In vitro safety and renoprotective effects of *Psilocybe natalensis* mushroom extracts in animal and human kidney cell lines

By

Mutavhatsindi Zwonaka Student no: 21007241

MSc in Life Sciences (Human Physiology)

Department of Life and Consumer Sciences

College of Agriculture and Environmental Sciences

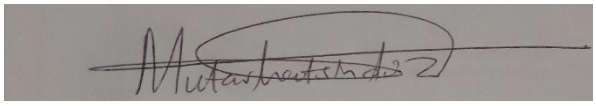
University of South Africa

Supervisor: Prof Sanah M. Nkadimeng

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Declaration

I, Mutavhatsindi Zwonaka, hereby declare that this dissertation is my own original work, except where otherwise indicated and acknowledged. It has not been submitted before for any degree or examination at this or any other university. All sources used have been properly cited and referenced in accordance with the required academic guidelines.



Signature: …….

Mutavhatsindi Zwonaka

Dedication

This dissertation is dedicated to my parents who are the silent designers of my future, whose devotion and selflessness laid the groundwork for all of my endeavours. You referred to me as "Professor" to my late father before I had ever seen a microscope. You would call and say "Professor" when I was just six years old. And if God permits, I hope to become one someday. You had a vision but no degrees. Where others saw a child drawing in the dirt, you saw a potential. I stand here today also as a living example of your legacy. Being your son makes me proud. Get plenty of rest, Dad. This degree? It is also yours too.

And to my mother, who is my first teacher, nurse, and cheerleader, who happens to provide for me by selling firewood before sunrise. Your sacrifices had ensured that I had clean shoes, a full stomach, and pocket money for school supplies. You sewed and cared for my uniform, reminding me to study hard. Your love was unconditional, and it has transformed scarcity into strength and poverty into purpose. This journey began with you, walking me to school barefoot and carrying my bag all the way. I carry your influence with me in all my endeavours.

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To my cousin, Mr. Tshishonga, thank you doesn't feel big enough. When funds ran dry, you stepped in quietly, steadily, without fanfare. You didn't ask for applause, just results. And here I am today finishing what I started because you held space for me when the world felt heavy. Your support wasn't just financial; it was just an emotional fuel. I owe you more than words can say. I promise to make you proud not just today, but for years to come.

List of abbreviations

ACEI: Angiotensin-Converting Enzyme Inhibitor

AH: cells treated with *P. natalensis* water extract 45 min after 10 μM doxorubicin.

AE: cells treated with *P. natalensis* ethanol extract 45 min after 10 μM doxorubicin.

AKI: Acute Kidney Injury

ARB: Angiotensin II Receptor Blocker

ATCC: American Type Culture Collection

BH: cells pre-treated with *P. natalensis* water extract 45 min before 10 μM doxorubicin.

BE: cells pre-treated with *P. natalensis* ethanol extract 45 min before 10 μM doxorubicin.

CKD: Chronic Kidney Disease

CLU: Clusterin

CVD: Cardiovascular Disease

CysC: Cystatin C

DMSO: Dimethyl Sulfoxide

Dox: Doxorubicin

DX-10 CONT: cells treated with 10 μM doxorubicin alone

DX-40: cells treated with 40 μM doxorubicin, positive control

E: Ethanol

eGFR: Estimated Glomerular Filtration Rate

ESA: Erythropoiesis-Stimulating Agent

ESRD: End-Stage Renal Disease

H: Hot-water extracts

HEK-293: Human Embryonic Kidney 293 cells.

HHD: Home Haemodialysis

ICHD: In-Center Haemodialysis

IL-6: Interleukin-6

IRI: Ischemia-Reperfusion Injury

KIM-1: Kidney Injury Molecule-1

KTx: Kidney Transplantation

LC_{50}: Lethal Concentration, 50%

MCC: Medicines Control Council (South Africa)

MEM: Minimal Essential Medium

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAG: N-acetyl-β-glucosaminidase

NO: Nitric Oxide

NORM: untreated, non-induced controls.

NOS: Nitric Oxide Synthase

PBS: Phosphate Buffered Saline

PD: Peritoneal Dialysis

PKD: Polycystic Kidney Disease

RAAS: Renin-Angiotensin-Aldosterone System

REC: Research Ethics Committee

ROS: Reactive Oxygen Species

RRT: Renal Replacement Therapy

SD: standard deviation

SKU: Stock Keeping Unit

SOD: Superoxide Dismutase

SSK: Sterile Substrate Kit

TAC: Total Antioxidant Capacity

TFF3: Trefoil Factor 3

TNF-α: Tumor Necrosis Factor-alpha

USRDS: United States Renal Data System

WHO: World Health Organization μg/mL:

micrograms per milliliters;

Abstract

Background: Psilocybin containing mushrooms commonly referred to as “magic mushrooms” have been used historically for their mind healing and are increasingly being investigated for therapeutic applications, including major depressive disorder and potential anti-inflammatory properties. However, their safety and potential renoprotective effects in kidney disease remain unclear. Doxorubicin, is a widely used chemotherapeutic agent known to induce nephrotoxicity through oxidative stress, increase nitric oxide production, activate pro-inflammatory pathways and apoptosis.

Aim: This study aimed to investigate the safety and morphological effects, and potential renoprotective and anti-inflammatory effects of ethanol and hot-water extracts of *Psilocybe natalensis (P. natalensis) mushroom* in an *in vitro* model of doxorubicin-induced kidney cell injury and oxidative stress on Vero monkey kidney cells and human embryonic kidney (HEK-293) cells

Methods: *Psilocybe natalensis* “magic” mushrooms were harvested, dried, ground into a fine powder and extracted using hot-water and 70% ethanol. When confluent, Vero and HEK-293 kidney cells were treated with extracts for 48 hours to assess the cytotoxicity. Doxorubicin was used as a positive-control, and untreated cells were used as control. To test renoprotective effect, confluent Vero and HEK-293 cells were induced with 10 μM doxorubicin 45 minutes before and after treatment with different concentrations of *P. natalensis* water and ethanol extracts over 24 hours. Mitochondrial activity was measured using Mosmann's tetrazolium-based colorimetric (MTT) assay to assess percentage cell viability. Tumor necrosis factor-alpha (TNF-α) levels were measured in induced human HEK-293 kidney cells using ELISA. DX-10 were control cells induced with doxorubicin (10 μM); non-induced normal cells were normal cells that were neither induced nor treated with extracts; 40-DX were positive control cells that were induced and treated with 40 μM doxorubicin. Morphology on normal and doxorubicin-induced cells was assessed using phase-contrast microscopy and 100 μM lense (100x magnification).

Results: The LC_{50} values indicated that the extracts were not cytotoxic compared to doxorubicin, with LC_{50} values below 20 µg/mL in Vero and HEK-293 kidney cells. Human HEK-293 kidney cells had lower cytotoxicity (LC_{50} > 200 µg/mL) with both water and ethanol extracts, while Vero kidney cells had LC_{50} of 199.50 ± 1.07 µg/mL with the ethanol extract. Doxorubicin inducing treatment (10 µM) significantly decreased percentage cell viability (p= 0.001) and increased nitrite oxide levels (p= 0.001) in both kidney cell types, with DX-40 significantly (p= 0.001) amplifying kidney cell injury effect even more. Hot-water and 70% ethanol extracts of *P. natalensis* enhanced %cell viability in doxorubicin-induced kidney cells in a dose-dependent manner, particularly when administered prior to doxorubicin exposure. Extracts also decreased induced-nitrite oxide levels dose-dependently, especially the 70% ethanol extract. Doxorubicin (10 µM) induced morphological changes, notably more severe in HEK-293 kidney cells. These effects of doxorubicin were not exacerbated on both kidney cells upon treatment with the mushroom extracts. Furthermore, the extracts reversed doxorubicin-induced TNF-α levels in a dose-dependent manner, with hot-water extracts showing more pronounced effects.

Conclusion: The study revealed that the hot-water and 70% ethanol extracts of *Psilocybe natalensis* mushroom were not toxic to the normal kidney cells used in the study and especially on human kidneys cells. The extracts protected against induced-kidney cell injury and did not worsen morphological alterations in kidney cells, while they also inhibited certain pro-inflammatory markers as indicated by induced-nitric oxide levels and TNF-α levels. Notably, hot-water and 70% ethanol extracts were found to be more effective, suggesting their potential for medicinal use in preventing kidney damage. Furthermore, the study aligns with previous research that has highlighted the antioxidant and anti-inflammatory properties of this psilocybin magic mushroom species, supporting its further investigation as a potential therapeutic agent for kidney function improvement in kidney disease conditions.

Key words: Psilocybin, *Psilocybe natalensis*, Doxorubicin, Kidney cell injury, Oxidative stress, Induced-Nitric Oxide, Nephrotoxicity, Renoprotective, Antioxidant, Vero cells, HEK-293 cells, Psilocybin extract.

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Chapter 1.

Introduction

1.1 Background

Kidney failure is a condition that occurs when a kidney becomes unable to filter waste product from the blood (Razmaria, 2016). Loss of renal function may result in electrolyte imbalance and disruption of homeostasis in affected individuals (Ellison and Farrar, 2018). Kidney failure is broadly classified into acute kidney injury (AKI) and chronic kidney disease (CKD). Acute kidney injury may be further dividend into prerenal, intrinsic (intrarenal), and postrenal causes (Mohsen *et al*., 2023). The treatment of chronic kidney disease (CKD) is costly, and the condition is associated with reduced quality of life as well as increased morbidity and mortality (Couser *et al*., 2011). According to the World Health Organization (WHO, 2021), approximately 1.5 million people world-wide died from endstage renal disease in 2020, representing about 2.7% of all deaths. The mortality rate associated with for end stage renal disease varies significantly by country and region, with some areas experiencing much higher rates than others (Hecking *et al*., 2014). Furthermore, CKD patients are 10 to 30 times more likely to have a risk of cardiovascular disease (CVD) than people without kidney diseases (Carmena *et al*., 2020).

Psilocybin-containing mushrooms commonly referred to as “magic mushrooms” have been used historically for their psychoactive effects (Nkadimeng *et al*., 2020). Psilocybin (4-phosphoryloxy-N, N-dimethyltryptamine), is a naturally occurring psychoactive compound found in magic mushrooms, has recently been shown to exhibit antidepressant effects (Shahar *et al*., 2024). Following ingestion, psilocybin is rapidly dephosphorylated by alkaline phosphate to form its active metabolite (Meshkat *et al*., 2025; Kraehenman *et al*., 2016). Magic mushrooms are not well-thought-out toxic in general, by means of a lethal dose of 280 mg/kg in rats and 17 mg/kg in humans (Nkadimeng *et al*., 2020; Van Amsterdam *et al*., 2011). Where there were reports of toxicity, reports showed that they were mainly used in combination with other drugs (Van Amsterdam *et al*., 2011). The mushroom effects are known to occur within 26 minutes of ingestion and to generally resolve within 4-6 hours (Kraehenman *et al*., 2015; Beck *et al*., 2025).

However, there was one incorrect clinical case report that has suggested a potential association between psilocybin mushroom and acute kidney injury after psilocybin mushroom ingestion (Austin *et al*., 2019). The case involved a fifteen-year-old male who presented with elevated creatinine and urea levels 36 hours following a consumption of what was reported to be *Psilocybe cubensis* mushrooms with his three friends. However, the other three male individuals who consumed the same mushrooms with him did not present any of these similar effects (Austin *et al*., 2019). The findings suggested that injection of the psilocybin mushrooms was clearly not the underlying reason for the

candidate's complications, in fact it suggested that the person had previously underlying kidney related diseases issues that existed before administrating the mushrooms with his friends. However, this incident, although incorrect, suggested the need for further investigation into the effects of the psilocybin containing mushroom consumption on renal function particularly in individuals where there may be pre-existing kidney-related conditions.

The kidneys play a key role in maintaining homeostasis through process such as glomerular filtration. Given this, it is important to investigate the potential effects of psilocybin containing mushrooms on kidney function (Triplitt, 2012). Kidney condition is a major global public health concern, as highlighted by the Global Kidney Health Atlas. Consequently, understanding the safety of psilocybin mushroom use in individuals with or at risk of kidney injury is important (Htay *et al*., 2018). However, the effects of psilocybin administration on kidney function, in both healthy and diseased states, remain unclear. This study therefore aimed to assess the effects and safety of psilocybin on kidney function using *in vitro* human and animal cell line models.

1.2. Problem statement

Psilocybin-containing mushrooms have gotten a lot of interest in the field of mental health and inflammation research, especially in studies looking at their possible advantages for depression and inflammatory diseases (Nkadimeng *et al*., 2020; Nkadimeng *et al*., 2021; Zeiss *et al*., 2021).

In 2019, Austin *et al*. (2019) conveyed a case report where four young men consumed what was reported as one of the *Psilocybin mushroom specie*. After consumption of the mushrooms, one of the young men experience kidney related complications. However, the other people who consumed the same mushrooms under the same conditions did not present any of these problems, indicating possible pre-existing renal injury conditions in the subject and not the mushroom specie that was consumed. This case study highlights a potential need to investigate if there could be challenges that could result from mushroom consumption in an existing renal injury condition. Currently there are no studies that have been done on the effects of psilocybin mushrooms use in an existing kidney problem condition.

Kidneys are along with the most important organs in the body, and they perform a key role in keeping and sustaining the normal and healthy functioning of the body. The maintenance of the body's internal environment's homeostasis, including control of the water, electrolyte, nitrogen, and acid-base balances, depends on the kidneys (James, and Evans, 2023.). They also regulate blood pressure (BP) and the formation of red blood cells (Ogobuiro and Tuma, 2023). As a conclusion, potential effects of psilocybin mushroom use on renal function warrant systematic investigation across various levels of analysis.

The safety and effects of the psilocybin mushrooms on normal kidney cells, function and morphology are also not fully known and need to be investigated. Moreover, molecules such as ROS and pro-inflammatory cytokines such as TNF-α and IL-6 have been implicated in many of both acute and chronic kidney related diseases (Ranneh *et al*., 2017). The effects of the psilocybin mushrooms on these parameters are not fully known especially in a kidney condition.

Safety and effects of psilocybin mushroom use in a kidney injury toxicity have never been done and not known. As a result, the study is critical and should be carried out in order to show any potential benefits and detrimental effects that may occur on the kidney following ingestion of magic mushrooms especially in pre-existing renal injury conditions. Despite growing interest in psilocybin mushrooms, their safety, mechanistic effects on renal function and the impact on oxidative stress and inflammatory biomarkers in context of pre-existing kidney injury remain unexplored.

1.3. Aims and objectives

1.3.1 Aim

The project aimed to investigate *in vitro* safety and renoprotective effects of *Psilocybe natalensis* (*P. natalensis*) extracts in animal and human kidney cell lines.

1.3. 2 Specific Objectives

The specific objectives were:

- Preparing hot-water and 70% ethanol extracts of *P. natalensis* mushroom.
- To assess the cytotoxicity and safety of *P. natalensis* mushroom extracts on normal human (HEK-293) and animal (Vero) kidney cell lines *in vitro*.
- To establish an *in vitro* model of doxorubicin-induced renal cell injury and oxidative stress in human and animal kidney cell lines.
- To evaluate the potential renoprotective effects of *P. natalensis* extracts against doxorubicin-induced cytotoxicity and morphological alterations in kidney cells.
- To investigate the effects of *P. natalensis* extracts on inflammatory and oxidative stress-related molecular markers, including TNF-α, in induced-injured human kidney cells.

1.4 Hypothesis

We hypothesized that:

- The *Psilocybe natalensis* mushroom extracts will be safe and not have cytotoxic effects on the normal kidney Vero and HEK-293 cells.
- The mushroom extracts will not exacerbate the doxorubicin-induced cell injury and oxidative stress induced nitric oxide (NO) on the treated cells compared to untreated cells.
- The mushroom extracts will decrease the doxorubicin-induced TNF-α levels compared to untreated cells.

Chapter 2

Literature Review

2.1 INTRODUCTION

More than 10% of people globally are suffering from chronic kidney disease (CKD), making it a major public health concern worldwide (Jha *et al*., 2013). In the year 2016, the unruly was rated 16th among the main causes of mortality and is projected to rise to 5th place by 2040 (Foreman *et al*., 2018). A notable burden on people, health care systems, health system infrastructure, the labour force, families, and societies, (CKD) is characterized by abnormal kidney function or structure for three months (Fraser and Blakeman., 2016). The most frequent cause of (CKD) is diabetic kidney disease (Alicic *et al*., 2017), other conditions that are prevalent comprise glomerulonephritis, autosomal dominant polycystic kidney disease, and hypertension (HTN) (Cornec-Le Gall *et al*., 2019). Kidney toxicity is an important factor in the course of renal disease, with reactive oxygen species (ROS) being a major contributor to kidney damage (Gwozdzinski *et al*., 2021). Reactive oxygen species can alleviate oxidative stress, inflammation, and cell death, resulting in kidney damage and disease (Ozbek, 2012). Serum creatinine, urea, and cystatin C, are well-known biomarkers of kidney damage and disease, indicating a reduction in kidney function (Benoit *et al.*, 2020). However, existing markers are limited, and new biomarkers are being developed to diagnose kidney disease early and more precisely (Mizdrak *et al*., 2022). Medications are often used to manage (BP), minimize proteinuria, and delay the course of kidney disease (Pugh *et al*., 2019).

Traditional therapies can be beneficial, but they have limitations, such as adverse effects and reduced efficacy in late disease stages (Qi *et al*., 2015). Traditional treatments, such as herbal cures and plant-based therapies, have been used to treat kidney condition, especially in Asian nations (Khan *et al*., 2022). Some traditional drugs have showed promise in lowering oxidative stress and inflammation (Forman and Zhang, 2021), but more research is needed to fully understand their efficacy and potential interactions with other treatments. The best chance of treating renal disease and enhancing patient outcomes may come from a multimodal approach that incorporates traditional therapy and lifestyle modifications. Since chronic inflammation is a major cause of kidney damage and the progression of the disease, it is essential to understand how inflammation affects renal pathology and therapy. Targeted pharmacological interventions are required to address the underlying mechanisms and optimize patient outcomes, furthermore, the etiology of chronic kidney disease remains unknown in a considerable proportion of affected people (Chu *et al*., 2020). Atypical urinalysis, renal tract anatomy, and estimated glomerular filtration rate (eGFR) are used to classify (CKD). Chronic kidney disease (CKD) stage 5 is the utmost severe stage and comprises affected role with end-stage

renal disease (ESRD) with an estimated glomerular filtration rate less than fifteen milliliters per minute per one point seven three square meters (Jha *et al*., 2013). Effective management of CKD requires addressing risk factors such as diabetes mellitus (DM) and hypertension (HTN) (Fraser and Blakeman., 2018). Only a handful of people with CKD progress to end-stage renal disease (ESRD), which calls for kidney transplantation (KTx) and/or renal replacement therapy (RRT) including dialysis. Dialysis can be administered either in-center (ICHD), in a hospital or satellite dialysis unit, or at home using peritoneal dialysis (PD) or home haemodialysis (HHD) (Tennankore *et al*., 2022).

Traditional therapy is a substitute for RRT for patients who are not likely to benefit from RRT in terms of survival or quality of life, or who choose not to get RRT, and it just provides a way of treating CKD symptoms (Phair *et al*., 2018). Renal replacement therapy was performed on over 2.62 million patients worldwide in 2010, and due to developments in obesity and diabetes mellitus as well as an aging population in many nations, the number is expected to quadruple by 2030 (Liyanage *et al*.,2015). The rising incidence of ESRD therapy can also be attributed to better access to ESRD services in industrialised nations (Stel *et al*., 2012). With 378 people per million of the total American population undergoing treatment for ESRD in 2016, the US has the second-highest occurrence rate of RRT globally, after Taiwan. (USRDS, 2018). Chronic kidney disease is becoming more prevalent in South Africa, and there is limited access to RRT, particularly in rural areas. According to studies, traditional remedy is recurrently employed as a supplemental or as substitute therapy for renal illness in Africa, underlining the need for more study on its efficacy and safety. The rising burden of kidney disease in Africa underlines the significance of creating effective preventive and treatment measures customized to the region's particular requirements.

Kidney illness has a multidimensional and extensive influence on the economy, families, individuals, and the healthcare (Ranasinghe, 2017). Reduced economic output, family hardship, healthcare expenses, higher mortality, and disparities and inequities are some of the main effects. (Mackenbach *et al*., 2011). In order to minimize the burden of kidney disease on people, families, and society at large, it is critical to address these effects and strive toward efficient prevention, early detection, and management measures as the illness's prevalence rises. This study's primary goal was to provide a review with an emphasis on the effects of kidney diseases in the last few decades, particularly focusing on the role of oxidative stress and inflammation in kidney disease progression and management.

2.2 Literature review

2.2.1 Kidney structure and function

The kidneys, are responsible for filtering the blood, maintain body's fluid and electrolyte stability, and controlling BP (Test, 2022). They are composed of several distinct structures, including nephrons, which are responsible for filtering and excreting waste products (Ridley, 2018), and they filter blood through a series of processes that take place within the nephrons (Balzer *et al*., 2022). Molecules such as water, electrolytes, and excess products are sieved into the Bowman's capsule, while bigger molecules such as proteins remain in the plasma (Mangan *et al*., 2018).

Nephron function proceeds from filtration to urine modification where blood enters glomerulus where plasma is filtered based on molecular size (RATE, G.F., 2023). The resulting filtrate passes through the proximal tubule which reabsorbs most of water, electrolytes, and nutrients (Zhuo and Li, 2013). A counter-current mechanism that establishes a medullary osmotic gradient in the loop of Henle that facilitates further water and electrolyte reabsorption (Capasso *et al*., 2019). The distal tubule and collecting duct make final adjustments to urine composition in response to the hormonal signals (Brunzel, 2021). These processes collectively maintain fluid volume, electrolyte balance, and acid base homeostasis. The kidneys regulate BP and erythropoiesis through secretion of renin and erythropoietin (Ellison and Ferrar, 2018). Mechanisms of drug-induced nephrotoxicity, as seen with doxorubicin, involve disruption of tubular function and oxidative stress in renal cells. Therefore, it is important to understand these cellular mechanisms in both Vero and HEK-293 kidney cells, in order to provide the physiological basis for the evaluation of cytotoxicity, morphological changes and inflammatory responses through TNF-α and NF-κB-p65, since this study aims at investigating the safety and renoprotective potential of *P. natalensis* extracts.

2.2.2 Kidney disease and injury mechanisms

Approximately 850 million individuals worldwide are affected by kidney diseases which is a huge global burden (Lv *et al*., 2019). According to Webster *et al*. (2017), the 12th largest cause of mortality in 2017 was CKD. In addition to the non-modifiable factors such as age and familial history of renal disease, modifiable lifestyle and metabolic factors can significantly elevate the risk of CKD. Obesity and smoking can contribute to the vascular and renal damage whereas diabetes and HTN remain the key causes of CKD progression because of their impact on glomerular injury and renal hemodynamic (Kazancioğlu, 2013). Kidney disease also has the significant financial impact and the yearly cost of treating renal failure is reported to be over $100 billion globally (Vanholder *et al*., 2021).

According to George *et al*. 2017) an estimated 13.9% of the African population suffers from CKD. The high incidence of risk factors includes DM, HTN, and infectious illnesses like HIV and hepatitis is primarily responsible for this burden (Upadhyay, 2022). Moreover, it is reported that the high incidence of undiagnosed and untreated renal disease in the area is partly the result the of limited access to healthcare and diagnostic services. In addition, the expense of treating kidney disease is frequently out of reach for many Africans especially those who are in low-income countries (Rayner *et al*., 2023).

In South Africa, kidney illness is also a serious public health concern which affects an estimated 10% of the population (Jha *et al*., 2013). While South Africans have greater access to healthcare and diagnostic services than many African nations, the expense of kidney disease treatment remains a substantial barrier for many patients, particularly those living in the rural regions or on low incomes countries (Liyanage *et al*., 2015). To reduce the burden of kidney disease in South Africa, a diversified strategies are required that include preventative measures, early identification and treatment and increased access to affordable healthcare services.

There are few well known diseases such as the AKI which is the medical term for a sudden and severe decline in kidney function that is brought on by a reduction in the blood supply to the kidneys or injury to the kidneys themselves (Yokota *et al*., 2018). Acute kidney injury can result in potentially fatal complications like uremia, electrolyte imbalances, and fluid overload (Di Lullo *et al.,* 2019). Nephrotic syndrome is another disease that is characterized by the damage to the glomeruli, the microscopic blood arteries in the kidneys in charge of filtering the blood, which then results in a number of symptoms that are known as nephrotic syndrome (Lionaki *et al*., 2019). Proteinuria, edema, and hyperlipidemia are some of the symptoms that are associated with nephrotic syndrome (Murray and Paolin, 2020). Kidney cysts arise as a consequence of polycystic kidney disease (PKD) which is a genetic disorder that can lead to high BP, kidney stones, and also the kidney cancer risks. Renal cell carcinoma is the most prevalent figure of kidney cancer which can manifest with signs that include blood in the urine, side or back discomfort, and weight loss (Bahadoram *et al*., 2022).

As indicated before, kidneys can play a key part in the body by filtering the blood, eradicating waste products and surplus fluid, and ensuring that the body's fluid and electrolyte levels are balanced at all times (Klahr, 2013). However, some diseases can harm the kidneys, decreasing their function which may lead to life-threatening effects (Raimundo and lopes, 2011). According to Zicarelli *et al*. (2021), CKD is a medical disorder that causes a progressive decrease of renal function over time. As a result, toxins and waste materials can accumulate in the blood as a result of this illness, which can then lead to a numerous number of problems (Dehghani *et al*., 2021). Changes in the electrolyte balance, fluid overload and HTN can all be consequences of chronic renal disease (khan *et al*., 2016).

These genetic mutations can lead to cellular effects such as oxidative stress and inflammation which can damage cells and tissues throughout the body (Chatterjee, 2016). Over time, chronic kidney disease can also lead to risk of cardiovascular condition and bone condition (Liu *et al*., 2014). Management of CKD is aimed at controlling complications and slowing down the disease progression, often through medications and lifestyle changes (Kalantar *et al*., 2021). The kidney is a significant endocrine organ, even though its job is to remove waste from the circulation. Furthermore, the kidney produces five essential hormones, enzymes, including erythropoietin, renin, Klotho, kallikrein, and 1, 25-dihydroxyvitamin D3 and these essential hormones are negatively affected in CKD (Haussler *et al*., 2016).

2.3 Oxidative Stress in kidney pathology

Oxidative stress is a contributor to the progression of CKD and complications. Reactive oxygen species overwhelm intrinsic antioxidants that leads to the oxidative damage, lipids, proteins, and DNA (Ozougwu, 2016). Both enzymatic and non-enzymatic antioxidants comprise the total antioxidant capacity and function to restore redox balance by neutralizing excess ROS (Gupta *et al*., 2021). The sustained oxidative stress triggers apoptosis in renal tissue when the balance fails it also contributes to the decline in glomerular filtration, HTN, diabetes-related nephropathy and other systematic complications such as atherosclerosis (Ogura and Shimosawa, 2014). The Elevated oxidative stress is associated with worse outcomes in patients with CKD, including cardiovascular disease and mortality (Shoji *et al*., 2012). Because of these interventions that target oxidative stress that are of interest in haemodialysis patients when low levels of ROS such as hydrogen peroxide and superoxide anion act as signaling molecule to maintain cellular homeostasis, excessive ROS production is pathological and drives renal injury (Antic *et al*., 2019).

Oxidative stress also impairs renal excretory function and ROS production in the kidney, actions which may also affect renal regulatory systems and change the water-electrolyte and acid-base balance (Fularski *et al*., 2024). Oxidative stress promotes tubulointerstitial fibrosis, podocyte death, and proteinuria (Frąk *et al*., 2024). Kidney damage is mediated by oxidative stress that is regulated by the Nrf2/Keap1 system, increased NOX generation, and an imbalance in autophagy signaling (Jia *et al*., 2024). The progression of CKD is significantly influenced by oxidative stress, which is also associated with endothelial dysfunction (Ebert *et al*., 2021). Through nitric oxide synthase (NOS), decreased renal afferent arteriole autoregulation, and elevated perfusion pressure, NO plays a significant role in kidney injury (Carlström, 2021). Ischemia/reperfusion injury induces tubular epithelial cell death that leads to loss of tubular integrity and impairment of reabsorption and secretion, and these structural and functional disruption contribute to acute kidney injury (Lasorsa *et al*., 2024). The development and course of AKI may be impacted by abnormal apoptosis and endoplasmic reticulum stress (Cheng *et al.,* 2024).

2.3.1 Oxidative Stress in Ischemia-Reperfusion Injury

Ischemia/reperfusion injury (IRI) is a frequent unfavourable issue during transplant procedures (Dery *et al*., 2023). It is distinguished by structural and functional alterations that take place in both stages (Robichaux *et al*., 2021). Ischemia/reperfusion injury involves several molecular pathways, with oxidative stress and ROS production as key contributors (Tejchman *et al*., 2021). Hypoxic parenchymal and endothelial cells produce more adhesion molecules and cytokines in response to ischemic damage, which causes systemic inflammation (Liu *et al*., 2013). Experimental generation of ROS mimics the IRI response, ROS scavengers guard against IRI, and post-ischemic tissues exhibit increased ROS production and its byproducts (Hofmann *et al*., 2022). Oxidative stress caused by IRI arises from non-enzymatic sources that includes hemoglobin and myoglobin, as well as enzymatic or enzyme-related activities (Tejchman *et al*., 2021). Reoxygenation activates neutrophils, CD4+ T cells, and platelets, resulting in enhanced ROS generation and mitochondrial damage (Ansari *et al*., 2018). Ischemia/reperfusion injury is a prominent cause of AKI in KTx, and organs with broader criteria are more vulnerable (Greite *et al*., 2020). The reactive oxygen species (ROS) overwhelms renal antioxidant defenses that leads to tubular kidney cell injury and impaired function and this mechanistic link between oxidative stress and post-transplant kidney cell injury formulates the rationality for this study that investigates renoprotective effects of *P. natalensis* extracts in animal and human kidney cell lines.

2.3.2 Oxidative Stress and Inflammation

Chronic inflammation raises levels of malondialdehyde, nitrite, and lipid peroxidation products (Ito *et al*., 2019). Elevated production of ROS cause oxidative stress and can upregulate expression of proinflammatory cytokine expression (Chatterjee, 2016). Genes implicated in inflammatory pathways exhibit altered expression due to activation of transcription factors by oxidative stress (Li and Chang, 2021). Proteins, lipids, and DNA/RNA are the primary targets of oxidative stress (Seixas *et al*., 2022). Reactive oxygen species in biological tissues have been shown to cause oxidative damage to biochemical constituents such as lipids, proteins, carbohydrates, and nucleic acids (Juan *et al*., 2021). This oxidative damage contributes to renal injury and the progression of CKD (Yan *et al*., 2021).

2.4 Biomarkers of renal injury relevant to *in vitro* models

Numerous renal biomarkers have been identified and measured as part of medication therapy for the treatment of various kidney-induced disorders (Xie *et al*., 2013). One of them is a delicate and precise damage marker of proximal tubule epithelial cells, kidney injury molecule-1 (KIM-1), is a sensitive and specific biomarker of proximal tubular

epithelial cell injury, which is shed into the tubular lumen in response to renal damage (Xie *et al*., 2013; Lim *et al., 2013).* Another one is of the urine markers is Trefoil factor 3 (TFF3) which is a short peptide hormone that is thought to be a sensitive markers of AKI (Yu *et al*. 2010). The other one is Urine B2M which is also recognized as marker of proximal tubular disfunction in drug-induced nephrotoxicity and it has been used as a marker of glomerular injury in rodent studies (Obert *et al*., 2021).

Cystatin C (CysC) is another biomarker that presents good sensitivity and specificity to monitoring acute and chronic renal deficits (Obert *et al*., 2021). It is considered to reliable marker of early-stage diabetic nephropathy and an effective diagnostic tool for the preclinical kidney disease (Barutta *et al*., 2021). Clusterin (CLU) levels are considerably higher in dedifferentiated tubular cells during AKI, as well as in PKD (Kavitha *et al.*, 2022). This marker is a potential predictor of tubulointerstitial renal lesions and end-stage kidney disease (ESKD) (Tan *et al*., 2016). N-acetyl-β-glucosaminidase (NAG), is a biomarker of proximal tubular damage that is persistent, sensitive, quantitative, and robust (Novak *et al*., 2023). It has been linked to an increase in kidney disorders such AKI, diabetic nephropathy, and chronic glomerular diseases (Kalantar-Zadeh *et al*., 2021).

2.5 5. Current treatments and limitations

Currently multiple approaches for classifying and diagnosing kidney disease are being summarized in Table 1. Medications such as Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptors blockers (ARBs) are frequently used to treat CKD (Shen *et al*., 2021). In CKD renin-angiotensin-aldosterone system (RAAS) overactivation promotes glomerular HTN, proteinuria, and renal fibrosis through angiotensin II-mediated vasoconstriction and proinflammatory signaling and when RAAS blockade slows CKD progression by attenuating these effects, and reduces albuminuria (Patel *et al*., 2017). They have been confirmed to lessen proteinuria, BP, and inflammation, which reduce the course of CKD (Impellizzeri *et al*., 2014). Enalapril, ramipril and lisinopril are a few instances of ACEIs, whereas losartan, valsartan, and irbesartan are examples of ARBs (Sturdivant and Allen-Thomas, 2023).

A group of drugs known as diuretics are known to increase urine production which then reduces fluid retention and edema (Bell and Mandalia, 2022). They are frequently employed in the management of fluid overload and HTN in the treatment of CKD (Shin and Lee, 2021). Diuretics such as spironolactone, hydrochlorothiazide, furosemide and hydrochlorothiazide (Arumugham, and Shahin, 2023). Erythropoiesis-Stimulating Agents (ESAs) are drugs that can encourage the body to produce more red blood cells, and they are frequently used to treat anaemia linked to CKD (Kidanewold *et al*., 2021). Epoetin alfa and darbepoetin alfa are two examples of ESAs (Portolés *et al*., 2021). Phosphate binders are drugs that are given to CKD patients to stop them from absorbing phosphate from their diet (Ogata *et al*., 2022). This is because phosphate build-up in the blood can contribute to bone disease and cardiovascular disease. Calcium carbonate, lanthanum

and sevelamer, lanthanum carbonate is type of phosphate binders (Nain *et al*., 2023). As a result, the management that are underlying the illness in kidney diseases and its consequences requires a comprehensive and varied approach (Mason and Bakus, 2010).

Additionally, research has shown that targeting ROS with antioxidants and plant-based therapies may be beneficial in reducing kidney disease progression (Daenen *et al.,* 2019; Sharma *et al*., 2023). Plant-based compounds such as curcumin and resveratrol as well as herbs like *Astragalus membranaceus* and *Cordyceps sinensis* have traditionally been used to reduce oxidative stress and inflammation that is associated with kidney disease (Akbari *et al*., 2022). Pre-clinical studies have demonstrated the potential benefits of plant-based therapies in kidney disease such as reducing urinary protein excretion and improving kidney microstructure in rats and these findings are limited to animal model studies, and the differences in metabolism, dosing, and disease heterogeneity mean that efficacy and safety in humans remain to be stablished in future studies (Khan *et al*., 2022). Additionally, plant-based therapies have been shown to reduce inflammatory biomarkers such as TNF-α and IL-1β, which may contribute to reduced inflammation in kidney disease (Elliott *et al*., 2022). Traditional medicine including herbal remedies and polyherbal formulations has also been used to combat kidney failure, particularly in Asian countries and numerous formulations have shown to reduce proteinuria and slow CKD progression in randomized controlled trials and large cohort studies in Taiwan (Jha, 2010).

Clinical trials on plant-based therapies for kidney disease are limited, but some studies have shown promising results and in randomized controlled trials and large cohort studies from China and Taiwan, Chinese herbal formulation such as Astragalus-based preparations has reduced 24-hour urinary protein excretion by 15-30% and slowed the decline in estimated glomerular filtration rate (eGFR) when added to standard care in patients with diabetic kidney disease (Khan *et al*., 2022). Combination therapy with the plant extracts and conventional therapies have been found to confer additive or synergistic Reno protective effects in affected person with CKD (Mohany *et al*., 2021). Additionally, some clinical trials have shown that plant-based therapies can reduce proteinuria which is a key indicator of kidney damage (Khan *et al*., 2022). Supplementary scientific examinations are desired to confirm the efficiency and welfare of plant-based therapies for kidney disease (Carrero *et al*., 2020).

Table 2.1: CKD Treatment Modalities: Dialysis, Medications, Traditional medicine, kidney transplantation, and Lifestyle Interventions

Treatment Method	Percentages of Cases
Kidney transplantation (KTx)	5-10% (of ESRD patients) (de Jong *et al*., 2022).
Traditional medicine	30-50% (in African nations) (Ozioma and Chinwe, 2019).
Life style modification	70-80% (of CKD patients) (Ojangba *et al.,* 2023).
Medications (e.g. Blood pressure management	49-60% (of CKD patients) (Judd and Calhoun, 2015).
Dialysis (RRT)	24% worldwide in 2010 (Caskey *et al*., 2011).

The use of traditional medicine in management of renal is a complex and requires further research (Jha, 2010). Although some traditional medications have shown potential benefits such as reducing oxidative stress or inflammation (Jin and Kang, 2024), it is also important to note that others have been shown to be nephrotoxic (Alobaidi, 2024). Consequently, studies in toxicity still remain essential in the discovery and development of herbal remedies, as ensuring safety is critical when targeting improvements in renal function, reductions in proteinuria and slowing down the CKD progression (Sharma *et al*., 2023). Improvement in the standardization of herbal remedies is essential (Wu *et al*., 2024). As a result, potential interactions with conventional medications may then be possible (Sherani *et al*., 2024). However, consideration of risks and benefits is important to ensure the safe use of traditional therapies in kidney disease.

2.5.1 Impact of Kidney Treatment on Patients

Kidney disease places a substantial burden on patients that promote growing emphasis on early detection and timely intervention (Kalantar-Zadeh *et al*., 2021) According to a 2024 review by George and Kengne, the developments in diagnosis, treatment, and prognosis of CKD have improved patient outcomes. However, CKD still remains a key public health concern, primarily in sub-Saharan Africa, where the prevalence is rising due to the factors such as hypertension, diabetes, and HIV/AIDS (Stanifer *et al.,* 2014). Research has also shown that sleep disorders are common in affected role with end stage renal disease and are associated with augmented mortality (Mixson *et al*., 2023). Furthermore, studies have explored the role of genetics and the gut microbiome in CKD progression, highlighting the complex interplay between various dynamics in the maturity and progression of the kidney disease (Wing *et al*., 2016).

Chronic kidney disease (CKD) has improved in the long-term survival since there are advances in paediatric care, intensive regimes such as dialysis and frequent clinic visits impose substantial psychological, emotional, and financial burden on families (Adamczuk and Roszkowska-Blaim, 2017; Viana *et al*., 2013). Paediatric nephrologists are expanding the care that include psychosocial support for patients and caregivers (Beach *et al*., 2021). This demonstrates the need for treatment strategies that target renal pathology but also mitigate treatment that are related to stress through integrated approaches such as psychosocial interventions, caregiver support programs, and less the burden for dialysis.

2.6 Psilocybin and its Therapeutic potential

Psilocybin is a naturally occurring hallucinogen that is found in *P. natalensis* mushrooms and it has been used in traditional medicine and spiritual practices for centuries particularly in indigenous communities (Mazdeh, 2024). Psilocybin is rapidly converted to psilocin after ingestion which normally acts on 5-HT2A receptors to alter mood, perception, and cognition that often produce profound subjective experiences (Spiers et al., 2024). Recent research is currently examining its therapeutic potential for treatment resistant depression and other mental health conditions. The native species *P. natalensis* has drawn interest for its psilocybin content in South Africa and potential pharmacological profile, though information on safety and efficacy remain limited (Maloka et al., 2022). While evidence that links psilocybin to kidney disease is scarce. Therefore, understanding renal clearance and potential nephrotoxicity is relevant for trials that involve patients with impaired renal function. There is no information currently on the safety and use of psilocybin mushrooms on kidney and existing kidney diseases, and investigating these effects will be beneficial.

2.7 Conclusion

Inflammation and oxidative stress are drivers of acute and chronic kidney disease, that contribute to tubular injury, fibrosis, and progression to ESRD. Doxorubicin induces renal damage through ROS generation and upregulation of pro-inflammatory cytokines such as TNF-α and IL-6, that mirrors mechanisms that are seen in drug-induced AKI. Current pharmacologic strategies that include ACEIs, ARBs, and antioxidants, show limited efficacy in preventing nephrotoxicity and carry adverse effects that restrict long-term use. Natural products with dual anti-inflammatory and antioxidant properties represent an alternative. While *P. natalensis* species are being studied for psychoactive compounds and emerging phytochemical analyses reveal polysaccharides, phenolics, and ergothioneine with demonstrated antioxidant and cytoprotective effects *in vitro* studies. However, no single study has evaluated *P. natalensis* extracts for their renoprotective activity, nor examined their effects on doxorubicin-induced renal cell injury in human HEK293 or Vero kidney cell models. Therefore, the study investigates *P. natalensis* hot-

water and 70% ethanol extracts for cytotoxicity and protective efficacy against doxorubicin induced oxidative stress and inflammation in Vero and HEK-293 kidney cells and establishing the safety and renoprotective potential of *P. natalensis* extracts will address the gap in natural product interventions for drug-induced nephrotoxicity.

Chapter 3

Materials and Methods

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3.1.1 Ethical and Regulatory Approval

The University of South Africa Health Research Ethics Committee authorized the research project's protocol, which was given the ethics approved number (2024/CAES_HREC/2655).

The Medical Control Council (MCC) and South Africa Health products Regulatory Authority (SAHPRA) from the South African Department of Health authorized the research project and granted permission license number POS 156/2024/2025 since psilocybin mushrooms are categorized as a schedule 7 substance in the country.

3.1.2 Mushroom growth and Extracts Formation

The Spore Spot Company, located in Durban, South Africa, identified and verified a growing uncontaminated substrate kit (SSK-2) and a spore print syringe of *Psilocybe natalensis* (*P. natalensis*) mushroom, also referred to as "Natal super strength" with SKU number NSS-1.



Figure 3.1: *Psilocybe natalensis* mushrooms in growth chamber.

Mushroom spores were inoculated onto sterile substrate in a triple-secured containment facility immediately upon arrival according to Nkadimeng at al (2020) method. Mycelium was transferred into a sterile monotub, as soon as it had colonized around 60% of the

substrate. The temperature was controlled by an air conditioner, and the humidity was supplied by a humidifier. The mushrooms were harvested and dried in an open oven at 35 to 36 °C after they had developed and produced fruit. Cover a day or two. Using a grinder, the dried mushrooms were ground into a fine powder. Extracts were prepared by dissolving 5 g of dry powder in a small sterile beaker with 50 mL of boiling distilled water, cold water, and 70% ethanol.



Figure 3.2: *Psilocybe natalensis* mushrooms, hot-water and 70% ethanol extracts.

The mixture was stirred for 5 minutes and then allowed to stand for 24 hours. Following 24 hours, the mixtures were filtered into small vials no. 6 (Lasec, Johannesburg, South Africa), weighed, and dried in an open oven at 30°C until required. The extracts' yield was measured, and extracts were stored in a dark until required.

3.2 Kidney Cells Culturing and Treatment with Extracts (Water and Ethanol)

3.2.1 Cell culture conditions

The HEK-293 and Vero kidney cell lines were obtained from Cellonex, Separation Scientific SA (Pty) Ltd and cultured using Dulbecco's modified Eagle's medium (DMEM) (Pan Biotech, Separations Scientific) complemented with 10% fetal bovine serum (Biocom Africa, South Africa) and 1% penicillin (100 units/mL) and streptomycin (100 µg/mL) (Separation Scientific, South Africa) at 37°C in an atmosphere with 5% CO_2 (HERAcell 150, Thermo Electron Corp., Separations Scientific, Johannesburg, South Africa). Before being employed in the tests, the cells were allowed to grown to approximately 80% confluence.

3.2.2 Cytotoxicity determination of *P. natalensis* extracts on the Vero and HEK-293 cells

The viability of the cells was assessed using a modified version of Mosmann's tetrazolium-based colorimetric (MTT) assay as described by Nkadimeng *et al*., (2020), Cells were separated at confluence, centrifuged for 5 minutes at 200× g, and then resuspended in growth medium to 1 × 10^4 cells/mL in each well of columns 2 through 12 of a sterile 96-well microtitre plate. For the assay, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS and 1% penicillium streptomycin. Cells were incubated in a 5% CO_2 incubator; the plates were incubated for 24 hours at 37°C. Following the removal of DMEM from the cells, 200 µL of extract in a predefined concentration range was reconstituted. The test extracts were serially diluted in DMEM. Extracts were cultivated on microtiter plates for 48 hours at 37°C in an incubator with 5% CO_2. Untreated cells (negative control) and positive control (doxorubicin chloride; Merck, Johannesburg, South Africa) were included.

Subsequent incubation, media was removed and 200 µL of phosphate buffered saline (PBS) (Whitehead Scientific, Johannesburg, South Africa) was used to rinse the cells and aspirate the medium (both with and without treatment). MTT was added (Inqaba Biotec, Pretoria, South Africa; stock solution of 5 mg/mL in PBS) to each well after adding 100 µL of medium, and then incubated for an additional 4 hours at 37 °C. To prevent disturbing the MTT crystals, the medium in each well was carefully removed after the MTT was incubated. 50 µL of Dimethyl sulfoxide (DMSO) was applied to each well in order to dissolve the MTT formazan crystals. MTT crystals were dissolved by gently agitating the plates. Absorbance was measured at 540 nm and 630 nm using a microplate reader (Thermo Scientific). The difference in absorbance, reflecting MTT formation, was used as an indicator of cell viability. The plate reader was blanked using the wells in column 1 that contained medium and MTT but no cells. Cell viability was expressed as a percentage using the formula % Viability = ((Sample Absorbance/control Absorbance) × 100). The studies were repeated at different times and in technical triplicate. The concentration of test extract causing 50% inhibition of cell viability relative to untreated control cells was defined as the lethal concentration 50 (LC_{50}). Dose-response data were analyzed by nonlinear regression. LC_{50} values were calculated from the fitted curve and reported as mean ± standard deviation.

3.2.3 Determining the effects of the extracts (both water and ethanol) in doxorubicin-induced HEK-293 and Vero kidney cells

Table 3.2: Experimental Groups for Doxorubicin-Induced Cytotoxicity Assay

Group Name	Cell Type	Extract Treatment	Purpose
NON-DX Normal	HEK-293 & Vero	None	Negative control. No doxorubicin, No treatment
DX-10 Control	HEK-293 & Vero	None	Injury control. Induced, untreated
DX-40 Positive Control	HEK-293 & Vero	None	High toxicity control. Confirms assay sensitivity
Extract treatment post-and pre-doxorubicin induced	HEK-293 & Vero	*P. natalensis* extracts at varying concentrations, added before doxorubicin	Test protective effect

In vitro nephrotoxicity was induced by exposing cells to 10 µM doxorubicin. This concentration shown that 10 µM produced significant reduction in cell viability in both HEK-293 and Vero cells without causing complete cytotoxicity. This level of injury has provided a reproducible model for evaluating the cytoprotective effects of testing extracts When confluent, the human and animal kidney cells were seeded 10 000 cells per 96 well and 100 000 cells per 6-well tissue culture treated plates and incubated in a 5% CO_2 incubator, the plates were incubated for 24 hours at 37°C. After 24 h of incubation, cells were treated with 10, 25, 50, and 75 µg/mL of 70% ethanol and water extracts of *P.*

natalensis mushrooms before and after a 45 min induction with 10 µM doxorubicin at 37°C.

3.2.3.1 Determining the effects of extracts (both water and ethanol) on doxorubicin-induced nitrite levels in the HEK293 and Vero kidney cells

From the above treated cells in 6-well plates, nitrite concentration in cell culture supernatant was quantified as an indicator of NO generation based on the Griess reaction (Sigma-Aldrich, Johannesburg, South Africa) after 24 hours of treatments. In short, 100 µL of Griess reagent was mixed with 100 µL of cell culture supernatant, incubated for 15 minutes, and the absorbance was assessed at 540 nm. The serially diluted standard curve was used to calculate the nitrite concentration. Measurement of nitrite was included to assess doxorubicin-induced nitrosative stress and inflammatory activation, as elevated NO production via inducible NOS is a downstream effect of NF-κB signaling in renal cell injury. Changes in nitrite levels were used to evaluate the potential suppression of induced inflammatory related -nitrite levels l of *P. natalensis* mushroom extracts treatment.

3.2.3.2 Determining effects of extracts on doxorubicin induced inflammatory cytokine (TNF- α) in human HEK293 kidney cells

The inflammatory mediator TNF-α was measured on the HEK-293 kidney cells, as this human kidney cell line was used to assess human-relevant inflammatory responses. The measurement was done using the enzyme-linked immunosorbent assay (ELISA). In order to do this, the 200 000 HEK-293 kidney cells were seeded into 25 cm tissue-cultured small tissue cultured flasks and incubated for 24 hours as it was previously described above. After removing the culture medium, cells were pre-treated with varying amounts of *P. natalensis* water and ethanol extracts for 45 minutes. Doxorubicin was then added to a final concentration of 10 µM, and cells were incubated for an additional 24 h. The human HEK-293 kidney cells were then subjected to the enzyme-linked immunosorbent assay (ELISA) after the medium was taken out. In order to facilitate antigen binding, media were then added to each well of the 96-well microplate that were coated with human TNFα, and the plates were then incubated for 90 minutes at 37ºC. Following incubation, the liquid was disposed away and each well received the 100 µL of biotinylated detection antibody working solution. The wells were then left in the dark at 37 ºC for 60 minutes in order to develop color as per manufacturer's instructions. 50 µL of stop solution was used to halt the reaction. A microplate reader was used to read the absorbance right away at 450 nm. By building up a standard curve from the absorbance values of the standards and interpolating sample absorbances, the analyte concentration was ascertained. The reader

was zeroed using the blank wells. The effects of *Psilocybe natalensis* extracts on inflammatory markers in the HEK-293 kidney cells were then evaluated to the assay's inclusion of negative (untreated cells) and positive (doxorubicin-treated) controls. Each independent experiment was repeated three times on different days and data was presented as mean ± SD from the independent experiments

3.3 Morphological analysis of HEK-293 and Vero kidney cells

Morphological analysis was performed using an inverted phase-contrast microscope that is equipped with a total magnification of 100 µM lense. For each treatment in 6-well plates, three non-overlapping fields per well were imaged for both Vero and HEK-293 kidney cells, and results were compared to negative controls (untreated kidney cells) and kidney cell injury controls (10 µM doxorubicin-treated kidney cells) as described in section 3.2.3. The assessment focused on kidney cell rounding, detachment, membrane blebbing, cytoplasmic granulation, and changes in monolayer confluence as indicators of cytotoxicity and stress. Observations were captured using a microscopic camera.

3.4 Statistical analysis on HEK-293 and Vero kidney cells

A linear regression analysis of the concentration-response curve displaying the percentage of viability versus the sample concentration of two independent experiments was used to identify the LC_{50} of the samples and the positive control doxorubicin. The findings were presented as mean ± standard deviations, and pairwise multiple comparison procedures using the Holm-Sidak method and one-way ANOVA analysis of variance by means of an interactive statistical program (Sigmastat, SPSS version 26, San Jose, CA, USA) were used to compare statistically significant values. Shapiro-Wilk was used for normality testing, while Brown-Forsythe was used for equal variance testing. A $p<0.050$ was deemed statistically significant.

Chapter 4

Results

4.1 Cytotoxicity effects of *Psilocybe natalensis* extracts on Vero cells and HEK-293 kidney cells

The LC_{50} values for *Psilocybe natalensis* extracts on both the Vero kidney cells and HEK293 cells are shown in Table 4.1. In the Vero monkey kidney cells, the hot-water extract showed lower LC_{50} values when compared to the 70% ethanol extracts which were 200 µg/mL. Effects of the extracts on the HEK-293 kidney cells were non-toxic with LC_{50} values that were above 200 µg/mL for both 70% ethanol extract and hot-water extract. Doxorubicin as a positive control used in this study, exhibited toxicity with LC_{50} values that was lower than 20 µg/mL in both the Vero and HEK-293 kidney cells.

Table 4.1: Cytotoxicity effects of *Psilocybe natalensis* extracts (LC_{50} µg/mL) on normal Vero and HEK-293 kidney cells

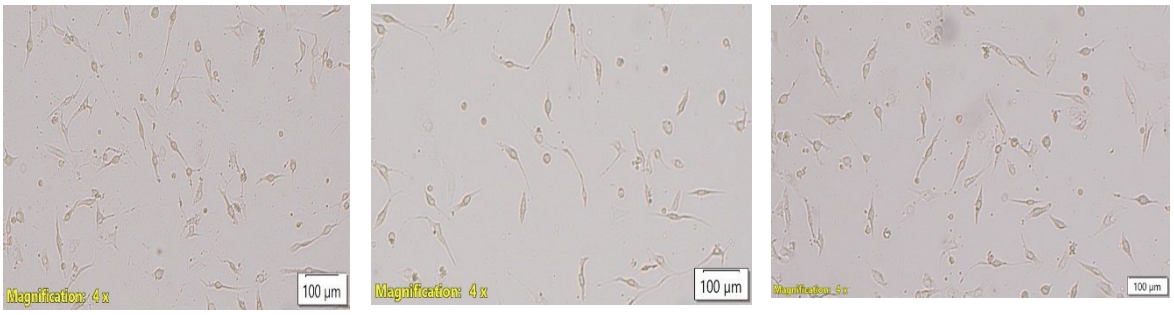
Samples	**Vero kidney cells LC_{50} (µg/mL)**	**HEK-293 cells LC_{50} (µg/mL)**
Hot-Water	53,1±1,53	251,65±4,58
70% Ethanol	199,50±1,07	205,90±8,99
Doxorubicin	11,04±1,33	19,70±1,24

4.2 Morphological effects of *Psilocybe natalensis* extracts on both Vero and HEK-293 kidney cells

Morphological results in Figure 4.1 showed that both the extracts of the 70% ethanol and water of *P. natalensis* did not induce any morphological changes compared to the control cells.

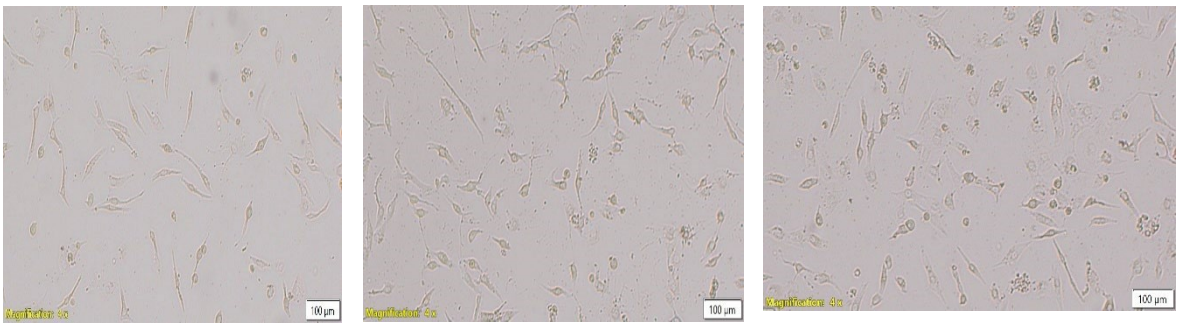
70% ethanol and hot-water treatment on Vero monkey kidney cells Ethanol

treatment

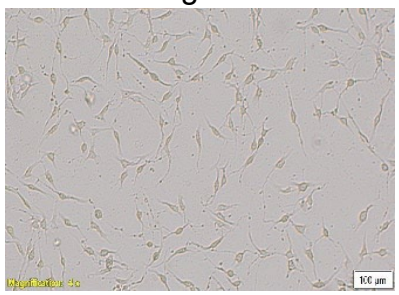


25 ug/mL 50 ug/mL 100 ug/mL **Water**

treatment



25 ug/mL 50 ug/mL 100 ug/mL



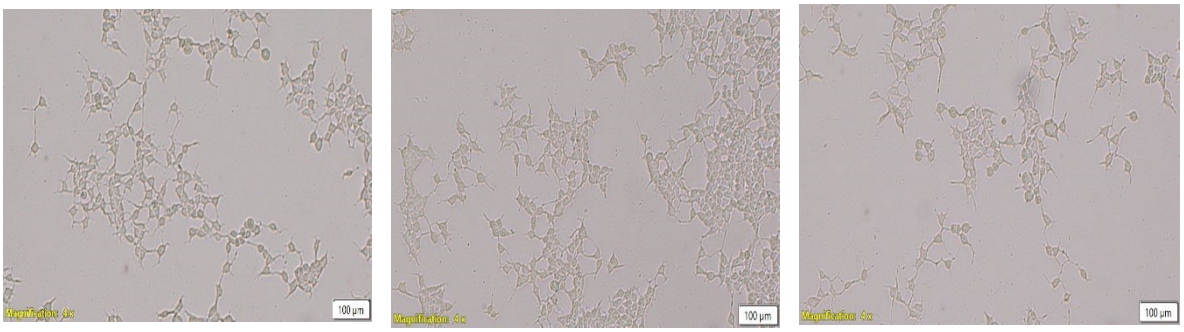
Control (non-treated)

Figure 4.1: Effect of *P. natalensis* water and 70% ethanol extracts on Vero kidney cell viability. Vero kidney cells treated with 25, 50, and 100 µg/mL of *P. natalensis* extracts. Control = untreated cells.

The results in Figure 4.2 have displayed non-altering effects of *P. natalensis* extracts on the morphological structure of the treated HEK-293 kidney cells when compared with the non-treated control on Hek-293 kidney cells.

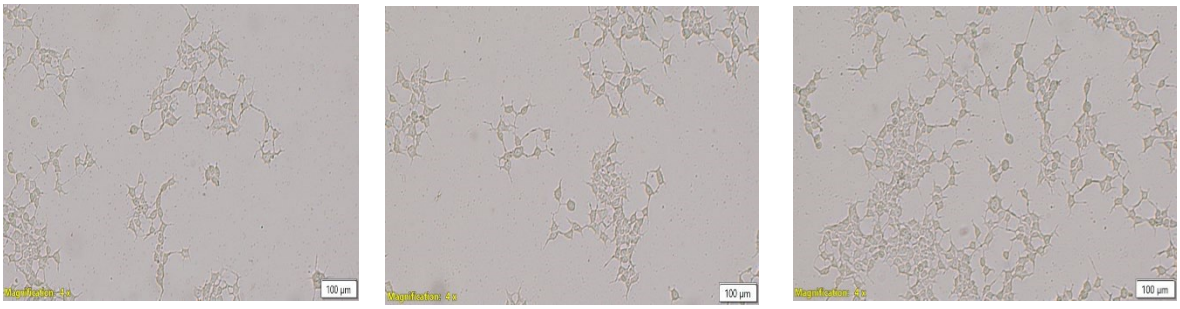
70% Ethanol and Water treatment on HEK-293 kidney cells Ethanol

treatment

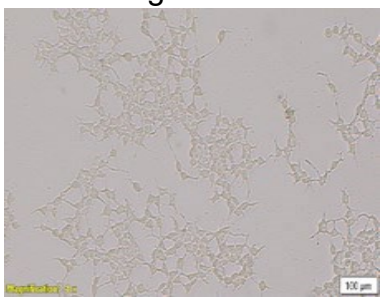


25 ug/mL 50 ug/mL 100 ug/mL

Water treatment



25 ug/mL 50 ug/mL 100 ug/mL

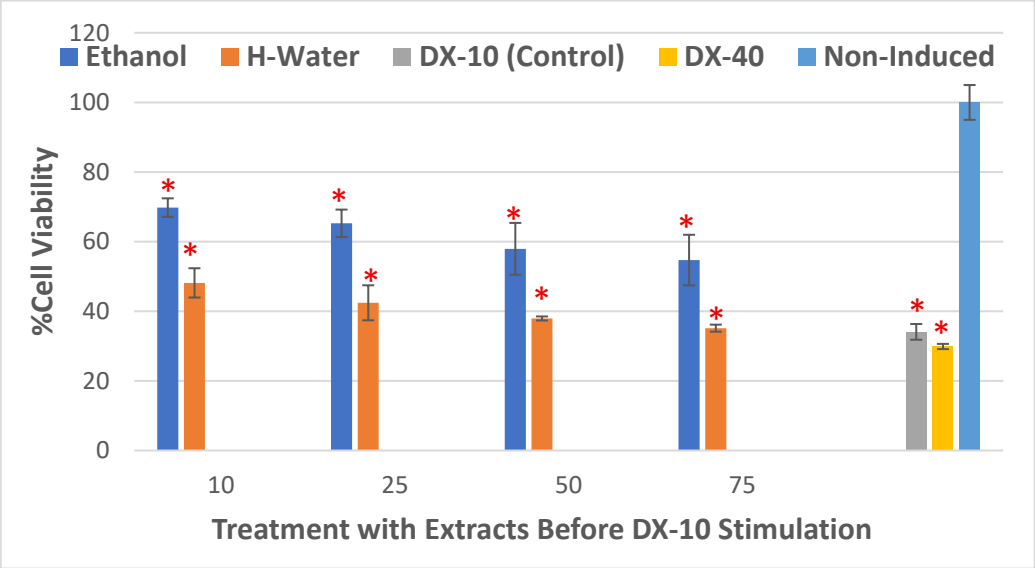


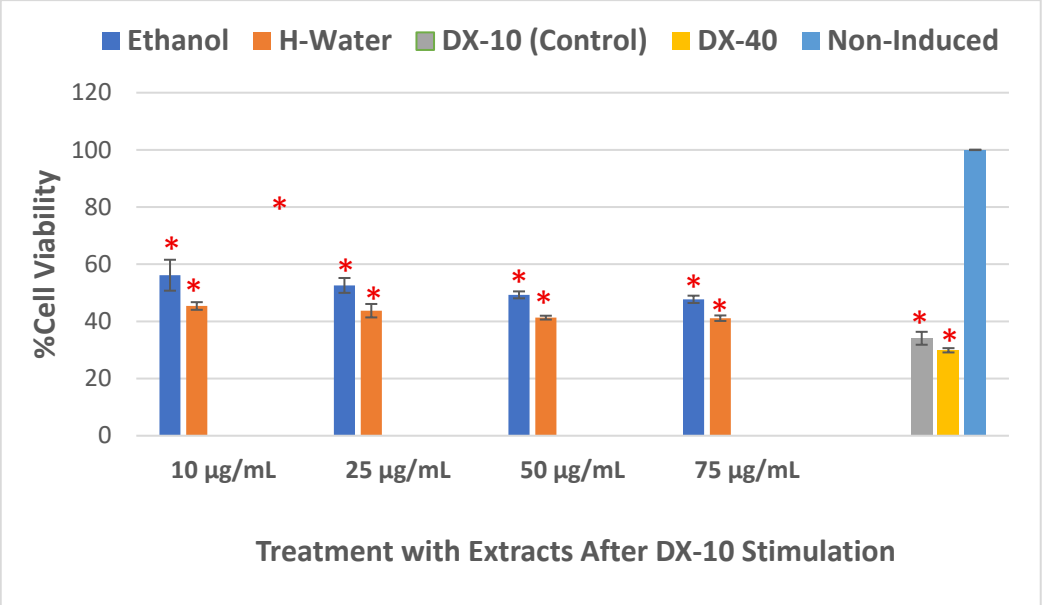
Control (non-treated)

Figure 4.2: Effects of *P. natalensis* extracts on HEK-293 kidney cells. HEK-293 cells were treated with 25, 50, and 100 µg/mL of *P. natalensis* water extract and 70% ethanol extract. Control cells were untreated.

4.3 Protective effect of *Psilocybe natalensis* extracts against doxorubicin-induced cytotoxicity in Vero and HEK-293 kidney cells

The results in Figure 4.3 shows that the treatment with 10 μM doxorubicin has significantly reduced % cell viability in Vero kidney cells (DX-10) compared to untreated controls (p= 0.001), indicating induction of cell death. The DX-40 which were used as positive control induced even a higher kidney cell injury on the Vero kidney cells and death signified by an even lower % cell viability which was significant (p = 0.001) when it was compared with both the DX-10 and the non-induced normal Vero cells. The extracts increased %cell viability of treated Vero kidney cells in a dose dependent manner. The cells that were induced with doxorubicin 45 min before treatment with extracts were non-significantly lower in %cell viability than the cells that were treated first and then induced with doxorubicin after 45 min after treatment, Figure 4.3. The 70% ethanol extract showed significantly greater protective effect than the water extract, with higher cell viability in doxorubicin-treated cells (p = 0.001). The water extracts had significant effects (p=0.001) with the smallest concentrations (10 and 25 μg/mL), Figure 4.3.





(A) Doxorubicin induced Before treatment (B) Doxorubicin induced After treatment

Figure 4.3: Protective effects of *Psilocybe natalensis* water and 70% ethanol extracts on the % viability of doxorubicin-induced Vero kidney cells 45 min before and after treatment with extracts. DX-10 cells: induced Vero kidney cells used as control. Non-induced kidney cells: Vero kidney cells that were not induced nor treated. DX-40 cells: Positive Control cells treated with 40 µg/mL doxorubicin: *: Statistically significant.

Figure 4.4 shows that 10 µM doxorubicin significantly increased cell injury and death in HEK-293 kidney cells, as evidenced by a reduction in % cell viability compared to untreated controls (p= 0.001). The positive control HEK-293 cells, treated with 40 µg/mL induced even much more significant (p= 0.001) cell death when it was compared to the DX-10 and the non-induced HEK-293 kidney cells.

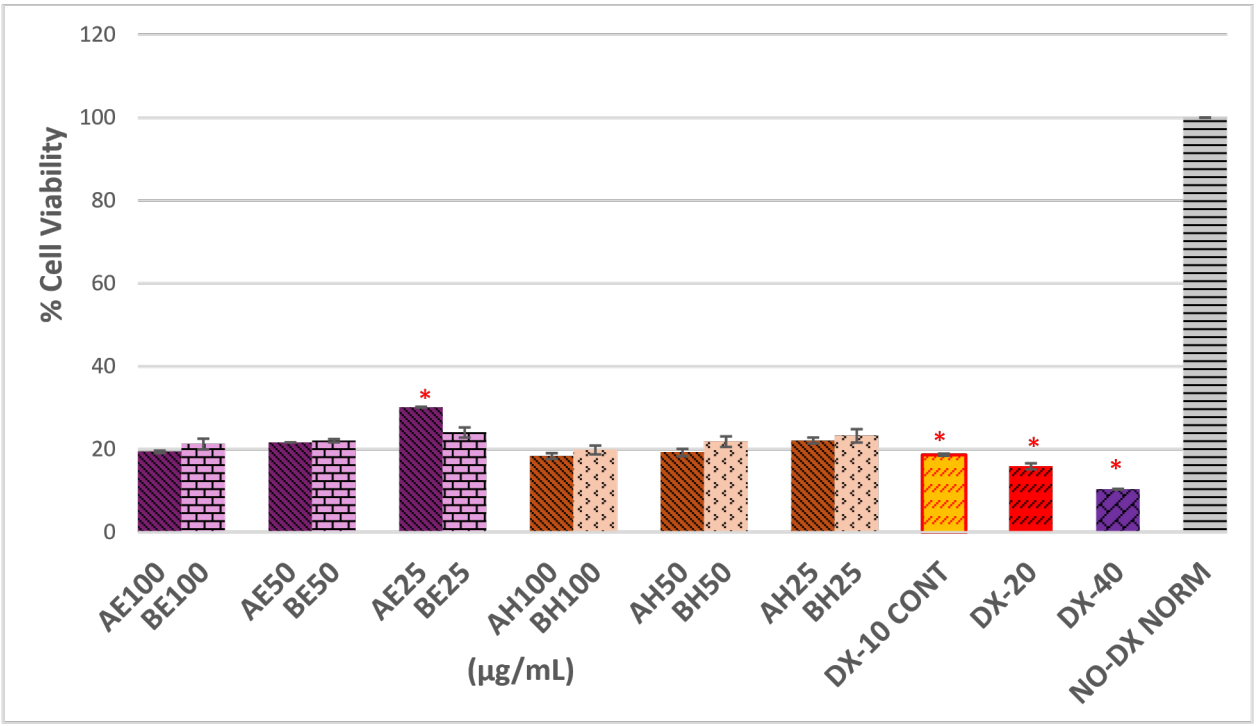


Figure 4.4: Protective effects of *Psilocybe natalensis* extracts on the % cell viability of DX-10-induced HEK-293 kidney cells treated 45 min Before (BH) and After (AH) stimulation. **Control**: DX-10; non-induced: HEK-293 kidney cells not induced nor treated; DX-40: positive control HEK-293 kidney cells induced with 40 µg/mL doxorubicin: *: statistically significant.

Treatment with *P. natalensis* extracts reversed the effects in a dose-dependent manner and the effects were much more pronounced with the 70% ethanol extracts, Figure 4.4. The HEK-293 kidney cells that were treated first for 45 minutes before induction with the doxorubicin had a slight non-significant increase in %cell viability when compared to the HEK-293 kidney cells that were induced first for 45 minutes with doxorubicin and only treated after 45 minutes with *P. natalensis,* but the difference was not statistically significant, Figure 4.4.

4.4 Inhibition by *Psilocybe natalensis* extracts on doxorubicin-induced nitrite in Vero and HEK-293 cells

The results from Figures 4.5 and 4.6 showed that doxorubicin has significantly increased nitrite oxide production in both Vero and HEK-293 kidney cells compared to untreated controls (p= 0.001). Treatment with 40 µg/mL doxorubicin [DX-40] produced a further significant increase in nitrite oxide levels (p= 0.001).

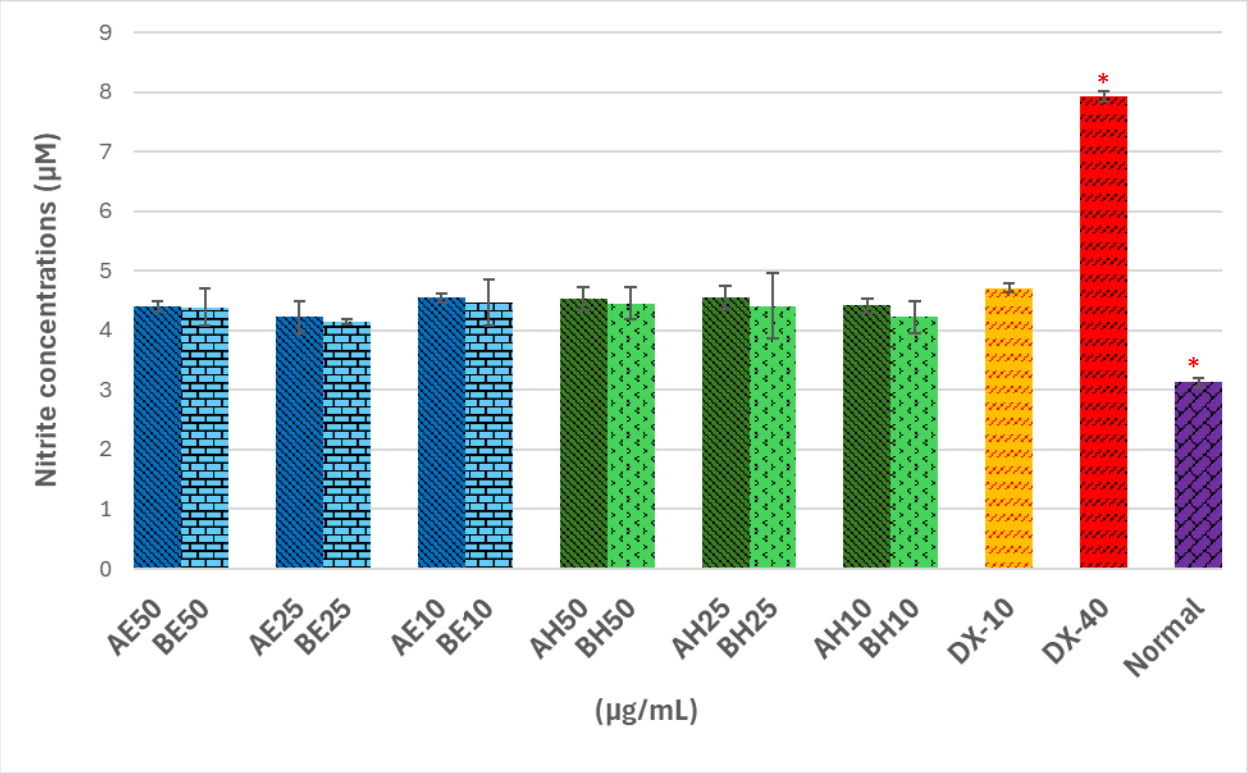


Figure 4.5: Inhibition of *Psilocybe natalensis* extracts on doxorubicin-induced nitrite content on the Vero kidney cells before and after 45 min stimulation. DX-10 cells: Control cells (10µM); DX-40 cells: positive control cells treated with 40 µg/mL; Normal cells: Vero kidney cells not stimulated nor treated with extracts: *: Statistically significant.

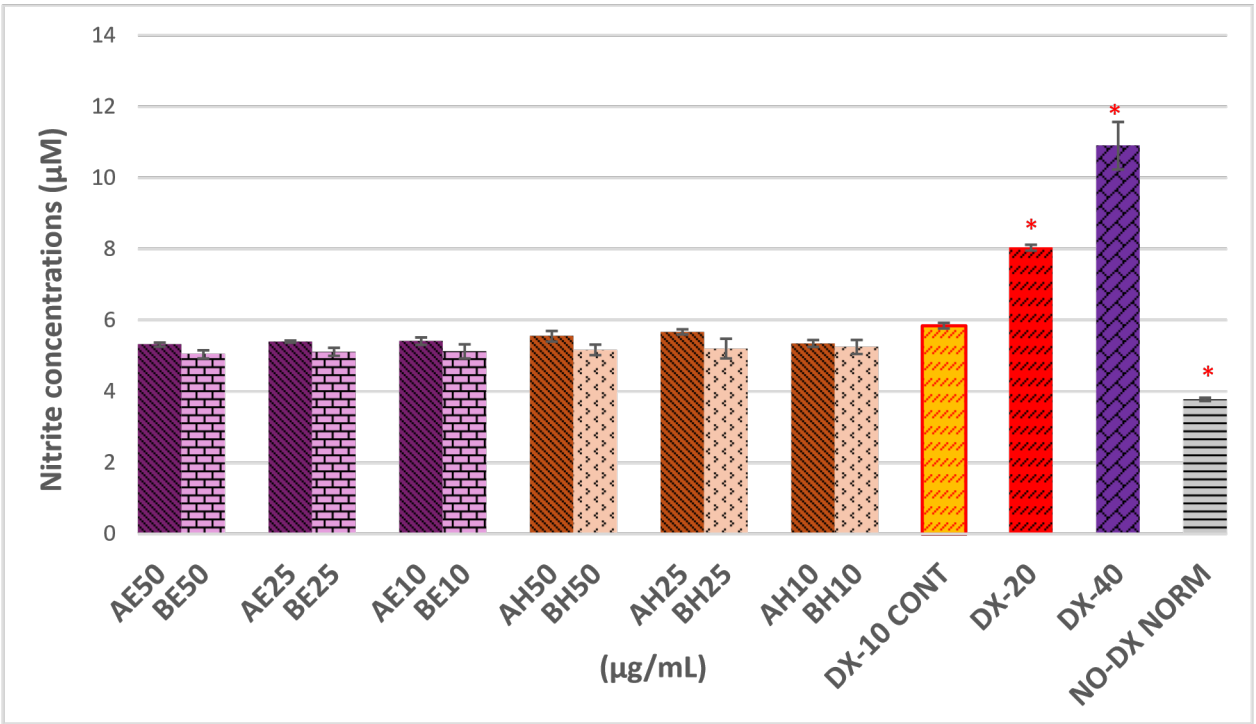


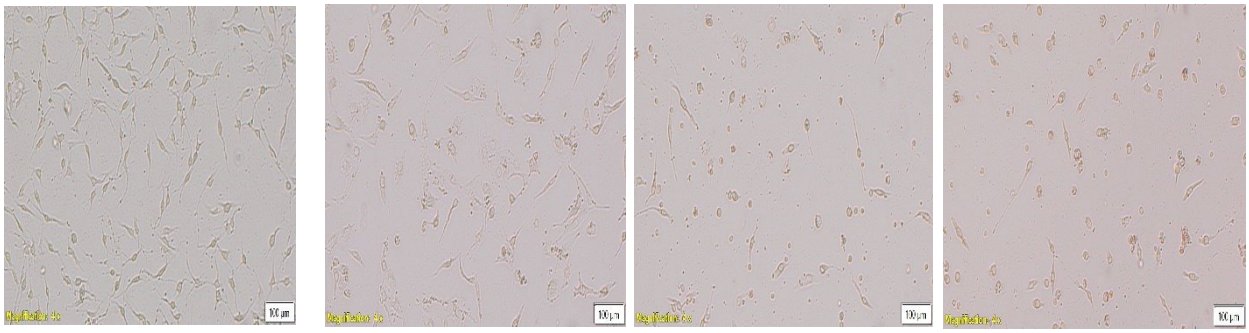
Figure 4.6: Inhibition of *P. natalensis* extracts on doxorubicin-Induced nitrite content on the HEK-293 kidney cells 45 min before and after stimulation with doxorubicin. DX-10 CONT cells: Control cells (10µM); DX-40 cells: positive control cells treated with 40 µg/mL; NO-DX NORM cells: HEK-293 kidney cells not stimulated nor treated with extracts: *: statistically significant.

Treatment with the water and 70% ethanol extracts of *P. natalensis* reduced the induced nitrite levels even though non-significant in both Vero and HEK-293 kidney cells, Figure 4.5 and 4.6. The inhibition effects were more with the cells that were induced first with doxorubicin for 45 minutes before treatment compared to the ones that were induced with doxorubicin only after been treated 45 minutes with the extracts. The inhibitory effects appeared to increase with extract concentration (dose-dependent) but was not statistically significant and hot-water extracts produced slightly higher inhibition than 70% ethanol extracts and that difference was also non-significant.

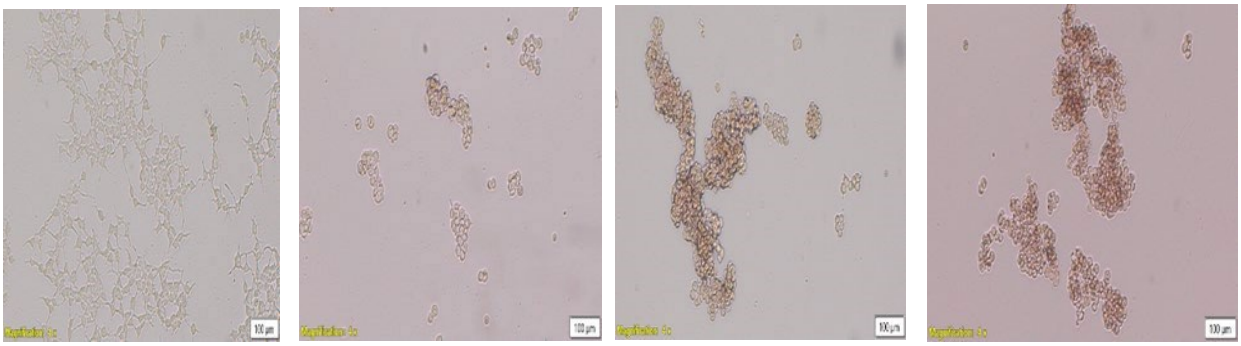
4.5 Morphological effects of *Psilocybe natalensis* extracts treatment in Vero and HEK-293 kidney cells induced with doxorubicin

Exposure to doxorubicin induced morphological changes in Vero cells in a concentration dependent manner. Cells treated with 20 µg/mL doxorubicin showed cell shrinkage/ rounding/ detachment, while treatment with 40 µg/mL caused more extensive detachment and nuclear condensation, as shown in Figure 4.7. The results in Figure 4.7 also showed that on the human HEK-293 kidney cells, stimulation with doxorubicin induced changed in morphology such as cell shrinkage/ rounding/ detachment with all the concentrations used from 10, 20 and 40 µg/mL. Doxorubicin also caused the induced HEK-293 kidney cells aggregation and detachment, and these effects were more pronounced with the 20DX cells and even more with the DX-40 treated cell which were positive controls used in the study and treated with 40 µg/mL of doxorubicin.

Vero kidney cells



NO DX (normal). 10 µM DX 20 µM DX 40 µM DX **HEK-293 kidney cell**

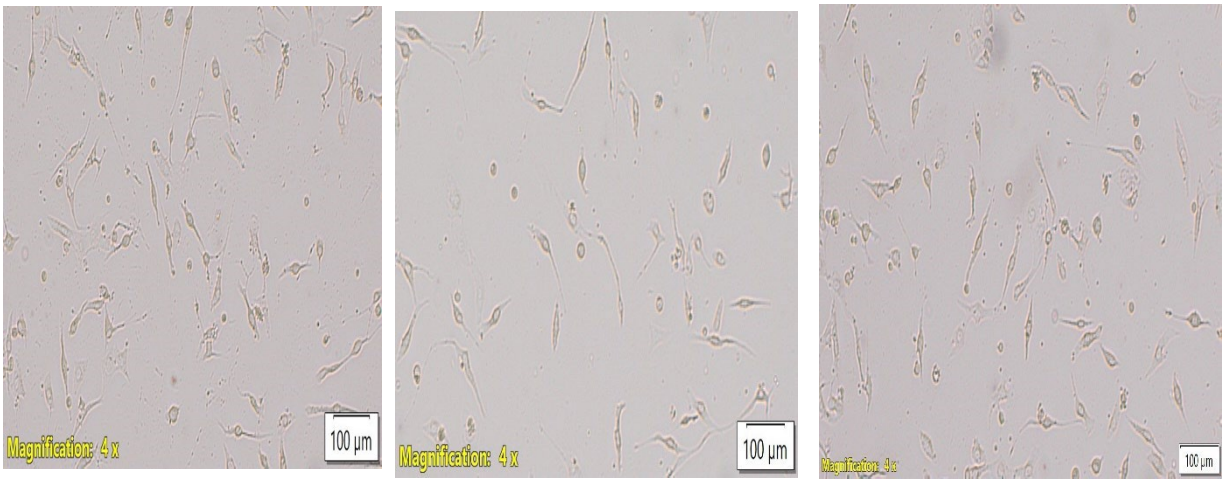


NO-DX (normal). 10 µM DX 20 µM DX 40 µM DX

Figure 4.7: **The morphological effects of doxorubicin on the Vero monkey kidney cells and HEK-293 kidney cells**. No=DX (normal) are cells that are neither induced nor treated with extracts. Vero and HEK-293 kidney cells induced with doxorubicin (10 µM, 20 µM, and 40 µM DX).

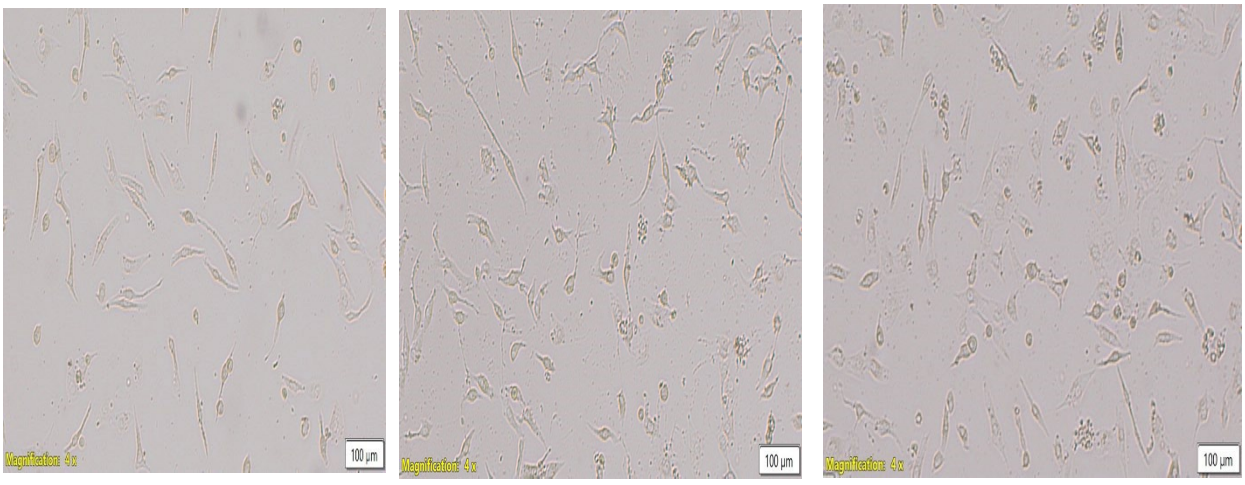
Vero monkey kidney cells induced with DX-10 (10µM)

70% Ethanol extracts



25ug/mL 50ug/mL 100 ug/mL

Hot-water extracts



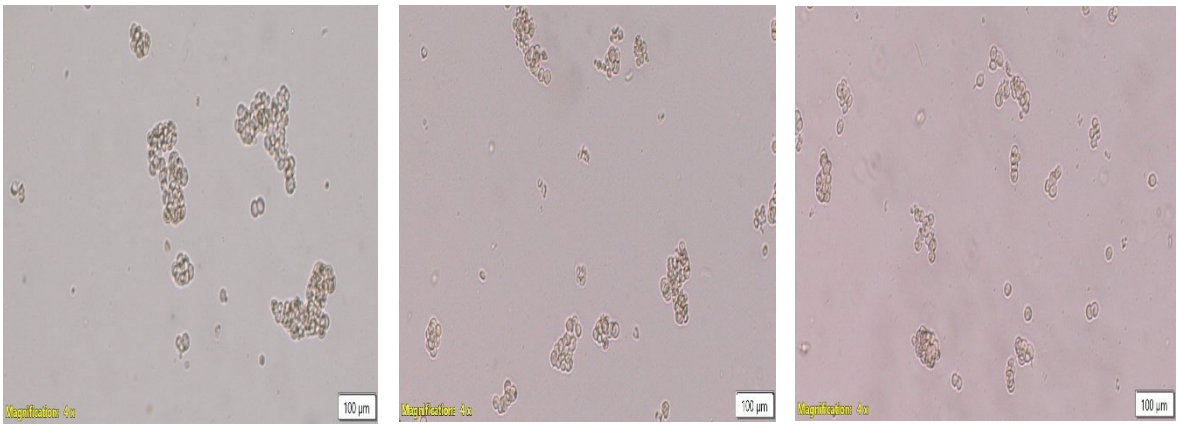
25 ug/mL 50ug/mL 100 ug/mL

Figure 4.8: Morphological effects of *Psilocybe natalensis* extracts (25, 50, and 100 µg/mL) on doxorubicin-induced Vero kidney cells.

Treatment with *P. natalensis* water and 70% ethanol extracts did not produce any observable morphological changes in Vero kidney cells at any concentration tested, as shown in Figure 4.8. In HEK-293 kidney cells pre-treated with doxorubicin, the extracts did not exacerbate the morphological alterations induced by 10 µM and 40 µM doxorubicin, Figure 4.9

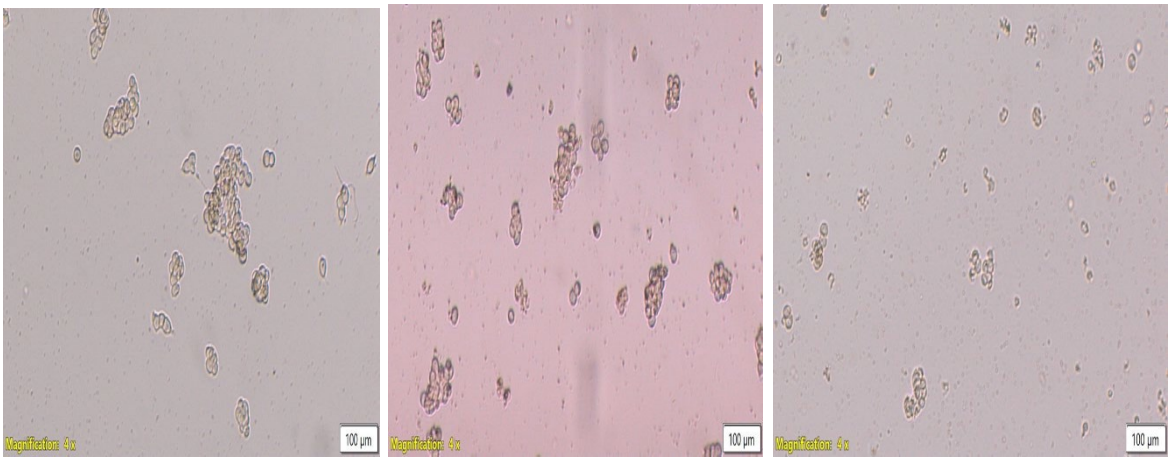
HEK-293 cells induced with DX-10 (10µM)

70% Ethanol extracts



25ug/ML 50 ug/ML 100 ug/mL

Hot- Water extracts



25 ug/ML 50 ug/ML 100 ug/ML

Figure 4.9: Morphological effects of *Psilocybe natalensis* extracts (25, 50, and 100 µg/mL) on doxorubicin-induced HEK-293 kidney cells.

4.6 Anti-inflammatory effects of *P. natalensis* extracts on doxorubicin-induced TNF-α levels on human HEK-293 kidney cells

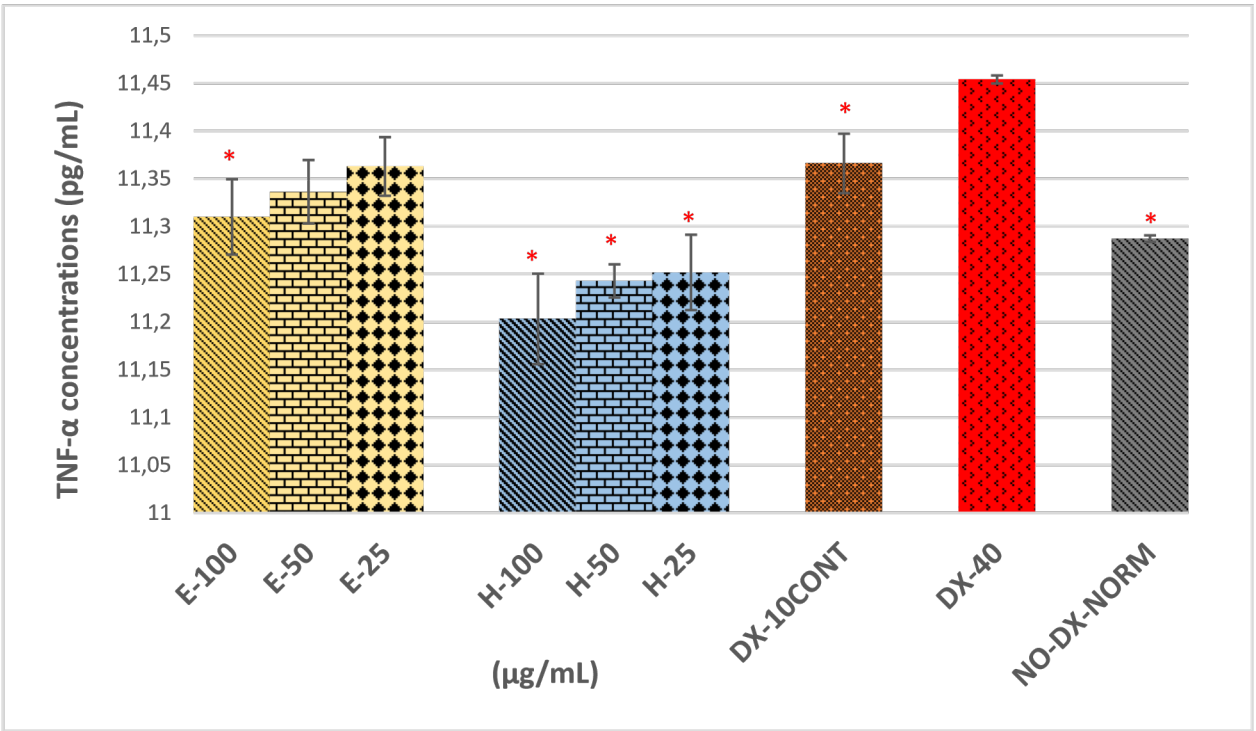


Figure 4.10: Anti-inflammatory effects of *Psilocybe natalensis* ethanol (E-100, E-50, E-25 µg/mL) and hot-water (H-100, H-50, H-25 µg/mL) extracts on doxorubicin induced TNF-α levels in human HEK-293 kidney cells. DX-10 CONT=kidney cells treated with 10 µM doxorubicin only; DX-40=positive control, 40 µg/mL doxorubicin; NODX NORM=untreated control kidney cells without doxorubicin or extract exposure. *: Statistically significant.

The results in Figure 4.10 above showed that treatment with doxorubicin induced a significant (p= 0.001) increase in TNF-α concentrations in the human HEK-293 kidney cells compared to the non-induced and non-treated cells, NO-DX-NORM cells. The DX40 cells increased the levels of TNF-α levels even higher and significantly higher (p= 0.001) than both DX-10 control and NO-DX normal cells, Figure 4.10. Treatment with the extracts reduced significantly with the water extracts and with the highest concentration of the 70% extracts, the levels of TNF-α concentrations. The decrease was in a dose dependent manner. The inhibitory effects were more pronounced with the water extracts, Figure 4.10.

Chapter 5

Discussion

5.1 Discussion

Kidney disease is increasingly documented as a significant global health issue, having oxidative stress that is being identified as a crucial factor in its progression (Daenen *et al*., 2019). One worth mentioning agent, doxorubicin which is commonly used in chemotherapy, has been associated with kidney cell injury of both Vero kidney cells and HEK-293 Kidney cells and nephrotoxicity predominantly through oxidative stress mechanisms (Lahoti *et al*. 2012). On the other hand, *Psilocybin* magic mushrooms, particularly *P. natalensis* have an antique contextual in psychoactive use, but recent studies have advocated that they offer therapeutic advantages particularly anti-inflammatory and antioxidant influences (Nkadimeng *et al*., 2021). Still, there is a sizeable gap in research concerning the affiliation between *Psilocybin* magic mushrooms and kidney disease. This opens a compelling area for investigation especially considering *Psilocybin* magic mushroom's possible capacity to influence oxidative stress and inflammation. Engaging in this line of analysis is particularly applicable to the study that is focused on extracts from *Psilocybe natalensis*. Therefore, this study seeks to play a potential significant part towards the potential use of *Psilocybe natalensis* extracts in the context of kidney disease, potential benefits and/or risks and thereby paving the way for future inquiry and potential therapeutic applications.

The results have shown that the extracts were relatively low to both normal Vero kidney cells and HEK-293 kidney cells, with LC_{50} values that were observed in comparison to the positive control, doxorubicin with LC_{50} below 20 µg/mL. The 70% ethanol extract was particularly non-toxic, with an LC_{50} value that was around 200 µg/mL and suggesting that the extracts were not cytotoxic for further therapeutic studies on the animal Vero kidney cells. However, these results also showed that the extracts were not cytotoxic on the human HEK-293 kidney cells with very high LC_{50} that were above 200 µg/mL signifying less toxicity. According to the Cancer Institute of the United States of America, the extracts with LC_{50} values stated above were considered toxic and the water and 70% ethanol extracts of *P. natalensis* used in the study will therefore not be considered toxic with these LC_{50} demonstrated in the study (Nkadimeng *et al*., 2020).

Both *P. natalensis* 70% ethanol and hot-water extracts have reduced doxorubicin-induced kidney cell injury and cell death in both Vero and HEK-293 kidney cells. The results showed a success induction of kidney cell injury and death which was demonstrated by a significant decrease in %cell viability of doxorubicin-induced kidney cells compared to non-induced kidney cells. Treatment with *P. natalensis* extracts increased % cell viability in a dose-dependent manner in doxorubicin-exposed Vero and HEK-293 kidney cells. The

70% ethanol extract produced greater effects than the hot-water extract at same concentrations level. These results were consistent with a protective effect of *P. natalensis* extracts against doxorubicin-induced cytotoxicity and the specific constituents responsible and their mechanism behind this remains to be determined. Potential mechanisms of actions behind this protective impact could entail oxidative stress pathway suppression, cellular signaling pathway regulation and cellular protective mechanism activation (Sadiq, 2023).

The more pronounced effects observed in Vero and HEK-293 kidney cells pre-treated with *P. natalensis* extracts before doxorubicin exposure indicate a potential protective effect against oxidative stress-induced kidney cellular injury. This effect could be mediated through the potential upregulation of antioxidant defenses or activation of cellular protective pathways, potentially enhancing resistance to subsequent oxidative stress (Vo *et al*., 2020). Pre-treatment with *P. natalensis* extracts had allowed the Vero and HEK293 Kidney cells to mount a more effective defense against doxorubicin-induced oxidative stress, thereby reducing the extent of kidney cell injury. The findings suggest that the extracts may modulate cellular signaling pathways, potentially contributing to the observed protective effects. Possibilities of the extracts in helping to modulate signaling pathways that are involved in cell survival and apoptosis such as the PI3K/Akt pathway may have been behind these effects, however this pathway was not directly assessed in this study, so any involvement of this pathway still remains speculative (Rommel *et al*., 2010). This pathway is known to regulate cell survival and proliferation, and its dysregulation is implicated in various diseases, which include cancer and neurodegenerative disorders (Advani and Kumar, 2024). In this study, %cell viability improved in kidney cells that were pre-treated with the extracts, suggested an enhanced survival under doxorubicin exposure. Apoptosis and modulation of signaling pathways were not directly assessed in this study. The protective effects observed in the study involve the activation of cellular defense mechanisms, although specific pathways such as Nrf2 were not directly evaluated in this study (Kensler *et al*., 2012). Nrf2 is a transcription factor that regulates the expression of antioxidant genes which include SOD, GPx, and catalase (He and Wen, 2020). When activating this pathway, the extracts could aid in enhancing the cells' natural antioxidant defenses and promote cell survival.

The current investigation also found that doxorubicin successfully induced a significant increase in nitrite oxide level thereby signifying increase in induced-NO in both animal Vero and HEK-293 kidney cells. This study found that *P. natalensis* extracts had a suppressing effect on the generated doxorubicin induced nitric oxide (NO) and pointing to a possible mechanism for relieving oxidative stress in doxorubicin-induced Vero and HEK-293 kidney cell damage. Nitric oxide is an important factor in the pathophysiology of oxidative stress and excessive production can cause the development of reactive nitrogen species, that can worsen cellular damage, especially when induced by pathological presence of oxidative. However, the changes were not statistically significant, so this mechanism to the observed protective effect remains to be fully investigated, especially

by measuring ROS in future (Roberts *et al*., 2010). The reduction in NO levels following treatment with *P. natalensis* extracts indicate modulation of oxidative stress pathways, potentially contributing to their renoprotective effects. Furthermore, the decrease in generated NO levels when using *P. natalensis* extracts proposed that these mushroom extracts may modulate oxidative stress pathways and subsequently donated to their renoprotective properties. The inhibition of induced-NO when utilizing *P. natalensis* extracts, as observed in the current study, had proposes a potential mechanism for alleviating oxidative stress in doxorubicin-induced Vero and HEK-293 kidney cell injury. It was also interesting to observe that the extracts had suppressing effects even though non-significant on the kidney cells that were induced first for 45 minutes compared to the ones that were induced after treatment. This finding aligns with the % cell viability data where it was slightly lower in kidney cells that were pre-treated with doxorubicin before mushroom extract exposure. However, the difference was not statistically significant, so it reflects a trend toward reduced kidney cell injury and death numbers rather than a confirmed effect. However, overall weather induced before or after, the extracts had lowering effect on the induced-NO which could be beneficial in kidney cell injury and oxidative stress.

The morphological changes such as reduced cell density and increased cell rounding have been detected in the study and they reflect the underlying cellular damage and kidney injury that are caused by doxorubicin and include adjustments in cell size, shape or structure and they are also symptomatic of apoptosis, necrosis, or other forms of kidney cell death (Ozbek, 2012). However, findings from this study demonstrated that extracts did not induce any morphological effects on the normal animal Vero and human HEK-293 kidney cells with all the concentrations that were used in the study. The study also showed that both water and 70% ethanol extracts did not exacerbate the doxorubicin induce morphological changes especially in comparison to the positive control (40-DX), which worsened the 10 µM doxorubicin control effects in both kidney cells. The morphological findings suggest that the extracts were not morphologically toxic to normal kidney cells and did not exacerbate doxorubicin-induced damage, rather than implying definite reversal of injury.

The significant increase in TNF-α levels after doxorubicin stimulation was induced implied that there was an inflammatory response and this is consistent with the doxorubicin-drug known nephrotoxic effects. The 40-DX, positive control confirmed that TNF-α levels increased with higher doxorubicin concentrations and also showed that the higher the concentration of doxorubicin, the more TNF-α pro-inflammatory response was induced. *Psilocybe natalensis* extracts reduced this reaction and that indicated that there are anti-inflammatory properties in the extracts. The suppressive effects on the human HEK-293 kidney cell were more pronounced with the water extracts. The 70% ethanol extracts anti-inflammatory effectiveness was with the 100 µg/mL concentration while the hot-water extracts were effective with all the doses. These findings proposed that there are possible therapeutic advantages in the extracts. These findings corroborate with the previous

research on *P. species* anti-inflammatory effects (Nkadimeng *et al*., 2020). The decrease in TNF-α levels by *P. natalensis* extracts suggested that these extracts modulate inflammatory pathways which then could contribute to their renoprotective properties and the specific involved pathways were not measured and they would require further investigation. The dose-dependent effect implied that the anti-inflammatory benefits are due to several pathways and substances and these anti-inflammatory activities were not identified in this study. This corroborates with the wide range of beneficial substances that are discovered in *Psilocybe species*, which include *Psilocybin*, antioxidants and phenolic compounds (Nkadimeng *et al*., 2020; Lee *et al*., 2018).

In summary, when comparing these effects of the extracts, the 70% ethanol extracts were safer and with greater protective impact on doxorubicin induced kidney cell injury. It is notable that the extracts of hot water remain effective at all levels of concentrations indicating that there is possibility of water-soluble chemicals. Given the significance of TNF-α in the evolution of kidney disease, these results encouraged more research into *P. natalensis* extracts as possible potential therapeutic agents for reducing inflammation related kidney damage (Daenen *et al*., 2019). The protective benefits of the extracts also potentially relied on their ability to deter oxidative stress pathways, following the reduction in induced NO upon treatment with extracts. Previous studies have shown that *P. natalensis* extracts had antioxidant, anti-inflammatory effects and reduced significantly the generation of ROS, which are known to cause kidney cell harm and death (Nkadimeng *et al.,* 2020; Shirley, 2021). This is accomplished by energizing antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase. By depressing oxidative stress, the *P. natalensis* mushroom extracts investigated in this study may play a role in protecting cellular components like DNA, proteins, and lipids.

70% ethanol was more resourceful solvent for extracting a broader spectrum of bioactive chemicals including *Psilocybin* and other phenolic substances, which might explain the reported effects (Nkadimeng *et al*., 2020). The improved solubility of these bioactive chemicals in 70% ethanol have resulted in more powerful extracts, contributing to the study's more dramatic effects (Plaskova and Mlcek, 2023). This is consistent with prior research done by Lee *et al*. (2018), who found that 70% ethanol extracts of *Psilocybe species* are more efficient in exhibiting antioxidant and anti-inflammatory properties (Lee *et al*. 2018). The difference in the effectiveness between 70% ethanol and hot-water extracts might potentially indicate that the bioactive molecules responsible for the renoprotective effects are water-insoluble, heat-sensitive making 70% ethanol a better solvent for extraction both applicable to the both Vero and HEK-293 kidney cells and this is only a possible explanation and was not directly tested. Additional research is needed to settle out the individual bioactive chemicals that are responsible for the reported benefits and to also optimize the extraction procedures for maximal efficacy. In this study, the stronger effects of 70% ethanol extracts suggested a notable possible *in vitro* protective activity against oxidative stress-induced damage in Vero and HEK-293 kidney cells.

Chapter 6

Conclusion, Limitations and Recommendations

6.1 Conclusion

This research study showed that *P. natalensis* mushroom extracts will not be considered toxic in Vero and HEK-293 kidney cells based on the LC_{50} and were safe at the concentrations used in the study, but broader safety cannot be inferred from two kidney cell lines alone. The study also demonstrated that the extracts were even safer on the human than on animal kidney cells thereby indicating lower *in vitro* cytotoxicity on human kidney cell line than the animal kidney cell line on these tested conditions. The study confirmed that doxorubicin reduced %cell viability and increased kidney cell injury and death which is consistent with its use in the *in vitro* model of kidney cell injury. Doxorubicin treatment elevated nitrite levels and TNF-α release, suggesting the increased in nitric oxide production and inflammatory response. However, this oxidative stress was not assessed as measurements were limited to the nitrite as an indirect marker. The study demonstrated the potential protective effects of the water and 70% ethanol extracts of *P. natalensis* psychedelic mushroom on the induced kidney cell injury and cell death on both animal Vero and human HEK-293 kidney cells which could be beneficial in a kidney disease condition. The extracts inhibited the induced-NO oxidative stress-related effects and did not invoke morphological effect on the induced and non-induced cells. The extracts reduced doxorubicin-induced nitric oxide production in the *in vitro* assays and showed no morphological effects on doxorubicin-treated or untreated kidney cells. While these findings may indicate potential protective effects under the tested conditions, further studies are needed to determine more relevance to kidney disease processes.

The extracts also demonstrated anti-inflammatory effects by suppressing the doxorubicin induced pro-inflammatory cytokine TNF-α levels. The 70% ethanol extract showed greater protection of %cell viability whereas the hot-water extract produced a greater reduction in TNF-α levels thereby indicating preliminary protective *in vitro* effects against doxorubicin induced HEK-293 kidney cell damage, which happens most likely through antioxidants and anti-inflammatory pathways that were not measured in this study. Finally, the study showed for the first time that the extracts of *P. natalensis* magic mushrooms will be considered safe, especially on the normal human kidney. The *P. natalensis mushroom* extracts demonstrated the potential therapeutic implications in preventing kidney damage which was shown by the extracts' capacity to protect against induced-kidney cell injury, alter oxidative stress pathways and induced-NO generation, not worsened morphological alterations of induced cells and inhibit pro-inflammatory TNF-α induced levels.by. The 70% ethanol extract had greater reductions in doxorubicin-induced nitric oxide and kidney

cell injury and death compared to the hot-water extract under the tested conditions and whether this reflects differences in antioxidant or anti-inflammatory compound content still needs to be investigated further. The hot-water extract on the other hand showed greater reduction with the pro-inflammatory TNF-α levels compared to the ethanol extracts. The greater effects of the hot-water and 70% ethanol extract suggest that they are more effective for medicinal invention and indicate that there are more antioxidants and anti-inflammatory compounds that are extracted with both 70% ethanol and water as indicated by previous photochemistry studies on the specific magic mushroom species. More investigations into underlying mechanisms of action are needed to elaborate the potential therapeutic effects in kidney cell injury and death, and oxidative stress.

6.2 Limitations

This study's limitations were restricted to an *in vitro* model where two kidney cell lines were used which may not reflect complex in vivo kidney physiology. Oxidative stress was assessed only through nitrite levels due to challenges with fluorescence-based assays, and no mechanistic studies of anti-inflammatory pathways were performed and nitrite data alone provided limited evidence of oxidative stress modulation. Future studies that are going to use oxidative stress markers, mechanistic pathways analysis and in vivo models are needed to corroborate these findings.

6.3 Recommendations

The study recommends that future studies should investigate key mechanistic pathways that include measuring specific markers of oxidative stress such as ROS levels, lipid peroxidation, and antioxidant enzyme activity and as well as evaluating NF-κB and Nrf2 signaling in doxorubicin-induced kidney cell injury. Phytochemical characterization of the *P. natalensis* extracts is also recommended to identify active compounds that are responsible for the effects that were observed. These findings should also be validated using *in vivo* studies in future with established animal models of kidney injury to assess translational relevance.

Chapter 7

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