore, to confirm the influence of wheat bran supplementation on the free radical scavenging properties of mushrooms.

MATERIALS AND METHODS

Mushroom spawn and bulk substrate preparation

The sugar cane tops were obtained from farms around the northern part of KwaZulu-Natal province, South Africa (UVS farm at longitudes 28°42'24.9"S 31°54'09.0"E). The bagasse substrate, WB supplement and test mushrooms (*P. ostreatus*) were all obtained at the South African Department of Agriculture and Rural Development. The mushroom strain (*P. ostreatus*) was pre-cultured on potato dextrose agar and incubated in the dark environment at 25 ºC and then maintained as working spawn cultures at 4 ºC.

The modified method outlined by Crisan and Sands (1978) was utilized for spawn preparation, whereby 1 kg of sorghum grains were soaked overnight in 1.5 l

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of water then excess water was drained. Gypsum ($CaSO_4 \cdot 2H_2O$), calcium carbonate ($CaCO_3$) and soaked grains were mixed, respectively, in a ratio of 4:1:300 g. The mixture was then packed into a 250 ml bottles and sterilized at 121 ºC. The mixture was then aseptically inoculated with 10 mm^2 of previously grown pure cultures of test mushroom strain (*P. ostreatus*). The inoculated bottles were incubated in the dark at ± 25 ºC for approximately two weeks. The sugar cane tops were milled but the bagasse substrate did not need further processing since it was already in a fine form. Tap water was added to the substrates to achieve 65 % moisture content using the following rule of thumb, 1 droplet to 2 droplets of water must be released when the substrate is squeezed. The substrates were then separately supplemented with various levels of WB, viz, 0 % WB, 2 % WB, 18 % WB and 20 % WB, respectively. After mixing supplements thoroughly with the base substrates, 1 kg of the resultant substrate was packed into polypropylene bags (22.5 cm × 30 cm) and compressed by hand to achieve compactness. The bagged substrates were pasteurized at 60 ºC to 65 ºC for 6 h and allowed to cool to room temperature.

Substrate inoculation, spawn running and fruiting

After cooling, the bagged substrates were inoculated with previously prepared pure grain spawn of the test mushrooms at the rate of 2 % of wet substrate under the lamina flow hood. The inoculated bags were incubated in a dark room at 25 ºC to 27 ºC until they became fully colonized by mycelia. The bags were then transferred to a fruiting room which was constructed from plastic film that was covered by a single layer of 30 % grey shade cloth on the outside. The mushrooms were fruited under ambient temperatures. The mushroom fruiting bodies were harvested and dried in the same tunnel with 30 % shade cloth which had varying temperatures depending on the weather, however the temperatures did not exceed ± 45 °C and thereafter were powdered for further analysis.

Heavy metal evaluation

The heavy metals were determined both in substrates and within the actual mushroom using modified methods of Lanre-Iyanda and Adekunle (2012).[12] The powdered mushrooms and substrates (sugarcane and bagasse) of about 0.5g was firstly ashed in muffle furnace which was at 250°C for the period of 12 hours. After ashing, the samples were then digested with 9 ml of aqua regia solution (HCL and HNO3 at ratio 3:1). The digested solution was then transferred into a 25 ml volumetric flask and the flask was filled up to the mark with deionized water. Thereafter, analysis of different elements was carried out using an atomic absorption spectrophotometer.

Estimated daily intake analysis

The estimated daily intake (EDI) of metals through consumption of 100 g fresh mushrooms by individuals of 65k g in body weight were calculated using following described formula[13]:

$$EDI = C\ metal\ X\ D\ mushroom\ intake \div BW\ average$$

Whereby, C = metal concentration in mg/kg; D = daily intake of mushroom in kg person^{-1}; BW= average body weight in kg person^{-1}

Evaluation of mushroom yield

The yield of mushrooms was calculated using a modified method from Morais *et al*. (2000), whereby the following equation was used: MY = [Weight of fresh mushroom harvested (g) per fresh substrate weight]. Hence the yield of mushrooms grown from differently supplemented substrates was attained.

DPPH scavenging activity

Slightly modified method by Ayeni *et al*. (2019) was to used, to confirm the DPPH radical scavenging ability of mushroom extract[14,15]. Each methanol mushroom extract had stock solution of (25mg/ml) of which was diluted into various concentrations ranging from 10-800 µg/ml. About 500µl of DPPH solution (0.1Mm) was mixed and incubated with 1ml of extract at various concentrations and kept in dark for 30min. Then the absorbance was read at 517nm, hence the percentage of scavenging ability was calculated using the following formula:

% Scavenging Activity = (Ac - As)/ Ac *100

Where: Ac = Absorbance of the control; As = Absorbance of the sample. BHT was used as the standard.

DNA cleavage assays

The ability of *P. ostreatus* mushrooms to protect pET30 plasmid DNA from damage caused by free radicals namely Fenton's reagent (30% H_2O_2 50Mm Ascorbic acid and 80Mm iron (III) chloride), was evaluated following previously stated methods.[14] The reaction was carried out in 96 well microtiter plate with the reaction mixture made up to a total volume of 10µl composed of 5µl pET30 plasmid DNA (concentration of 2.4 µg), 0.5 µl Fenton's reagent , 4 µl of various mushroom extract (2mg/ml) dissolved in DMSO solvent and the final volume of the reaction mixture was brought up to 10 µl using distilled water. The reaction mixture was incubated for 30 min at 25°C in the absence of light. Immediately after incubation the reaction mixtures were mixed with 5 µl loading dye, (0.25% bromophenol blue dye in 50% glycerol). Total mixture of each sample loaded into 1% agarose gel (1 g of agarose dissolved in 100ml in TAE buffer). Electrophoresis was carried out at 100 volts for 45 min, thereafter the nicked and native DNA were visualized under UV light.

Data analysis

All experiments were repeated in triplicate. Data generated were calculated using SPSS original version 6.0 and GraphPad Prism version 5.0. The results are reported as mean ± S.E.M. The statistical differences were determined using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. The values were considered statistically significant where $p \leq 0.05$. Furthermore, Pearson's correlation analysis ($p < 0.05$) was employed to understand the relationship between supplement level and the concentration of heavy metals.

RESULTS AND DISCUSSION

The current study evaluated the concentration of nine heavy metals within both substrates supplemented with WB (Table 1) and within the *P. ostreatus* mushroom grown on these substrates (Table 2). During Mushroom cultivation supplementation with WB can be the source of minerals such as magnesium (Mg), zinc (Zn), manganese (Mn), iron (Fe) and phosphorus (P) for the mushrooms [10]. Metals such as Cd, Pb, Cr and Al are known as non-essential metals since they are toxic for both humans and the environment even at minimum concentrations.[16,17] The results displayed a varying concentration of the nine metals, as noted, however metals such as Al and Fe were observed to be in greater concentrations for all the substrates (sugar cane and bagasse) as well as within the mushrooms cultivated on these substrates. The high concentration of Al and Fe within all the substrates can be possibly be traced back to the source of the substrates i.e. sugar cane, which has been shown by previous researchers to retain and absorb heavy metals.[18,19] Furthermore, bagasse has also been found to be a potent bio-sorbent of metallic pollutants.[20] The results observed in Table 1 indicated that the sugarcane tops contained different heavy metals at varying concentrations. Metals which were in high concentration within sugar cane tops were Al at 871.39 ±44.71, Fe at 609.43±25.88 followed by Mn at 153.02±2.19.

Whereas metals such as cadmium (Cd), chromium (Cr) and copper (Cu) were observed to be in lower concentrations within sugar cane tops. The addition of WB supplement into substrates significantly influenced concentrations of some heavy metals within substrates,

Table 1: Heavy metal concentration of base substrate sugarcane tops and bagasse supplemented with varying levels of WB (mg/kg) prior to mushroom cultivation.

WB-Sugarcane tops substrate heavy metal analysis					
Substrate composition	**Al**	**Cu**	**Cd**	**Fe**	**Mn**
0% WB-Sugarcane	588.16 ± 63.85^{a}	3.90 ± 0.15^{a}	3.04 ± 0.50^{a}	370.15 ± 29.15^{a}	184.99 ± 7.65^{b}
2% WB-Sugarcane	790.41 ± 66.18^{ab}	4.04 ± 0.22^{a}	2.51 ± 0.06^{a}	495.27 ± 23.38^{b}	151.69 ± 3.41^{a}
18% WB-Sugarcane	819.0 5± 40.93^{b}	5.11 ± 0.28^{ab}	2.52 ± 0.15^{a}	549.68 ± 17.19^{bc}	140.10 ± 2.30^{a}
20% WB-Sugarcane	871.39 ± 44.7^{1}b	5.67 ± 0.31^{b}	2.39 ± 0.02^{a}	609.43 ± 25.8^{8}c	153.02 ± 2.1^{9}a
	Ni	**Pb**	**Cr**	**Zn**	
0% WB-Sugarcane	13.94 ± 0.64^{a}	7.30 ± 1.70^{a}	4.1 2± 2.90^{a}	16.14 ± 0.61^{a}	
2% WB-Sugarcane	13.48 ± 1.90^{a}	9.80 ± 1.83^{a}	2.27 ± 1.06^{a}	18.56 ± 1.40^{a}	
18% WB-Sugarcane	12.99 ± 2.09^{a}	11.7 6± 2.20^{a}	5.24 ± 1.49^{a}	29.73 ± 0.31^{b}	
20% WB-Sugarcane	14.21 ± 0.98^{a}	10.94 ± 1.31^{a}	3.78 ± 0.11^{a}	32.8 4± 1.40^{b}	
Bagasse-WB substrate heavy metal analysis					
Substrates composition	**AL**	**CU**	**Cd**	**Fe**	**Mn**
0% WB-Bagasse	3345.66 ± 253.13^{b}	0.44 ± 2.15^{a}	3.51 ± 0.10^{a}	3476.32 ± 436.66^{a}	103.89 ± 6.74^{a}
2% WB-Bagasse	2433.11 ± 75.26^{a}	7.11 ± 0.77^{a}	4.05 ± 0.21^{a}	2837.58 ± 332.^{a}	131.79 ± 29.14^{a}
18% WB-Bagasse	2298.90 ± 24.08^{a}	8.54 ± 0.21^{a}	2.16 ± 1.47^{a}	2296.20 ± 58.83^{a}	107.20 ± 1.32^{a}
20% WB-Bagasse	2306.79 ± 125.79^{a}	9.02 ± 0.46^{a}	1.56 ± 0.88^{a}	2015.87 ± 179.42^{a}	122.23 ± 4.73^{a}
	Ni	**Pb**	**Zn**	**Cr**	
0% WB-Bagasse	27.29 ± 3.24^{b}	22.17 ± 7.96^{a}	103.48 ± 81.64^{a}	36.41 ± 2.35^{a}	
2% WB-Bagasse	16.71 ± 2.71^{ab}	14.45 ± 2.77^{a}	21.40 ± 1.76^{a}	26.04 ± 2.28^{a}	
18% WB-Bagasse	12.51 ± 1.20^{a}	10.97 ± 0.51^{a}	34.90 ± 1.13^{a}	14.96 ± 2.11^{a}	
20% WB-Bagasse	12.67 ± 0.07^{a}	14.11 ± 2.93^{a}	37.11 ± 1.78^{a}	20.04 ± 2.32^{a}	

Superscript with different letter(s) are significantly different ($P \leq 0.05$) within same column. Superscript with different letter(s) are significantly different ($P \leq 0.05$) within same column.

Table 2: Heavy metal concentration within *P. ostreatus* grown on sugarcane tops supplemented with varying levels of WB (mg/kg).

Heavy metal analysis on *P. ostreatus* grown with the sugarcane tops					
Substrates composition	**AL**	**CU**	**Cd**	**Fe**	**Mn**
0% WB-Sugarcane	46.21 ± 4.35^{b}	10.37 ± 0.52^{ab}	7.64 ± 4.77^{a}	330.39 ± 220.94^{a}	12.80 ± 5.37^{a}
2% WB-Sugarcane	48.58 ± 2.98^{b}	10.28 ± 0.19^{ab}	4.87 ± 0.52^{a}	117.54 ± 5.99^{a}	26.04 ± 1.75^{a}
18% WB-Sugarcane	10.15 ± 3.17^{a}	8.60 ± 0.39^{a}	4.46 ± 0.19^{a}	155.30 ± 59.66^{a}	13.56 ± 6.23^{a}
20% WB-Sugarcane	18.46 ± 3.30^{a}	12.64 ± 1.13^{b}	5.52 ± 1.12^{a}	698.99 ± 469.53^{a}	9.83 ± 2.42^{a}
	Ni	**Pb**	**Cr**	**Zn**	
0% WB-Sugarcane	6.69 ± 0.19^{a}	4.82 ± 0.17^{a}	18.94 ± 10.68^{a}	90.98 ± 2.70^{a}	
2% WB-Sugarcane	4.54 ± 0.31^{a}	3.99 ± 0.76^{a}	6.17 ± 0.15^{a}	110.56 ± 2.39^{b}	
18% WB-Sugarcane	3.61 ± 0.40^{a}	3.3 ± 0.73^{a}	11.76 ±7.82^{a}	89.314 ± 4.74^{a}	
20% WB-Sugarcane	5.98 ± 3.28^{a}	3.16 ± 0.17^{a}	90.31 ± 70.79^{a}	95.78 ± 1.66^{a}	
Heavy metal analysis on *P. ostreatus* grown with the bagasse supplement					
Substrates composition	**AL**	**CU**	**Cd**	**Fe**	**Mn**
0% WB-Bagasse	12.63 ± 1.16^{a}	5.02 ± 0.33^{a}	3.79 ± 0.16^{a}	115.11 ± 3.18^{a}	26.98 ± 2.28^{a}
2% WB-Bagasse	21.7 ± 4.77^{a}	5.49 ± 0.86^{a}	4.43 ± 0.07^{ab}	117.94 ± 8.55^{a}	30.85 ± 1.20^{a}
18% WB-Bagasse	34.46 ± 1.86^{a}	5.82 ±0.36^{a}	3.96 ± 0.04^{a}	145.46 ± 23.75^{a}	8.82 ± 0.56^{a}
20% WB-Bagasse	23.25 ± 7.92^{a}	17.67 ±10.33^{a}	5.20 ± 0.35^{b}	995.85 ± 870.04^{a}	30.17 ± 21.21^{a}
	Ni	**Pb**	**Zn**	**Cr**	
0% WB-Bagasse	1.93 ± 0.34^{a}	0.67 ± 0.17^{b}	98.79 ± 4.53^{a}	3.29 ± 0.05^{a}	
2% WB-Bagasse	2.42 ± 0.14^{a}	*<DL	94.33 ± 9.18^{a}	5.86 ± 1.97^{a}	
18% WB-Bagasse	4.0 5± 0.22^{a}	*<DL	86.98 ± 2.30^{a}	9.97 ± 1.69^{a}	
20% WB-Bagasse	29.53 ± 26.15^{a}	*<DL	100.49 ± 6.60^{a}	944.83 ± 871.21^{a}	

Superscript with different letter(s) are significantly different ($P \leq 0.05$) within same column.

hence it was evident from Table 1 that the addition of WB ($p < 0.05$) caused a significant increase in concentration of Fe, Zn and Cu within sugar cane tops (Table 1).

However, the addition of WB showed no significant influence on heavy metals such as Cd, nickel (Ni), Pb and Cr. The results in Table 2 suggest that the uptake of heavy metals by the *P. ostreatus* was not significantly influenced by the varying levels of WB or Baggase supplementation, with the exceptions of Al and Cu.. For Cu only 20% WB had contrary outcomes. Metals such as Al, Fe and Zn were in higher concentration within the *P. ostreatus* mushroom compared to other metals (Table 2). In Table 2, the metals which appeared to be at a high concentration within the *P. ostreatus* was Fe (995.85 ± 870.04) and Zn (100.49 ± 6.60), while Pb (2%, 18% and 20% WB) was found to be below the detection limit of 0.01 mg/kg. Table 2 further shows that the addition of varying levels of supplements had no statistically significant effect on the concentration of heavy metals such as Al, Cu, Fe, Mn, Ni, Zn and Cr within mushrooms were not influenced by WB. The observed data was in line with previously obtained data for the bagasse substrate, this might be due to the natural abundance of these metals within the environment (Opaluwa *et al*., 2012). The

results in Table 3 show that there was no major significant effect on supplementing the bagasse substrates with WB in terms of heavy metal concentration. Regarding Al it was observed that the control (0%) had significantly ($p < 0.05$) higher concentration (3345.66 ± 253.13) compared to other levels of supplementation. For Ni, the control also had significantly higher concentration (27.2 9± 3.24) compared to other levels of supplementation. Some heavy metals such as Pb and Cd within *P. ostreatus* mushroom were significantly influenced by addition of varying levels of WB since the control culture (0%) had a minimum concentration of Cd. This study found that the accumulation of heavy metals within *P. ostreatus* varied for different metals and for mushrooms grown on different substrates. It was observed that metals such as Fe and Zn were in high concentrations within *P. ostreatus* mushrooms grown on both sugarcane tops and bagasse substrates (Table 2). This was in line with the findings of Zhu *et al.* (2011) showing that Fe and Zn are found in higher concentrations within mushrooms.[21] The Zn metal concentration ranged from 89.314 mg/kg to 110.56 mg/kg for *P. ostreatus* grown on sugar cane tops, and for *P. ostreatus* grown on bagasse substrate it ranged from 86.98 mg/l to 100.49 mg/l which was above the WHO permissible limit of 60 mg/kg.[19,22] Concentrations of Fe and Zn indicate that the *P. ostreatus* mushrooms may have high affinity towards Zn and Fe.

In general, when comparing metal concentration for mushrooms grown on both substrates it was observed that the metals which were higher for sugar cane tops grown mushrooms (AL, Fe and Zn) and were slightly lower for the bagasse grown mushrooms. This corroborates with the findings o f O gbo a nd O khuoya (2011) w ho stated that the biosorption of metals by species vary depending on the type of metal, metal concentration and most importantly the composition of the substrate.[22] To trace the source of heavy metals found within mushrooms, the transfer factor (TF) is used since it is one of the important factors which indicate the ability of metals to be transferred from substrate into mushroom. The TF > 1 indicates that the mushroom gains metals from the soil/substrate, while a TF < 1 means that the mushroom excludes metals from the substrates.[23] The results of this study indicate that the mobility of metals varies with type of metal and type of substrate. Hence, it was observed in Figure 1 that some elements had high mobility, and some had lower mobility. Heavy metals such as Zn, Cd, Cu and Cr, had TF > 1; although Cu and Cr on sugar cane tops substrate had TF >1, however on bagasse they had TF < 1. Other metals such as Fe, Al, Ni, Pb and Mn had TF < 1. Such differences in the transfer or biosorption of heavy metals may be due to factors such a variation in the composition of substrates,[4] type of metal and concentration.[5,22,24] According to researchers in the field, transfer factor does not necessarily represent the risk of a heavy metal, but it shows the possible source of contamination.[25] This study can then conclude that the nonessential metal, Cd, found in mushrooms is likely to have been transferred or absorbed by the mushroom from both substrates that were used for cultivation.

Furthermore, the study indicated that supplementing the substrates with WB does not significant correlate with the concentration of most metals found both in substrates and within mushrooms. For heavy metals found in substrates it was observed that the addition of WB into substrates significantly correlated with the concentration of heavy metals found in substrates such as sugar cane tops (Cu, r=0.982; Zn, r=0.996) and bagasse (Zn, r=0.993; Fe, r=-0.977) (Table 4). Addition of WB in substrates caused some negative correlation with concentration of heavy metals found in mushrooms. It was observed that there was negative correlation of WB and Al (r=-0.980) in mushrooms grown in sugar cane tops supplemented with WB. Also, some negative correlation was observed on Fe (r=-0.978) and Pb (r=-0.982) for mushrooms grown on bagasse supplemented with WB (Table 2). Such negative correlation may be linked to the fact that the fungi usually produce oxalic acid as means of immobilizing metals ions and complexes into insoluble oxalates which cause reduced bioavailability of these metals hence tolerance to the metals is increased.

Further analysis confirmed all the heavy metals tested within the mushrooms are that were below or within the recommended daily intake (RDI) set by regulatory body such as the FAO/WHO (Table 4). Nonetheless, caution needs to be exercised when consuming in following daily intake limits to avert any health risk associated with the consumption of these mushrooms might be reduced.

Some authors have indicated on previous studies that the practice of supplementation of substrate is beneficial in order to obtain satisfying yields together with good development of mushrooms [26]. Similarly, in this study that the addition of wheat bran as supplement into mushroom growing substrate contributed greatly into an increase in mushroom yield for both substrates (Figure 2). It was observed that as supplements were increasing also the yield increased up to certain point then had some slightly decrease. These findings corroborate with study conducted by Moonmoon *et al.*, 2011 who also found similar trend in terms of addition of supplements such as wheat barn, maize flower and rice bran as supplements to improve mushroom yield.[27] Thus, the practice of adding wheat bran as supplement seems to be beneficial for improving mushroom yield, however its effect on improving the therapeutic property of the mushroom (*P. ostreatus*) is not yet well recognized.

The results on Tables 5 and 6 indicated that *P. ostreatus* extracts were effective in scavenging DPPH radicals in a dose dependent manner, meaning the increase in concentration of the extract resulted in higher percentage in radical scavenging activity. Such activity might probably be due to the fact that *P.ostreatus* mushroom is rich in phenolic compounds which significantly contributes to numerous biological pathways, which could possibly scavenge free radicals [28]. By definition IC_{50} is the concentration of antioxidant required to scavenge 50% of DPPH radical, therefore smaller IC_{50} is ideal since it correspond

Table 3: Pearson's coefficient of correlation among supplement (WB), mushrooms and concentration of various heavy metals.

Concentration of Heavy metals									
Supplemented Substrate only	**Cu**	**Zn**	**Al**	**Cd**	**Fe**	**Mn**	**Ni**	**Pb**	**Cr**
WB-Sugarcane	0.982*	0.996**	0.787 (ns)	-0.707 (ns)	0.884 (ns)	-0.682 (ns)	-0.076 (ns)	0.855 (ns)	0.530 (ns)
WB-Bagasse	0.558 (ns)	0.993**	-0.621 (ns)	-0.947*	0.977*	0.682 (ns)	-0.806 (ns)	-0.712 (ns)	-0.863 (ns)
Mushrooms in WB Supplemented substrates	**Cu**	**Zn**	**Al**	**Cd**	**Fe**	**Mn**	**Ni**	**Pb**	**Cr**
P. ostreatus grown in WB-Sugarcane	0.171 (ns)	-0.402 (ns)	-0.980*	-0.553 (ns)	0.871(ns)	-0.446 (ns)	-0.881 (ns)	-0.926 (ns)	0.832 (ns)
P. *ostreatus* grown in WB-Bagasse	0.821 (ns)	-0.221 (ns)	0.6164 (ns)	0.5208 (ns)	-0.978*	-0.442 (ns)	0.914 (ns)	-0.982*	0.696 (ns)

Ns, Correlation is not significant; *Correlation is significant at the 0.05 level (two-tailed); **Correlation is significant at 0.001 level (two-tailed); WB, Wheat bran.

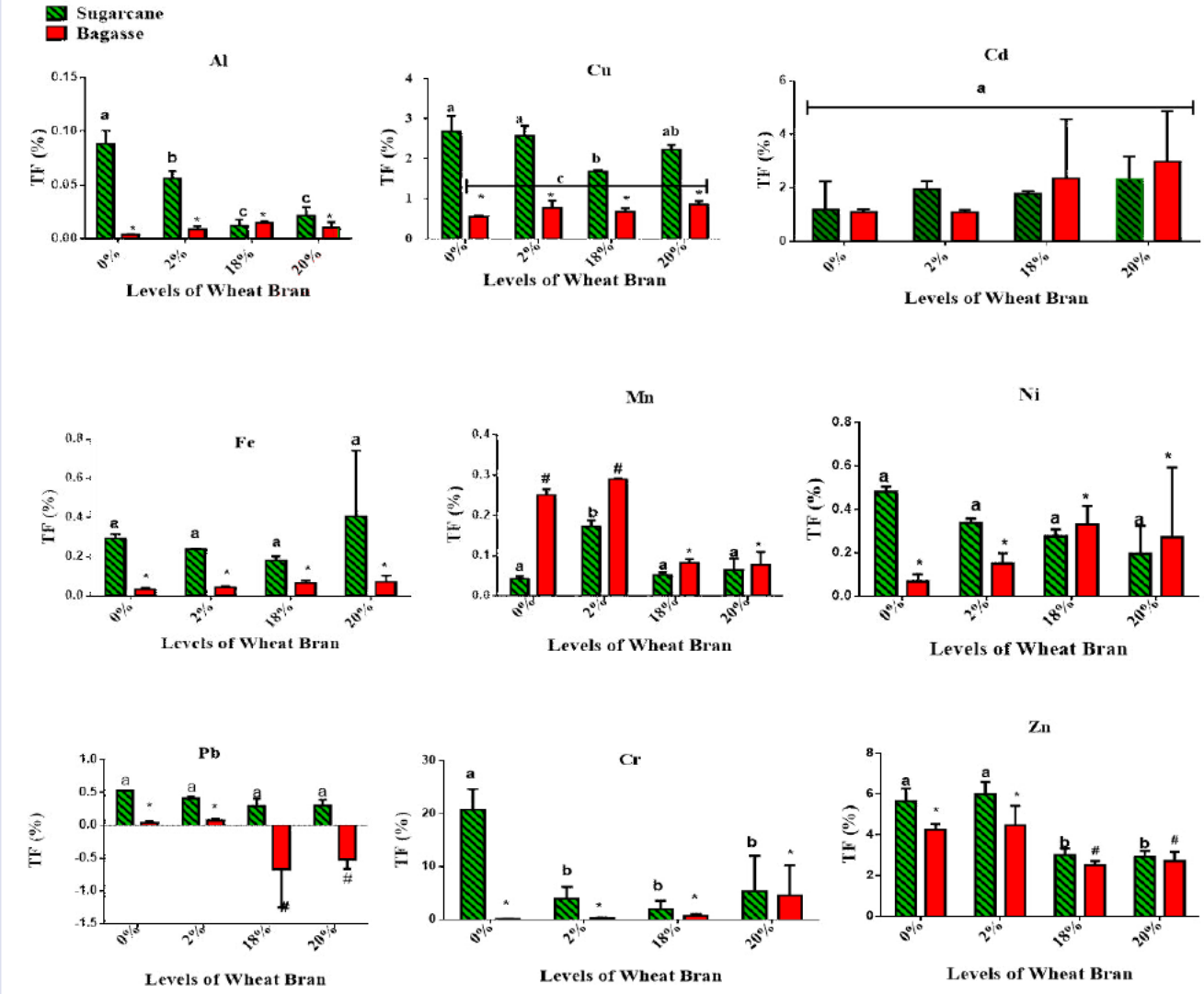

Figure 1: Transfer factor (TF) of heavy metals from sugar cane tops and bagasse into *P. ostreatus* mushroom.

higher antioxidant activity of plant extract [29]. In-terms of the IC_{50} it was noted that mushrooms grown on un-supplemented substrates had a more significant IC_{50} value when compared to the mushrooms grown on supplemented substrate. Furthermore, it was observed that an increase in supplement resulted in higher IC_{50} (not ideal), which could probably be influenced by different factors such as the content of phenolic compounds since they are the major compounds in mushrooms antioxidants which can scavenge free radicals.[30,31] Hence, Gąsecka *et al.*, 2016 have previously stated that substrates could have some influence on phenolic content of the mushrooms.[32] Therefore, our clearly demonstrate that the type and content of substrates used in growing mushooms, may directly influence the antioxidant properties and phenolic content of the resultant mushrooms.

Radicals such as hydroxyl radicals have the potential to damage DNA, lipids and proteins.[14,33–35] Results on Figure 3 proved that exposing pET30 plasmid DNA to Fenton's reagent caused complete damage of plasmid DNA. This is due to hydroxyl radical since it reacts with nitrogen bases of DNA causing production of both base radicals and sugar radicals which then react and results in breakage of sugar phosphate leading to DNA damage.[36] The results on Figure 3 showed that addition of WB on different mushroom extract had no significant protective role on DNA protection. As expected, sugarcane mushroom extract grown in the absence of WB successfully prevented DNA damage caused by Hydroxyl radical (Figure 3). The DNA protective efficiency of this mushroom extract may probably be due to compounds such as phenolics and flavonoids which usually prevent production of ROS by forming complex with cations such as CU^{+2} and Fe^{+3} which participate in the formation of hydroxyl radical [36]. Other extract did not show DNA protective activity probably due to lower content of these compounds (phenolics and flavonoids), hence previous studies by Fatima *et al.* (2018) indicated that extract with less phenols and flavonoids were not found to be effective in DNA protection. Nonetheless, results of this study indicate that mushroom extract have the potential antioxidant activity.

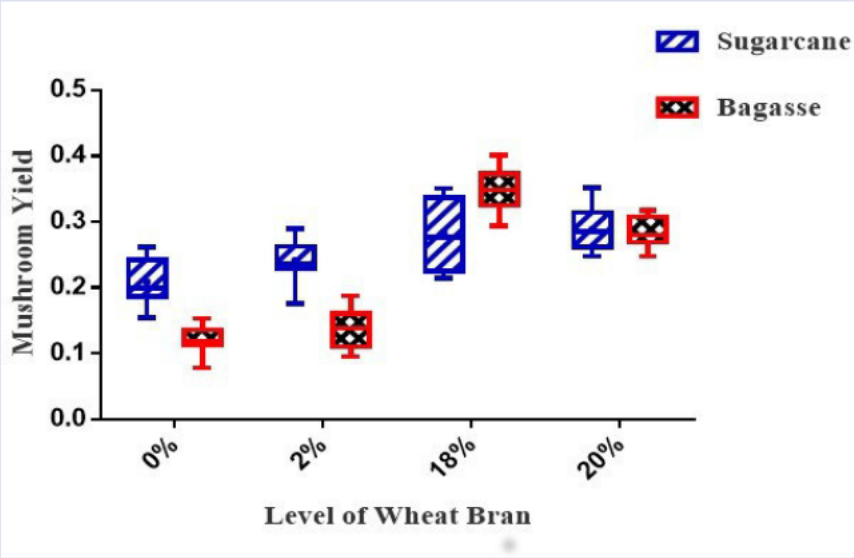

Figure 2: Increasing levels of wheat bran supplementation directly influence production yield of *P. ostreatus*.

Table 4: Estimated Daily Intake (EDI) of metals from consuming 0.1 kg of fresh mushroom by the 65 kg body weight individual (mg/kg day-1 bw). The *P. ostreatus* mushroom grown on Sugar cane tops supplemented with varying levels of WB.

P. ostreatus mushroom grown on Sugar cane tops					
Heavy metals	0% WB	2% WB	18% WB	20%WB	RDI (mg/day)
Al	0.07	0.07	0.02	0.03	-
Cu	0.02	0.02	0.01	0.02	0.9 (Ros et al., 2011)
Cd	0.01	0.007	0.007	0.008	0.007 (FAO/WHO)
Fe	0.51	0.18	0.24	1.08	8.0-18.0 (Ros et al., 2011)
Mn	0.02	0.04	0.02	0.02	1.8-2.3 (Ros et al., 2011)
Ni	0.01	0.006	0.006	0.009	0.13-0.4 (US RDA)
Pb	0.007	0.006	0.005	0.005	0.025 (FAO/WHO)
Cr	0.03	0.009	0.018	0.14	0.02-0.2 (US RDA)
Zn	0.14	0.17	0.14	0.15	8.0-11.0 (Ros et al., 2011)
P. ostreatus mushroom grown on Baggase cane tops					
Heavy metals	0% WB	2% WB	18% WB	20%WB	RDI (mg/day)
Al	0.02	0.03	0.05	0.04	-
Cu	0.008	0.008	0.009	0.03	0.9 (Ros et al., 2011)
Cd	0.006	0.007	0.006	0.008	0.007 (FAO/WHO)
Fe	0.18	0.18	0.22	1.53	8.0-18.0 (Ros et al., 2011
Mn	0.04	0.05	0.01	0.05	1.8-2.3 (Ros et al., 2011)
Ni	0.003	0.004	0.006	0.05	0.13-0.4 (US RDA)
Pb	0.001	*<DL	*<DL	*<DL	0.025 (FAO/WHO)
Cr	0.005	0.009	0.02	1.45	0.02-0.2 (US RDA)
Zn	0.15	0.15	0.13	0.15	8.0-11.0 (Ros et al., 2011)

*<DL (Below detection limits); Recommended Daily intake (RDI);

Table 5: Percentage scavenging activity of DPPH by *P. ostreatus* cultivated on bagasse with varying levels of wheat bran supplementation.

Conc (ug/ml)	0% WB	2% WB	18% WB	20% WB	BHT
10	61.24 ± 0.83	44.38 ± 0.33	49.62 ± 0.33	48.55 ± 1.06	89.67 ± 0.28
25	67.02 ± 1.95	45.96 ± 0.42	52.67 ± 0.10	48.59 ± 0.11	89.71 ± 0.11
50	65.13 ± 0.53	48.69 ± 0.64	55.88 ± 0.33	50.50 ± .17	89.81 ± 0.05
100	67.96 ± 11.62	57.56 ± 0.61	64.62 ± 0.23	55.34 ± 0.23	90.97 ± 1.14
250	74.58 ± 0.29	80.09 ± 0.37	74.00 ± 0.96	64.54 ± 0.17	89.71 ± 0.29
500	84.72 ± 3.07	84.61 ± 3.18	79.50 ± 0.54	77.02 ± 0.36	85.92 ± 3.68
800	84.72 ± 0.71	84.50 ± 0.14	71.85 ± 0.08	85.95 ± 0.54	88.60 ± 1.00
IC_{50}	1.89	29.76	13.09	27.40	6.98

(n = 3, X ± SEM), IC50 – Inhibitory concentration

Table 6: Percentage scavenging activity of DPPH by *P. ostreatus* cultivated on sugar cane tops with varying levels of wheat bran supplementation.

Conc (ug/ml)	0% WB	2% WB	18% WB	20% WB	BHT
10	50.36 ± 0.100	50.02 ± 0.16	47.27 ± 0.84	46.06 ± 0.84	89.67 ± 0.28
25	52.81 ± 0.100	52.66 ±1.08	50.17 ± 0.29	51.90 ± 3.15	89.71 ± 0.11
50	57.06 ± 0.24	56.31 ± 0.30	56.19 ± 0.25	55.29 ± 0.33	89.81 ± 0.05
100	63.80 ± 0.38	65.57 ± 0.65	67.98 ± 0.57	65.84 ± 0.83	90.97 ± 1.14
250	75.52 ± 0.36	84.03 ± 0.79	87.80 ± 1.58	84.33 ± 0.48	89.71 ± 0.29
500	70.47 ± 1.70	87.38 ± 1.21	90.28 ± 0.75	87.68 ± 0.36	85.92 ± 3.68
800	67.10 ± 0.53	84.44 ± 0.41	89.64 ± 0.59	81.05 ± 0.83	88.60 ± 1.00
IC50	9.191139	15.2869	18.55854	18.23718	6.98

(n = 3, X ± SEM), IC50 – Inhibitory concentration

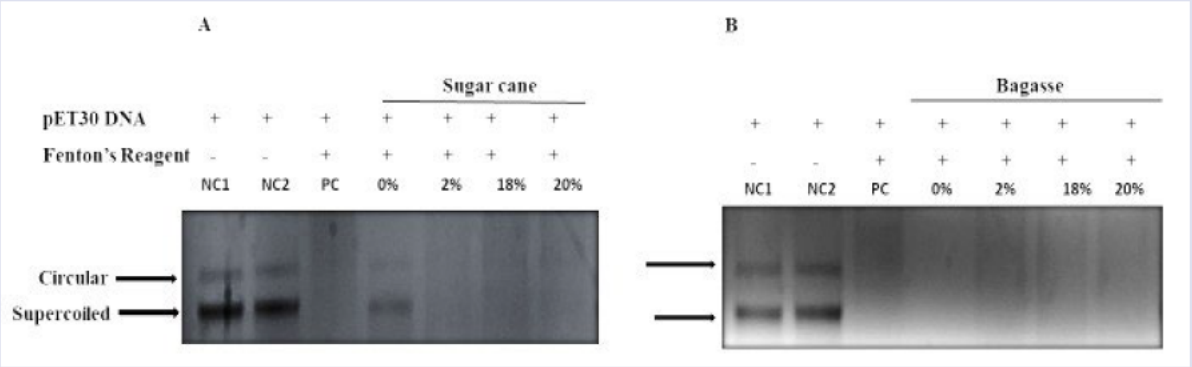

Figure 3: Plasmid DNA protective ability of *P. ostreatus* mushrooms grown on substrates namely sugar cane (A) and bagasse (B) supplemented with varying levels of wheat bran. The pET30 plasmid was incubated with extract in the absence (-) and in the presence (+) of DNA oxidizing reagent (Fenton's reagent). Lane NC1 (Negative control with DNA only); Lane NC2 (Negative control with DNA and DMSO); Lane PC (Positive control with DNA and Fenton's Reagent); Lane 0% - 20% [Extract with DNA and Fenton's reagent; (Extracts are from mushrooms grown on substrates supplemented with 0%, 2%, 18% and 20% wheat bran, respectively)].

CONCLUSIONS

This study found that sugar cane tops and bagasse substrates were enriched with heavy metals which were probably as a result of emissions by industries, fertilizers and pesticides which farmers use during sugar cane farming. This study showed that the *P. ostreatus* mushroom absorbs the heavy metals from the substrates but at varying rates, meaning that *P. ostreatus* mushrooms have different affinities for different metals. Supplementation of the substrate with WB influenced some of the metals within both substrates and the mushrooms. This data further supports the literature stating that the absorption of metals by species is influenced by substrate composition. The addition of the WB supplements to mushroom resulted in significantly higher yield, however, inversely reduced antioxidant reducing activity of the grown mushroom. This suggests that bagasse and sugar cane tops supplementation does not influence heavy metal accumulation in mushroom cultivation. Further studies are still required to investigate the presence of phenolic and flavonoid compounds which are capable of scavenging free radicals.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

AUTHOR'S CONTRIBUTIONS

The manuscript was written and read by all the authors mentioned. OJP, MBCS and SSM structured and designed the experiments, wrote and proof read the paper for possible corrections. SSM and NLG conducted the laboratory experiments, collected and analyzed data.

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CHAPTER 3

The Effect of Supplementing Mushroom Growing Substrates on the Bioactive Compounds, Antimicrobial Activity, and Antioxidant Activity of *Pleurotus ostreatus*

The previous chapter focused on the absorption of heavy metals together with the influence of WB on the radical scavenging activities of *P. ostreatus* mushrooms.

However, this published article (chapter 3) mainly focused on the influence of wheat bran supplements on mushrooms bioactive compounds, biological activity, and mushrooms productivity. This article provided new insights on the benefits of adding wheat bran supplements to improve the quality, content of bioactive compounds, productivity, antimicrobial activity, and antioxidant properties of *Pleurotus ostreatus* mushrooms.

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Research Article

The Effect of Supplementing Mushroom Growing Substrates on the Bioactive Compounds, Antimicrobial Activity, and Antioxidant Activity of *Pleurotus ostreatus*

Senzosenkosi Surprise Mkhize[1], **Mthokozisi Blessing Cedric Simelane**[2], **Ishmael Nkoana Mongalo**[3] and **Ofentse Jacob Pooe**[1]

[1]*Discipline of Biochemistry, Westville Campus, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa*
[2]*Department of Biochemistry, University of Johannesburg, P.O. Box 524, Auckland Park 2006, South Africa*
[3]*College of Agriculture and Environmental Science (CAES) Laboratories, University of South Africa, Private BagX06, Johannesburg 0710, South Africa*

Correspondence should be addressed to Ofentse Jacob Pooe; pooeo@ukzn.ac.za

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Pleurotus ostreatus mushroom contains important bioactive compounds and has several biological activities; however, mushroom growing substrates have major influence on chemical and functional characteristics of the mushroom. Hence, the study aimed to evaluate the influence of supplementing mushroom growing substrates with wheat bran (WB) towards yield/productivity, bioactive compounds, and antimicrobial and antioxidant activity of *P. ostreatus*. The mushroom was cultivated on sugarcane substrates supplemented with increasing levels of WB (0%–20%). The mushroom extracts were screened for bioactive compounds using gas chromatography-mass spectrometry (GC-MS). Antimicrobial activity was carried out using microplate assay, while antioxidant potential was investigated using reducing power assay. The addition of supplements on mushroom growing substrates had an influence on mushroom yield; hence, higher supplementation (18% and 20%) produced higher yield. The GC-MS revealed several bioactive compounds with known activity, such as vitamin E, phenol, fatty acids, and terpenoids. Concentration-dependent antioxidant activity was observed; hence, extracts at higher concentrations gave significantly higher reducing power. The *P. ostreatus* extract had antimicrobial activity against all the tested organisms, with *S. aureus* showing high susceptibility to most of the extracts. However, mushrooms grown on bagasse substrates supplemented with 14% (0.02 mg/ml) and 20% WB (0.08 mg/ml) proved to have better antimicrobial activity on *Escherichia coli*. The difference in susceptibility demonstrates that substrates type and composition could have an influence on bioactive compounds found within mushrooms, also influencing medicinal properties of edible mushroom. Thus, supplementing mushroom growing substrates not only improve yield, but also can contribute to bioactive compounds with medicinal potential.

1. Introduction

Mushrooms are usually defined as the macrofungi with fruiting body which could be either above (hypogenous) or underground (epigeous) and could be seen with naked eyes; hence, it can be picked [1, 2]. The mushrooms are being gradually recognized as important food due to their contribution towards human health, disease, and nutrition [2]. Hence, they are being utilized throughout the world as food, drugs, and tonics [3]. Among all the cultivated mushrooms, oyster mushroom species are one of the most cultivated mushrooms worldwide following *Agaricus bisporus* [4]. This is due to the fact that *Pleurotus* spp. are easily cultivated at low production cost with high yield and biological efficiency [5]. Furthermore, the *Pleurotus* spp. could be easily cultivated on a number of readily available substrates [6].

These saprophytic fungi have been reported to contain numerous metabolites, which exhibit many important

pharmacological activities [7]; thus, oyster mushrooms have been recognized to be highly medicinal due to their content of bioactive metabolites, which could potentially be used to produce a variety of important pharmaceutical products [8]. The bioactive metabolites such as phenolic compounds, polyketides, terpenes, and steroids, which are usually found in *Pleurotus* mushrooms, have previously been reported to be medically active in several therapies [4]. For example, compounds such as pentadecane and Phenol, 2,4-bis (1,1-dimethylethyl), which could be found in *P. ostreatus* mushrooms have both antioxidant and antimicrobial properties [9, 10]. Furthermore, it is noteworthy that natural antioxidants such as phenolic compounds are in high demand due to their potential in the treatment of several diseases such as diabetes and cardiovascular disorders and anticancer, anti-inflammatory, and antimicrobial activities, besides their potential applications in the food and pharmaceutical sectors [11–14].

It is important to also note that the type or characteristics of the growing substrates significantly influence the content of certain bioactive compounds within the mushrooms [15, 16]. Therefore, it is important to select the good substrates, which could increase the content and variations of metabolites such as phenolics in mushrooms [17]. Furthermore, mushroom growing substrates have previously been supplemented with different supplements in order to promote rapid growth and productivity of mushrooms [18, 19]. Supplements such as wheat bran have been used as a source of carbohydrates and nitrogen to the main substrates [20], since most of the substrates do not have enough nitrogen required by mushroom [21]. Hence, it has been established that wheat bran supplement improves yield/productivity of *P. ostreatus* mushrooms [18]. It was therefore the aim of the study to evaluate the influence of wheat bran supplement towards mushroom productivity, content of bioactive compounds, and biological activity of oyster mushroom. Thus, information scarcity on utilizing wheat bran supplements to improve quality, content of bioactive compounds, productivity, antimicrobial activity, and antioxidant property of *Pleurotus ostreatus* mushrooms would be fixed. Hence, an alternative source of antibiotic towards the resurgence of multidrug resistance and nosocomial infections could potentially be established from such mushrooms. Therefore, the aim of the current work is to compare the antimicrobial and antioxidant activity of mushrooms cultivated at different quantities of sugar cane bagasse and sugar cane tops and, furthermore, to identify and quantify possible compounds which could well explain biological activity of such extracts using gas chromatography-mass spectrometry.

2. Materials and Methods

2.1. P. ostreatus Mushroom Cultivation Procedure

P. ostreatus mushrooms were cultivated on locally available substrate/waste materials namely sugar cane bagasse and sugar cane tops. Few modifications from the methods by [18] were incorporated in the process of mushroom cultivation. The four-step process was followed for the cultivation of *P. ostreatus*: (a) preculturing of *P. ostreatus* on PDA, (b) spawn preparation, (c) substrate preparation and inoculation, and (d) *P. ostreatus* fruiting. The test *P. ostreatus* mushroom was obtained from Cedara College of Agriculture at Pietermaritzburg in Kwa-Zulu Natal (South Africa), where it was previously identified and characterised.

The *P. ostreatus* mushroom strain was initially precultured on potato dextrose agar (PDA) and thereafter incubated under dark environment at ±25°C till mycelia fully covered the PDA plate. The precultured *P. ostreatus* strain was then stored at 4°C, hence maintained as mother culture for further processing. *P. ostreatus* mushroom spawn was prepared following a modified method outlined by [22] using birds seed grains that was soaked in overnight in distilled. Briefly 4 g of the soaked birds seed grains were mixed with 1g gypsum ($CaSO.\ 4{\cdot}2H_2O$) and 300 g calcium carbonate (CaCO3) and autoclaved. The grains were then inoculated aseptically with the previously grown mushroom cultures and thereafter incubated under dark environment at ±25°C, until mycelia fully colonized the bird seed grains. The prepared mushroom spawn was also stored at 4°C for further processing. The locally available substrates (sugarcane bagasse and sugar cane tops) were sparingly sprinkled with H_2O till 65% moisture was achieved. Thereafter, the substrates were supplemented with wheat bran at levels of 0%, 2%, 18%, and 20% wheat bran (WB), respectively. These levels of supplements were thoroughly mixed with the substrates and then pasteurised at 60–65°C for six hours and allowed to cool at room temperature [23]. The pasteurised substrates were then inoculated with the previously prepared spawn, which was later incubated under dark till mycelia fully colonised the substrates. Once the substrates were fully colonized by mushroom mycelia, they were removed from dark environment into the fruiting room that was made of 30% shade cloth. The *P. ostreatus* mushrooms fruited under ambient temperatures at constant fogging to achieve 60% moisture which is ideal for fruiting of oyster mushrooms.

2.2. Carbon to Nitrogen Ratio (C/N) of Supplemented Substrates and Mushroom Yield/Productivity. The supplemented substrates (sugar cane tops and sugar cane bagasse) were analysed for total carbon and nitrogen composition following a modified method by [24]. The C and N within substrates were analysed using CHN analyser (Leco, Moenchengladbach, Germany) following the combustion method, whereby 3 mg of dried substrates were analysed in triplicate. Thereafter, the C/N ratio was calculated from the mean of the results obtained and used to confirm the degree of condensation of organic compounds. The yield of the produced mushrooms was calculated according to method extrapolated from [18]; hence, the following equation was used:

$$\mathrm{MY} = \left[\frac{\text{Weight of fresh mushroom harvested}\,(g)}{\text{fresh substrate weight}}\right]. \quad (1)$$

2.3. Preparation of Mushroom Extracts. A slightly modified method by Chowdhury et al. [25] was used for the extraction and preparation of mushroom extract. The freshly harvested

mushrooms were sundried under transparent tunnel with 30% shade cloth. Then the dried mushrooms were milled into powder using a Scientec hammer mill, resulting into a 2 mm mesh powder. Thereafter, 100 g of powdered mushroom was dissolved into 250 ml methanol and incubated in shaker set at 200 rpm for 24 h at 25°C. The mushroom extract was filtered using Whatman No. 1 filter paper and evaporated to semidryness using fume mode. The semidry *P. ostreatus* mushroom extract was then stored at 4°C for further analysis.

2.4. Screening of Bioactive Compounds Using Gas Chromatography-Mass Spectrometry (GCMS). Well established method by Daffodil et al. [26] was used to screen for the bioactive compounds within the mushrooms. For GCMS analysis, 3 mg of extract was mixed with 10% methanol and 90% dichloromethane (DCM) and shaken to dissolve into a homogenous mixture. The GC-MS analysis of the extract was conducted using the Shimadzu GC-MS solutions system, whereby the gas chromatography interfaced with the mass spectrometer (GC-MS) which had Elitel and a fused silica capillary column (30 mm × 0.25 mm 1D X1 μMdf, consist of 100% Dimethyl poly siloxane). Electron ionization system with ionizing energy of 70 eV was used for the GCMS detection. The carrier gas named Helium (99.999%) was used at the constant flowrate of 1 ml/min, with the injection volume of 8.00 μl, and split ratio of 10 : 1.250°C was the injector temperature and 28°C being the ion source temperature. The oven was programmed at 110°C (2 min isothermal) and was increasing at 10°C/min to 200°C and 5°C/min to 280°C, then ended with 9 min isothermal which was at 280°C. The 70 eV was used for the mass spectra with scanning interval of 0.5 seconds, having fragments from 45 to 450 Da. The GC had the running time of 36 min in total at which the relative amount in percentage of each component was calculated using comparison of the average peak area with the total areas. The GC-MS mass spectrum was interpreted using database of National Institute Standard Technology (NIST), which has more than 62000 patterns in their library.

2.5. Antimicrobial Activity Assay. The minimum inhibitory concentration (MIC) of mushroom extracts was determined for *Escherichia Coli*, *Staphylococcus aureus*, *Candida albicans,* and *Cryptococcus neoformans* using the microplate dilution assay [27]. About 100 μl of nutrient broth was added to all the 96 well microtitter plates. About 100 μl of mushroom extract (5 mg/ml) dissolved in 1% DMSO was thereafter serially diluted throughout the rows in the 96 well microtitter plates. About 100 μl bacterial culture set at 0.5 McFarland standard was then added to all the wells of the 96 well microtitter plates which were incubated at 37°C for 24 hours. The *p*-Iodo-nitrotetrazolium violet (INT) solution (40 μl of 0.2 mg/ml) was thereafter added into 96 well microtitter plates and incubated at 37°C for 30 min. The red colour indicated the growth of the microorganism, fermenting INT into formazan. MIC was recorded as the lowest concentration of mushroom extract that completely inhibited the microbial growth of organisms.

2.6. Reducing Power Assay. The *P. ostreatus* ability to reduce free radicals was evaluated using modified method reported by Ayeni et al. [28]. About 2.5 mL of the methanolic mushroom extracts at various concentrations (10–800 μg/ml) were mixed with 0.2 M phosphate buffer (pH 6.6) together with 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, and thereafter, 10% trichloroacetic was added, and the solution was centrifuged for 10 min at 1000 rpm. Immediately after centrifugation, 2.5 ml of the upper layer of the solution was taken out and mixed with equal ratio of distilled water and $FeCl_3$ (0.5 mL, 0.1%). Absorbance was measured at 700 nm (96 well plate reader); hence, higher absorbance indicated better reducing power of mushroom extracts.

2.7. Statistical Analysis of Results. All experiments were repeated independently, in triplicate. Data generated were calculated using SPSS original 6.0 and Graph Pad Prism. The results are reported as mean ± S.E.M. The statistical differences were determined using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. The values were considered statistically significant where $p \leq 0.05$.

3. Results and Discussion

3.1. The C/N Ratio of Growing Substrates Together with P. ostreatus Yield. The *Pleurotus* mushrooms have the greatest advantage of being cultivated in a simple way within tropical climates. Such mushrooms can be grown on both lignin and cellulose rich substrates which are readily obtainable [29]. Even though the *Pleurotus* mushrooms have such advantages, it should be emphasised that the growth and yield performance of *P. otreatus* are dependent on the carbon to nitrogen ratio (C/N) of the growing substrates [30]. Hence, previous studies have reported that substrates should be supplemented with nitrogen and carbon source to obtain optimum C/N for the growth of mushroom [31]. Therefore, mushroom growing substrates should be formulated in such a way that they contains balanced C/N ratio [30]. In our study, two different substrates (sugar cane tops and sugar cane bagasse) were supplemented with varying levels of wheat bran (0%, 2%, 14%, 18%, and 20%) in order to obtain an optimum C/N ratio which would improve the growth and yield performance of *P. otreatus* mushrooms. From the results on Figure 1(a), it is noted that the unsupplemented substrates (0%) and lower levels of WB supplemented substrates (2%) had significantly higher C/N, when compared to higher levels of supplementations (14%, 18% and 20%). This was especially observed for bagasse supplemented with 0% and 2% WB, which had significantly ($p < 0.05$) higher C/N ratio (96 : 1 and 94 : 1), when compared to bagasse supplemented with 14%, 18%, and 20% WB. Similar trend was also observed for the sugar cane supplemented substrates, with the exception being the fact

that sugar cane substrates had lower C/N ratio when compared to bagasse substrates. The obtained C/N ratio for higher levels of supplementation for bagasse substrates was in line with an ideal C/N value reported by Duprat [32] who stated that an ideal C/N value for *P. ostreatus* grown agro-industrial waste should range from 25 to 50 : 1. The sugar cane substrates had an ideal range for C/N ratio regardless of the effect of wheat bran supplementation. Our study confirms that as C/N ratio decreases, the yield could potentially increase; hence, it was noted that higher levels of supplementation that had lower C/N ration and hence produced better yield (Figure 1(b)), when compared to the lower levels of supplementation which had higher C/N ratio. Therefore, increasing the levels of wheat bran supplement in mushroom growing substrates might have probably resulted in balanced or optimum C/N, hence influencing the yield performance of *Pleurotus* spp. mushroom. Our findings were in line with the results reported by [33] who stated that substrates with lower C/N ratios result in higher mushroom yield. The C/N ratios that were obtained, were influenced by the level of wheat bran supplemented into the substrates; hence, it was observed that for both substrates, an increase in wheat bran level resulted in the increase in mushroom yield (Figure 1(b)). Such findings corroborates with the findings of [34–36], who reported that an increase supplementation level within the base substrates result in increased mushroom yield, which is of great interest to mushroom farmers.

3.2. The Antioxidant Property of P. ostreatus Mushroom. Mushrooms have been reported to have protective role just like many plants, they potentially protect organisms against oxidative stress; hence, they produce phytocompounds with antioxidant activity, which prevent formation of free radicals, which forms diseases due to the reactive oxygen species (ROS) and reactive nitrogen species (RNS) [37]. Hence, previous authors have stated that the consumption of Oyster mushrooms may protect the human body since Oyster mushrooms have radical scavenging activity [38]. The present study revealed that methanolic extract of *P. ostreatus* mushroom grown on both unsupplemented and wheat bran supplemented substrates (sugar cane tops and sugar cane bagasse) have antioxidant property. Figures 2(a) and 2(b) indicate that *P. ostreatus* mushrooms have reducing power, which was concentration-dependent; hence, the reducing power was observed to be increasing as the concentration increased throughout. Such trend of concentration dependency of reducing power of *P. ostreatus* mushroom is in line with the results obtained by [39], who also demonstrated that the reducing power of methanol extract from five ear mushrooms was dose-dependent. Thus, the observed reducing power (ability) of *P. ostreatus* mushroom might be due to the fact that mushrooms have the hydrogen donating ability which breaks the free radical chain [40]. When comparing the reducing power of different supplement levels, it was observed from Figures 2(a) and 2(b) that unsupplemented (0%) substrates had higher reducing power followed by lower supplementation (2% WB) compared to the rest of supplemented substrates. The highest reducing power was observed at the concentration of 800 μg/ml, with a reducing power of 42% observed on unsupplemented (0%) sugar cane bagasse. The unsupplemented (0%) sugar cane tops were also observed to have higher reducing power of 61% with a concentration of 800 μg/ml, which was similar to the reducing power of the control namely ascorbic acid (A. A), which also had a reducing power of 61% at 800 μg/ml. Such variations in reducing power of differently supplemented substrates was probably due to the differences in the amount of reductones such as phenolics and flavonoids, which have antioxidant ability through breaking free radical chain by donating a hydrogen atom [41].

3.3. Antimicrobial Activity of P. ostreatus Mushroom Extracts. Besides the antioxidant property (reducing power) of mushrooms, the current study also confirmed that the *P. ostreatus* mushroom also have the antibacterial and antifungal activity as observed on Tables 1 and 2. The results in Tables 1 and 2 reveal that *P. ostreatus* grown on wheat bran supplemented substrates have the antimicrobial potential. The methanolic extract of *P. ostreatus* mushroom grown on differently supplemented substrates inhibited *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Cryptococcus neoformans* with MIC values ranging from 2.5 mg/ml to 0.08 mg/ml. The results in Table 1 indicate that mushroom extracts grown on sugarcane tops had moderate antibacterial activity for *S. aureus* (MIC range from 0.31–0.16 mg/ml) in all levels of WB supplementation, and 20% WB showed moderate antibacterial activity towards *C. albicans* (0.31 mg/ml). However, mushroom extracts grown from sugarcane bagasse had good antibacterial activity for supplements such as 14% (0.02 mg/ml) and 20% WB (0.08 mg/ml) for *E. coli* bacteria. Furthermore, moderate antibacterial activity was also noted for sugar cane bagasse on bacteria such as *S. aureus* (0.31–0.63 mg/ml), *C. albicans* (0.31–0.63 mg/ml), and *C. neoformans* (0.16, 0.31, and 0.63 mg/ml). These results indicate that *P. ostreatus* extract had better activity; hence, the auth [27] have reported that the lower the MIC, the better the activity, and other researchers have stipulated and classified the antimicrobial activity of plant extract as good (MIC < 0.1 mg/mL), moderate (0.1 ≤ MIC ≤ 0.625 mg/mL), and weak (MIC > 0.625 mg/mL) [42]. Thus, such findings support the reports of previous studies which stipulated that *Pleurotus ostreatus* mushrooms have antibacterial and antifungal properties [43].

In general, it was noted that supplementation of substrates with wheat bran had some impact on the antimicrobial activity (antibacterial and antifungal) of *P. ostreatus* as observed that the mushrooms grown on unsupplemented substrates (0%) had MIC values which were different to other supplemented substrates; however, other mushrooms grown on supplemented substrates had MIC values which were similar to the MIC values of unsupplemented substrates. For example, it is observed in Table 2 that *Staphylococcus areus* was equally inhibited by *P. ostreatus* extract grown on unsupplemented (0%) and supplemented (2% and 18% WB) bagasse substrates since the MIC value of 0.63 mg/ml was noted; however, the only exception was only

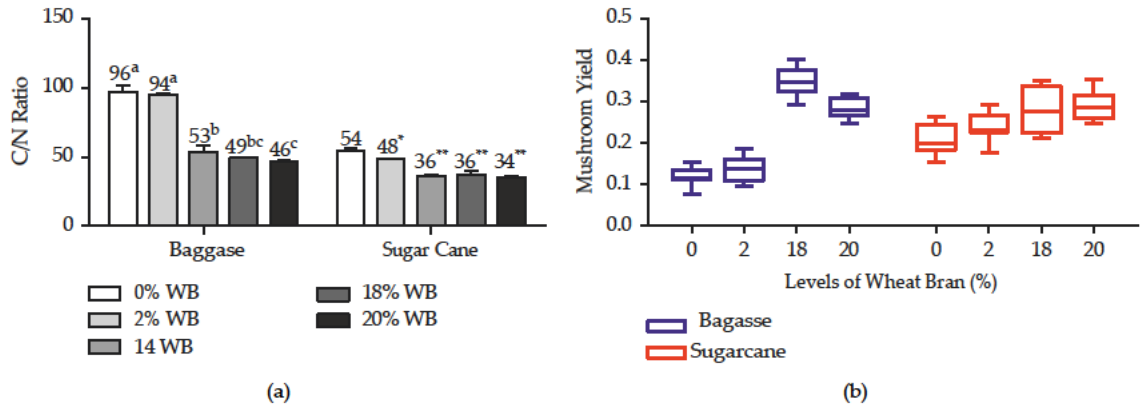

Figure 1: (a) The C/N ratio of mushroom growing substrates, which was supplemented with increasing levels of WB and (b) the yield of *P. ostreatus* mushroom, which was grown on various supplemented substrates. C/N: carbon to nitrogen; WB: wheat bran.

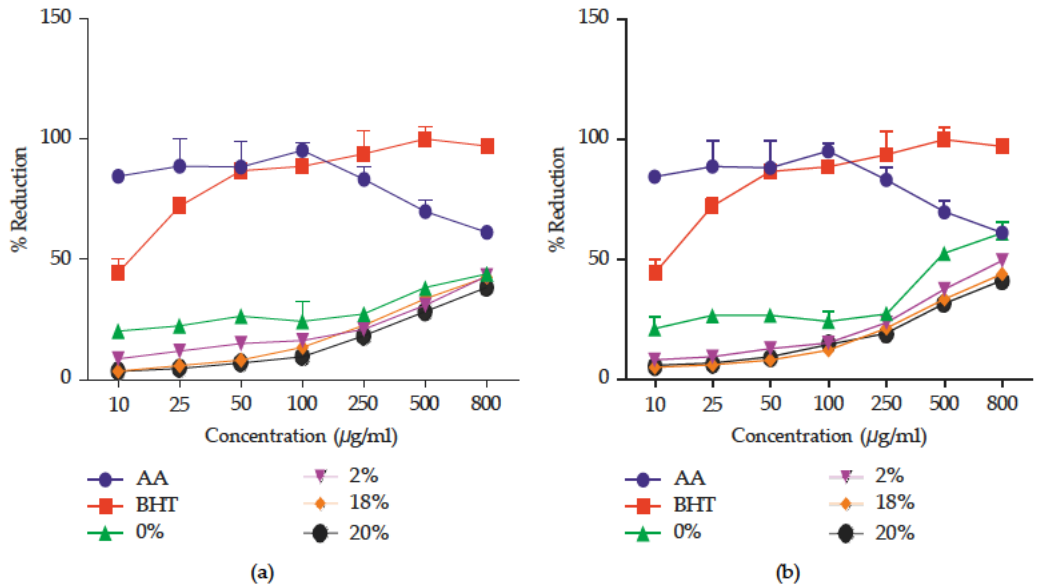

Figure 2: The Reducing power of *P. ostreatus* mushroom grown on various supplemented substrates: (a) sugarcane bagasse base substrates supplemented with different levels of wheat bran); (b) sugar cane tops base substrates supplemented with different levels of wheat bran).

Table 1: Minimum inhibitory concentration (MIC) (mg/ml) of methanol extract of *P. ostreatus* mushroom grown on sugar cane tops supplemented with varying levels of wheat bran.

Supplement (WB)	Test organisms			
	E. coli	*S. aureus*	*C. albicans*	*C. neoformans*
0%	2.50	0.31	1.25	2.5
2%	2.5	0.16	1.25	1.25
14%	2.5	0.31	2.5	1.25
18%	2.5	0.31	2.5	1.25
20%	1.25	0.16	0.31	1.25
Control drugs				
Vancomycin	0.002	0.001	—	—
Streptomycin	0.025	0.013	—	—
Amphotericin	—	—	0.012	0.004

Table 2: Minimum Inhibitory concentration (MIC) (mg/ml) of methanol extract of *P—ostreatus* mushroom grown on bagasse substrates supplemented with varying levels of wheat bran.

Supplement (WB)	Test organisms			
	E. coli	*S. aureus*	*C. albicans*	*C. neoformans*
0%	2.50	0.63	2.5	1.25
2%	1.25	0.63	0.31	0.63
14%	0.02	0.16	0.31	0.16
18%	2.5	0.63	0.31	0.31
20%	0.08	0.31	0.63	2.5
Control drugs				
Vancomycin	0.002	0.001	—	—
Streptomycin	0.025	0.013	—	—
Amphotericin	—	—	0.012	0.004

Table 3: GCMS profiling of methanolic extracts of *P. ostreatus* mushroom grown from sugarcane substrates supplemented with varying levels of wheat bran.

Supplement (%)	Peak	RT (min)	Compound	Molecular formula	Area %	Height %	Mol wt
0	1	12.894	Phytol acetate	C22H42O2	0.75	1.30	338
	2	12.990	2-Pentadecanone, 6,10,14-trimethyl-	C18H36O	0.30	0.57	268
	5	22.868	Pentadecanal-	C15H30O	0.43	0.86	226
	6	25.160	cis-11,14-Eicosadienoic acid, tert-butyldimethylsilyl	C26H50O2Si	0.42	0.32	422
	9	26.022	2-Methyl-Z,Z-3,13-octadecadienol	C19H36O	1.18	1.77	280
	11	28.844	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-	C27H46O2	0.85	1.13	402
	13	30.417	beta.Tocopherol.gamma.-Tocopherol	C28H48O2	8.93	9.33	416
	14	30.759	Vitamin E	C28H48O2	54.94	53.50	416
	16	32.388		C29H50O2	13.20	11.74	430
2	3	7.555	Pentadecane	C15H32	1.59	6.21	212
	5	10.002	Hexadecane	C16H34	1.48	5.45	226
	6	12.336	Hexadecane	C16H34	0.88	2.99	226
	10	14.946	Heptadecan	C17H36	0.47	1.35	240
	13	17.584	Hexadecanamide	C16H33NO	2.54	5.54	255
	15	18.395	beta.-sitosterol	C29H50O	1.09	1.13	414
	16	18.622	beta-sitosterol	C29H50O	13.47	8.16	414
	17	18.795.	gamma-sitosterol	C29H50O	16.41	9.22	414
	18	18.949	gamma-sitosterol	C29H50O	14.46	5.67	414
	19	20.285	9-Octadecenamide, (Z)-	C18H35NO	11.27	18.32	281
	23	21.017	Phenol,2,2′methylenebis[6-(1,1-dimethylethyl	C23H32O2	1.71	3.33	340
	26	23.734	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-,	C33H54O3	1.67	0.68	498
	27	27.430	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	C31H52O	8.30	3.58	440
18	7	30.400	beta.-Tocopherol	C28H48O2	4.24	5.83	416
	8	30.726	gamma.-Tocopherol	C28H48O2	52.30	39.73	416
	12	32.365	alpha.-Tocopherol-.beta.-D-mannoside	C35H60O7	12.59	9.61	592
20	2	26.620	Cyclotrisiloxane, hexamethyl-	C6H18O3Si3	2.25	6.09	222
	3	26.665	Periplocymarin	C30H46O8	3.88	2.96	534
	5	30.445	beta-Tocopherol	C28H48O2	3.19	4.67	416
	6	30.590	Heptadecafluorononanoic acid, hexyl ester	C15H13F17O2	2.43	3.28	548
	7	30.749	gamma-Tocopherol	C28H48O2	51.78	36.74	416
	10	31.945	4-Methoxy-2(1H)-quinolone	C10H9NO2	4.31	5.24	175
	11	32.365	alpha.-Tocopherol-.beta.-D-mannoside	C35H60O7	3.05	7.53	592

Table 4: GCMS profiling of methanolic extracts of *P. ostreatus* mushroom grown from sugarcane bagasse substrates supplemented with varying levels of wheat bran.

Supplement (%)	Peak	RT (min)	Compound	Molecular formula	Area %	Height %	Mol wt
0	2	27.675	1,1,3,3-Tetraallyl-1,3-disilacyclobutane	C14H24Si2	7.30	5.36	248
	3	28.701	Octadecanoic acid, 7-hydroxy-, methyl ester	C19H38O3	8.57	6.90	314
	5	30.770	gamma.-Tocopherol	C28H48O2	49.87	44.31	416
	6	30.885	Ginsenol	C15H26O	6.94	14.33	222
	7	32.372	alpha.-Tocopherol-.beta.-D-mannoside	C35H60O7	5.99	8.90	592
2	1	26.558	Silane, dimethyl(docosyloxy)butoxy-	C28H60O2Si	5.99	6.95	456
	3	30.790	beta.-Tocopherol	C28H48O2	17.97	22.96	416
	6	32.395	alpha.-Tocopherol-.beta.-D-mannoside	C35H60O7	3.05	6.22	592
14	3	7.545	Pentadecane	C15H32	2.12	3.93	212
	4	9.989	Hexadecane	C16H34	2.04	3.54	226
	8	14.920	Heptadecane	C17H36	0.64	0.93	240
	14	20.974	Phenol, 2,2′-methylenebis[6-(1,1-dimethylethyl)	C23H32O2	0.46	0.63	340
18	1	5.091	Undecane	C11H24	0.39	0.56	156
	4	7.050	1,3-Dioxolane, 4-[[(2-methoxy-4-octadecenyl)oxy] methyl]-2,2-dimethyl-	C25H48O4	0.66	0.68	412
	5	7.554	Pentadecane	C15H32	0.94	1.81	212
	7	9.999	Hexadecane	C16H34	1.63	2.36	226
	9	11.150	Heptadecane	C17H36	0.41	0.79	240
	11	12.332	Hexadecane	C16H34	0.77	1.28	226
	13	13.595	Heptadecane	C17H36	0.50	0.39	240
20	3	30.806	gamma-Tocopherol	C28H48O2	37.39	38.33	416

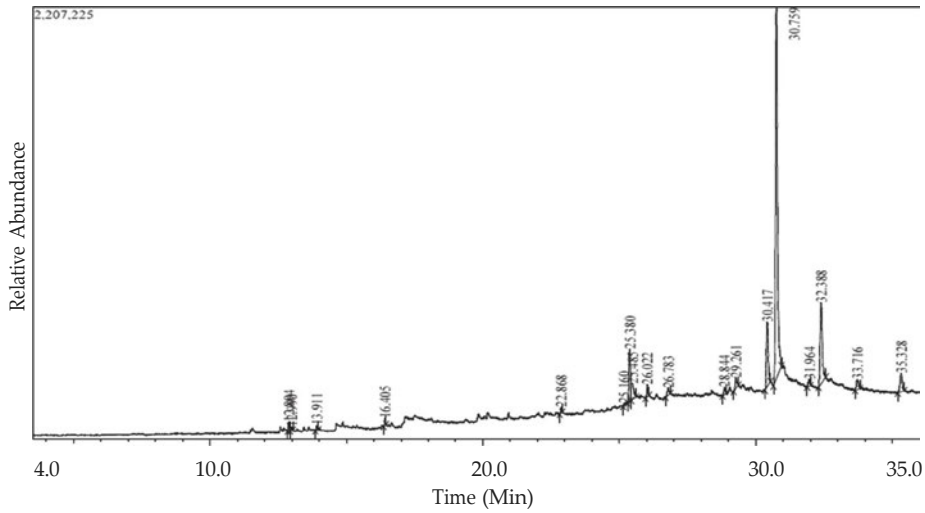

Figure 3: GC-MS chromatogram of methanolic extract of *P. ostreatus* mushroom cultivated from sugar cane substrates supplemented with various levels of wheat bran.

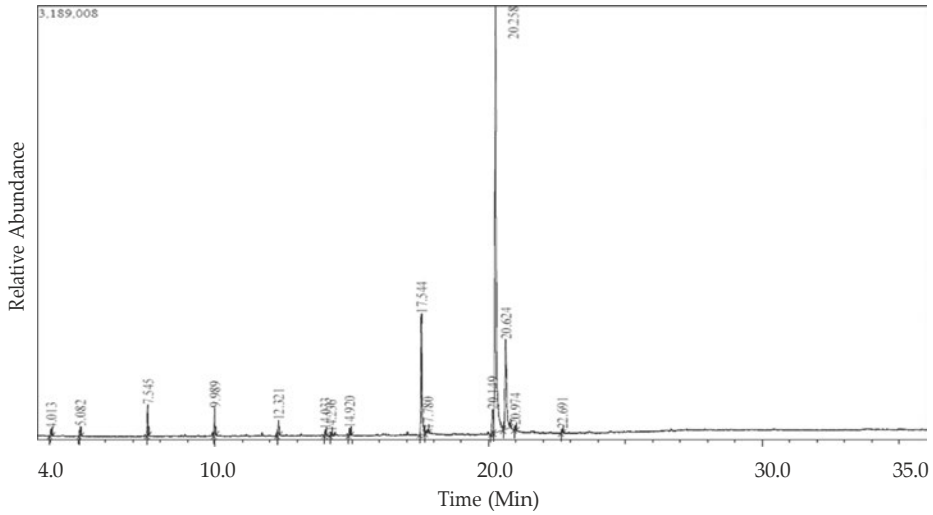

Figure 4: GC-MS chromatogram of methanolic extract of *P. ostreatus* mushroom cultivated from sugarcane bagasse supplemented with various levels of wheat bran.

observed on 14% WB (MIC of 0.16 mg/ml) and 20% WB (MIC of 0.31 mg/ml). Such similarities and differences in terms of inhibitory activities of *P. ostreatus* extract were probably due to their content in total phenols and flavonoids as Barros et al. [44] had previously stipulated that the antimicrobial activity of different mushrooms was directly correlated with their content of total phenols and flavonoids.

3.4. GCMS Analysis of Compounds within P. ostreatus. The GC-MS results in Tables 3 and 4 also testify that the mushrooms grown on various supplemented substrates have varying and some have similar compounds which might have played a role in both antimicrobial activity and antioxidant activity, which was observed in our study. Therefore, it could be stipulated that the high antibacterial potency observed for mushrooms grown in bagasse supplemented with 14% WB (MIC of 0.02 mg/ml) and 18% WB (MIC of 0.08 mg/ml) could be due to the presence of compounds such as Pentadecane, Hexadecane, Heptadecane, and Phenol, 2,2′-methylenebis [6-(1,1-dimethylethyl), which are known to have antibacterial activity. Furthermore, the GC-MS analysis have detected several compounds from *Pleurotus ostreatus* mushroom grown on different substrates (sugar cane tops and sugar cane bagasse) with varying levels of wheat bran supplements. Some of these compounds have the known biological activities; however, others do not have known biological activities. The results in Tables 3 and 4 indicate compounds of known activities; hence, compounds which did not have known activities were not included within the Tables.

However, the GCMS screening on Tables 3 and 4 indicates that *Pleurotus ostreatus* grown on different substrates with various levels of wheat bran supplementation produced

many compounds of which some are similar. The mushrooms grown on sugar cane (Table 3) supplemented with 0%, 18%, and 20% WB had similar compounds such as vitamin E compounds namely beta Tocopherol, gamma Tocopherol, and Alpha-Tocopherol, which are known to have antioxidant property [45]. Furthermore, mushrooms grown on sugarcane bagasse (Table 4) supplemented with 0%, 2%, and 20% WB also had vitamin E compounds. Besides the vitamin E compounds, there were plenty of other bioactive compounds detected by GCMS analyzer, including phenols, terpernoids, fatty acids, and fatty acids derivates as profile chromatogram on Figures 3 and 4 have highlighted some of these compounds found on methanolic extract of *P. ostreatus* mushroom. Compounds such as heptadecane, hexadecane, pentadecane, dibutyl phthalate, cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy- and ß-sitosterol which were detected on some of the *Pleurotus ostreatus* mushrooms are well known to have antimicrobial activity [46–51]. Such findings prove that some of the activities observed such antioxidant and antimicrobial were mainly due to the presence of the above-mentioned compounds. However, in vivo studies need to be explored to validate such activities.

4. Conclusions

Based on the findings of the study, it can be concluded that *P. ostreatus* mushroom produces better yield under a lower C/N ratio, which is influenced by the levels of supplement added into mushroom growing substrates. Furthermore, wheat bran supplement has some influence on the content of bioactive compounds within the *P. ostreatus* mushroom; hence, higher levels of supplementation caused the decrease in antioxidant potential (Ferric reducing power) of *P. ostreatus*. Therefore, this means the addition of supplements in mushroom growing substrates has the advantage of better yield but with decreased antioxidant property; however, little or no supplements has advantage of higher antioxidant potential but reduced yield. Further research needs to be conducted to confirm the correlation between the content of mushroom compounds towards different substrates.

Data Availability

The supplementary data used to support the results of the research study can be obtained through corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest for this work. There are no conflicts of financial interest and personal relationship that could influence this paper's reported work.

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CHAPTER 4

Bioprospecting the Biological Effects of Cultivating *Pleurotus ostreatus* Mushrooms from Selected Agro-Wastes and Maize Flour Supplements.

The previous article (chapter 3) proved that supplementing mushroom-growing substrates with wheat bran did not only improve mushroom yield, but, it also contributed to bioactive compounds with medicinal potential.

This article (chapter 4) was based on the effect of maize four supplements on the mushroom's bioactive compounds, antioxidant properties, antimicrobial properties, and antimalarial properties of the mushroom. Therefore, the medicinal benefits of adding maize four to the mushroom-growing substrates were established. To the best of our knowledge, no data support the impact of using maize flour as a supplement on variables such as the bioactive components, and antioxidant, antibacterial, and antimalarial properties of *P. ostreatus* mushrooms.

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Research Article

Bioprospecting the Biological Effects of Cultivating *Pleurotus ostreatus* Mushrooms from Selected Agro-Wastes and Maize Flour Supplements

Senzosenkosi Surprise Mkhize,[1] **Mthokozisi Blessing Cedric Simelane**,[2] **Nkoana Ishmael Mongalo**,[3] **and Ofentse Jacob Pooe**[1]

[1]*Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa*
[2]*Department of Biochemistry, University of Johannesburg, P.O. Box 524, Auckland Park 2006, South Africa*
[3]*College of Agriculture and Environmental Science (CAES) Laboratories, University of South Africa, Private Bag X06, Johannesburg 0710, South Africa*

Correspondence should be addressed to Ofentse Jacob Pooe; pooeo@ukzn.ac.za

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Pleurotus mushrooms are valuable food supplements with health and environmental restorative potential. In this paper, we sought to evaluate the biological activities and profile the bioactive compounds found in *Pleurotus ostreatus* cultivated from agro-waste supplemented with maize flour. We investigated carbon to nitrogen (C/N), antimicrobial, antioxidant, and antimalarial potential for the varying supplementation during mushroom cultivation. GCMS was utilized for screening bioactive compounds found in *P. ostreatus*. Changes in supplementation directly correlate with changes in compound profiling. Nonetheless, some compounds were found to be common amongst the tested mushrooms, including pentadecanoic acid; 9,12-octadecadienoic acid, methyl ester; pentadecanoic acid, methyl ester; octadecanoic acid; and diisooctyl phthalate. The highest antimicrobial potential against Gram-positive *Staphylococcus aureus* was observed when maize flour supplements were increased to 12% and 18%. Our data demonstrated that the observed antioxidant (DPPH, ABTS, and reducing power) and antimicrobial activity could emanate from various supplementation conditions. Furthermore, supplementation has an impact on the mushroom yield and phytochemical profiles of the produced mushroom.

1. Introduction

Mushrooms have established significant value in humans as they are increasingly incorporated into humans since they have profound functional and nutraceutical importance [1]. Amongst the most cultivated mushrooms, the oyster (*Pleurotus*) mushroom ranks as the third most cultivated species following the button and shiitake mushrooms [2]. The *Pleurotus* mushrooms have an added advantage of worldwide distribution since they can grow in temperate to tropical regions with temperatures ranging from 12 to 32°C [3]. Furthermore, the process of cultivation of oyster mushrooms is useful for reducing environmental pollution, and hence it is deemed as one of the environmentally friendly procedures since the mushroom produces mycelia which degrade lignocellulosic waste via a complex enzyme system [4]. For the mushroom mycelia to degrade and utilize lignocellulosic waste for its growth, specific nutrients are needed, and hence the mushroom-growing substrates should be supplemented to increase mushroom yield and improve rapid growth [5, 6] and nutritional property, and hence the nutritional contents of mushroom are influenced by the composition of growing substrates [7].

It has been documented that *Pleurotus* spp. need to be grown on substrates containing carbon, nitrogen, and inorganic compounds for growth [8]. Hence, the addition of supplements is a necessity to enhance the production of oyster mushrooms; however, the supplementation ratio should be of a certain limit to avoid the possibilities of contamination [9] and yield reduction [10]. Therefore, the substrates utilized for mushroom growth should have a well-balanced carbon-to-nitrogen (C/N) ratio that is supplied by nutrients within the substrates [11]. For example, the *P. ostreatus* mushroom requires an optimal C/N ratio for better growth on the substrate [12]. Hence, it was one of the objectives of the study to find an optimal C/N ratio of *Pleurotus ostreatus* mushroom using maize flour as a supplement. However, numerous mushroom species are well known to produce a variety of metabolites that have antioxidant, antimicrobial, antitumor, antihypertensive, antiplatelet aggregation, antihyperglycaemic, antigenotoxic, and antiviral activities [13]. It is worth noting that recently mushrooms have been reported to have compounds such as anthraquinones, flavonoids, and steroids which possess antimalarial activity [14]. Some of these metabolites from mushrooms such as phenolic compounds can be affected by different factors such as substrates and supplements used during cultivation [15]. Some authors have stipulated that supplements such as wheat bran are rich in compounds that have antioxidant activities [16], and hence Magdziak et al. [15] have recently found that the addition of 20% WB caused little effect on the synthesis of low-molecular-weight organic acids within *P. citrinopileatus*.

Hence, our study only focused on growing *Pleurotus ostreatus* mushrooms on sugarcane waste (sugarcane leaves and sugarcane bagasse) substrates supplemented with maize flour, which has been rarely used when compared to other supplements such as wheat bran. To our knowledge, no information confirms the effect of utilizing maize flour as a supplement on factors such as mushroom bioactive compounds, antioxidant properties, antimicrobial properties, and antimalarial properties of the mushroom. Hence, the medicinal benefits of adding maize flour to the mushroom-growing substrates could probably be achieved.

2. Materials and Methods

2.1. The Field and Laboratory Cultivation of P. ostreatus Mushroom. Two different agro-waste substrates (sugarcane bagasse and sugarcane top) were used for the cultivation of *P. ostreatus* mushrooms. A slightly modified method by Mkhize et al. [17] was adopted during the cultivation of the *P. ostreatus* mushroom. The *P. ostreatus* mushroom was obtained from the KwaZulu-Natal (KZN) Department of Agriculture and Rural Development (DARD). (i) Mushrooms were precultured on the media named Potato Dextrose Agar (PDA), incubated under dark conditions for a week at ±25°C, and then stored at −4°C until further used. (ii) Approximately 4-5 pieces of previously grown *P. ostreatus* mushroom on PDA were then inoculated into the fully prepared bird seed grain, and hence mushroom spawn was obtained through the adoption of slightly modified method from Mkhize et al. [9]. (iii) Mushroom-growing base substrates (sugarcane tops and sugarcane bagasse) were thereafter prepared by soaking in water till moisture of 65% was achieved, and the pH of the substrates was balanced by adding standardized formulas of 1% gypsum ($CaSO_4{\cdot}2H_2O$) and 15% $CaCO_3$, and thereafter the substrates were supplemented with maize flour, that is, 0% (no maize flour), 8%, 12%, and 18%, respectively. After supplementation, the substrates were pasteurized at temperatures around 60–65°C for six hours and thereafter given time to cool up to room temperature and inoculated with previously prepared *P. ostreatus* mushroom spawn. After inoculation, the substrates were incubated within the dark environment under ambient temperature (±25°C) until the mycelia colonized the entire substrate. (iv) The fully mycelial colonized substrates were taken out of the dark environment and were stored in the fruiting room which was constructed of 30% gray shade cloth and was timely fogged to maintain 60% moisture, which promoted rapid fruiting of *P. ostreatus* mushroom. The mushrooms were harvested and sun-dried under 30% gray shade cloth.

2.2. The Carbon-to-Nitrogen (C/N) Ratio of Maize Flour-Supplemented Substrates and P. ostreatus Mushroom Yield. The substrates (sugarcane tops and sugarcane bagasse) were initially screened for total carbon and nitrogen, adopting a modified method from Amoah-Antwi et al. [18]. The content of C and N within the abovementioned substrates was analyzed with the combustion method using a machine called CHN analyzer (Leco, Moenchengladbach, Germany). About 3 mg of oven-dried substrates was analyzed in triplicate, with the C/N ratio calculated from the mean of the obtained results.

The mushroom yield was calculated following a method by Mkhize et al. [9]; hence, the following formula was utilized to calculate the mushroom yield: MY = weight of fresh mushroom harvested/weight of fresh substrate, where MY = mushroom yield in grams.

2.3. P. ostreatus Mushroom Extraction. The *P. ostreatus* extract was prepared through the adoption of a slightly modified method by Chowdhury et al. [19]. The mushrooms which were sun-dried under 30% shade cloth were milled using a milling machine of 30 mm sieves. The mushroom powder of 100 g was then weighed and mixed with 250 ml of methanol solvent, which was thereafter allowed to be shaken at 200 rpm for 24 hours under room temperature (±25°C). After 24 hours, the mixture was allowed to be filtered using Whatman No. 1 filter paper and the filtrate was allowed to evaporate to dryness under the fume hood. The dried extract was thereafter stored in a dark environment under ambient temperature for future analysis.

2.4. Screening of the Bioactive Compounds within P. ostreatus Extract. Screening of bioactive compounds within the mushroom extract was done using gas column chromatography (GCMS) following a modified method by Daffodil

et al. [20]. Firstly, 3 mg of the extract was dissolved in 10% methanol and thereafter mixed with 90% dichloromethane (DCM) and shaken until homogeneity was achieved. The GCMS analysis of mushroom extract was conducted using the Shimadzu GCMS QP2010 SE. The gas chromatograph was interfaced with a mass spectrometer which was equipped with a Zebron ZB-5MSplus column (30 m × 0.25 mm, 0.25 μm). The GCMS detection was through an electron ionization system that had an ionizing energy of 70 eV. The method introduced helium gas as one of the carrier gases, which was set to be at a constant flow rate (1 ml/min), with an injection volume of 8.00 μl and a split ratio of 10 : 1. The injection temperature was at 250°C and the temperature of the ion source was at 28°C. Oven temperature and time were programmed as follows: 110°C (isothermal for 2 min) and was increased at about 10°C/min to 200°C, thereafter 5°C/min to 280°C, and finally ended with 9 min isothermal which was at 280°C. The mass spectra were acquired with 70 eV ionization energy employed, with a scanning interval of 0.5 seconds, in the mass range of 45 to 450 da. The running time for GC was 36 min in an overall state, whereby the relative amount (%) of each component was calculated by comparing its average peak area to the total area. Firstly, all peak areas were added together to obtain the total area of the peaks; thereafter, to calculate the % of any compound in the mixture, its individual area was divided by the total area of the peaks and multiplied by 100. A database from the National Institute of Standard Technology (NIST) mass spectral database 2010 (v11) [21] was used to interpret the GCMS mass spectrum; hence, the database had more than 62000 patterns within the library.

2.5. The P. ostreatus DPPH Scavenging Activity (Antioxidant Activity). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is said to be a stable nitrogen radical that has an absorption around 517 nm. Antioxidant compounds or extracts must be able to give an electron or a hydrogen atom, which therefore converts DPPH into a more stable and diamagnetic molecule [22]. The antioxidant activity of *P. ostreatus* was evaluated using a modified method from Ayeni et al. [23], whereby mushroom extracts were prepared in numerous concentrations which ranged from 10 to 800 μg/ml. An amount of 500 μl of DPPH (0.1 Mm) was mixed with 1 ml of a mushroom extract, which was then allowed to undergo incubation at room temperature for 30–60 minutes under dark conditions. Afterward, the absorbance of the mixture was read using a UV spectrophotometer set at 517 nm. The lower absorbance observed denoted that the extracts had higher radical scavenging activity and vice versa.

The following formula was used to calculate the antioxidant activity of mushroom extracts: % scavenging activity = (Ac − As)/Ac ∗ 100, where Ac = absorbance of the control and As = absorbance of the sample.

2.6. ABTS Radical Scavenging Assay. The method by Re et al. [24] was followed for evaluating the ABTS radical scavenging activity of *P. ostreatus* mushrooms, whereby 7 mM of ABTS stock solution was mixed with 2.45 mM potassium persulfate. The mixture of ABTS and potassium persulfate was incubated in the dark, at room temperature for a period of 12–16 h before further use. The ABTS solution was thereafter diluted with 5 mM phosphate-buffered saline (pH 7.4) to achieve an absorbance of 0.70 ± 0.02 at 730 nm. Afterward, 10 μL of mushroom extract was added into 4 mL of the diluted ABTS solution, then left for 30 min, and thereafter the absorbance was measured.

The ABTS radical-scavenging activity of the *P. ostreatus* mushroom extracts was calculated using the following formula: $S\% = (\text{Acontrol} - \text{Asample})/\text{Acontrol}) \times 100$, where Acontrol represents the absorbance of the control (ABTS solution in the absence of mushroom extracts) and Asample represents the absorbance of the test mushroom extracts. The IC50 values were thereafter calculated for every sample.

2.7. P. ostreatus Reducing Power Assay. The ability of *P. ostreatus* mushrooms to reduce free radicals was evaluated following a method from Ayeni et al. [23]. 2.5 mL of *P. ostreatus* mushroom extract (10–800 μg/ml) and 2.5 mL of 0.2 M phosphate buffer (pH 6.6) were mixed with another 2.5 ml of 1% potassium ferricyanide. The whole mixture was allowed to be incubated at temperatures of 50°C for 20 minutes and thereafter was mixed with 10% trichloroacetic acid. The solution was thereafter centrifuged at 1000 rpm for 10 minutes. Approximately 2.5 ml of the supernatant was immediately pipetted out of the solution and mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). The absorbance of the solution was observed at 700 nm, and hence higher absorbance denoted better reducing power of the *P. ostreatus* extracts.

2.8. Antimicrobial Screening Activity. Microorganisms, namely, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Cryptococcus neoformans*, were used to screen for the antimicrobial properties of *P. ostreatus* extract, whereby the microplate dilution assay was utilized for minimum inhibitory concentrations (MICs) [25]. The nutrient broth of about 50 μl was added into 96 micro-titer plates, and then 50 μl of *P. ostreatus* extract (5 mg/ml) that was previously dissolved with 1% DMSO solvent was serially diluted downwards in the rows in 96 micro-titer plates. The microbial cultures were grown at 37°C for 24 hours, and then 50 μl of the microbial cultures was set at 0.5 McFarland standard and inoculated into 96 micro-titer plates which was thereafter incubated overnight at temperatures of 37°C. About 40 μl of p-iodonitrotetrazolium violet (INT) reagent (0.2 mg/ml) was thereafter added into all 96 well micro-titer plates and incubated back for 30 minutes at 37°C. Then, the minimal inhibitory concentration (MIC) was recorded as the lowest concentration of *P. ostreatus* extract that managed to inhibit the microbial growth of the above-mentioned microorganisms.

2.9. In Vitro Plasmodium falciparum Asexual Compound Activity Screening. The Institute for Advanced Medical Research and Training provided the *Plasmodium falciparum*

parasites, which were thereafter stored at 37°C within the human erythrocytes (O+/A+). The human erythrocytes were mixed with the complete culture medium which consisted of RPMI 1640 medium (Sigma-Aldrich) that was supplemented as follows:

(i) 20 mM D-glucose (Sigma-Aldrich)
(ii) 25 mM HEPES (Sigma-Aldrich)
(iii) 200 μM hypoxanthine (Sigma-Aldrich)
(iv) 0.2% sodium bicarbonate
(v) 24 μg/ml gentamicin (Sigma-Aldrich)
(vi) 0.5% AlbuMAX II.

The abovementioned medium was supplemented in a gaseous environment which consisted of 5% O_2, 5% CO_2, and 90% N_2. The *P. ostreatus* mushroom extract of various concentrations was used to treat the in vitro ring stage erythrocytic *P. falciparum* parasite. The treated parasite cultures included the genotypic drug-sensitive strain, namely, NF54 (200 μl at 1% haematocrit, 1% parasitaemia). The assay included the positive control, namely, chloroquine diphosphate (CQ, 1 μM), and the negative control, namely, RPMI media. The parasite was grown in 96 plates and incubated for 96 hours at 37°C under a gaseous environment with 5% O_2, 5% CO_2, and 90% N_2. After an incubation period of 96 hours, 100 μl of *P. falciparum* parasite was mixed with 100 μl of SYBR Green lysis buffer and thereafter incubated at room temperature for a period. The fluorescence was then measured using a GloMax®-Explorer Detection System with Instinct® Software.

The activity of the extracts was classified below to prioritize them for determination of full dose response: good activity (IC50 that was below 10 μg/ml), moderate activity (IC50 that was between 10 and 20 μg/ml), and no/minimal activity (IC50 that was above 20 μg/ml).

2.10. Data Analysis. The experiments were conducted in triplicate to ensure the accuracy of the obtained data. Statistical packages such as SPSS, original 6.0, and GraphPad Prism were used for capturing and analyzing the data. The one-way analysis of variance (ANOVA), which was followed by Tukey–Kramer multiple comparison tests, was a method of analysis used to determine the statistical difference, with values considered statistically significant when ≤ 0.05.

3. Results and Discussion

Amendment of mushroom-growing substrates through the addition of nitrogen and carbohydrate-rich supplements has significant effects on mushroom quality and yield [26]. Thus, controlling the C/N ratio within the compost (substrate) becomes important to obtain profitable mushroom yields [27]. Hence, within the current study, various levels of maize flour supplements were added into mushroom-growing substrates to improve the yield and medicinal properties of *P. ostreatus* mushrooms. The results depicted in Figure 1 indicate that the yield of *Pleurotus* mushroom was influenced by the addition of maize flour supplement, and hence it was noted that 0% MF (no maize flour) had a significantly lower yield when compared to the rest of the treatments for both substrates.

The two substrates (sugarcane top and bagasse) revealed varying yields of mushrooms, with sugarcane substrates producing higher yields for higher supplementation (12% and 18% MF) when compared to lower supplementation (8% and 0% MF). For the bagasse substrates, the yield was significantly higher for all supplement levels when compared to 0% MF (control). Such differences in yield could be attributed to different factors such as growth factors, the presence of carbohydrates that are not complex [28], and the optimal C/N ratio within the growing substrates. Hence, previous researchers such as Alborés et al. [29] have reported that a lower C/N ratio produces mushrooms of higher yield when compared to substrates with a higher C/N ratio. The results in Figure 1 corroborate with the previous findings, and hence it was noteworthy that bagasse substrates had quite higher C/N ratio when compared to sugarcane top that resulted in mushrooms grown from bagasse to have lower yields compared to sugarcane which gave higher yields. The observed results prove that substrates with low C/N ratios turn to support fruit body formation (yield) better than substrates with high C/N ratios as stipulated by Okere et al. [30]. Furthermore, Chang and Miles [31] recommend a C/N ratio of 32-150 as being the most appropriate in the cultivation of *Pleurotus* spp. Other authors such as Cueva et al. [32] observed the best results when the C/N ratio ranged from 37 to 53 for the mushroom strain 768/12. Thus, the results proved that the composition of the substrates probably influences the mushroom C/N ratio and mushroom yield, and hence it was noted in Figure 1(a) that unsupplemented (0%) substrates (both bagasse and sugarcane) had higher C/N ratio compared to the supplemented substrates. Furthermore, the yield in Figure 1(b) was higher for supplemented substrates compared to the unsupplemented substrates (0%), which means adding supplements to substrates promotes the mushroom yield.

Besides influencing yield, the composition of mushroom-growing substrates and other factors such as pH, growing conditions, and genetic factors may influence the metabolic pathways of *P. ostreatus* mushrooms, thus influencing the phenol content of the mushroom [33, 34]. Hence, during cultivation, the majority of the mushrooms turn to produce some of these valuable secondary metabolites such as phenolic compounds which have health beneficial roles that include antimicrobial, antioxidant, anti-inflammatory, antiallergic, antimutagenic, and cardio protective benefits [35, 36]. Based on the findings in Figure 2, it was observed that the cultivated *P. ostreatus* mushrooms had the antioxidant property, as noted that they can scavenge the free radicals (2,2-diphenyl-1- picrylhydrazyl (DPPH)) in a concentration-dependent manner. Amongst the methanolic extracts tested, it was revealed that all mushroom extracts including the control (0% MF) showed to have antioxidant activity, with maximum activity noted at the highest concentrations (250 μg/ml and 500 µg/ml). For the mushrooms grown in bagasse, it was observed that the *P. ostreatus* mushrooms grown on unsupplemented (0%

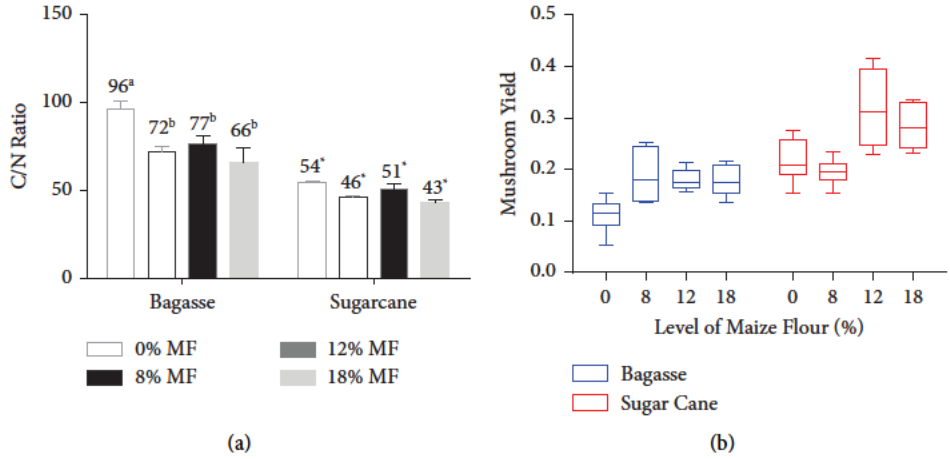

Figure 1: (a) C/N ratio of mushroom-growing substrates, which were supplemented with increasing levels of MF, and (b) the yield of *P. ostreatus* mushroom, which was grown on various supplemented substrates. C/N ratio: carbon-to-nitrogen ratio; MF: maize flour.

MF) bagasse had slightly greater antioxidant activity, especially at lower concentrations when compared to other levels of supplementation. In regard to sugarcane-grown mushrooms, it was also noted that *P. ostreatus* mushrooms grown on 12% MF had slightly greater antioxidant activity, especially at higher concentrations (100–500 μg/ml). The variation in the scavenging of free radicals (DPPH) was probably due to the content of phenolic compounds since the antioxidant activity of the mushroom correlates with the content of phenolics. In general, such findings prove that the locally available substrates (sugarcane and bagasse), which are of low cost, can potentially be used to grow nutraceutical mushrooms with improved antioxidant activity.

Several publications have indicated that most human diseases are caused by the uncontrolled production of reactive oxygen species (ROS) and other free radicals [37], and hence cells are endlessly exposed to a large number of stressful conditions which lead to the generation of free radicals such as $^{\cdot}OH$ and $^{\cdot}O^{2-}$ that cause oxidative damages in biological systems [38]. Hence, free radicals implicate the progression of several health conditions which include diabetes, cancer, atherosclerosis, cardiovascular diseases, and neurodegenerative disorders [39]. However, *Pleurotus* mushrooms are said to be rich in antioxidants that increase the antioxidative capacity of plasma and therefore minimize the risk of the abovementioned diseases [40]. Thus, numerous biochemical assays have been utilized for screening the antioxidant properties of various mushrooms, one of them being the DPPH assay (the most popular and frequently used) [41] and the other being the ABTS assay. The antioxidant properties of *P. ostreatus* mushrooms were further evaluated using ABTS assay (Table 1), and hence the activity of *P. ostreatus* against ABTS radicals was determined using IC50, which corresponded to the concentration of mushroom extracts that were able to scavenge 50% of ABTS. Therefore, a higher IC50 value denotes the lower antioxidant activity of *P. ostreatus* extracts [42], while a lower IC50 value indicates higher antioxidant activity [43]. The results of the study stipulated that *P. ostreatus* mushrooms grown on sugarcane substrates turn out to have lower potency (>2.5 μg/ml) in scavenging ABTS radicals when compared to the *P. ostreatus* mushrooms cultivated on bagasse substrates. It was further observed that the unsupplemented (0% MF) substrates had lower radical scavenging activity (>2.5 μg/ml) when compared to the maize flour-supplemented bagasse substrates. This was probably due to the presence of bioactive compounds within *P. ostreatus* mushrooms, and thus a previous study by Gupta et al. (2018). also specified that the concentration and efficacy of bioactive compounds vary with the type of mushroom, the type of substrate on which the mushroom was grown, fruiting conditions, and storage conditions of mushrooms. The highest ABTS scavenging activity was observed on the *P. ostreatus* mushroom that was cultivated on sugarcane bagasse supplemented with 12% MF (5.6 μg/ml), followed by 8% MF (7.6 μg/ml), which shows that maize flour supplement affected the antioxidant activity of *P. ostreatus* mushroom. This means that the maize flour boosted the phenolic content of mushrooms since previous research indicated that the maximum ABTS scavenging activity is usually associated with the total phenolic content of mushroom extracts, and hence phenols are considered a major antioxidant component [44]. The ABTS radical scavenging activity observed for *P. ostreatus* mushrooms was probably due to the hydrogen-donating ability and chain-breaking ability of *P. ostreatus* mushroom extracts [45], which means *P. ostreatus* mushrooms grown on maize flour supplemented substrates potentially minimize the risk of human diseases.

Amongst the antioxidant activities of any substance, reducing power is also considered as one of the significant indices [46], which depends on the nature of the compound and its property to prevent the propagation of free radicals, which is achieved through the transference of protons into radical species [23]. All mushroom extracts from differently supplemented substrates were evaluated for their ability to have reducing power, and hence their ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) was monitored. The results in Figure 3 revealed that all extracts have the

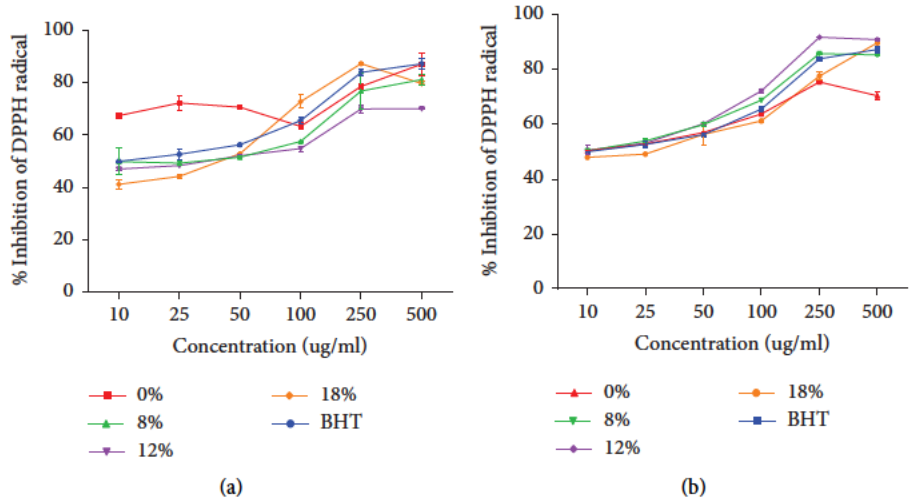

Figure 2: The DPPH radical scavenging activity of *P. ostreatus* mushrooms cultivated on (a) bagasse and (b) sugarcane base substrates supplemented with varying levels of maize flour supplements.

Table 1: Antioxidant activity (ABTS) of methanolic *P. ostreatus* mushroom extract grown on maize flour-supplemented bagasse and sugarcane substrates.

ABTS IC50 (μg/ml): mean IC50 value ± SD		
Maize flour supplement	Bagasse	Sugarcane
0%	>2.5	>2.5
8%	7.6 ± 0.09	>2.5
12%	5.6 ± 0.01	>2.5
18%	44.2 ± 0.01	>2.5
Ascorbic acid	0.32 ± 0.00	

reducing capacity in a concentration-dependent manner, and hence as the concentration increases, the reducing capacity increases. The mushroom extract grown on bagasse substrate supplemented with 18% MF had higher reducing power when compared to other extracts, which might have been due to its content of phenolic and flavonoid compounds which usually play a great role in antioxidant activities [47]. For the mushroom extract grown on sugarcane substrates, it was noteworthy that the unsupplemented substrates (0% MF) had slightly higher reducing power when compared to the supplemented substrates, and this probably meant that the supplements (maize flour) on sugarcane substrate caused some slight decrease in the reducing capacity of the supplemented mushroom extracts. In general, from the results in Figure 3, it could be stipulated that the reducing capacity of the *P. ostreatus* mushroom varies for both substrates and for all supplement levels, and this is an indication that there are some variations in the number of reductones (flavonoids and phenolics) that contribute to the reducing ability (antioxidant) [48], and hence the observed findings are also in line with Mkhize et al. [5] who also observed that the unsupplemented substrates demonstrated higher reducing capacity compared to the supplemented base substrates, which was the case in the current study for the *P. ostreatus* grown on sugarcane base substrates.

Besides the antioxidant activity, it has been documented that several mushroom extracts also possess antimicrobial activity [49], which is due to unique compounds such as alkaloids, peptides, flavonoids, tannins, terpenoids, proteins, and anthraquinones [50]. The study revealed that *P. ostreatus* mushrooms which were grown on sugarcane tops and sugarcane bagasse have antimicrobial activity (0.08-2.5 mg/ml) against various microorganisms; however, the observed activities were noted to vary depending on the type of microorganisms and the level of maize flour supplementation used for substrate supplementation. Hence, it was noted in Table 2 that *P. ostreatus* mushrooms grown in sugarcane top substrates had weaker activity (MIC > 0.625 mg/ml) against *E. coli* microorganisms and *C. neoformans* but had improved or moderate activity (0.1 < MIC < 0.625 mg/ml) and good activity (MIC < 0.625 mg/ml) against *S. aureus* bacteria. Such differences in susceptibility for different microorganisms might be because some microorganisms such as *E. coli*, *C. albicans*, and *C. neoformans* are considered Gram-negative, while other microorganisms such as *S. aureus* are said to be Gram-positive. Thus, the Gram-negative bacteria are known to consist of a cell wall that has an outer membrane made of lipopolysaccharides, which potentially prevents substances from invading the cell wall [51]. The better inhibition of the Gram-positive *S. aureus* bacteria compared to the Gram-negative bacteria corroborates with the findings of Gezer et al. [52] who achieved a similar trend for edible mushrooms found in Turkey. It was further noted from Table 2 that supplementation of sugarcane substrates with MF supplement influenced the susceptibility of certain microorganisms, and higher supplementation such as 12% MF and 18% MF resulted in increased *S. aureus* susceptibility when compared to lower supplements (0% MF and 8% MF). Thus, moderate activity (0.3 mg/ml and 0.16 mg/ml) was observed at lower levels of MF supplementation, whereas good activity (0.08 mg/ml) was observed at higher levels of MF supplementation. Such

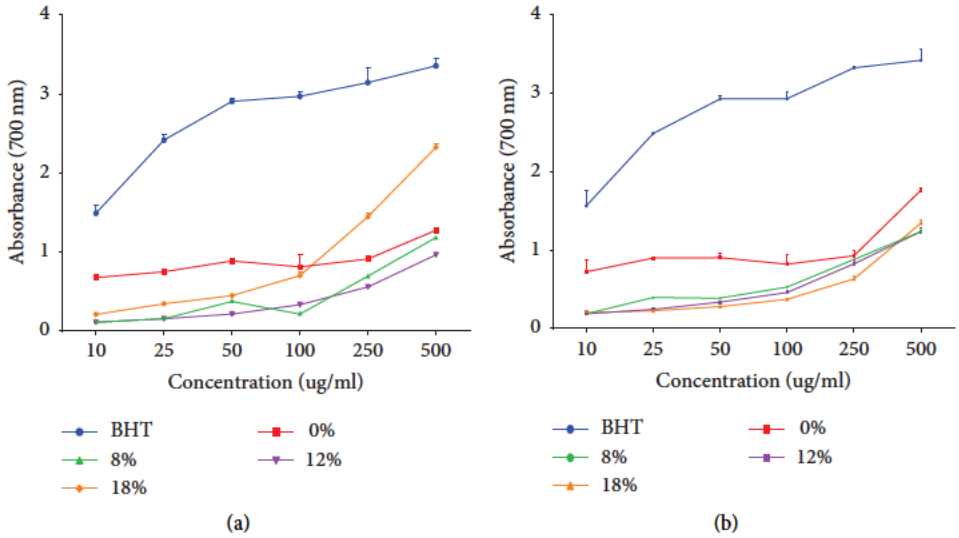

Figure 3: Reducing power of *P. ostreatus* grown on (a) bagasse and (b) sugarcane substrates supplemented with maize flour supplement.

Table 2: Minimum inhibitory concentration (MIC) of methanol extract of *P. ostreatus* mushrooms grown on sugarcane substrates supplemented with varying levels of maize flour.

MF supplement level	Test organisms			
	E. coli	*S. aureus*	*C. albicans*	*C. neoformans*
0%	2.50	0.31	1.25	2.5
8%	2.5	0.16	2.5	2.5
12%	2.5	0.08	0.63	1.25
18%	2.5	0.08	1.25	1.25
Control drugs				
Vancomycin	0.002	0.001	—	—
Streptomycin	0.025	0.013	—	—
Amphotericin	—	—	0.012	0.004

Table 3: Minimum inhibitory concentration (MIC) of methanol extract of *P. ostreatus* mushrooms grown on bagasse substrates supplemented with varying levels of maize flour.

MF supplement level	Test organisms			
	E. coli	*S. aureus*	*C. albicans*	*C. neoformans*
0%	2.5	0.63	2.5	1.25
8%	2.5	0.63	0.31	2.5
12%	0.16	0.63	2.5	2.5
18%	0.31	1.25	1.25	2.5
Control drugs				
Vancomycin	0.002	0.001	—	—
Streptomycin	0.025	0.013	—	—
Amphotericin	—	—	0.012	0.004

Table 4: The *in vitro* antimalarial activity of *P. ostreatus* mushrooms cultivated on sugarcane substrates with varying levels of maize flour supplement (at 20 and 10 μg/ml).

Dual screening of mushroom extracts		
	Asexual parasites, SYBR Green	
MF supplement level	% inhibition ± SD	
	20 μg/ml	10 μg/ml
0%	0.00 ± 7.27	2.74 ± 1.97
8%	3.71 ± 8.93	6.58 ± 1.58
12%	1.45 ± 4.87	5.11 ± 1.17
18%	7.04 ± 4.16	4.55 ± 2.20
CQ (1 μM)	100	100

Table 5: The *in vitro* antimalarial activity of *P. ostreatus* mushrooms cultivated on bagasse substrates with varying levels of maize flour supplement (at 20 and 10 μg/ml).

Dual screening of mushroom extracts		
	Asexual parasites, SYBR Green	
MF supplement level	% inhibition ± SD	
	20 μg/ml	10 μg/ml
0%	0.00 ± 10.59	0.00 ± 4.10
8%	0.00 ± 8.46	0.00 ± 2.18
12%	3.83 ± 6.40	3.02 ± 4.74
18%	0.00 ± 3.37	0.00 ± 4.63
CQ (1 μM)	100	100

variation in antimicrobial activity might be probably due to the type and amount of bioactive compounds which vary with environmental conditions such as soil nutrients, precipitation, and temperature, which influence the content of secondary metabolites and biological activities of the plants [53]. Hence, Kim et al. [54] also testified that the high inhibition observed for RD mushroom extract was due to high phenolic compounds.

Furthermore, the results in Table 3 also proved that supplementation of bagasse substrates with MF caused a major influence on the susceptibility of different microorganisms towards mushrooms grown on varying levels of supplemented bagasse substrates. For example, *E. coli* was less or weakly sensitive to lower levels of MF (0% and 8% MF), whereby at higher levels of MF supplementation (12% and 18% MF), moderate susceptibility of *E. coli* was observed. However, unsupplemented and low supplementation (0%, 8%, and 12%

Table 6: GCMS profiling of methanolic extracts of *P. ostreatus* mushroom grown on bagasse substrates supplemented with varying levels of maize flour supplement.

MF supplement level (%)	Peak	RT (min)	Compound	Molecular formula	Area %	Height %	Mol wt
0	1	26.553	4-Pyrimidinecarboxylic acid, 2,6-bis[(tert-butyldimethylsilyl)oxy]	$C_{23}H_{46}N_2O_4Si_3$	12.38	12.62	498
	2	27.675	1,1,3,3-Tetraallyl-1,3-disilacyclobutane	$C_{14}H_{24}Si_2$	7.30	5.36	248
	3	28.701	Octadecanoic acid, 7-hydroxy-, methyl ester	$C_{19}H_{38}O_3$	8.57	6.90	314
	4	29.336	Dimethylmalonic acid, dodecyl pentafluorophenyl ester	$C_{23}H_{31}F_5O_4$	8.94	7.58	466
	5	30.770	gamma.-Tocopherol	$C_{28}H_{48}O_2$	49.87	44.31	416
	6	30.885	Ginsenol	$C_{15}H_{26}O$	6.94	14.33	222
	7	32.372	alpha.-Tocopherol-.beta.-D-mannoside	$C_{35}H_{60}O_7$	5.99	8.90	592
8	1	4.449	Succinimide	$C_4H_5NO_2$	0.13	0.18	99
	2	4.721	Octanoic acid	$C_8H_{16}O_2$	0.02	0.05	144
	14	10.994	4-Trifluoroacetoxytridecane	$C_{15}H_{27}F_3O_2$	0.02	0.06	296
	16	11.875	Tetradecanoic acid	$C_{14}H_{28}O_2$	0.11	0.25	228
	17	12.155	n-Nonadecanol-1	$C_{19}H_{40}O$	0.01	0.05	284
	18	12.543	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	0.02	0.06	256
	23	13.827	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	0.12	0.31	270
	25	14.170	Cyclopentadecanone, 2-hydroxy-	$C_{15}H_{28}O_2$	0.14	0.20	240
	27	14.320	Dibutyl phthalate	$C_{16}H_{22}O_4$	0.26	0.48	278
	28	14.658	Pentadecanoic acid	$C_{15}H_{30}O_2$	13.49	7.94	242
	33	15.345	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$	0.01	0.03	266
	35	15.523	cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	0.05	0.09	310
	36	15.635	cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	0.08	0.14	310
	39	16.256	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	0.15	0.34	296
	41	16.490	Eicosane	$C_{20}H_{42}$	0.01	0.03	282
	42	16.628	Methyl stearate	$C_{19}H_{38}O_2$	0.02	0.06	298
	44	17.157	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	7.61	10.60	280
	47	17.430	Palmitoleic acid	$C_{16}H_{30}O_2$	5.96	14.0	254
	48	17.568	Octadecanoic acid	$C_{18}H_{36}O_2$	2.74	3.32	284
	50	17.810	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	17.810	1.19	294
	54	18.086	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	0.59	0.45	280
	64	18.780	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentyl	$C_{25}H_{42}O_2$	0.04	0.07	374
	69	19.510	9,12-Octadecadien-1-ol, (Z,Z)-	$C_{18}H_{34}O$	0.02	0.02	266
	77	20.269	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	0.05	0.09	281
	86	22.651	Diisooctyl phthalate	$C_{24}H_{38}O_4$	0.15	0.30	390
	87	22.895	Ethanol, 2-(9,12-octadecadienyloxy)-, (Z,Z)-	$C_{20}H_{38}O_2$	0.01	0.02	310
	91	23.838	cis-9,10-Epoxyoctadecan-1-ol	$C_{18}H_{36}O_2$	0.01	0.02	284
	126	34.330	Ergosta-7,22-dien-3-ol, (3.beta.,5.alpha.,22E)-	$C_{28}H_{46}O$	0.10	0.08	398
	129	35.041	7,22-Ergostadienone	$C_{28}H_{44}O$	0.04	0.06	396
	131	35.830	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	$C_{30}H_{50}O$	0.31	0.24	426
12	1	26.330	Ethanol, 2-[2-[2-[2-[p-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy] ethoxy]ethoxy	$C_{22}H_{38}O_5$	24.38	14.19	382
	2	26.555	Silane, dimethyl(docosyloxy)butoxy-	$C_{28}H_{60}O_2Si$	24.38	27.30	456
	3	30.801	N-(6,7,9,10,12,13,15,16-Octahydro-5,8,11,14	$C_{16}H_{23}NO_6$	27.54	31.25	592
	4	32.395	alpha.-Tocopheryl acetate	$C_{31}H_{52}O_3$	7.49	14.56	472
	5	34.245	1-Propanol, 3-(octadecyloxy)-	$C_{21}H_{44}O_2$	15.74	12.69	328

Table 6: Continued.

MF supplement level (%)	Peak	RT (min)	Compound	Molecular formula	Area %	Height %	Mol wt
18	2	11.887	Tetradecanoic acid	$C_{14}H_{28}O_2$	0.11	0.26	228
	4	13.130	Pentadecanoic acid	$C_{15}H_{30}O_2$	2.65	4.69	242
	5	13.839	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	0.13	0.32	270
	7	14.328	Dibutyl phthalate	$C_{16}H_{22}O_4$	0.12	0.26	278
	11	16.170	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	2.94	6.71	294
	12	16.260	8-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	0.65	1.48	296
	13	16.361	Dihydroqinghaosu	$C_{15}H_{24}O_5$	0.14	0.23	284
	14	16.624	Methyl stearate	$C_{19}H_{38}O_2$	0.16	0.40	298
	17	17.324	Octadecanoic acid	$C_{18}H_{36}O_2$	2.38	2.69	284
	23	18.215	Ethyl 9,12-hexadecadienoate	$C_{18}H_{32}O_2$	0.19	0.29	280
	33	21.724	(Z)6,(Z)9-Pentadecadien-1-ol	$C_{15}H_{28}O$	0.23	0.39	224
	37	22.630	Diisooctyl phthalate	$C_{24}H_{38}O_4$	0.23	0.45	390
	38	23.399	4,22-Stigmastadiene-3-one	$C_{29}H_{46}O$	0.11	0.22	410
	55	34.995	Dasycarpidan-1-methanol, acetate (ester)	$C_{20}H_{26}N_2O_2$	0.15	0.16	326

Table 7: GCMS profiling of methanolic extracts of *P. ostreatus* mushroom grown on sugarcane substrates supplemented with varying levels of maize flour supplement.

MF supplement level (%)	Peak	RT (min)	Compound	Molecular formula	Area %	Height %	Mol wt
0	1	12.894	Phytol, acetate	$C_{22}H_{42}O_2$	0.75	1.30	338
	2	12.990	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	0.38	0.57	268
	3	13.911	5,9,13-Pentadecatrien-2-one, 6,10,14-trimethyl	$C_{18}H_{30}O$	0.39	0.68	262
	4	16.405	Cyclopentadecanone	$C_{15}H_{28}O$	0.45	1.03	224
	5	22.868	Pentadecanal-	$C_{15}H_{28}O$	0.43	0.86	226
	6	25.160	cis-11,14-Eicosadienoic acid, tert	$C_{26}H_{50}O_2Si$	0.42	0.32	422
	7	25.380	2-Isoamyl-6-methylpyrazine	$C_{10}H_{16}N_2$	6.59	7.61	164
	8	25.485	p-Cresyl isovalerate	$C_{12}H_{16}O_2$	2.82	2.27	192
	9	26.022	Butyldimethylsilyl	$C_{19}H_{36}O$	1.18	1.77	280
	10	26.783	8-Nonene-2,4-diol, 8-methyl-, (R∗,S∗)-	$C_{10}H_{20}O_2$	1.09	1.05	172
	11	28.844	2-Methyl-Z,Z-3,13-octadecadienol	$C_{27}H_{46}O_2$	0.85	1.13	402
	12	29.261	(Phenylthio)acetic acid, tridec-2-ynyl ester	$C_{21}H_{30}O_2S$	1.58	1.65	346
	13	30.417	.beta.-Tocopherol	$C_{28}H_{48}O_2$	8.93	9.33	416
	14	30.759	.gamma.-Tocopherol	$C_{28}H_{48}O_2$	54.94	53.50	416
	15	31.964	Hexahydropyrimidine-2,4,6-trione, 5-ethyl-1-methyl	$C_{27}H_{34}N_4O_3$	0.93	1.03	462
	16	32.388	Vitamin E	$C_{29}H_{50}O_2$	13.20	11.74	430
	17	33.716	Ethyl-3.alpha.,5.alpha.-cyclocholest-22(E)-en-6-one Octadecanoic acid, 7-hydroxy-, methyl ester	$C_{29}H_{46}O$	1.66	1.47	410
	18	35.328	Cholest-6-one, 3-chloro- 3-chloro-17-(1,5-dimethylhexyl)-10,13-dimethylhexadecahydrocyclopenta [a]phenanthren-6-one $$ 3-Chlorocholes	$C_{27}H_{45}C_lO$	3.42	2.68	420
8	1	6.581	2,4-Decadienal	$C_{10}H_{16}O$	0.02	0.07	152
	2	9.690	Tridecanoic acid, 12-oxo-	$C_{13}H_{24}O_3$	0.01	0.04	228
	3	9.829	Trifluoroacetic acid,n-tridecyl ester	$C_{15}H_{27}F_3O_2$	0.03	0.12	296
	6	12.162	n-Nonadecanol-1	$C_{19}H_{40}O$	0.03	0.13	284
	7	13.175	Pentadecanoic acid	$C_{15}H_{30}O_2$	2.81	5.42	242
	8	13.731	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$	0.01	0.05	266
	11	14.656	Pentadecanoic acid	$C_{15}H_{30}O_2$	17.39	15.40	242
	13	15.515	cis-10-Heptadecenoic acid	$C_{17}H_{32}O_2$	0.06	0.15	268
	9	14.177	Palmitoleic acid	$C_{16}H_{30}O_2$	0.19	0.31	254
	15	15.814	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	0.05	0.12	652
	17	16.168	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	0.10	0.41	294
	18	16.260	8-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	0.03	0.13	296
	20	16.615	Methyl stearate	$C_{19}H_{38}O_2$	0.01	0.03	298
	21	17.311	Ethyl 9,12-hexadecadienoate	$C_{18}H_{32}O_2$	58.53	31.17	280
	23	17.511	Octadecanoic acid	$C_{18}H_{36}O_2$	2.48	4.80	284
	24	17.620	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl	$C_{21}H_{36}O_4$	0.54	1.23	352
	25	17.754	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	1.32	1.66	294
	32	18.297	9,17-Octadecadienal, (Z)-	$C_{18}H_{32}O$	1.01	1.36	264
	37	19.189	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl	$C_{35}H_{68}O_5$	0.03	0.09	568
	42	20.251	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	0.06	0.14	281
	44	21.744	9,12-Octadecadienoyl chloride, (Z,Z)-	$C_{18}H_{35}NO$	0.15	0.46	281
	47	22.643	Diisooctyl phthalate	$C_{24}H_{38}O_4$	0.08	0.25	390
	49	22.965	Decanedioic acid	$C_{10}H_{18}O_4$	0.01	0.04	202
	50	23.396	Ergost-25-ene-3,5,6,12-tetrol, (3.beta.,5.alpha.,6.	$C_{28}H_{48}O_4$	0.04	0.12	448
	57	28.148	15,17,19,21-Hexatriacontatetrayne	$C_{36}H_{58}$	0.04	0.11	490
12	1	26.330	Ethanol, 2-[2-[2-[2-[p-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy] ethoxy]ethoxy	$C_{22}H_{38}O_5$	24.38	14.19	382
	2	26.555	Silane, dimethyl(docosyloxy)butoxy-	$C_{28}H_{60}O_2Si$	24.38	27.30	456
	3	30.801	N-(6,7,9,10,12,13,15,16-Octahydro-5,8,11,14	$C_{16}H_{23}NO_6$	27.54	31.25	592
	4	32.395	alpha.-Tocopheryl acetate	$C_{31}H_{52}O_3$	7.49	14.56	472
	5	34.245	1-Propanol, 3-(octadecyloxy)-	$C_{21}H_{44}O_2$	15.74	12.69	328

Table 7: Continued.

MF supplement level (%)	Peak	RT (min)	Compound	Molecular formula	Area %	Height %	Mol wt
	1	4.727	Octanoic acid	$C_8H_{16}O_2$	0.10	0.30	144
	2	4.818	2-Hydroxy-gamma-butyrolactone	$C_4H_6O_3$	0.06	0.17	102
	3	4.880	Benzoic acid	$C_7H_6O_2$	0.06	0.11	122
	5	5.888	Nonanoic acid	$C_9H_{18}O_2$	0.12	0.28	158
	6	6.242	2,4-Decadienal, (E,E)-	$C_{10}H_{16}O$	0.06	0.19	152
	7	6.562	2,4-Decadienal	$C_{10}H_{16}O$	0.09	0.25	152
	8	7.120	Decanoic acid, silver(1+) salt	$C_{10}H_{19}AgO_2$	0.02	0.07	278
	10	9.575	Dodecanoic acid	$C_{12}H_{24}O_2$	0.04	0.09	200
	12	9.782	Fumaric acid, ethyl 2-methylallyl ester	$C_{10}H_{14}O_4$	0.06	0.20	198
	13	9.821	n-Pentadecanol	$C_{15}H_{32}O$	0.07	0.26	228
	14	9.855	Diethyl phthalate	$C_{12}H_{14}O_4$	0.04	0.14	222
	16	11.883	Tetradecanoic acid	$C_{14}H_{28}O_2$	0.30	0.92	228
	17	12.155	Z-5-Nonadecene	$C_{19}H_{38}$	0.04	0.18	266
	18	12.626	Pentadecanoic acid	$C_{15}H_{30}O_2$	0.01	0.04	242
	19	13.211	Pentadecanoic acid	$C_{15}H_{30}O_2$	4.51	6.68	242
	21	13.830	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	0.04	0.15	270
	25	14.322	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	$C_{16}H_{22}O_4$	0.44	0.83	278
	30	15.420	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	0.01	0.03	436
18	31	15.530	cis-10-Heptadecenoic acid	$C_{17}H_{32}O_2$	0.07	0.16	268
	36	16.258	9-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	0.06	0.20	296
	37	17.280	Ethyl 9,12-hexadecadienoate	$C_{18}H_{32}O_2$	49.82	25.51	280
	38	17.330	14-Pentadecenoic acid	$C_{15}H_{28}O_2$	5.94	18.75	240
	39	17.476	Octadecanoic acid	$C_{18}H_{36}O_2$	1.54	3.21	284
	44	17.808	9-Octadecyne	$C_{18}H_{34}$	0.41	0.61	250
	57	20.167	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	0.24	0.60	294
	58	20.246	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	0.08	0.20	281
	59	20.886	Isopropyl linoleate	$C_{21}H_{38}O_2$	0.06	0.18	322
	63	21.738	9,12-Octadecadienoyl chloride, (Z,Z)-	$C_{18}H_{31}ClO$	0.09	0.22	298
	64	21.830	cisZ-11,12-Epoxytetradecan-1-ol	$C_{14}H_{28}O_2$	0.02	0.04	228
	66	22.641	Diisooctyl phthalate	$C_{24}H_{38}O_4$	0.09	0.26	390
	67	22.883	8,11-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	0.03	0.10	294
	70	23.836	Isopropyl linoleate	$C_{21}H_{38}O_2$	0.02	0.08	322
	72	24.626	Rhodopin	$C_{40}H_{58}O$	0.19	0.35	554
	77	25.666	8-Hexadecenal, 14-methyl-, (Z)-	$C_{30}H_{50}$	0.04	0.08	410
	79	26.609	Squalene	$C_{30}H_{50}$	0.02	0.06	410
	85	28.880	9(11)-Dehydroergosteryl benzoate	$C_{35}H_{46}O_2$	0.98	1.58	498
	122	37.993	Globulol	$C_{15}H_{26}O$	0.05	0.09	222

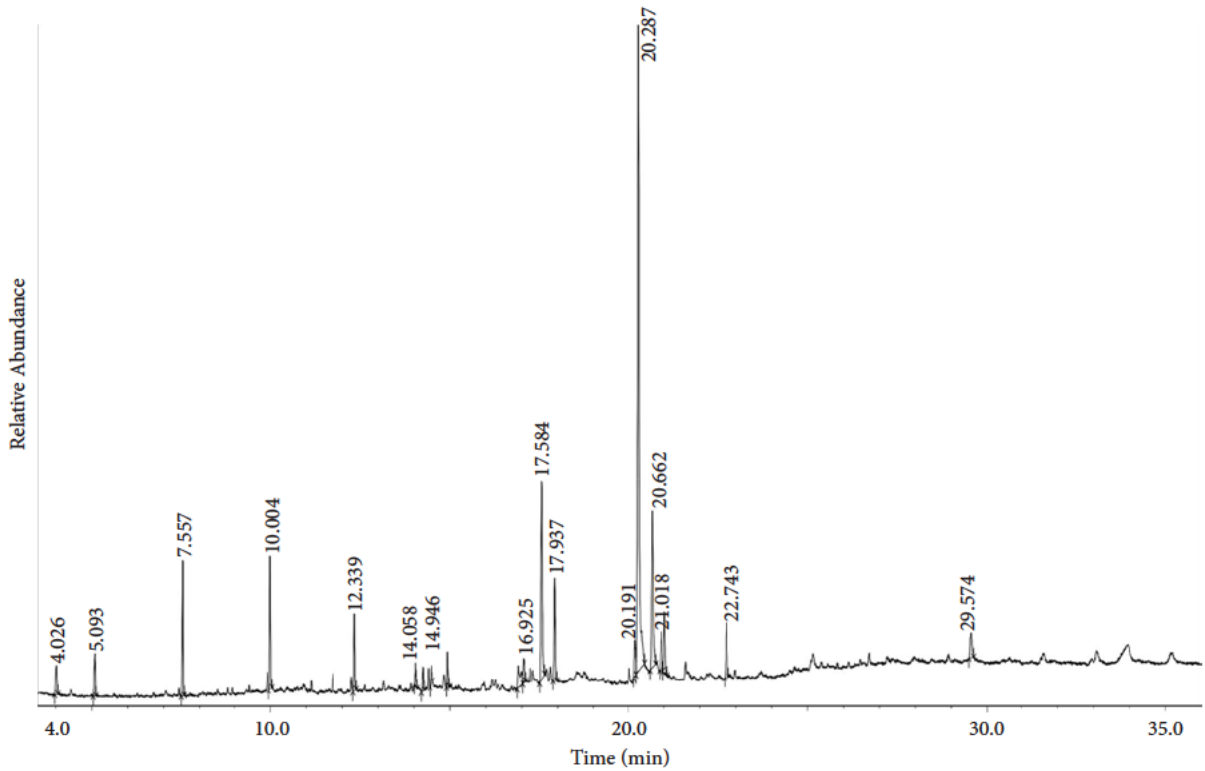

FIGURE 4: GCMS chromatogram of methanolic extract of *P. ostreatus* mushroom cultivated on sugarcane substrates supplemented with maize flour supplement.

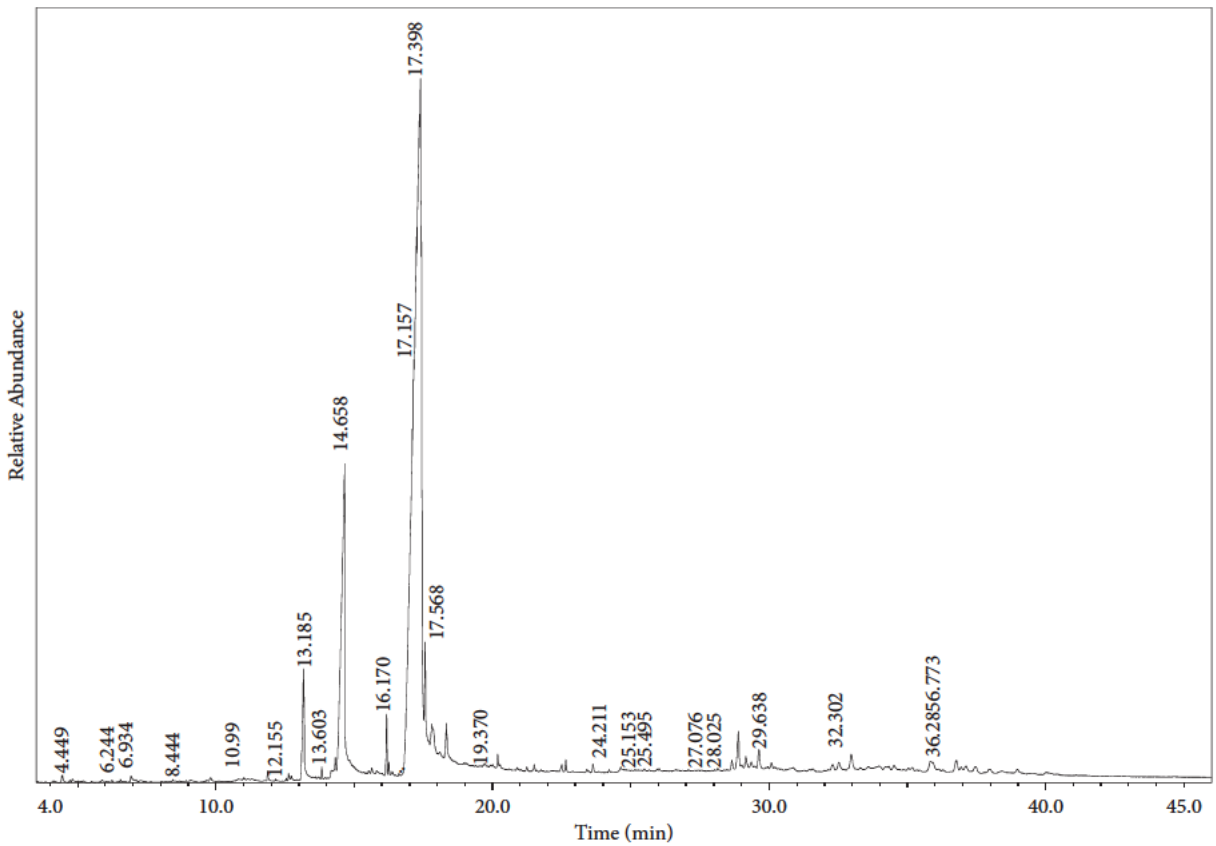

FIGURE 5: GCMS chromatogram of methanolic extract of *P. ostreatus* mushroom cultivated on bagasse substrates supplemented with maize flour supplement.

MF) had moderate activity towards *S. aureus* when compared to 18% MF. It was also noted that *P. ostreatus* extracts have lower activity towards *C. albicans* and *C. neoformans*. Such variations in antimicrobial activity could potentially be due to variations in the concentrations of bioactive metabolites such as phenolics and flavonoids, which are known to have strong biological activity [55]. The antimalarial potential of mushroom extracts was evaluated in the current study; thus, it was worth noting that all mushroom extracts had weak anti-plasmodium activity (Tables 4 and 5), which contradicts to the literature that stated that mushrooms possess antimalarial activity [14]. Previous researchers have obtained possible antimalarial activity of various mushrooms such as *Pleurotus ostreatus* [56], *G. lucidum,* and *T. pfeilii* [57]. The possible reason for such weak antimalarial activity observed in the current study could be attributed to the fact that cultivated edible mushrooms are known to have lower concentrations of secondary metabolites [58], since the mushroom-growing substrates may not necessarily provide all nutrients required by the mushrooms [59].

The abovementioned biological activities (DPPH, ABTS, reducing power, and antimicrobial) of *P. ostreatus* mushroom could be due to the types of compounds present within the extracts. Hence, Tables 6 and 7 and the chromatogram in Figures 4 and 5 prove that all *P. ostreatus* extracts had varying biological active compounds of known activity. These compounds were selected since they had known activities such as antioxidant and antimicrobial. Hence, GCMS results in Tables 6 and 7 proved that various levels of MF supplementation result in variations in biologically active compounds within the mushroom. However, some compounds such as pentadecanoic acid; 9,12-octadecadienoic acid methyl ester; pentadecanoic acid methyl ester; octadecanoic acid; and diisooctyl phthalate were found to be the most common ones amongst certain supplement levels. The majority of these compounds such as pentadecanoic acid, benzoic acid, Z-5-nonadecene, and dihydroqinghaosu are well known to have antimicrobial activity [60] and some antimalarial activity [61]. Furthermore, the antioxidant activity of some of these compounds such as 9-octadecenoic acid methyl ester [62], 9,12-octadecadienoic acid, methyl ester [63], and squalene [64] have been reported by various authors.

Hence, this proves that the type of supplement used on the growing substrates has a major influence on the type of compounds present within the mushroom, which could potentially influence the biological activity of mushrooms. However, it could be recommended that the bioactive compounds from these mushroom extracts should be purified, isolated, and characterized based on their biological activity, which could be either in vitro or in vivo. It could prove to be useful in filling the gap of using synthetic compounds for medicinal purposes since synthetic antioxidants such as BHT have proven to cause chronic cytotoxicity at high concentrations [65].

4. Conclusion

The study revealed that maize flour supplements promote mushroom yield and better C/N ratio, which are optimal for the growth and yield of *P. ostreatus* mushrooms. The observed biological activities of the mushroom extracts were linked to the level of maize flour supplement used upon growing substrates. Hence, it could be concluded that supplementing the mushroom-growing substrates with maize flour supplements potentially influences the biological activities of the mushrooms, such as antioxidant and antimicrobial activity, probably through promoting variations in bioactive metabolites found in mushrooms. However, a correlation study needs to be further conducted to fully understand the abovementioned phenomenon. The observed activities were potentially contributed by a variety of compounds such as phenolics, flavonoids, vitamin E, and other compounds which were synthesized by various mushroom extracts. Some of these compounds such as pentadecanoic acid, benzoic acid, Z-5-nonadecene, dihydroqinghaosu, 9-octadecenoic acid, methyl ester, 9,12-octadecadienoic acid, methyl ester, and squalene, to name a few, had known biological activities. Hence, it can be recommended for future studies that some of the natural compounds, especially the ones with known antimicrobial, antimalarial, and antioxidant activity, should be isolated and purified, which would greatly minimize the use of synthetic compounds such as butylated hydroxytoluene (BHT) that have great toxicity within the human body. Furthermore, the aspects of gene-encoding enzymes that synthesize a plethora of potential secondary metabolites should be explored to gain more bioactive compounds which could potentially be novel in fighting against pandemic diseases such as cancer, diabetes, malaria, and sexually transmitted diseases to name a few. Furthermore, future studies should focus on the activation of silent genes through various strategies such as the one-strain compound approach, which could also promote novel natural new compounds that have various biological activities.

Data Availability

The study data in this paper come from the image classification datasets of the Kaggle platform and Baidu AI Studio platform: Large Scale Fish dataset (https://www.kaggle.com/crowww/a-large-scale-fish-dataset); Weather dataset (https://aistudio.baidu.com/aistudio/datasetdetail/13165); Flowers Recognition dataset (https://www.kaggle.com/alxmamaev/flowers-recognition); and Fruits 360 dataset (https://www.kaggle.com/moltean/fruits).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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CHAPTER 5

Characterization and Biological Evaluation of Zinc Oxide Nanoparticles Synthesized from *Pleurotus ostreatus* Mushroom.

The objective of this published article was to biosynthesize zinc oxide nanoparticles (ZnO NPs) using *P. ostreatus* to obtain a simple, eco-friendly method of ZnO NPs synthesis. Additionally, the antibacterial activity and cytotoxicity towards HepG2 and Hek293 cells were evaluated for the *P. ostreatus* synthesized ZnO NPs. In this chapter, the *P. ostreatus* synthesized ZnO NPs were characterized and their antibacterial, antioxidant, and anticancer potential were analyzed.

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Characterization and Biological Evaluation of Zinc Oxide Nanoparticles Synthesized from *Pleurotus ostreatus* Mushroom

Senzosenkosi Surprise Mkhize [1], Ofentse Jacob Pooe [1,*], Sandile Khoza [2], Ishmael Nkoana Mongalo [3], Rene Khan [4] and Mthokozisi Blessing Cedric Simelane [5,*]

[1] Discipline of Biochemistry, Westville Campus, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa
[2] Department of Chemistry, University of Zululand, Kwa-Dlangezwa, Empangeni 3886, South Africa
[3] College of Agriculture and Environmental Science (CAES) Laboratories, University of South Africa, Private BagX06, Johannesburg 0710, South Africa
[4] Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa
[5] Department of Biochemistry, University of Johannesburg, Auckland Park 2006, South Africa
* Correspondence: pooeo@ukzn.ac.za (O.J.P.); msimelane@uj.ac.za (M.B.C.S.)

Abstract: This study aimed to biosynthesize zinc oxide nanoparticles (ZnO NPs) using *Pleurotus ostreatus* to achieve a simple ecofriendly method, and further evaluate antimicrobial activity and cytotoxicity towards HepG2 and Hek293 cells. The nanoparticles were characterized through UV-Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), transmission and scanning electron microscopy (TEM and SEM), selected area electron diffraction (SAED), X-ray diffraction (XRD), and dynamic light scattering (DLS). The minimal inhibitory concentration (MIC) for antimicrobial activity and MTT assay for cytotoxicity were conducted in vitro. The study revealed an efficient, simple, and ecofriendly method for synthesis of ZnO NPs that have antimicrobial activity. UV-Vis showed peaks at 340 and 400 nm, and the bioactive compounds found in the mushroom acted as capping, reducing, and stabilizing agents. TEM characterized NPs as an amorphous nanosheet, with preferential orientation as projected by SAED patterns. The spherical and agglomerated morphology was observed on SEM, with EDX proving the presence of Zn and O, while XRD indicated a crystallite size of 7.50 nm and a stable nature (zeta potential of −23.3 mV). High cytotoxicity on Hek293 and HepG2 cells was noted for ZnO NPs. The study provides an alternative, ecofriendly method for biosynthesis of ZnO NPs that have antibacterial activity and potential use in cancer treatment.

Keywords: ZnO nanoparticles; green synthesis; *Pleurotus ostreatus*; antimicrobial

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1. Introduction

Nanotechnology is a rapidly growing field of science, with numerous applications in areas such as engineering and biomedical science [1]. Its applications have triggered research on the synthesis of nanomaterials that are biocompatible, environmentally friendly, fast, safe, efficient, and cost-effective [2]. Two methods (traditional and green synthesis) have been used for the synthesis of nanoparticles; however, the traditional system has fallen out of favor due to its adverse effects such as producing excessive carbon dioxide, which contributes to greenhouse effects and also poses danger to the scientists involved in the synthesis [3]. This has caused a major shift towards green-synthesis methods. Hence, microorganisms (bacterium, fungi, including mushrooms, and yeast) and plants are now used for the green synthesis of nanoparticles [4]. Recently, ZnO nanoparticles have been synthesized successfully using plant extracts [5] and mushroom extracts, thus emphasizing the development of green-synthesis methods. Green synthesis has been

proven to be a non-toxic, pollution-free, environmentally friendly, economic, sustainable [6], clean, safe, and cost-effective method [3].

Nanoparticles (NPs) are regarded as microscopic particles having a size ranging from 1 to 100 nm [7]. They exhibit unique properties due to the quantum confinement effect; however, the influence of shapes and structures of the nanoparticles affects the reactivity and toughness of the nanomaterial [8]. These attributes of nanoparticles are due to their variations in shape, surface-to-volume ratio, size, and composition. These properties render their unique physical, chemical, and biological properties that may potentially be of benefit in numerous sectors such as medicines, agriculture, pharmaceuticals, electronics, and cosmetics [9]. Hence, nanoparticles have shown great potential in biomedical applications in managing infectious diseases that are usually caused by multidrug resistant organisms [10]. Currently, antimicrobial-resistant diseases, including hospital-acquired Gram-negative bacterial infections, and their associated morbidity and mortality, have grown at an alarming rate [11]. Hence bacteria such as *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Enterobacter* are considered as multidrug-resistant bacterial species causing nosocomial infections [12]. The rise in multidrug-resistant pathogens is significantly affecting the global economy and causing catastrophic effects in the health care system worldwide, thus resulting in increases in mortality and morbidity, a loss of certainty regarding orthodox drugs, and a rise in treatment costs [12].

Previously, multidrug-resistant pathogens, such as *Enterococci* and *Pseudomonas*, were associated with rapid nosocomial infections. Additionally, *Enterococci* has been recognized as the second-leading cause of nosocomial infections worldwide [13] (Bhonchal Bhardwaj, [13] 2020), whereas *P. aeruginosa* was found to contribute to 10–11% of all nosocomial infections [14]. There have been many attempts to eradicate multidrug-resistant bacteria; however, one of the major problems is that some organisms such as *P. aeruginosa* become more virulent when co-cultured with other Gram-positive bacteria [12,15]. Other researchers have suggested that *P. aeruginosa* is aggravated during coinfection with *E. faecalis* [16]. Therefore, it is paramount to find alternative effective and safe antibacterial responses, especially those with multiple mechanisms of resistance [17–19].

Recently, Gaglio et al. [18] explored the use of mushrooms as an antimicrobial agent since mushrooms produce a large number of bioactive metabolites with therapeutic potential, as reported earlier by [19]. These metabolites produced by mushrooms have immunomodulatory, cardiovascular, antifibrotic, antidiabetic, antitumor, liver protection, anti-inflammatory, antiviral, antioxidant, and, mostly, antimicrobial properties [20]. Thus, mushrooms may be a good alternative source of natural antibiotics having compounds with a low molecular weight (LMW) or high molecular weight (HMW) [21]. The numerous bioactive compounds found in mushrooms render mushrooms a new potential source for the synthesis of NPs [22]. This finding follows a major focus on NPs, which showed that NPs prevent microbial drug-resistance in some cases. This is because their mode of action is via direct contact with the bacterial cell wall, meaning there is no necessity to penetrate the cell. Hence, this provides hope that NPs do not promote bacterial resistance [23]. Zinc oxide (ZnO) NPs are mostly known to have distinct antibacterial, antimicrobial, and antifungal properties compared to other oxides [24]. However, the synthesis of zinc oxide NPs using *P. ostreatus* mushroom has been rarely explored in the past because ZnO NPs have been mainly produced using approaches such as the sol-gel, solvothermal, hydrothermal, and micro-emulsion methods [25], and solution combustion synthesis [26]. Based on the conditions and method used for synthesis, ZnO can be obtained in the form of knots, needles, nanorods, nanosheets, or nanoplates [26]. Thus, the shape of the nanoparticle usually affects its properties, such as its tensile strength, hardness, and stiffness, and its functions [27].

Hence, the present study focused on the synthesis of ZnO NPs using *P. ostreatus* mushrooms to evaluate both the antioxidant and the antibacterial effects against the mul-

tidrug resistance bacteria (*P. aeruginosa* and *E. faecalis*). This study proved to be advantageous in finding ways to eliminate the regime of multidrug-resistance, especially since mushroom-derived NPs are known to have high stability, longer shelf life, water-solubility, and good dispersion properties [22]. This technology of synthesizing nanoparticles using mushrooms is very promising for the synthesis of non-toxic, ecofriendly, and mostly stable nanomaterials [28,29]. Hence, this study promotes mushrooms as an alternative source for the synthesis of nanoparticles using a very cost-effective, non-toxic, and ecofriendly synthesis method for ZnO NPs. Previous researchers have only used green-synthesis methods for the production of CuO NPs using actinomycetes bacteria [30]. Hence the main objective of this study was to synthesize stable and biocompatible ZnO NPs, which would be an efficient antimicrobial and potential anticancer agent, using a fast, safe, cost-effective, and ecofriendly green method. This was achieved through incorporating a rarely used *P. ostreatus* mushroom as a capping and reducing agent during green synthesis.

2. Materials and Methods

2.1. Cultivation of P. ostreatus Mushrooms

Mushrooms were cultivated using locally available sugar cane bagasse waste material. The modified method outlined by Mkhize et al. [31] was utilized for the whole process of growing mushrooms. Briefly, the mushroom strain was pre-cultured on potato dextrose agar (PDA) and incubated at ambient temperatures, and thereafter maintained as mushroom culture in a refrigerator at 4 °C for further processing. Mushroom spawn was prepared using the modified method adopted from Mkhize et al. [32], whereby bird seed grains were soaked in distilled water overnight, and the excess water was drained. The grains were mixed with gypsum and calcium carbonates at a ratio of 4:1:300 g, and then sterilized in an autoclave. The sterilized grains were aseptically inoculated with previously grown mushroom cultures, and after that incubated in a darkroom set at ±25 °C for 2–3 weeks until mycelia fully colonized the grains. The spawn was inoculated into sterilized sugar cane bagasse, then incubated in the dark until mycelia fully colonized the bagasse. After full colonization, the colonized bags were moved from the dark into a fruiting room, which was constructed from plastic film covered by a single layer of 30% grey shade cloth on the outside. Then, mushrooms were allowed to fruit under ambient temperatures with constant fogging to provide 60% moisture to the mushrooms. The mushroom fruiting bodies were harvested and dried in the same tunnel with 30% shade cloth, with varying temperatures depending on the weather. However, the temperatures did not exceed ±45 °C. Mushrooms were then powdered for further analysis.

2.2. Synthesis of Mushroom ZnO NPs (Zinc Oxide Nanoparticles)

The mushroom powder (10 g) was dissolved in 100 mL of distilled water and allowed to boil for approximately 30 min, then filtered using Whatman filter paper, and the filtrate was stored at 4 °C for further analysis. The modified method of Muhammad et al. [33] was used for mushroom ZnO nanoparticle synthesis, whereby mushroom extract was used as the capping agent/reducing agent. Specifically, 0.2 M zinc nitrates ($ZnNO_3$) and 4 M sodium hydroxide (NaOH) solution were prepared by dissolving $ZnNO_3$ and NaOH at a ratio of 1:1 in distilled water. About 25 mL of 4 M NaOH was pipetted dropwise into 25 mL of 0.2 M $ZnNO_3$ under constant stirring with a magnetic stirrer. The pH of 13 was maintained via the addition of distilled water. A quantity of 10 mL of the mushroom extract was added to the solution mixture and heated at temperatures of 60–80 °C for 2 h. Within intervals of 30 min, a solution mixture of 3 mL was taken out for UV-Vis analysis at the wavelength of 200–800 nm using a Shimadzu spectrophotometer. The color change of the solution was gradually observed, which possibly indicated the formation of mushroom ZnO NPs. After heating while stirring with a magnetic stirrer, the solution mixture was allowed to cool at room temperature, and thereafter centrifuged at a speed of 3000

rpm for 15 min. The precipitate obtained was washed by adding distilled water and again centrifuged. The obtained precipitate was dried at 60 °C overnight. The mushroom ZnO NP powder was used for further analysis, such as analysis of morphology, structure, characterization, and antioxidant and antibacterial activity.

2.3. Characterization of ZnO NPs

For the characterization of NPs, different methods were utilized, as modified from different researchers such as Mickymaray [11] and Sharma et al. [9]. The ZnO NPs were characterized using a UV-visible spectrophotometer set at 300–600 nm with the operating resolution of 1 nm. The Fourier transform infrared (FTIR) spectroscopic analysis of mushroom ZnO NPs was evaluated using a spectrum 65 FTIR spectrometer to determine the vibrational bonding between mushroom ZnO NPs and phyto-compounds attached to surfaces of the NPs. The FTIR spectrum was recorded at the wave number range of 380 to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1}. X-ray diffraction (XRD) analysis was performed using a powder X-ray diffractometer that had Cu-kα radiation of 1.5418 Å, and a nickel monochromator in the range of 2θ from 10° to 80°. XRD was mainly performed to evaluate the phase of ZnO NPs. TEM was performed by dispensing a drop of ZnO NP solution into a carbon-coated 200 mesh copper grid and evaporated at room temperature before examination using TEM. Further characterization was also performed using an energy dispersive X-ray spectrometer (EDS) attached to a scanning electron microscope (SEM), which was used to identify and map elements within the synthesized ZnO NP powder. The dynamic light scattering (DLS) technique using a Zetasizer was employed to determine ZnO NPs' stability using the zeta potential of the nanoparticles.

2.4. Antimicrobial Activity Assay

The minimum inhibitory concentrations (MICs) of mushroom ZnO NPs were determined for *Enterococcus faecalis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Mycoplasma hominis*, *Staphylococcus aureus*, and *Bacillus cereus* using the microplate dilution assay modified from Soyingbe et al. [34]. The 2-fold serial dilution of nanoparticles was prepared in a 96-well microtiter plate using nutrient broth. A quantity of 50 µL of nutrient broth was added to all the 96-well microtiter plates. About 50 µL of mushroom ZnO NPs (5 mg/mL) dissolved in 1% DMSO was thereafter serially diluted downwards throughout the rows in the 96-well microtiter plates. A quantity of 50 µL bacterial culture set at 0.5 McFarland standard was added to all the wells of the 96-well microtiter plates, which were incubated at 37 °C for 24 h. The p-Iodo-nitrotetrazolium violet (INT) solution (40 µL of 0.2 mg/mL) was then added to 96-well microtiter plates and incubated at 37 °C for 30 min. The MIC was recorded as the lowest concentration of mushroom ZnO NPs that completely inhibited the microbial growth of organisms.

2.5. DNA Cleavage Studies

A modified method of Rajabi et al. [35] was followed for the DNA cleavage assay, whereby the ability of synthesized ZnO NPs to digest bacterial DNA was tested. The mushroom-synthesized ZnO NPs of various concentrations (30–100 µg/mL) interacted with the pET30 plasmid DNA. The reaction for DNA cleavage was conducted in a 96-well microtiter plate, whereby the reaction mixture was made up to 10 µL. The mixture was composed of pET30 plasmid DNA (2.4 µg), various concentrations of ZnO NPs (3.0–100 µg/mL), H_2O, and H_2O_2 for the positive control instead of ZnO NPs. The reaction mixtures were incubated at 37 °C for 60 min. After incubation, the mixtures were mixed with 0.25% bromophenol blue dye in 50% glycerol. Then, electrophoresis was carried out at 100 volts for 45 min; after that, the electrophoresis bands were observed under UV light.

2.6. Cytotoxicity Assay

In this study, the MTT assay was used to evaluate the cytotoxic effect of mushroom-synthesized ZnO NPs, and that of methanolic *P. ostreatus* mushroom extract on two cell lines, namely, human embryonic kidney (Hek293) and hepatocellular carcinoma (HepG2). A modified method from [36] was adopted, whereby the HepG2 and Hek293 cells were grown in 25 cm^2 flasks using complete culture medium (CCM; DMEM with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin/fungizone) and incubated at 37 °C and 5% CO_2. Confluent flasks were trypsinized to resuspend the cells, and the cells were counted using the trypan blue method. The cells were transferred into a sterile 96-well microtiter plate (15,000 cells/well, 200 µL CCM per well) and allowed to attach overnight. The medium was removed, and adherent cells were treated in triplicate with the prepared extracts (0–2000 µg/mL, 200 µL/well); a 4000 µg/mL stock solution of the mushroom ZnO NPs and *P. ostreatus* mushroom extract was used to prepare the treatment dilutions. A vehicle control (1% DMSO in CCM) was included. After 24 h, the treatment medium was discarded and replaced with 20 µL MTT salt solution (5 mg/mL in 0.1 M PBS) and 100 µL CCM at 37 °C. After a 4 h incubation, the MTT salt was removed, and the formazan crystals were solubilized by adding 100µL of DMSO to each well and incubated for an hour at 37 °C. The absorbance of the samples was read at 570 nm/690 nm using a Bio-Tek µQuant spectrophotometer (USA). The cell viability was calculated using the following formula:

$$\text{Cell viability} = ((\text{absorbance of sample})/(\text{absorbance of control}) \times 100)$$

Furthermore, the cell viability vs. log treatment concentration curve was analyzed using nonlinear regression analysis (GraphPad Prism V5.0, GraphPad PRISM®, La Jolla, CA, USA). The 25% inhibitory concentration (IC_{25}) was extrapolated from the curve; at this concentration, cells retained 75% cell viability. The cell viability at the respective MIC was also extrapolated from the curve.

2.7. Statistical Analysis of Results

All experiments were repeated in triplicate. Statistical analysis data generated were calculated using Graph Pad Prism. The statistical differences were determined using a one-way analysis of variance (ANOVA), followed by a Tukey–Kramer multiple comparison test; results are reported as mean ± S.E.M. The values were considered statistically significant where $p \leq 0.05$.

3. Results and Discussion

The UV-Vis spectrum of *P. ostreatus*-synthesized ZnO NPs displayed absorption peaks in the range between 300 and 600 nm, as displayed in Figure 1A. The observed absorption peaks of 340 and 400 nm confirmed the formation of ZnO NPs. Previous studies also obtained absorption peaks similar to those of our study, obtaining absorption peaks between 300 and 400 nm, which were reported to be typical of ZnO NP peaks [37]. Thus, this confirms the presence of ZnO NPs in our mushroom-synthesized nanoparticles. The results of the study were also in line with the findings of Manimaran et al. [38], who obtained an absorption peak of 350 nm for *P. djamor* mushroom-synthesized ZnO NPs.

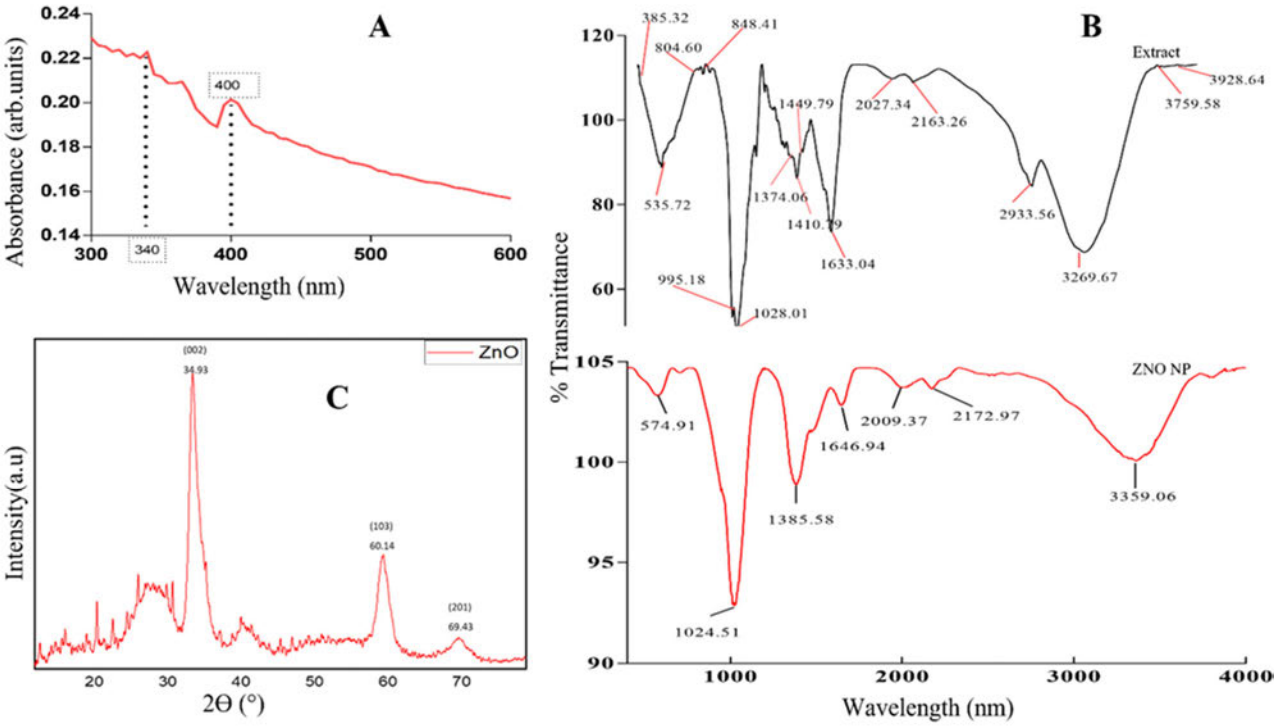

Figure 1. Characterization of *P. ostreatus*-synthesized ZNO NPs: (**A**) UV-Vis spectrum of the synthesized ZnO NPs; (**B**) FTIR spectrum of ZnO NPs; and (**C**) XRD pattern of ZnO NPs.

Figure 1B shows the Fourier-transform infrared spectroscopy (FTIR) spectra of the *Pleurotus ostreatus* mushroom extract and synthesized ZnO NPs. Hence FTIR was used to identify functional groups present within *Pleurotus ostreatus* mushroom extract and within synthesized ZnO NPs. FTIR spectra showed a broad band at 3291.06 cm^{-1} for a mushroom extract, which shifted to 3359.06 cm^{-1} on synthesized ZnO NPs. These broad bands correspond to the O-H stretching vibrations, which were attributed to the alcohol or phenols found in mushrooms that might be transferred to ZnO NPs during synthesis. Furthermore, the mushroom extract had absorption bands of 2117.60 cm^{-1}, which also shifted to 2172.97 and 2009.37 cm^{-1} within ZnO NPs due to C=C interactions. The presence of C=O (amide1) was observed for the absorption band of 1633.04 cm^{-1} in the mushroom extract. This absorption band was slightly shifted to 1646.94 cm^{-1} in ZnO NPs. This shift was an indication that mushroom extract contained functional groups that could act as a capping and reducing agent in the synthesis of ZnO NPs. The peaks of 1410.79 and 1028.01 cm^{-1} were noted in the mushroom extract, which shifted to 138.58 and 1024.51 cm^{-1} within the spectra of mushroom-synthesized ZnO NPs, indicating the bending alkane vibrations of C-H (138.58 cm^{-1}) and amine C-N (1024.51 cm^{-1}) stretching vibrations. The peaks (1024.51 and 574.91 cm^{-1}) observed on the fingerprint region of ZnO NPs probably corresponded to the ZnO stretching vibrations; metal oxides are known to have absorption peaks between 600 and 400 cm^{-1} [39]. Such findings corroborate with other findings reported by Daumann et al. [40], who also established a deep absorption band from 610 cm^{-1} to a lower wavelength corresponding to ZnO. The observed peak at 574.91 cm^{-1} was attributed to the ZnO stretching band, meaning the *P. ostreatus* extracts successfully acted as capping and stabilizing agents in the synthesis of ZnO NPs. The mushroom-synthesized ZnO NPs were further analyzed with XRD to determine the crystallinity and purity of the nanoparticles, as indicated in Figure 1C. The diffraction pattern of ZnO NPs at three theta angles of 34.93, 60.14, and 69.43, which are indexed to (002), (103) and (201) planes, were observed, as shown in Figure 1C. Thus, the XRD pattern of the study was in line with the research conducted by Manimaran et al. [38], where *P. djamor* mushrooms were used for

the synthesis of ZnO NPs. The average particle size of the sample was found to be 7.50 nm, derived from the full width at half-maximum (FWHM) of the more intense peak; hence, this corresponded to the 002 plane located at 34.93°.

The elemental composition of the green-synthesized ZnO NPs was determined using EDX spectrum analysis. The EDX spectrum shown in Figure 2C revealed the presence of Zn and O within the samples, as expected, with no traces of impurities. Hence, a strong signal in Zn and O regions was observed, confirming that mushroom extracts were able to reduce and stabilize Zn^{+} ions, and that ZnO NPs were synthesized. Previous research conducted by Preethi et al. [41] also proved that mushrooms can act as a reducing agent in synthesizing ZnO NPs. The study indicated that the percentage composition of zinc was 65.93% and that of oxygen was 34.07%. This result agrees with the findings of Mohana and Sumathi [42], who found the percentage composition of zinc was 66.16% and that of oxygen was 33.84% for ZnO NPs synthesized by *Agaricus bisporus* mushroom extract. The results of ZnO mapping shown in Figure 2A,B proved that zinc and oxygen were present in the synthesized ZnO NPs, which further verifies that the mushroom-synthesized ZnO NPs had no impurities.

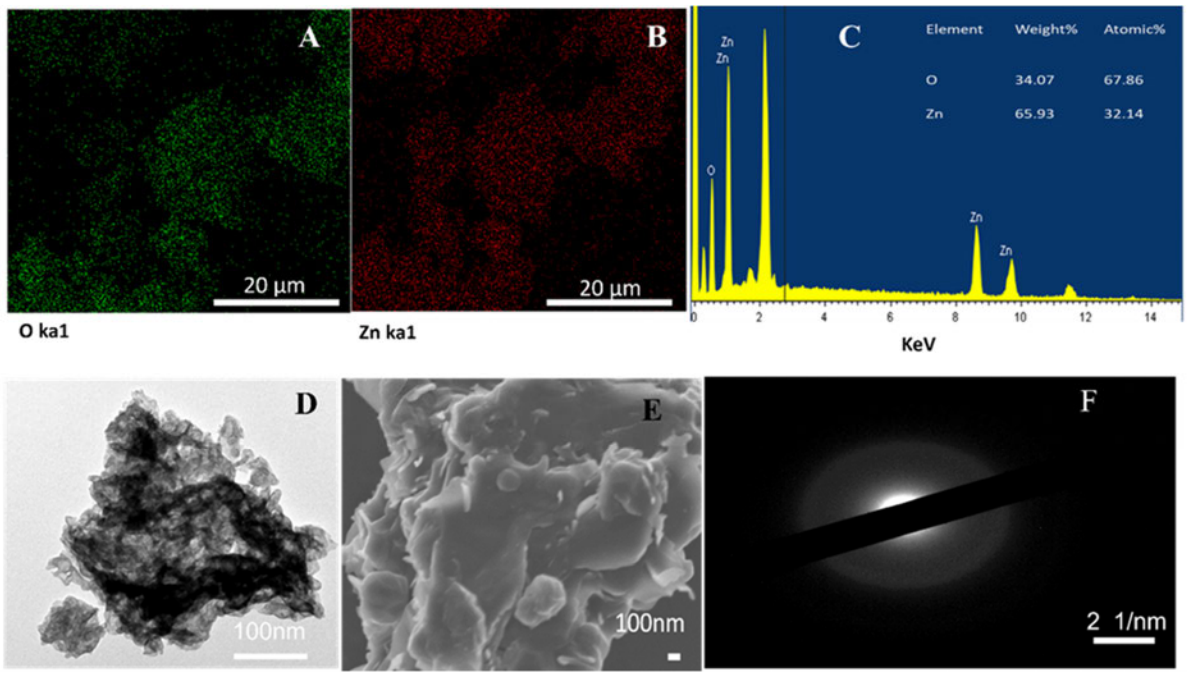

Figure 2. Structural characterization of ZnO NPs by electron microscopy prepared from *P. ostreatus*: (**A**,**B**) are EDS elemental maps (20 kV) of oxygen (Ka1) and zinc (Ka1) emission lines, respectively; (**C**) EDX analysis (20 kV) of O Ka1 and Zn Ka1; (**D**) TEM images of synthesized ZnO NPs; (**E**) SEM micrograph image of synthesized ZnO NPs; and (**F**) SAED pattern of ZnO NPs.

Figure 2D shows the transmission electron microscopy (TEM) images of the amorphous ZnO NPs, which had dark spots that are probably due to the stacking of a large number of layers of nanosheets. This indicates that the synthesized ZnO NPs were probably agglomerated, since ZnO nanoparticles are also prone to forming agglomerations that are either hard or soft, where hard agglomeration is caused by the chemical reaction of the surface groups and soft agglomeration is caused by other physical effects [43]. Furthermore, the selected area electron diffraction (SAED) pattern in Figure 2F shows bright rings, which confirm the preferential orientation of mushroom-synthesized ZnO crystals.

The SEM images in Figure 2E indicate that the synthesized ZnO NPs have a spherical morphology and are agglomerated together. Furthermore, as expected, the green-synthe-

sis method is known to usually cause agglomeration in NPs because the green-synthesized NPs mostly have a high surface area, allowing NPs to stick together [44]. However, polarity and the electrostatic attraction of ZnO NPs may also have contributed to the agglomeration of ZnO NPs [39]. The surface charge and stability of *Pleurotus ostreatus*-mediated ZnO NPs were examined using the zeta potential (Figure 3). In this study, the *Pleurotus ostreatus*-synthesized ZnO NPs were observed to be negatively charged with good stability; thus, the zeta potential was found to be −23.3 mV. These results suggest that the synthesized ZnO NPs had negatively charged capping molecules, which are responsible for the stability of the synthesized nanoparticles [45]. These findings corroborate well with the results reported by Mohana and Sumathi [42], who found the zeta potential of *Agaricus bisporus*-mediated ZnO NPs to be −20.5 mV, with good stability and a negative charge. Other researchers also confirmed that particles with charges of or above −25 mV and +25 mV tend to be more stable [46].

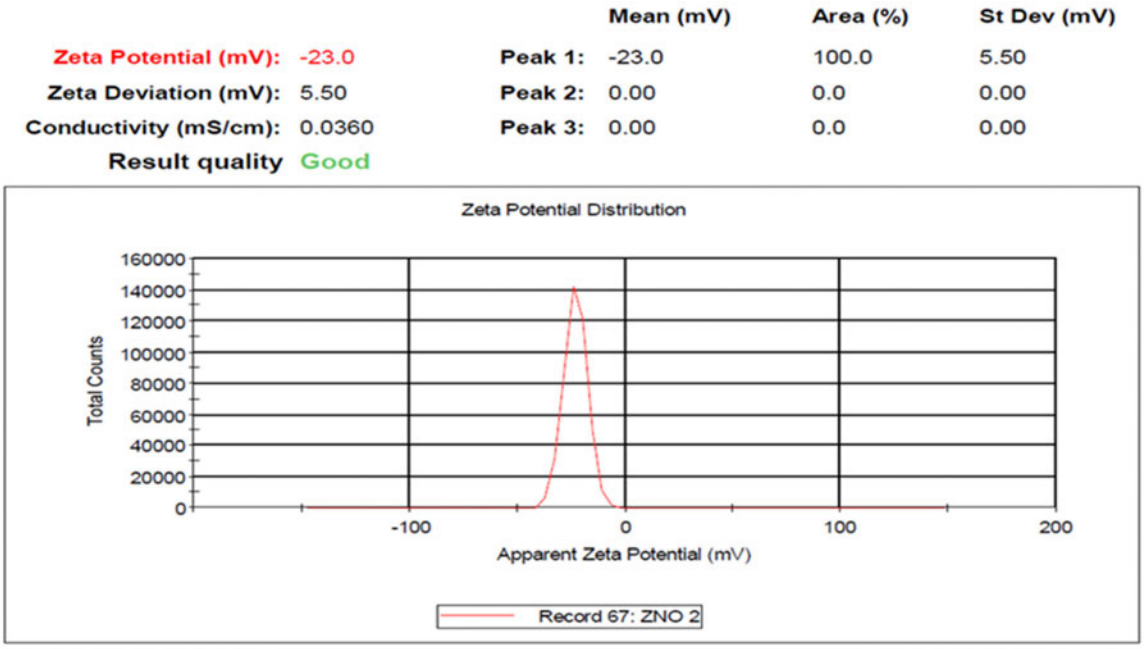

Figure 3. Zeta potential analysis of the biosynthesized ZnO NPs.

Most pathogenic microorganisms, such as those of clinical isolates, have been reported to exhibit a higher level of resistance towards antibiotics that are particularly used in medical care facilities [47]. In addition to the clinical isolates, the bacterial strains associated with foodborne outbreak infections also proved to have higher levels of resistance towards antibiotics [48]. The literature states that zinc oxide nanoparticles can be used as antimicrobial agents against pathogenic microorganisms [49]. In the present study, the antibacterial activity of mushroom-synthesized ZnO NPs was investigated for both Gram-positive (*C. albicans*, *B. cereus*, *S. aureus*, and *E. faecalis*) and Gram-negative (*K. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, *E. coli*, and *M. catarrhalis*) bacteria using the 96-well microtiter method to determine the MIC, as indicated in Table 1. The results in Table 1 indicate that both the mushroom extract and *Pleurotus ostreatus*-synthesized ZnO NPs exhibit antimicrobial activity against all tested microorganisms (Gram-positive and Gram-negative).

However, when comparing mushroom extract with mushroom-synthesized ZnO NPs, the study showed that mushroom-synthesized ZnO NPs had enhanced antimicrobial activity for almost all the tested microorganisms. Therefore, this means that mushroom antibacterial activity is enhanced through nanoparticle synthesis. This activity of mushroom-synthesized ZnO NPs may be attributed to the fact that both Gram-positive

and Gram-negative microorganisms contain a cell wall that is negatively charged, hence causing interactions of the bacterial cell wall with positively charged ions released by nanoparticles [50]. Previous researchers have also noted that the ZnO NPs interact with the plasma membrane of bacteria through electrostatic attraction; hence, membrane permeability may be disrupted through metal ions (Zn^{2+}), resulting in damage to bacterial DNA leading to cell death [51]. The results indicate that different microorganisms have varying susceptibility towards mushroom-synthesized ZnO NPs, probably due to differences in the polarity of the cell membranes of these bacteria [52].

To further confirm possible antibacterial activity of synthesized ZnO NPs, DNA cleavage assay was conducted in our study. Figure 4 shows that mushroom-synthesized ZnO NPs potentially damage bacterial DNA in a concentration-dependent manner. Lane C1 indicates pure bacterial DNA, whereas lane C2 shows DNA treated with H_2O_2 (completely denatured DNA), and the remainder of the lanes indicate DNA treated with mushroom-synthesized ZnO NPs at increasing concentrations of 3.0, 6.25, 12.5, 25.0, 50.0, and 100 μg/mL. It can be noted from Figure 4 that mushroom-synthesized ZnO NPs were able to digest/damage bacterial DNA; hence, higher concentrations of ZnO NPs, such as 50 and 100 μg/mL, degraded the DNA structure. This observed DNA cleavage mechanism may be responsible for the antibacterial effects attained in other microorganisms. As expected, other researchers also found that DNA digestion can potentially be considered as an antimicrobial mechanism employed by nanoparticles [53]. These results suggest that ZnO NPs may compromise membrane permeability of bacteria, therefore causing Zn^{2+} to enter the bacterial cytoplasm and causing damage to bacterial DNA, as reported by Murali et al. [51].

Table 1. Antimicrobial activity of mushroom ZnO NPs (MIC in mg/mL) against selected pathogenic microorganisms.

Microorganisms	Mushroom ZnO NPs	Mushroom Extract	* Control Drug
P. aeruginosa	0.04	0.04	0.003
E. faecalis	0.08	1.25	0.006
C. albicans	0.31	0.63	0.003
K. pneumoniae	0.16	0.63	0.008
B. cereus	0.63	1.25	0.004
P. vulgaris	0.31	0.16	0.001
S. aureus	0.63	0.63	0.004
E. coli	0.16	0.63	0.001
M. catarrhalis	1.25	1.25	0.003
M. hominis	0.16	0.63	0.004

* Control drug: gentamycin for all bacterial strains and amphotericin for *Candida albicans*.

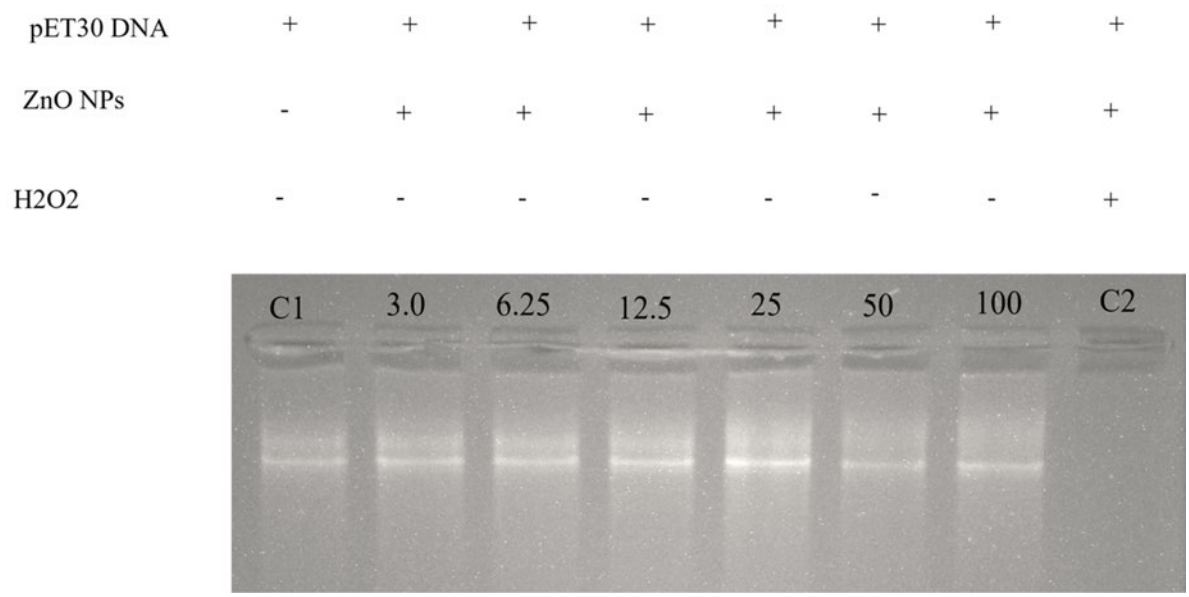

Figure 4. The gel electrophoresis image of DNA cleavage of *P. ostreatus*-synthesized ZnO NPs. C1 (control with pure pET30 DNA), C2 (control with DNA+ H_2O_2), lanes 3.0–100 represent increasing concentrations of *P. ostreatus*-synthesized ZnO NPs in μg/mL.

The cytotoxicity of mushroom-synthesized ZnO NPs and mushroom extract was evaluated in vitro using HepG2 and Hek293 cell lines, which are the two frequently used cell lines for cytotoxicity testing. These cell lines were selected due to the vital role of liver cells in cellular metabolism and glucose homeostasis. In addition, the liver also detoxifies xenobiotics (including drugs) to water-soluble metabolites for excretion by the kidney [54]. The conversion of the yellow tetrazolium salt into purple formazan crystals by mitochondrial dehydrogenases only occurs in viable cells; thus, the intensity of formazan absorbance is directly proportional to the percentage of viable cells in the sample [55]. The mushroom extract (0–2000 μg/mL) caused a variable response in HepG2 cells, with cell viability above 100% for all treatments (Figure 5A). In contrast, the mushroom extract induced a dose-dependent decline in cell viability for the Hek293 cells, with the lowest cell viability of 22% recorded at 2000 μg/mL (Figure 5B). The mushroom-synthesized ZnO NPs induced similar cytotoxic responses for the HepG2 (Figure 5C) and Hek293 (Figure 5D) cells. There was a sharp decline in cell viability from approximately 97% at 16 μM to 12% at 100 μM in HepG2 cells, and 94% and 22% cell viability was observed in Hek293 cells at the same concentrations. The observed results for mushroom-synthesized ZnO NPs are consistent with the findings of Anitha et al. [56], who stipulated that ZnO NPs have the potential to cause cytotoxicity in many cells, including HepG2 cells.

Cell viabilities below 20% were recorded from the concentrations of 1000–2000 μg/mL treatments in both HepG2 (Figure 5C) and Hek293 cells (Figure D). The decreased cell viability indicates a diminished capacity of mitochondrial dehydrogenases to convert the yellow tetrazolium salt into purple formazan crystals [55]. Thus, the lower IC50 values in Hek293 and HepG2 cells following ZnO treatment (Table 2) suggest increased sensitivity of the liver and kidney cells to this exposure (Figure 5C,D). In particular, these values denote mitochondrial toxicity since cell viability decreased. Recently, Li et al. [57] showed that ZnO NPs promote ROS generation, inducing mitochondrial dysfunction, and may impair mitochondrial biogenesis in cardiomyocytes [55,57]. The ROS generation may be attributed to interference at complex III of the electron transport chain [58]. The acute cytotoxicity of the mushroom-synthesized ZnO NPs may be due to parameters such as particle size, surface area, and surface reactivity. Previous studies have identified these parameters for other nanoparticles [59]. For example, Panda et al. [60] noted that smaller-

sized nanoparticles are more toxic when compared to larger nanoparticles; however, it should also be considered that other factors can influence the toxicity of nanoparticles, as in the case of metallic particles [61].

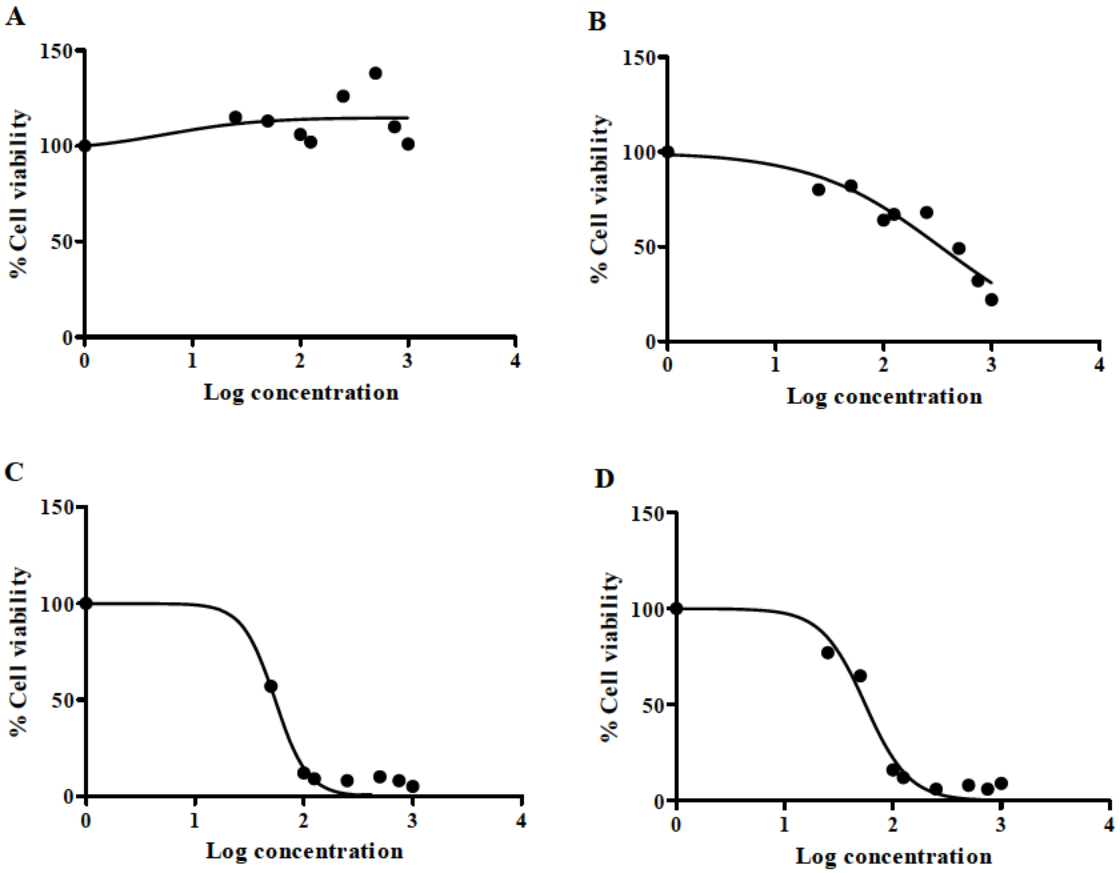

Figure 5. The dose response curves derived from the MTT assay. (**A**) The mushroom extract was not cytotoxic to HepG2, with increased cell viability occurring with increased concentrations. (**B**) Cell viability decreased gradually in Hek293 cells treated with increasing mushroom extract concentration. (**C**) ZnO mushroom nanoparticles decreased cell viability significantly between 10 and 100 µg/mL in HepG2 cells, with more than 80% cell death from 1000 to 2000 µg/mL. (**D**) The Hek293 cell response to ZnO mushroom nanoparticles indicates cytotoxicity at all concentrations.

Table 2. The extrapolated IC25 and IC50 concentrations for HepG2 and Hek293 cells.

Sample	Cell Lines	IC25 (mg/mL)	IC50 (mg/mL)
Mushroom extract	Hek293	0.074 (74 µg/mL)	0.335 (335 µg/mL)
	HepG2	-	-
Mushroom ZnO NPs	Hek293	0.034 (34 µg/mL)	0.056 (56 µg/mL)
	HepG2	0.037 (37 µg/mL)	0.055 (55 µg/mL)

In this study, the ISO norm that requires >75% cell viability for biomedical products was applied [61]. Thus, an IC25 where HepG2 and Hek293 cells retained 75% viability was applied for the mushroom ZnO NPs and mushroom extract to be deemed safe. The IC25 for the *P. ostreatus* mushroom extract was 0.074 mg/mL in Hek293 cells (Table 2) and could not be calculated for HepG2 cells (Table 2), which retained cell viability (Figure 5A), indicating the concentrations retained 75% cell viability in both cell lines in accordance

with the ISO norm. At the MIC for *P. aeruginosa* (0.04 mg/mL), the Hek293 and HepG2 cell viability was around 83% and 112%, respectively. Thus, the *P. ostreatus* mushroom extracts conformed to the ISO standard for the *P. aeruginosa* MIC, indicating that the mushroom extract would be safe to use for treating *P. aeruginosa* infections. However, the MIC for *P. vulgaris* exposed to mushroom extract (0.160 mg/mL) yielded 63% cell viability in Hek293, scoring below the ISO standard. Interestingly, the mushroom extract was not cytotoxic to HepG2 cells at the *P. vulgaris* MIC. Since all other MIC values for the mushroom extract were higher than the *P. vulgaris* MIC, the data suggest that a cytotoxic effect can manifest in Hek293 cells, but not HepG2 cells.

Acceptable ISO values for the Hek293 and HepG2 cells were 0.034 and 0.037 mg/mL, respectively (Table 2). Thus, the liver and kidney cells appear more sensitive to the mushroom-synthesized ZnO NPs (Figure 5C,D). Therefore, it was not surprising that the 67% and 70% cell viability for Hek293 and HepG2 cells, respectively, at the *P. aeruginosa* MIC (0.04 mg/mL), was slightly below the ISO norm. Similarly, approximately 33% and 24% cell viability was extrapolated for the MIC of *E. faecalis* (0.08 mg/mL) in Hek293 cells and HepG2 cells, respectively. The mushroom-synthesized ZnO NPs induced an MIC of 0.160 mg/mL for *K. pneumonia*, *E. coli*, and *M. hominis*, corresponding to cell viabilities of 10% (Hek293 cells) and 4% (HepG2 cells). Further increases in MIC, as indicated for other pathogenic microorganisms (Table 1), were also overtly cytotoxic to both cell lines. This suggests that the mushroom-synthesized ZnO NPs may be a potential anticancer agent if appropriately administered so that it can have specific activity; hence, future studies should confirm the anticancer property and cell proliferation as influenced by the ZnO NPs. Other mushrooms have been reported to be able to synthesize ZnO NPs, such as *P. djamor* [38], *P. floridanus* [62], and *Agarius bisporus* [41], in addition to *P. ostreatus*. To the best of our knowledge, this is one of the first reports demonstrating the biotechnological potential for synthesizing stable, biocompatible, ecofriendly, and antimicrobial ZnO NPs using *P. ostreatus*.

4. Conclusions

This paper presents the successful synthesis of ZnO NPs from *P. ostreatus* mushrooms, and characterization of ZnO NPs on UV-Vis spectra confirmed the peaks of ZnO NPs at 340 and 400 nm. The synthesized ZnO NPs were observed to be spherical and agglomerated with high stability. Thus, the mushroom was able to act as a reducing, capping, and stabilizing agent in the synthesis of ZnO NPs. The green-synthesis method proved beneficial in fighting against Gram-positive and Gram-negative bacteria, which are a major concern in the health sector. This is the first report of ZnO NP biosynthesis using the edible *P. ostreatus* mushroom. Hence, an alternative safe, cost-effective, and eco-friendly biosynthesis method to produce ZnO NPs can be achieved through mushroom-mediated synthesis. Further research can be conducted on other nanoparticles using the *P. ostreatus*-mediated biosynthesis method. Overall, minimal cytotoxicity to HepG2 cells (cell viability above 75% in accordance with the ISO standard) was observed for the *P. ostreatus* mushroom extract. Thus, the liver function of xenobiotic metabolism possibly resulted in the detoxification of the cytotoxic compounds in the mushroom extract. However, cell viability below the ISO standard at MIC above 0.160 mg/mL indicates that the mushroom extract should be used with caution due to its potential cytotoxicity to kidney cells. The mushroom-synthesized ZnO NPs were acutely cytotoxic to both Hek293 and HepG2 cells. Further studies should investigate the cytotoxic effects of the *P. ostreatus* mushroom extracts in vivo using a rat or mouse model, since metabolism in the liver may reduce the toxic effects in kidney cells that were elucidated in this study. The cytotoxicity data also demonstrate antiproliferative activity of the mushroom-synthesized ZnO NPs in HepG2 cells. Thus, further investigation of potential anticancer effects of the *P. ostreatus* mushroom extract and mushroom-synthesized ZnO NPs is warranted and recommended.

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CHAPTER 6

General Discussions and Conclusions

6.1 General Discussions

Mushroom cultivation offers a quick, high-profit, environmentally friendly, cost-effective solution for poverty-stricken countries, with its high market price and environmental benefits. Mushrooms are also widely recognized as a delectable, nutritious, and medicinal food worldwide (Shakil et al., 2014), consequently, mushroom cultivation has become popular worldwide, including in African countries (Anchang, 2014). Even though mushrooms play such an important role within poor communities, the biggest obstacle faced by the communities consuming mushrooms is the issue of heavy metals since mushrooms are capable of bio-accumulating heavy metals from soil and any substrates they are grown on (Ab Rahman et al., 2022). Hence, the study sought to evaluate the concentration of different heavy metals absorbed by *P. ostreatus* from local agro-waste substrates since communities around South Africa consume mushrooms that are grown from these local agro-industrial wastes.

The findings of the study will guide nutritional purposes and warn communities if these edible mushrooms contain high levels of toxic metals that are beyond the WHO or FAO recommended daily intake. Our study observed that the accumulation of heavy by *P. ostreatus* varied depending on the type of heavy metals and the type of substrates used. Therefore, this means numerous factors influenced the accumulation of heavy into *P. ostreatus* mushrooms. According to the observed results, metals such as Fe and Zn were noted to have a high affinity to be absorbed by *P. ostreatus*, regardless of the type of substrates used. Due to the mushroom's strong requirement for iron (Fe) throughout its metabolic activities, it can therefore be said that the *P. ostreatus* mushroom may have the greatest capacity to bioaccumulate metals such as Fe (Umeo et al., 2020). Hence, compounds such as mannoproteins that are usually found in *P. ostreatus* cause the retention of siderophore-iron chelates within the cell wall, increasing mushroom iron uptake (Umeo et al., 2020). Furthermore, the *P. ostreatus* mushrooms were

observed to contain essential elements like Zn and Fe, which are crucial for human health and metabolic processes, meaning the *P. ostreatus* grown from agro-waste can be an alternative food to fight malnutrition (Dunn et al., 2007).

The study also revealed that heavy metals were present within the *P. ostreatus* mushrooms, but at moderate or tolerable concentrations, since they were below or within the recommended daily intake (RDI) recommended by WHO. Therefore, *P. ostreatus* grown from agro-wastes can be deemed to be safe for consumption, however, caution needs to be exercised to avoid health risks that could arise due to exceeding the recommended RDI by FAO/WHO.

Supplementing mushroom-growing substrates effectively improved the C/N ratio and mushroom yield, hence higher levels of supplementations were observed to enhance *P. ostreatus* yield. These findings implied that the addition of supplements into substrates resulted in a balanced C/N ratio which is known to greatly affect mushroom production or yield. Previous studies have stipulated that the substrates with lower C/N promote better yield of mushrooms, compared to substrates with high C/N ratio (Hoa & Wang, 2015). The obtained results could benefit the mushroom cultivators since obtaining a good yield is of great interest to the mushroom cultivators.

Even though the practice of adding supplements into mushroom growing substrates proved to be very beneficial in obtaining a good yield, contrary results were obtained pertaining to improving the antioxidant activity of *P. ostreatus* mushrooms. For the antioxidant activity of mushrooms against DPPH radicals and reducing power assay, the mushrooms grown on un-supplemented substrates had high radical scavenging activity when compared to *P. ostreatus* grown from the supplemented substrates. It was noted in Chapter 2 and Chapter 4 for DPPH and Chapters 3 and 4 for reducing power assay that mushrooms grown on un-supplemented substrates had an enhanced antioxidant activity compared to mushrooms grown on supplemented substrates. This phenomenon might have been caused by the presence of phenolic compounds, which are often influenced by the composition of the growing substrates (Gąsecka et al., 2016). For example, ions of some metals such as Zn, usually form a complex

with polyphenols (Clarisse et al., 2000), which therefore results in a reduced or decreased antioxidant activity due to limited availability of free radicals for donation (Fontes Vieira et al., 2013). Previous studies by Yokota et al., (2016), have also stipulated that high concentrations of metals such as Fe within the fungi generally caused a decrease in mushroom antioxidant activity. Therefore, it could be stipulated that the low antioxidant activity that was observed for the mushrooms grown on supplemented substrates was probably influenced by a high concentration of Fe within mushrooms.

The study revealed variations in the radical scavenging activities between mushrooms grown on different substrates. Such observed variations may potentially be due to variations in bioactive compounds such as phenolic compounds that closely correlate with the antioxidant activity of the mushrooms. The observed antioxidant activities suggest that the local agro-waste could potentially be used as an alternative for producing mushrooms with therapeutic properties against various diseases such as diabetes, cancer, and heart diseases, however, further research is needed to confirm this phenomenon.

Besides the antioxidant activity observed from *P. ostreatus* mushrooms, antimicrobial activity against bacterial and fungal microorganisms was noted. Chapter 3 and Chapter 4 revealed that *P. ostreatus* grown on wheat bran and maize flour-supplemented substrates have antimicrobial potential against drug-resistant microorganisms, such as *E. coli*, *S. aureus*, and *C. albicans*. However, there were some variations in the antimicrobial activity of different mushroom extracts, hence some had weaker activity (MIC > 0.625 mg/ml), moderate activity (0.1 < MIC < 0.625 mg/ml), and others had greater antimicrobial activity (MIC < 0.625 mg/ml).

The antimicrobial activities were noted to vary depending on the type of microorganisms and the level of supplementation. For example, the *P. ostreatus* mushrooms grown from sugar cane tops had weaker activity (MIC > 0.625 mg/ml) against *E. coli* and *C. neoformans* but had moderate activity (0.1 < MIC < 0.625 mg/ml) and good activity (MIC < 0.625 mg/ml) against *S. aureus* bacteria. The variations in the antimicrobial activity of *P. ostretus* mushrooms grown with different substrates might probably be due to the presence of a broad spectrum of

antimicrobial compounds that were observed for GCMS results in Chapters 3 and 4. Therefore, it could be concluded that the addition of supplements into growing substrates contributes towards variations in the bioactive compounds. Thus, future studies could focus on the extraction and purification of these compounds, which could be beneficial to humans since these compounds could act as an alternative natural source of antibiotics against multidrug-resistant bacteria and fungi. Even though a broad spectrum of compounds was observed using GCMS, however, there was a possibility of the presence of nonvolatile, polar, or thermally labile compounds since the GCMS is not capable of analyzing the above compounds. It could therefore be of future interest to incorporate other methods such as liquid chromatography/mass spectrometry (LC/MS), which is known to determine highly polar compounds.

The DNA protection ability of *P. ostreatus* mushroom extracts was evaluated against hydroxyl radicals which induce damage to pET30 plasmid DNA. The hydroxyl radicals that are generated by the Fenton reaction induce oxidative breaks in DNA strands and form an open circular DNA (Golla and Bhimathati, 2014). The observed results revealed that the addition of WB supplements to different substrates had no significant effects on protecting the pET30 plasmid DNA against hydroxyl radicals. The only protection that was observed was on the *P. ostreatus* grown from the un-supplemented (0% WB) sugar cane tops, meaning this treatment produced mushrooms with enough concentration of phenolics and flavonoids that are known to prevent the formation of ROS (Golla & Bhimathati, 2014). The supercoiled plasmid DNA was slightly retained via *P. ostreatus* grown from the un-supplemented (0% WB) substrates (Chapter 2), which probably means the DNA damage was decreased. Such protection could potentially increase if the concentration of extracts is increased further since it has been reported in the above chapters that the antioxidant activity of *P. ostreatus* mushroom is concentration-dependent. The antimalarial activity of *P. ostreatus* mushroom was observed to be weak for all the extracts, which possibly means that *P. ostreatus* did not produce enough secondary metabolites with antimalarial properties. These findings corroborate the phenomenon that was stipulated by former researchers who stipulated that cultivated edible

mushrooms produce lower concentrations of secondary metabolites (Abugri & Mcelhenney, 2013). However, some compounds with antimalarial activity such as Dihydroqinghaosu (Youyou, 2004), were observed within the *P. ostreatus* grown from sugar cane bagasse supplemented with 18% MF. This compound was probably present at lower concentrations that cannot exhibit enough antimalarial activity. It can therefore be recommended for future studies that the observed compound should be extracted, purified, and tested both in-vitro and in-vivo for potential antimalarial activity.

The *P. ostreatus* mushrooms were further explored for the biosynthesize of nanoparticles (ZnO NPs), using a cost-effective, simple, and eco-friendly method to obtain stable and biocompatible ZnO NPs. The study revealed that *P. ostreatus* mushrooms successfully synthesized the ZnO NPs, which was the very first time the *P. ostreatus* mushroom was incorporated into the biosynthesis of ZnO NPs (Chapter 5). The green synthesized ZnO NPs were characterized with UV-Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), transmission and scanning electron microscopy (TEM and SEM), selected area electron diffraction (SAED), X-ray diffraction (XRD), and dynamic light scattering (DLS). With the above-mentioned characterization techniques, it was proven that the rarely used *P. ostreatus* produced bioactive compounds such as phenols which potentially acted as reducing and capping agents during the synthesis of ZnO NPs. For example, FTIR revealed certain vibrations and interactions that correspond to some functional groups that could potentially act as a capping and reducing agent. Hence O-H stretching vibration, C=C interactions, C=O (amide), C-H, and amine C-N stretching vibrations were observed in both the mushroom extracts and in the synthesized ZnO NPs, which shifted within the synthesized nanoparticles. The elemental composition of the mushroom synthesized nanoparticles indicated that the synthesized ZnO NPs contained Zn and O elements, as expected, with no traces of impurities. Thus, the strong signal of Zn and O regions was noted, which confirmed that *P. ostreatus* mushroom extract managed to reduce and stabilize Zn^{+} ions, hence ZnO NPs were synthesized. The TEM showed that the ZnO NPs were amorphous with dark spots which probably meant there was stacking

of nanosheets, and the SEM indicated that synthesized nanoparticles had spherical morphology with agglomeration, since ZnO nanoparticles are also prone to forming agglomeration. Interestingly, the biosynthesized ZnO NPs were observed to be very stable since a zeta potential of −23.3 mV was obtained, hence when the zeta potential is more negative than −30 mV (or more positive than +30 mV), the nanoparticles particles are regarded as stable, since they will repel each other (Fernández et al., 2016).

The *P. ostreatus* synthesized ZnO NPs proved to be advantageous in combating both the Gram-positive (*C. albicans*, *B. cereus*, *S. aureus*, and *E. faecalis*) and Gram-negative (*K. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, *E. coli*, and *M. catarrhalis*) microorganisms. This activity was slightly higher for the synthesized nanoparticles, when compared to the actual mushroom extracts, meaning that the nanoparticles enhanced the antimicrobial activity. This proves that the green synthesized ZnO NPs using *P. ostreatus* mushroom could potentially be a starting point in the search for an alternative source of antibiotics against multi-drug resistant microorganisms. The study showed that the *P. ostreatus* synthesized ZnO NPs manage to act as an antibacterial agent through the DNA cleavage mechanism, hence the bacterial DNA was observed to be damaged by the effect of ZnO NPs. Hence, nanoparticles disrupt the membrane permeability of the bacteria, which causes Zn^{2+} to intrude into the bacterial cytoplasm and damage the DNA (Murali et al., 2021). The acute cytotoxicity to both Hek293 and HepG2 cells was noted, which means the green ZnO NPs have anti-cancer potential if they could be appropriately administered, however, this nanoparticle still needs to be further studied so that its full anticancer property can be fully elucidated.

6.2 General Conclusions

The study found that *P. ostreatus* mushrooms grown from locally available substrates are safe for public consumption due to their moderate heavy metal content which is within WHO-recommended daily intake. It is worth noting that extra caution needs to be exercised to avoid health risks that could arise due to exceeding the recommended daily intake set by FAO/WHO. It could also be concluded that adding supplements (wheat bran and maize flour) to mushroom-

growing substrates not only improves mushroom yield, it also promotes the production of bioactive compounds within the mushrooms. Thus, the addition of supplements proved to influence the biological activities of the *P. ostreatus* mushrooms. The biological activities were observed to vary depending on the type and the level of supplement added to the growing substrates. It should be noted that supplements promoted mushroom yield for some substrates, however, negatively decreased biological activities on radicals' assay such as DPPH and reducing power probably due to suppressed antioxidant genes and elevated yield-related genes. The added supplements might have suppressed antioxidant genes while those responsible for yield probably became elevated. However, this phenomenon still needs to be researched further in future studies.

This research laid the basis for the establishment of new natural antibiotics, anti-cancer, and antioxidant agents, together with the establishment of a fast, safe, cost-effective, and ecofriendly method for the biosynthesis of biological active nanoparticles. Hence, better or moderate antibacterial activities were observed against multidrug-resistant bacteria such as *S. aureus*, especially for mushrooms grown on substrates with higher maize flour supplementation. The cost-effective, safe, and eco-friendly method of synthesizing the biologically active ZnO NPs using rarely used *P. ostreatus* mushrooms was established. Therefore, mushrooms can not only be produced for consumption but could also be used for other purposes such as medicinal use and nanoparticle synthesis which have medical properties as well.

6.3 Future Studies and Recommendations

It could be recommended for future studies that the natural bioactive compounds that were observed from *P. ostreatus* mushroom extracts should be extracted, purified, characterized, quantified, and profiled based on their biological activities. Thus in-vivo, studies should be further performed to establish the therapeutic potential of the natural compounds from *P. ostreatus* mushrooms grown from supplemented substrates. Therefore, the regime of multidrug-resistant bacteria and other diseases such as cancer, diabetes, etc. could be

minimized. Since the results of the study indicate potential bioactive compounds with antimicrobial activity, it should be recommended that in-silico drug-likeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis could further be explored for the observed bioactive compounds. Future studies should also focus on finding methods that are easy and cheap to activate the silent genes that are responsible for the production of natural bioactive compounds, which could also promote novel natural compounds that have various therapeutic activities.

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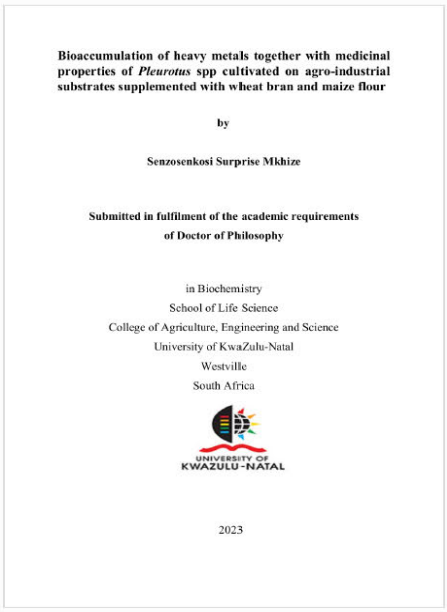

Bioaccumulation of heavy metals together with medicinal properties of *Pleurotus* spp cultivated on agro-industrial substrates supplemented with wheat bran and maize flour

by

Senzosenkosi Surprise Mkhize

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in Biochemistry
School of Life Science
College of Agriculture, Engineering and Science
University of KwaZulu-Natal
Westville
South Africa

UNIVERSITY OF KWAZULU-NATAL

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