Oral *Tulbaghia violacea* extract-based nano-ZnO administration in piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake

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DECLARATION

I, Yamkela Mbenga declare that this dissertation, hereby submitted for the degree of Master of Science in Animal Science at North-West University, is my work and has not been submitted to any other institution for an award. Where other sources have been used; they have been appropriately cited and acknowledged.

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GENERAL ABSTRACT

The high costs of conventional protein sources such as soya bean meal (SBM) have prompted the search for a cheaper alternative and readily available protein sources like marula seed cake (MSC) for pig production, particularly by smallholder farmers in Southern Africa. Marula seed cake (MSC) has a high crude protein and amino acid content similarly to SBM. Due to its high content of protein and residual oil, both of which provide ideal conditions for the growth and proliferation of toxigenic fungi, MSC is however naturally contaminated with mycotoxins. Mycotoxins are known to induce deleterious effects and cause economic losses in pigs by causing reduced growth, immunosuppression and other effects. Hence the need to find strategies to counter their deleterious effects in pigs fed MSC-supplemented diets. This study was aimed at investigating ameliorative effects of *T. violacea* bulb extract bio-fabricated zinc oxide (ZnO) nanoparticles (Nano-ZnO) in piglets fed diets supplemented with naturally mycotoxin-contaminated MSC. Nano-ZnO was phyto-synthesized using the aqueous leaf extract of *T. violacea* as reducing and stabilizing agent. The synthesized ZnO NPs were characterized by techniques including X-ray diffraction (XRD), UV–visible spectroscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) coupled with energy dispersive X-ray (EDX) spectrometer. The morphological studies confirmed that the as-prepared ZnO NPs were spherical, with an average particle diameter of 45.26 nm. The PXRD pattern indicated single-phase hexagonal ZnO NPs with high crystallinity, while the absorption spectra showed evidence of quantum size effect. The Nano-ZnO were applied in a piglet feeding trial by orally administering (gavaging) them to piglets fed diets supplemented with naturally-mycotoxin contaminated MSC. A total of 60, 4-week old weaned Large White piglets were randomly allocated to two (2) iso-energetic and iso-nitrogenous diets formulated by replacing SBM (Control; 0% MSC) (Group A) with MSC (Treatment; 20% MSC) (Group B). Once-daily, Group A piglets (0% MSC) were orally drenched with 10 mL of the vehicle (25% ethanol in water; 25 parts ethanol: 75 parts water, v/v) whilst Group B piglets (20% MSC) were similarly orally drenched with an equivalent volume of the: vehicle (25% ethanol) (Group B1), 50 mg/L of bulk ZnO in 25% ethanol (Group B2), 50 mg/L of chemically-synthesised Nano-ZnO in 25% ethanol (Group B3), and 50 mg/L of green (*T. violacea* bulb extract-fabricated) Nano-ZnO in 25% ethanol (Group B4). Average feed intake (FI) and body weight gain (BWG) were measured weekly, and then feed conversion efficiency (FCE) was calculated by dividing the BWG by the FI. On day 33, blood was collected for serum analysis, and all animals were slaughtered after which carcass and meat quality measurements were taken. The

data showed that dietary incorporation of 20% MSC decreased BWG and FCE ($P < 0.001$). Meanwhile, dietary inclusion of 20% MSC increased weekly FI while 20% MSC with green Nano-ZnO decreased FI, BWG and FCE ($P < 0.001$). Furthermore, the results demonstrated that dietary inclusion of 20% MSC did not affect the overall FI, BWG and FCE, while green Nano-ZnO decreased these parameters ($P < 0.001$). Also, results showed that dietary incorporation of 20% MSC decreased hot carcass weight (HCW) ($P < 0.001$), cold carcass weight (CCW) ($P < 0.001$), foreleg weight ($P = 0.001$) and hind leg weight ($P < 0.001$) whilst 20% MSC with green Nano-ZnO elicited further deleterious effects. Nevertheless, dietary inclusion of 20% MSC had no significant effects on the weights of the shoulder, ham, belly, and rib and back lengths, while 20% MSC with green Nano-ZnO decreased shoulder weight ($P = 0.001$) and ham weight ($P < 0.01$). Neither 20% MSC inclusion nor administration of green Nano-ZnO had any effect on meat quality parameters ($P>0.05$), except for meat temperature that was decreased ($P<0.001$) and meat yellowness that was increased ($P < 0.05$) after 24 hours of cold storage whilst water holding capacity (WHC) was decreased by green Nano-ZnO administration ($P<0.01$). Lastly, neither dietary inclusion of 20% MSC nor oral administration of green Nano-ZnO had any effect on serum biochemical parameters ($P>0.05$) whilst green Nano-ZnO administration decreased serum albumin ($P <0.05$), cholesterol ($P <0.05$), and amylase enzyme activity ($P = 0.001$). It was then concluded that MSC could be utilized as an alternative protein source in weaned piglet diets with no need for an ameliorative strategy in the form of green Nano-ZnO.

Keywords: piglets; zinc oxide nanoparticles; *Tulbaghia violacea*; marula seed cake.

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DEDICATION

I dedicate this dissertation to my mother, **Tandiwe Tryphina Mbenga**.

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PEER-REVIEW ARTICLE FROM THIS DISSERTATION

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NOMENCLATURE

AFs	Aflatoxins
ANFs	Anti-Nutritional Factors
CP	Crude Protein
FMs	Fumonisins
MSC	Marula Seed Cake
Nano-ZnO	Zinc Oxide Nanoparticles
NPs	Nanoparticles
OTs	Ochratoxins
SBM	Soybean Meal
SSA	Sub-Saharan Africa
TRCs	Trichothecenes
ZnO	Zinc Oxide
ZEAs	Zearalenones

CHAPTER 1: INTRODUCTION

1.1 Background

Animal products such as milk and meat are important sources of human dietary protein and energy (Schönfeldt and Hall, 2012). In the present decade, there has been a global yearly increase in the demand for animal-based food. By 2050, it is projected that growth in global population especially the doubled inhabitants of Sub-Saharan Africa (SSA) will result in a substantial increase in the consumption of animal products and tremendous pressure on animal production (Leridon, 2020a) . The increase in demand for animal protein is presently evident in South Africa where data on the per capita consumption of white meat shows a significant increase from 21.48 kg to 40.04 kg between 2001 and 2017. Similarly, the per consumption of red meat increased from 18.96 kg in 2001 to 27.74 kg in 2017 (DAFF, 2017b). In addition, the rise in population and its increasing demand for animal protein, places an ongoing demand on agriculture, especially in the livestock sector, for food security and safety (WHO, 2017).

To ensure the agricultural system is not overwhelmed by the demand for animal products, it is essential to integrate smallholder farmers. Therefore, present day research needs to focus on quality, consistency and cost of animal feed, a major drawbacks faced by smallholder non-ruminant farmers (Davis and D'Odorico, 2015). Soybean meal (SBM) is a widely used source of conventional dietary protein for animals with a relatable optimal amino acid profile. While SBM is frequently utilized in commercial production systems, its high cost is a major deterrent for smallholder farmers. It is therefore essential to develop alternative affordable protein rich source as animal feed, such as marula (*Sclerocarya birrea caffra*) seed cake. This alternative protein source has a comparable nutritional value to conventional protein sources, and is readily available in SSA at reasonable prices, without compromising the nutritional requirements of the animals (Martens *et al*., 2012, Mthiyane and Mhlanga, 2017a).

Marula seed cake (MSC) is a residual by-product of oil extraction from the dry seeds of the marula fruit, from a tree that belongs to the *Anacardiaceae* family (Chirwa and Akinnifesi, 2008, Mlambo *et al*., 2011b). The marula tree typically grows in high-lying areas with minimal sub-zero periods in the winter found throughout Eastern and Southern Africa (Mariod and Abdelwahab, 2012). Research has been conducted on the utilization of MSC as a dietary protein supplement for beef cattle (Mlambo *et al*., 2011a), goats (Mlambo *et al*., 2011b), dairy cattle (Mdziniso *et al*., 2016), broiler chicken (Mthiyane and Mhlanga, 2017a, 2018a), sheep (Malebana, 2018), Japanese quills (Mazizi *et al*., 2019a, 2020a) and in pig production

(Hlongwana *et al.*, 2021a, Mabena *et al.*, 2022). Also, it has been tested *in situ* for ruminant diets (Nkosi *et al.*, 2019, Muya *et al.*, 2020). According to the above-mentioned studies, MSC is a decent alternative plant-based protein source that can substitute SBM in livestock production, and its nutritive value was reported to improve livestock productivity, health status, and meat quality. However, there is sparsity of data on the use of MSC as an alternative protein source in pigs. Therefore, the present study postulates MSC as a potential alternative protein source in pig production.

One of the major challenges with feeding MSC in non-ruminant animals is its natural contamination by toxigenic mycotoxins such as T-2 toxin and deoxynivalenol (Mthiyane and Mhlanga, 2017a). The high proportion of concentrates in the diets of non-ruminant animals, such as pigs and poultry, is one of the reasons why they are more susceptible to mycotoxins (Bryden, 2012b). Feed contaminated with mycotoxin if consumed by animal or man can result in carcinogenic, dermatoxic, estrogenic, hemorrhagic, hepatotoxic, immunotoxic, mutagenic, nephrotoxic, neurotoxic, and teratogenic health effects (Simpson *et al.*, 2001a, Cunha *et al.*, 2018a). Furthermore, mycotoxin intoxication has been reported to significantly impair body weight gain, feed intake, and feed conversion efficiency due to feed refusal, growth retardation, and even weight loss in pigs (Bryden, 2012b). It is therefore essential to explore methods to exclude or ameliorate mycotoxin effects in animals used as food. One of such reported method involves the use of nanomaterials such as zinc oxide

Previous studies have demonstrated the great potential of different forms of zinc oxide showing unique antioxidant properties (Kovacic and Somanathan, 2013, Muthuvel *et al.*, 2020), a possible solution to the effects caused by mycotoxins. Zinc oxide, a multifunctional metal salt resulting from unique physical and chemical properties, can be in different particle sizes, which can either be in bulk particles or be reduced to be in nanoparticles (Kołodziejczak-Radzimska and Jesionowski, 2014). The nanoparticles of zinc oxide are synthesized using various techniques, that is, using the chemical, physical, green or biological synthesis (Fakhari *et al.*, 2019). However, the physical and chemical-based synthesis of nanoparticles are expensive, potentially unsafe, and possibly hazardous, not to mention the complicated separation process, high pressure, and energy requirements (Rodriguez-Sanchez *et al.*, 2000).

Green synthesis of nanoparticles is eco-friendly and poses no threats to animals and the environment. Therefore, it is hypothesised that MSC could serve as an alternative protein source in pig production and the adverse health effects caused by mycotoxins present in MSC

could be mitigated by supplementing with zinc oxide nanoparticles synthesized using *T. violaceae* bulb extract. This study presents the first-time application of MSC and *T. violaceae* phyto-mediated zinc oxide nanoparticles in pig production, which necessitates the utilization of both products to mitigate feed costs, one of the current problems faced by pig farmers in Southern Africa.

1.2 Problem statement

The pork industry is one of the primary sources of human dietary protein. However, the high cost of feeds due to imported and expensive conventional protein sources like soybean meal limits pork production, particularly by smallholder farmers in Southern Africa (Lekule and Kyvsgaard, 2003). Soybean production in the SSA region does not meet the requirements for both the human food and livestock feed industries, hence the price increases (Enahoro *et al.*, 2019). This has necessitated the investigation of alternative protein sources such as MSC, that are cost-effective and readily available in this region (Valbuena *et al.*, 2015). This agro-waste by-product from the oil extraction of the marula seed kernels has numerous positive effects on beef cattle (Mlambo *et al.*, 2011a), goats (Mlambo *et al.*, 2011b), dairy cattle (Mdziniso *et al.*, 2016), broiler chickens (Mthiyane and Mhlanga, 2017a, 2018a), sheep (Malebana, 2018) and Japanese quails (Mazizi *et al.*, 2019a, 2020a). There is limited literature regarding the use of MSC in pig production.

MSC is predisposed to natural contamination by secondary metabolites of filamentous fungi known as mycotoxins (Mthiyane and Mhlanga, 2017a). Mycotoxins are naturally occurring toxins in foodstuffs such as maize, peanuts, almonds, figs, and several other foods (Keller *et al.*, 2013). Several mycotoxins have overlapping toxicities on livestock including pigs. The continuous intake of even chronic low-doses of mycotoxin-contaminated feeds increases the risk of varieties of health hazards ranging from mycotoxicosis, carcinogenic, mutagenic, teratogenic, oestrogenic, neurotoxic, immunotoxic to death (Bennett, 1987, Simpson *et al.*, 2001b, Ratcliff, 2002b, Cunha *et al.*, 2018b). It is therefore essential to explore measures to prevent mycotoxins and their health effects in pigs.

Several techniques have been explored to eliminate mycotoxins in animal feeds. The most widely used and successful are clay particles, such as bentonites and zeolites (Dal Pozzo *et al.*, 2016). However, clay absorbents have a tendency to bind minerals and vitamins from the feed (Brown *et al.*, 2014, Bhatti *et al.*, 2018). This necessitates the search for an effective technique to deal with mycotoxins in pigs, such as nano-biotechnology. While prior studies suggest that

zinc oxide nanoparticles may offer a low-cost, promising, and effective method to ameliorate the adverse effects of mycotoxins in livestock, there is presently a scarcity of data on the effects *T. violaceae* phyto-mediated zinc oxide nanoparticles in pig production.

1.3 Justification

The challenge faced by pig farmers and the industry in SSA has provided an opportunity for the evaluation of alternative protein sources, such as MSC, that are indigenous and could be utilized for improvement of pig production to counteract the issue of food insecurity and alleviate poverty in this region. MSC is readily available in abundance and an affordable protein source for livestock in SSA. According to (Mlambo *et al.*, 2011b), it has an outstanding crude protein content (470 g/kg DM) and a high-grade amino acid composition similar to that of soya bean meal, except for lysine (Mthiyane and Mhlanga, 2017a). Additionally, it has high oil content (EE: 343.5-411.32 g/kg DM) (Mthiyane and Mhlanga, 2017a, Malebana et al., 2018a) which has 10 times more oxidative stability compared to olive oil (Mariod and Abdelwahab, 2012) and is highly rich in both saturated and monounsaturated fatty acids (Mthiyane and Mhlanga, 2017a, Malebana *et al.*, 2018a, Mthiyane and Hugo, 2019). Thus, incorporating MSC into piglet diets, not only would piglets receive much-needed protein, energy, and other nutrients, but it would also improve the shelf life and health status of pork. This would subsequently benefit humans. These nutritional properties and stability prove that MSC could be a more favourable potential substitute for SBM in piglet diets in Southern Africa and elsewhere where marula trees occur in abundance. This seed cake has been utilized in pig production, where it reduced the average daily gains and consequently resulted in poor feed conversion efficiency (Mabena *et al.*, 2022).

Anti-nutritional factors (ANF) in non-ruminant animals cause health problems, such as mycotoxicosis, particularly in cases where alternative protein sources are used (Martens *et al.*, 2012). Hence, the need for dietary supplementation with *T. violaceae* bulb-extract based zinc oxide nanoparticles that has properties that would ameliorate the negative effects caused by mycotoxins present in MSC. The *T. violaceae* plant has sulphur compounds which are precursors for the synthesis of cysteine (Kubec *et al.*, 2013a). Cysteine is a rate-determining precursor for glutathione synthesis, the major antioxidant molecule responsible for mopping up and quenching toxic substances in body cells (Mthiyane, 2006). Furthermore, nanoparticles on their own have a great potential to reduce the toxic effects of mycotoxins (Kaushik *et al.*, 2009), with metal oxide nanoparticles showing unique anti-oxidant properties (Kovacic and

Somanathan, 2010), particularly that of zinc-oxide. More antioxidant properties could be achieved by green synthesis, which is cheap, easy to prepare, environmentally friendly, and poses no threats to animals.

Upon completion of this project, a diet that will meet the nutritional requirements for piglets and address the problem of mycotoxin intoxication will be formulated. Also this diet will improve the anti-oxidative status, which will give the ability to quench the free radicals caused by mycotoxins. The availability of such cost-effective feed will significantly benefit pig and consumer's health, pig farmers, the pork industry, and the South African feed industry economically. In evaluating these protein-energy-rich diets with a possible solution to mycotoxin contamination, it is important to investigate the effects of oral *T. violacea* bulb extract-based zinc oxide nanoparticles on performance, meat quality, biochemical and physiological parameters in piglets fed diets supplemented with natural mycotoxin-contaminated MSC.

1.4 Aim

1.4.1 Broad objective

To investigate ameliorative effects of *T. violaceae* bulb extract bio-fabricated zinc oxide nanoparticles in piglets fed diets with natural-mycotoxin contaminated marula seed cake.

1.5 Objectives of the study

1.5.1 Specific objectives

(a) To synthesize and characterize zinc oxide nanoparticles from *T. violacea* bulb extract-based biological/green method and using chemical route of synthesis.

(b) To determine the ameliorative effects of oral administration of *T. violacea* extract-based green zinc oxide nanoparticles on growth performance, carcass characteristics, meat quality and serum parameters in piglets fed diets supplemented with natural mycotoxin-contaminated marula seed cake.

1.6 Hypotheses of the study

(a) **H_0**: *T. violacea* bulb extract will not enhance the stabilization and increase the agglomeration of zinc oxide nanoparticles during synthesis.

H_1: *T. violacea* bulb extract will significantly enhance the stabilization and decrease the agglomeration of zinc oxide nanoparticles during synthesis.

(b) **H_0**: Oral administration of *T. violacea* bulb extract-based green ZnO nanoparticles will not decrease feed intake and not increase growth performance, carcass characteristics, meat quality and serum parameters in piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

H_1: Oral administration of *T. violacea* bulb extract-based green ZnO nanoparticles will significantly decrease growth performance, carcass parameters, meat quality and serum parameters in piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

1.7 Components of the dissertation

This study has 5 chapters in an article format, which are as follows:

Chapter 1 contains the general introduction, which provides background and motivation for this study.

Chapter 2 contains the literature review which gives an overview of how population growth impacts food security, on the use of pig production to alleviate food insecurity, the use of alternative protein sources for pig production and the potential impact of MSC, how each major class of mycotoxins affects the pig production, and the use of nanoparticles as a possible way to ameliorate the negative effect of these mycotoxins on pigs.

Chapter 3 is a research chapter that has the first objective, the synthesis (green and chemical) and characterization of ZnO nanoparticles.

Chapter 4 is a research chapter objective 2, which investigates the ameliorative effects of oral administration of *T. violacea* extract-based green zinc oxide nanoparticles on growth performance, carcass characteristics, meat quality and serum parameters in piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

Chapter 5 gives a general discussion, concludes the dissertation, reflects on the research done, presents comments, and gives recommendations for future prospective studies.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In 2015, FAO reported that 22.8 percent of the population in sub-Saharan Africa (SSA) is undernourished, resulting from the ever-growing population in this region (FAO, 2015). This region is now expected to be accompanied by a doubling of population size by 2050 (Leridon, 2020b), which is expected to severely impact the food demand and the consumption of animal protein as a matter of such (Mthiyane and Mhlanga, 2017b, Fujioka, 2021a). According to DAFF (2019), South African meat consumption has drastically increased over the years, mainly white meat, due to its relevance to human nutrition and food safety. This significant rise in animal protein intake due to rapid growth in population size has imposed a tremendous strain on animal production and the animal feed industry (WHO, 2017). Therefore, it has become increasingly important to utilize species that can quickly meet this increased animal protein demand.

According to Mekuriaw and Asmare (2014), pigs which have a relatively higher feed conversion efficiency and faster growth rate than other farm species can serve as a source for improving food security in SSA. Despite this potential benefit, pig production is plagued by the lack of affordable feed resources in the region. Resource-limited farmers or smallholder farmers are compelled to use imported and expensive commercial feedstuffs that are relatively unaffordable and in short supply (Mthiyane and Mhlanga, 2017a, Chivandi *et al.*, 2012). Due to the exorbitant costs and scarcity of conventional protein sources, research has intensified in finding local alternative and inexpensive protein sources (Ncobela and Chimonyo, 2015) such as Marula seed cake (MSC), that can sustainably ensure improved pork productivity without compromising the nutritional demands of animals and production of protein-rich pork, predominantly by smallholder farmers in SSA (Hlongwana *et al.*, 2021a, Mabena *et al.*, 2022).

Marula seed cake is an agro-waste that is a by-product of oil extraction from the dry seeds of the ripe fruits of the marula tree (*Sclerocarya birrea A. Rich*). The use of MSC as an alternative protein source has been cited in the literature. The findings claimed that the composition of MSC makes it a good potential source of plant-based protein that can replace nutritional SBM in animal production (Mthiyane and Mhlanga, 2017a). However, the major challenge with feeding MSC in non-ruminant animals is the natural contamination by anti-nutritional factors that is the presence of tannins, saponins, phytate-phosphate, oxalate although they occur in low

concentrations (Malebana *et al.*, 2018a), and the prevalence of mycotoxins particularly Deoxynivalenol and T-2 toxins (Mthiyane and Mhlanga, 2017a).

Various filamentous fungi produce mycotoxins, usually of the *Aspergillus* and *Fusarium* genera. The high proportion of cereals in non-ruminant diets, especially pigs, makes them more vulnerable to mycotoxins. Exposure to high or continuous chronic low doses of mycotoxin-contaminated feed could lead to cancerous, teratogenic, mutagenic, oestrogenic, immunotoxic, or neurotoxic effects in pigs (Ratcliff, 2002b, Cunha *et al.*, 2018b). Consequently, this brought attention to finding effective ways to mitigate the negative effects of mycotoxins on piglets and pigs, including the use of nanoparticles.

Nanoparticles are any material in which one of their dimensions, either length, breadth, or height falls within 1 to 100 nm (Jeevanandam *et al.*, 2018). They have many interesting properties in biological research, including anti-microbial (Fernando *et al.*, 2018), anti-oxidative properties (Bedlovičová *et al.*, 2020). Nanoscale measurements have a broad surface area to volume ratio, and thus very specific properties. Literature is abundant on zinc oxide nanoparticles (ZnO-NP's) because of their vast bandwidth and strong exciton-binding capacity (Agarwal *et al.*, 2017). The use of ZnO-NPs in animal diets enhances weight gain, decreases feed intake, and improves feed conversion (poultry) (Ahmadi *et al.*, 2013, Zhao *et al.*, 2014), and no adverse effect on the growth of weanling pigs (Li *et al.*, 2016a). The ZnO nanoparticles that are currently reported by literature on animal nutrition have been synthesized through the physical and chemical techniques. However, physical and chemical synthesised nanoparticles make use of large rate of toxic chemicals with extreme environmental incompatibility (Parveen *et al.*, 2016). This has intensified the interest in the use of less costly, environmentally friendly and phytochemical-rich nanoparticles synthesised through various plant parts.

Tulbaghia violacea, commonly known as wild, sweet or society garlic, is an evergreen traditional medical plant that is growing, bulbous, perennial indigenously grown in the prolonged drought areas of South Africa (Harris, 2004). *T. violaceae* is high in thiol compounds and displayed strong antimicrobial properties (Ranglová *et al.*, 2015, Netshiluvhi and Eloff, 2016). These compounds are responsible for the glutathione synthesis which confer anti-oxidative effect in the animal cell (Mthiyane, 2006). There is scarcity of data which emphasizes the synthesis of ZnO nanoparticles using the *T. violacea* plant, the use of green synthesized nanoparticles on animal nutrition, and the use of *T. violaceae* synthesised nanoparticles on pig

production. This chapter focuses on giving a review on how population growth impacts food security, on the use of pig production to alleviate food insecurity, the use of alternative protein sources for pig production, and the potential impact of MSC to substitute SBM, how each major class of mycotoxins affects the pig production, and the use of phyto-encapsulated nanoparticles as a possible way to ameliorate the negative effect of these mycotoxins on pigs.

2.2 Human population growth and the role of pig production in food security in Southern Africa

The outlook for global food security is influenced by rising food demand, particularly in developing countries (Alston *et al.*, 2009). The predicted growth in the world population is expected to be accompanied by a doubling of the size of sub-Saharan Africa (SSA) by 2050 (99% increase) (Leridon, 2020a). The growing population of humans in this region presents socio-economic challenges for developing countries. Due to the high demand for animal-derived foods, population growth affects food security. Notwithstanding having the fastest population growth rate worldwide, Sub-Saharan Africa continues to represent a significant share, with one in four people in this region suffering from food insecurity due to low agricultural production (Prosekov and Ivanova, 2018).

Livestock production is an integral part of many countries' agricultural sectors because it provides high-quality animal protein food while also providing various socioeconomic benefits to communities (Capper, 2013). Due to their contribution to food security and nutrition, animal-derived food products will continue to be in high demand to feed this rapidly expanding human population (Godfray *et al.*, 2010). Amongst other contributing factors, pork production would be one of the most important ways to improve food security in this region. According to Mekuriaw and Asmare (2014), pigs have a relatively high feed conversion efficiency and high growth rate compared to other farm animals, making them a suitable tool for boosting food security for poor households in SSA (Prosekov and Ivanova, 2018).

Additionally, pigs are capable of transforming non-conventional animal feed into valuable by-products (Rodríguez-Estévez *et al.*, 2010). Pork, bacon, chops, and ham are high-quality proteins for human consumption that are produced from pigs, making them valuable for household food security, on their own or in exchange with grains. It provides an additional source of minerals and vitamins, but its primary role is to provide essential amino acids, which the human body is incapable of synthesizing and must be provided in a diet. Moreover, it is a cheaper source of animal protein for diets compared to beef, mutton, and chevon (Ironkwe and

Amefule, 2008). Genetically, pigs can convert feed to meat more efficiently than ruminants (DAFF, 2015a), so they can feed several consumers compared to other livestock species.

Pork remains the most consumed meat type worldwide, and global pork imports have increased by slightly more than 5% per year over the last eight years. In particular, South Africa is no exception; meat imports have increased significantly over the last decade, with poultry and pork accounting for the majority of the increase in import levels. In South Africa, pork remains a significant industry within the larger meat complex, accounting for only 7% of cumulative meat consumption in 2013 (BFAP, 2014). The South African pig industry contributes less than 0.5% to the world's pig production (Visser, 2014) and only 2.05% to South Africa's primary agricultural sector (DAFF, 2015b). Across the nine provinces of South Africa, pork is produced, with Limpopo and North-West being the top producers (Visser, 2014, DAFF, 2015b). Pigs can be produced under various production systems, providing the opportunity for people from various backgrounds to become pig farmers.

2.3 Pig feeding and nutrient requirements

For pigs to remain healthy and achieve their physiological demands, they must receive feed that is nutritious, palatable, and free of established gross contaminants, physical or toxic anti-nutritional factors and microorganisms (van Beers-Schreurs, 2002). Providing adequate nutrition to pigs at all stages of development is essential to pig safety. Feeding follows a staggered pattern due to nutritional demands changing as pigs grow (Heugten and Kempen, 2002). Consequently, nutrients are required for animal maintenance, growth and reproduction (Hu *et al.*, 2015). For improved production and productivity, animal nutritionists formulate feed that satisfies all of the pigs' nutritional requirements and are classified into six essential groups, namely, carbohydrates, fats, proteins, minerals, vitamins, and water.

All these nutrients required have significant roles, as they are all essential. Energy from carbohydrates and fats sources are crucial to the body's heat. It is also needed for physical activity such as walking, eating, and other energy-intensive tasks while pigs are involved (Pretorius *et al.*, 2007). According to Newton *et al.* (2005), proteins are necessary for pigs to build lean muscle, reproduce, and repair damaged tissues. Each protein comprises simpler compounds called amino acids, including lysine and methionine, which play a crucial role in pigs' growth and meat (protein) synthesis. Minerals which are classified as micro, macro, and trace elements all have a confirmed role in the physiological functioning of pigs and are ranked based on amounts required in their diets (O'Dell and Sunde, 1997)

Vitamins are essential nutrients that facilitate growth, maintenance, and health in animals. Every vitamin plays a well-defined metabolic role in the animal, and their importance depends on the animal's physiological status (McDowell, 2012). A significant part of a pig's diet is water. It is both a solvent and a primary transport medium for nutrients, chemical energy, metabolites, and waste products among cells and organs. Water also plays a central role in acid-base homeostasis and is the basis for chemical reactions (oxidation and hydrolysis). Pig producers are constantly challenged to provide a properly balanced diet because pigs are expected to grow to market weight in the shortest possible time frame without compromising good carcass characteristics in order to maximize profits (Kim *et al.*, 2010). Balanced diets for pigs should provide ample quantities of energy and protein to satisfy the requirements of pig nutrients, determined by certain factors (**Fig 2.1**). The NRC estimates nutrient requirements for various body weights of growing piglets at standard conditions (**Table 2.1**).

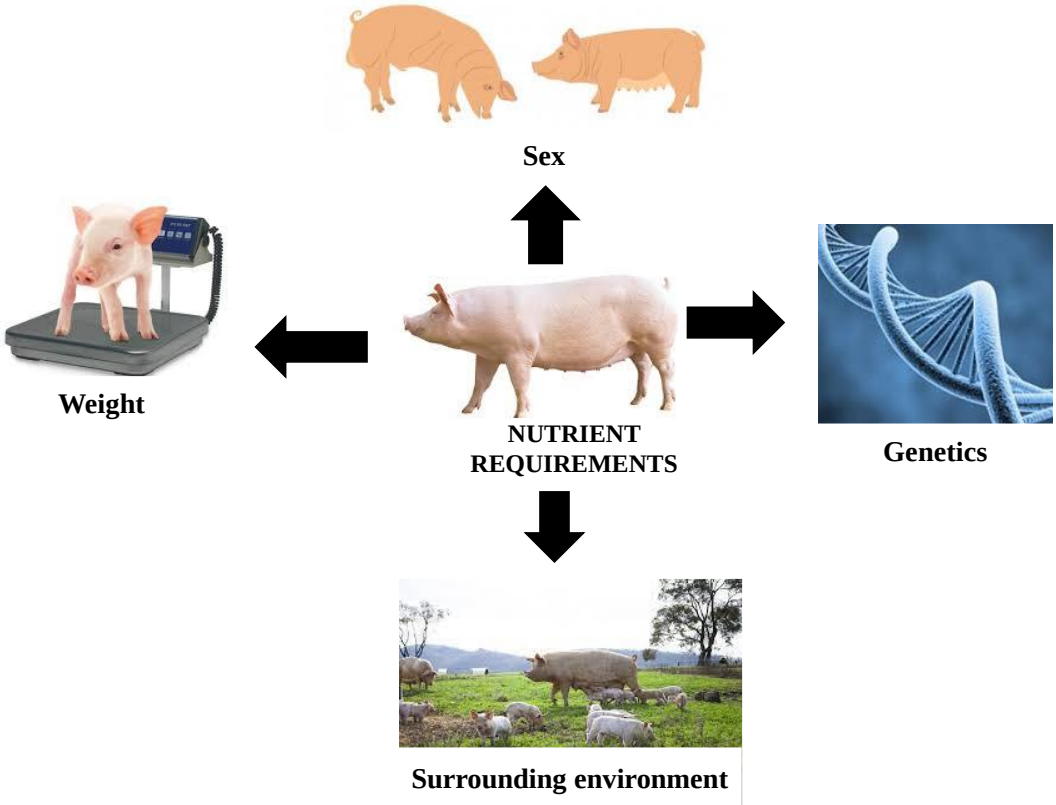

Figure 2. 1: Factors highly influencing the nutrient requirements in pigs.

Table 2. 1: Dietary nutrient requirements for growing pigs fed ad libitum feed (90% Dry Matter)

Nutrients	**Body Weight Range (kg)**						
	5-7	**7-11**	**11-25**	**25-50**	**50-75**	**75-100**	**100-135**
	Amino acids Total basis (%)						
Arginine	0.75	0.68	0.62	0.50	0.44	0.38	0.32
Histidine	0.58	0.53	0.48	0.39	0.34	0.30	0.25
Isoleucine	0.88	0.79	0.73	0.59	0.52	0.45	0.39
Leucine	1.71	1.54	1.41	1.13	0.98	0.85	0.71
Lysine	1.70	1.53	1.40	1.12	0.97	0.84	0.71
Methionine	0.49	0.44	0.40	0.32	0.28	0.25	0.21
Methionine + Cysteine	0.96	0.87	0.79	0.65	0.57	0.50	0.43
Phenylalanine	1.01	0.91	0.83	0.68	0.59	0.51	0.43
Threonine	1.05	0.95	0.87	0.72	0.64	0.56	0.49
Tryptophan	0.28	0.25	0.23	0.19	0.17	0.15	0.49
Valine	1.10	1.00	0.91	0.75	0.65	0.57	0.49
	Mineral elements						
Na	0.40	0.35	0.28	0.10	0.10	0.10	0.10
Cl	0.50	0.45	0.32	0.08	0.08	0.08	0.08
Mg	0.04	0.04	0.04	0.04	0.04	0.04	0.04
K	0.30	0.28	0.26	0.23	0.19	0.17	0.17
Cu	6.00	6.00	5.00	4.00	3.50	3.00	3.00
I	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Fe	100	100	100	60	50	40	40
Mn	4.00	4.00	3.00	2.00	2.00	2.00	2.00
Se	0.30	0.30	0.25	0.20	0.15	0.15	0.15
Zn	100	100	80	60	50	50	50

Adapted from (NRC, 2012).

2.3.1 Use of conventional protein sources and their limitations

The common conventional dietary protein sources for livestock in Sub-Saharan Africa are canola, cotton, groundnut, sesame, sunflower, palm kernel, groundnut, and soybean meal (Shipton and Hecht, 2005) due to their high amino acid profile and nutritional composition that is close to ideal (**Table 2.2**). In South Africa, however, SBM is the most commonly used plant-derived conventional protein source in the livestock feed industry (Acheampong-Boateng *et al.*, 2016). This protein source is an excellent source of lysine, arginine, and tryptophan as compared to other dietary protein sources. Also, SBM is deficient in cysteine, threonine, and high protein content making it more suitable for pigs and poultry (Baker, 2009).

Due to extreme climate change among other factors, soybean production in SSA does not meet up to the demand for SBM as required for human consumption and livestock feed industries, obligating the area to rely on extremely expensive imports (Sihlobo and Kapuya, 2016). For comparison purposes, the BFAP (2014) estimated that South Africa would need to import approximately one million tons of SBM by 2019 to compensate for a shortfall in local production in 2018. Furthermore, a rise in feed costs negatively impacts the intensification of animal production, making meeting the growing demand for animal products a challenge.

On the other hand, canola meal is a by-product of canola seed oil extraction (Khattab and Arntfield, 2009). However this by-product has been utilized in pig diets for a long time, its incorporation levels have been limited due to nutritional considerations, which stemmed mostly from early research that indicated deleterious impacts of dietary canola meal inclusion in pig diets (Mejicanos *et al.*, 2016). The presence of anti-nutritional factors (ANF; notably glucosinolates) in canola meals was responsible for the detrimental effects.

Therefore, smallholder farmers are adversely affected by not being able to afford this quality feedstuff and, as a consequence, the performance of their animals gets compromised. Also, the utilization of food rejects and the use of traditional plants as protein sources with unknown nutritional effects and value compromises the performance of animals. The scarcity of dietary protein supplies for livestock feed mandates that research be aimed at seeking alternatives to insufficient supply of SBM contributes to high costs, which translates into expensive animal feed (Malebana *et al.*, 2018a). Now, this mandates research to look for better inexpensive alternative protein sources that do not the compromise animal's production and health status.

Table 2. 2: Chemical composition of commonly utilized conventional protein sources in animal diets.

Components	Soybean meal	Rapeseed meal	Cotton seed meal	Sunflower Oil Cake	Canola seed meal
Dry matter	_	88.70	91.9	_	89.6
Crude protein	44.40	33.70	30.9	35.61	39.0
Ether extract	31.82	2.30	0.60	1.07	_
Neutral detergent fibre	15.51	28.30	49.2	43.86	32.2
Acid detergent fibre	9.5	19.60	33.4	27.59	18.6
Crude fibre	6.75	12.40	26.2	28.85	_
Ash	6.65	_	6.03	7.83	7.6

Source: Banaszkiewicz (2011), Dalle Zotte *et al*. (2013), Rodríguez *et al*. (2013), Maison (2014), Jannathulla *et al*. (2018).

2.3.2 Use of alternative protein sources and their limitations

Researchers are exploring the potential of seeds/nuts from indigenous fruit-bearing trees as alternative animal protein source, such as marula, macadamia, baobab and mucuna seed cakes (Mthiyane and Mhlanga, 2017a, Mthana, 2018, Nkosi *et al.*, 2019). Alternative protein sources are frequently equivalent to other conventional protein sources in terms of nutritional value, particularly their high crude protein as shown in **Table 2.3**, and energy content, and can thus be used to formulate rations (Gowda and Sastry, 2000, Raj *et al.*, 2016). The insufficient supply hampers the utilization of these protein sources in livestock production due to seasonal production and climate variations (Acheampong-Boateng *et al.*, 2016), and the occurrence of anti-nutritional factors (Martens *et al.*, 2012).

In addition to providing protein, leaf and seed meals also contain vitamins, minerals, oxycarenoids, and bioactive compounds that function at the cellular level (Melesse *et al.*, 2013). Previous research has shown that baobab seeds have high nutritional value. Anti-nutritional factors found in baobab seeds, for example, include oxalate (10%), phytate (2%), tannins and saponins (3-7%), all of which impair digestive efficiency and nutrient utilization in non-ruminants (Bale *et al.*, 2013).

The majority of small-holder farmers do not understand their animals' dietary/nutrient requirements, nor do they know how nutritious these alternative feedstuffs are. For pig diets, oil seed cakes have the potentials to be used as protein feedstuffs and energy sources, respectively (Hlongwana *et al.*, 2021a). However, these seed cakes consist of anti-nutritional factors (ANFs) such as tannins, lectins, gossypol, protease inhibitors, saponins, phytic acid, and mycotoxins at different concentrations (Francis *et al.*, 2001). Such alternatives may have deficient nutritional content due to fibre-based nitrogen and the abundance of such ANFs (Shayo and Udén, 1999). These compounds are believed to suppress or bind protein enzymes and decrease its digestibility.

Table 2. 3: The nutritional composition of some alternative protein sources

Components	Marula seed cake	Macadamia nut meal	Baobab nut meal	Watermelon seed cake	Mucuna seed cake
Dry matter	94.66	92.0	91.4	95.5	89.8
Crude protein	47.0	13.6	25.4	25.4	30.0
Ether extract	34.35	16.4	10.9	7.84	4.5
Neutral detergent fibre	33.8	49.9	41.8	_	_
Acid detergent fibre	16.8	39.4	37.8	_	_
ADL	9.44	3.00	19.72	_	_
Crude fibre	_	_	_	27.4	8.8
ASH	5.43	_	_	2.70	3.4

Source: Siddhuraju and Becker (2001), Mustafa and Alamin (2012), Mthiyane and Mhlanga (2017a), Nkosi *et al.* (2019).

2.3.3 Anti-nutritional factors and strategies to resolve them in alternative protein sources for pigs

Anti-nutritional factors (ANFs) are substances that, either by themselves or by their metabolic products, reduce the nutritional value of animal feed. These ANFs, also known as 'secondary metabolites' in plants, have been shown to be biologically active to an exceptional degree (Gemede and Ratta, 2014). Plants produce a wide range of chemicals, from simple to very complex, with many of them having been identified and characterized. Plenty of them are biotic and abiotic pressures, and more than 1200 groups are designed to combat herbivory. They do not participate in the primary plant metabolic processes for cell growth and reproduction (Makkar *et al.*, 2007).

A substantial portion of the protein used in pig feed is derived from legumes, cereals, and seed meals, which contain high concentrations of macro and micronutrients, also naturally contaminated by anti-nutritional factors. Tannins, phytic acid, saponins, gossypol, lectins, amylase inhibitors, protease inhibitors, and goitrogens are major anti-nutritional factors found in edible crops (Akande *et al.*, 2010). A significant concern is the interaction of anti-nutritional factors with nutrients because they limit the bioavailability of nutrients. Furthermore, other substances found in legumes and cereals, such as trypsin inhibitors and phytates, contribute to reduced protein digestibility and mineral absorption. In addition, mycotoxins are among the foremost anti-nutritional factors, whose impact differs with different species and the concentration of the toxins. Anti-nutrients decrease the bioavailability of other feedstuffs in a significant way, particularly the availability of micronutrients and minerals (Samtiya *et al.*, 2020).

There are various conventional approaches and techniques to combat the level of these anti-nutrient factors and approaches to combat their effect in animals. For example. fermentation, germination, debranning, autoclaving, soaking, and other processing methods and techniques are used to mitigate the anti-nutrient content of feeds (Martens *et al.*, 2012). Alternatively, feed additives can be used to counteract the effect caused by these anti-nutritional factors, such as prebiotics, probiotics, and enzymes (Lucio *et al.*, 2021). A variety of methods, when applied individually or in combination, have the potential to minimize or combat their impact on animal performance and production.

2.3.3.1 Mycotoxins and their effect in pigs

The bioactive compounds, mycotoxins, are produced by filamentous fungi or molds (Conte *et al.*, 2020). They could occur in food at all stages of the food chain due to contamination by toxic fungal genera particularly *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* (Keller *et al.*, 2005, Piacentini *et al.*, 2019). A review study by (Yang *et al.*, 2020b) along with others confirms the presence of mycotoxins with biological activity, such as aflatoxin, ochratoxin, fumonisin, zearalenone, deoxynivalenol, citrinin, patulin, and ergot alkaloids, in a significant proportion of feed samples. Such compounds are commonly reported in foodstuffs such as maize, peanuts, almonds, figs, and a number of other foods and feeds (Fung and Clark, 2004). They are physically and thermodynamically stable substances that can withstand milling, drying, cooking, and baking, and their consumption can result in mycotoxicosis in both animals and humans (Sainz *et al.*, 2015, Buszewska-Forajta, 2020).

Mycotoxins are more likely to be found in non-ruminant livestock such as pigs and poultry, which consume a high proportion of cereal-based feeds (Wan *et al.*, 2020b). They exhibited overlapping toxicity toward animals and microbes (Bennett, 1987). These toxins may be carcinogenic, immunotoxic, mutagenic, neurotoxic, oestrogenic, or, teratogenic, depending on their precise nature (Ratcliff, 2002a). This section of the review classifies and goes through the economic impact of the mycotoxins, and further discusses the effect these mycotoxins have on pigs and pig production.

2.3.3.1.1 Their classification (types) and economic significance

The five major parent mycotoxins of importance to animal health include the *aflatoxins*, *fumonisins*, *trichothecenes*, *ochratoxin*, *zearalenone* (Pal *et al.*, 2015). Mycotoxins can cause economic loss by causing reduced production, growth, immunosuppression and agalacia, as well as other effects (Bryden, 2012a, Oswald *et al.*, 2005, Wild and Gong, 2009). They have economic and commercial side effects in the sense that both productivity and the nutritional value of the infected feedstuff is affected, also affecting the production and health of the animals produced by this feed (Ratcliff, 2002a).

2.3.3.1.1.1 Classification

2.3.3.1.1.1.1 *Aflatoxins (AFs)*

Aspergillus genera are the primary producers of aflatoxins (AFs) particularly *Aspergillus flavus*, *A. nomius* and *A. parasiticus* species (Ali *et al.*, 2005, Alcaide-Molina *et al.*, 2009). A number of agricultural commodities prone to contamination by AFs including cocoa beans, cotton seeds, fruits, peanuts, maize, sorghum, spices, rice, and vegetables (Makun *et al.*, 2012).

In South Africa, the levels of occurrence of aflatoxins in animal feed ranges from 0.81-156 ppb (Mngadi *et al.*, 2008, Mwanza, 2012). The primary aflatoxin molecules to affect animal production and health are grouped as difurocoumarocyclopentenone group (AFB1, AFB2, AFM1, and AFM2) and difurocoumarolactone group (AFG1 and AFG2) (Ismail *et al.*, 2018, Benkerroum, 2020) their biochemical structures are presented in **Figure 2.2**.

AFB1 are classified as a class 1 human carcinogen by The International Agency for Research on Cancer with AFG1, and AF Milk 1 (AFM1) (IARC, 2002, Turner *et al.*, 2009, Wild and Gong, 2009). AFB1 is the most significant aflatoxin synthesized by toxigenic species and has attracted the most attention (Bennett and Klich, 2003). AFG2 and AFB2 are classified as probable human carcinogens in Group 2 (IARC, 2002, Reddy *et al.*, 2010). AFM1 is a hydroxylated AFB1 metabolite predominantly identified mostly in animal tissues and body fluids such as milk and urine (Richard, 2007, Wild and Gong, 2009, Reddy *et al.*, 2010). AFM1 is considered to be both hepatotoxic and carcinogenic, but its toxicity and carcinogenic risk are reported to be 8-10 times lower than AFB1 (D'Mello *et al.*, 1997, Wild and Gong, 2009).

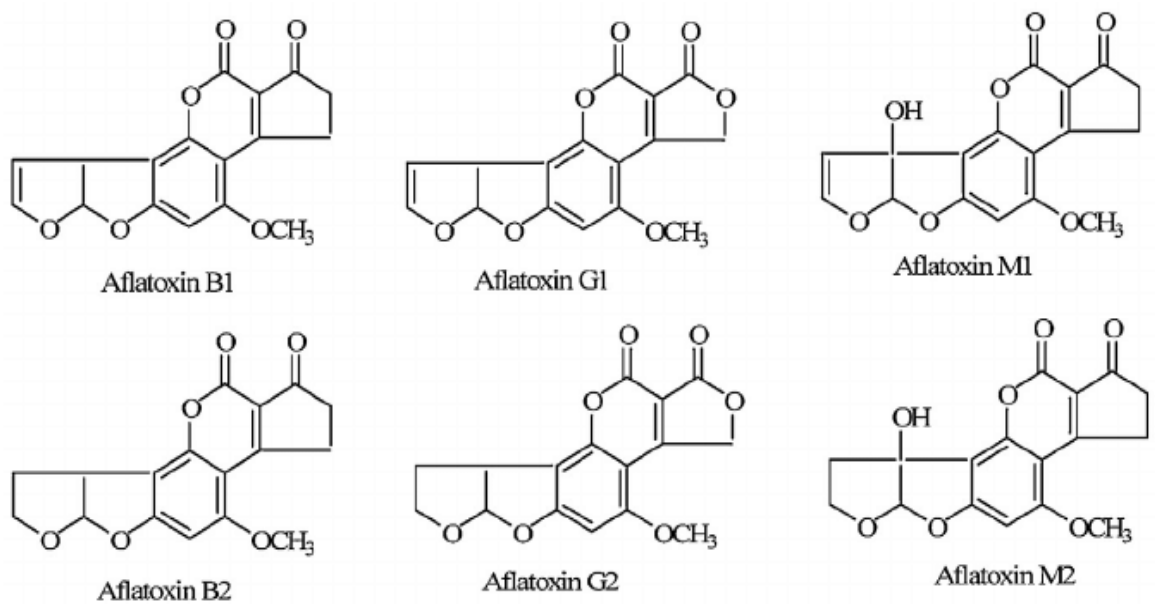

Figure 2. 2: Biochemical structures of Aflatoxin B1, B2, G1, G2, M1, M2 (Dhanasekaran *et al.*, 2011).

2.3.3.1.1.1.2 *Fumonisins (FMs)*

Fumonisins (FMs) are typically categorized as Fusarium toxins because they are produced by numerous organisms in this genus, with *F. verticillioides* and *F. proliferatum* being the primary producing species. *A. niger*, on the other hand, was discovered to also produce FMs (Streit *et al.*, 2012). Fumonisins were initially found in South Africa by Gelderblom *et al.* (1988). They are a class of non-fluorescent mycotoxins hypothesized to be synthesized by the condensing of amino acids into an acetate-derived precursor (Bennett and Klich, 2003, Sweeney and Dobson, 1999).

The B-series FMs (FBs), which include fumonisins B1, B2, B3, and B4 (**Figure 2.3**), are the most important of the 16 fumonisin analogs known to date (Streit *et al.*, 2012, da Rocha *et al.*, 2014). It is known that fumonisin B1 (FB1) is the most toxic and most prominent member of the FM family and is regarded as potentially human carcinogenic. (Marroquín-Cardona *et al.*, 2014). Fumonisin B2 (FB2) is also toxicologically relevant. In animals, consumption of FB infected feed may cause severe illnesses in horses, pigs, and rabbits which are considerably more sensitive than cattle and poultry (CAST, 2003, Marin *et al.*, 2013). FB1 is associated with pulmonary oedema in pigs, which is usually accompanied by reduced feed intake, dyspnea, fatigue, cyanosis, and death (CAST, 2003, da Rocha *et al.*, 2014, Groopman *et al.*, 2014).

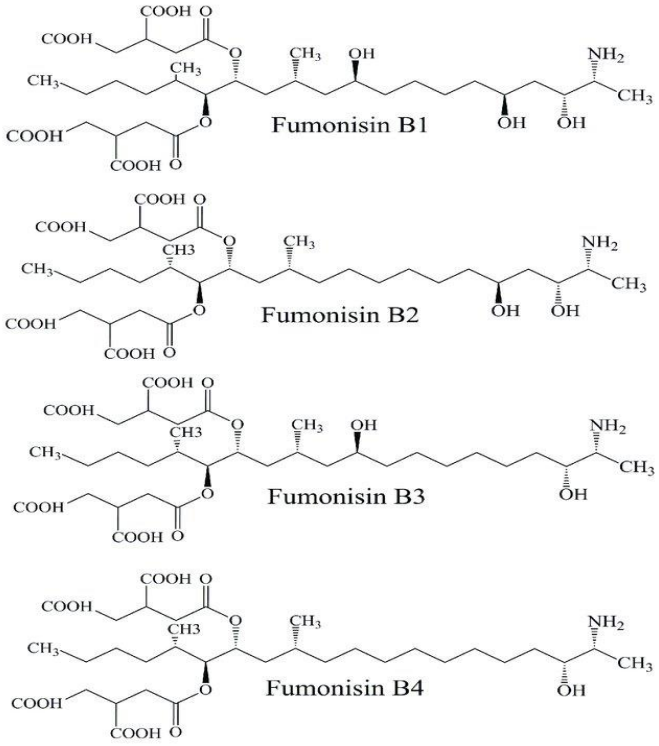

Figure 2. 3: Biochemical structures of Fumonisins B1, B2, B3 and B4 (Kostić *et al.*, 2019).

2.3.3.1.1.1.3 *Zearalenone (ZEN)*

Zearalenone (ZEN), formerly known as F-2 toxin, is a Fusarium mycotoxin produced primarily by *F. graminearum* but also by *F. culmorum*, *F. cerealis*, and *F. equiseti*, among others, and has derivatives α-Zearalenol (α-ZEL) and β-Zearalenol (β-ZEL) (Marin *et al.*, 2013, da Rocha *et al.*, 2014). The biochemical structure is presented (**Figure 2.4**). When ZEN shares structural similarities with the female sex hormone estradiol, it is then categorized as a nonsteroidal estrogen (Mahato *et al.*, 2021). Due to its chemical feature, it can bind estrogen receptors, causing deleterious consequences in both animals and humans such as reproductive problems and hyperestrogenism (CAST, 2003, da Rocha *et al.*, 2014). According to IARC (2012), ZEN belongs to group three, which implies that its carcinogenicity is not classifiable. In livestock, reproductive disorders include enlarged reproductive tracts in pigs, abnormal ovulation, fibrosis on uterine walls, and abnormal foetal development (Keller *et al.*, 2015).

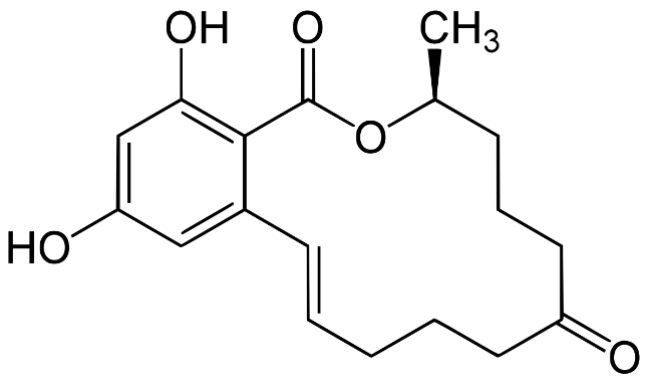

Figure 2. 4: Biochemical structure of Zearalenone (Kostić *et al.*, 2019).

2.3.3.1.1.1.4 *Trichothecenes (TRCs)*

Trichothecenes (TRCs) are primarily produced by Fusarium species, but not solely, as some *Cephalosporium*, *Myrothecium*, *Stachybotrys*, and *Trichoderma* species also produce these mycotoxins. These fungal metabolites are a broad class, containing more than 150 structurally linked compounds categorized into four chemical groups (A to D), with the most chemical structures of the most common TRCs (**Figure 2.5**) (CAST, 2003, Marin *et al.*, 2013) . Type A and B TRC's are crucial. Type A-TRCs are mostly constituted of HT-2 and T-2 toxins, whereas type B-TRCs are mostly comprised of deoxynivalenol (DON), its derivatives 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), and nivalenol (NIV) (Juan *et al.*, 2013). HT-2 and T-2 are the most toxic members of type A-TRCs, despite

their low prevalence. DON is the most common trichothecene found in animal feed worldwide (Meier, 2021).

In animals, they inhibit protein synthesis and DNA synthesis and have been found to weaken cellular immunity (Streit *et al.*, 2012, Groopman *et al.*, 2014). Low milk production, low egg and milk production, bloody diarrhoea, haemorrhaging, oral lesions, and death can occur in some cases where animals have been intoxicated with TRCs. Although it is not among the most acutely toxic TRCs, DON is considered very relevant to animal science because it is highly incidental (CAST, 2003, Streit *et al.*, 2012, Groopman *et al.*, 2014). Pigs tend to be more severely affected by DON exposure, as it causes feed refusal, vomiting, and anorexia in addition to the symptoms previously described (CAST, 2003). Furthermore, excessive levels of exposure result in increased susceptibility to infections and decreased animal performance (CAST, 2003, Marin *et al.*, 2013). Considering its carcinogenicity to humans, IARC has classified DON as unclassifiable (group 3) (IARC, 2012).

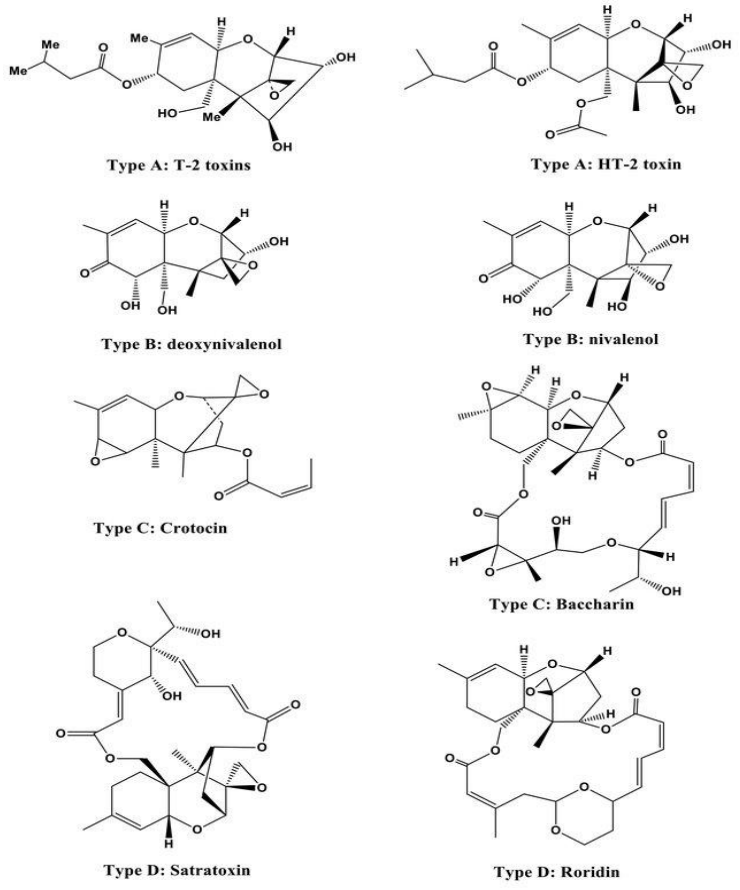

Figure 2. 5: Biochemical structures of Trichothecenes types (A, B, C and D) (Adhikari *et al.*, 2017).

2.3.3.1.1.1.5 *Ochratoxins (OTs)*

The Ochratoxins (OTs), OTA, OTB and OTC (**Figure 2.6**), are primarily produced by fungi of the genera *Aspergillus* and *Penicillium*, specifically *A. ochraceus*, *A. carbonarius*, *P. verrucosum*, and P. *nordicum* (CAST, 2003, Milani, 2013, Piotrowska *et al.*, 2013, de Rocha *et al.*, 2014). OTAs are known to produce considerable nephrotoxicity in exposed animals as a result of exposure to natural amounts in feed, as the kidneys are the primary target organ (Marin *et al.*, 2013, Marroquín-Cardona *et al.*, 2014). In fact, OTAs are known to cause endemic nephropathy in pigs (Marin *et al.*, 2013, Marroquín-Cardona *et al.*, 2014).

When consumed at high dietary doses, this toxin can damage the liver and necrose the intestinal and lymphoid tissues (CAST, 2003, Groopman *et al.*, 2014). OTAs play a role in a fatal kidney illness that is endemic in Balkan nations (Balkan endemic nephropathy) and have been classified as potentially carcinogenic, according to human toxicology (group 2B) (CAST, 2003, Marin *et al.*, 2013, Marroquín-Cardona *et al.*, 2014). Additionally, public health concerns have been raised concerning the transfer of OTA to animal-derived foods (Streit *et al.*, 2012).

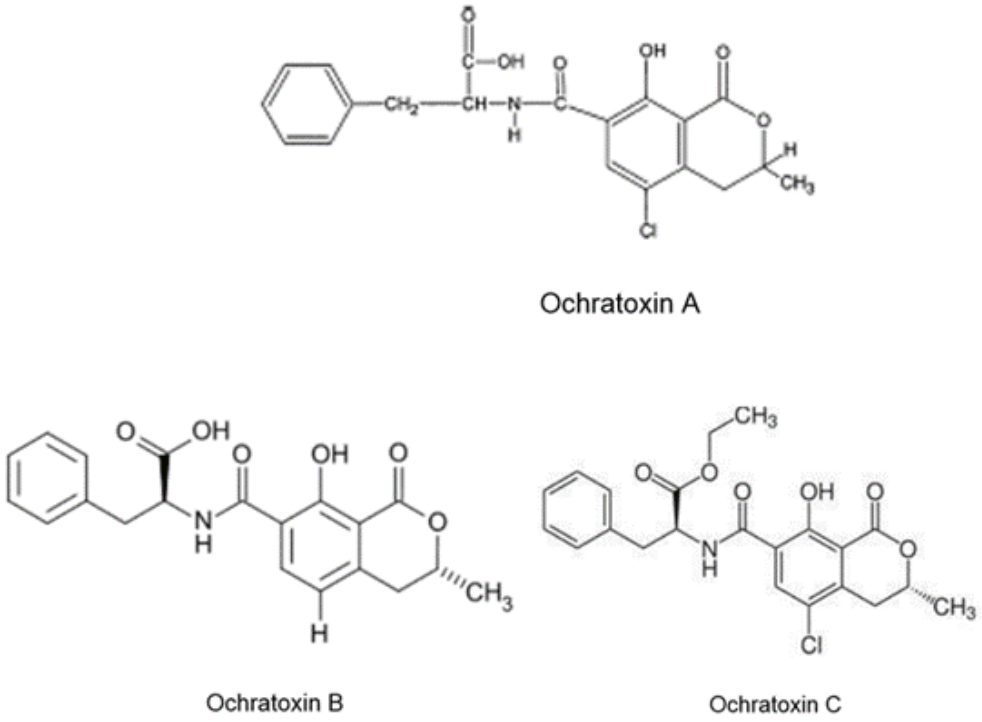

Figure 2. 6: Biochemical structures of Ochratoxins types (A, B and C) (Ibrahim and Menkovska, 2018).

2.3.3.1.1.2 Economic Significance of mycotoxins

Several studies conducted worldwide have demonstrated the economic impact of mycotoxin contamination. Mycotoxins contaminate more than 25% of the world's food and feed, resulting in significant economic losses and negative consequences for human and animal health, affecting household security, income, productivity, and livelihood (Enyiukwu *et al.*, 2014). Animals consuming mycotoxin-contaminated feeds may experience adverse health effects such as stunted growth, impaired immunity, significantly reduced disease resistance, chronic and acute disease, and, in the severe scenario, they may lead to death. In addition to decreased animal performance caused by mycotoxins, there are other economic consequences. The economic impacts of the three most common mycotoxins (aflatoxins, deoxynivalenol, and fumonisins) in agriculture, particularly in livestock production include the costs of mitigation techniques, and so as research methods and regulations (Oliveira *et al.*, 2014, Pinotti *et al.*, 2016, Smith *et al.*, 2016). Economic concerns also include the cost of regulatory and research programs to identify ways to mitigate human and animal health risks.

Animal production is adversely affected by these secondary metabolites, which is a global concern for the livestock industry (Marroquín-Cardona *et al.*, 2014, Pinotti *et al.*, 2016). Thus, health barriers to the feed supply chain, including mycotoxins, impose significant restrictions on animal production systems (Grenier *et al.*, 2016, Pinotti *et al.*, 2016). These metabolites cause disruptions in the feed industry by affecting the quality of the product. Mycotoxin pollution results in diminished product quality in feed sectors and may affect the removal and disposal of heavily polluted crops. These industries suffer high economic losses as a result of mycotoxin contamination. Also, afflicted livestock through the consumption of heavily intoxicated crop species, including disease, morbidity, mortality, and contamination of animal products (Zain, 2011). As a result, agricultural producers experience significant economic losses due to a reduction in quality and quantity of animal products (Bhat and Vasanthi, 2003). Even the infected feed and animal products may be transferred to human diet (Richard, 2007, Krska *et al.*, 2008), resulting in protein-energy deficiency, in particular among young children (Katerere *et al.*, 2008, Wild and Gong, 2010).

2.3.3.1.2 Factors affecting mycotoxin occurrence in pig feeds

Initially, mycotoxin contamination can occur at farm locations (before or during harvest), during transportation, storage, and even during livestock feed production. It can also happen at the livestock farm before the pigs consume the feed (Udomkun *et al*., 2017). In addition, feed components may also influence mycotoxin contamination in pig feed, thus affecting the final level of contamination. Therefore, to protect pigs from exposure to mycotoxins, feedstuffs and feeds must be measured for mycotoxin occurrence and concentration before the pigs' consumption.

The production and occurrence of mycotoxins are affected by multiple factors, and they can be classified physically, chemically, or biologically. Relative humidity, temperature, insect infestation, and other environmental factors conducive to the colonization and development of microorganisms that produce mycotoxins would be considered physical factors; chemical factors would include fertilizers; and biological factors would include the association of the toxigenic organisms with the substrate (D'Mello *et al*., 1997). These factors are clearly presented (**Figure 2.7)**, and so as to how they get transmitted along the food chain (Cinar and Onbaşı, 2019).

Moisture, water, temperature, time, and composition are all essential factors in the development of fungi and production of fungi metabolites (Iram *et al*., 2016, Hassan *et al*., 2017, Nayak *et al*., 2017). The optimal growth temperature for mycotoxin production differs by species, the strains, and medium of growth, and these factors can be summarized as climatic factors. According to Streit *et al*. (2012), regardless of the severity of rain or dry conditions, the impacts of weather conditions on mycotoxin occurrence were observed to increase across various mycotoxin species. Most fungi grow best at temperatures ranging from 5 to 35°C, with 25°C being ideal (Hassan *et al*., 2017, Leggieri *et al*., 2017), influenced by the post-harvest drying temperature (Hawkins *et al*., 2005).

In the agri-value chain, high moisture content (5–25%) increases fungal development and the production of mycotoxins (Hassan *et al*., 2017). Several fungus species thrive at water activities ranging from 0.87 to 0.99, however, no mycotoxins are identified at 0.93 (Leggieri *et al*., 2017). The greater the fungal colonies with the larger diameter with increasing water activity, the longer the incubation/storage time occurred. **Table 2.4** presents optimum temperature and water activity for the growth of the significant mycotoxins in feedstuff.

In general, mycotoxins are synthesized under acidic conditions (Sulyok *et al.*, 2007, Sandoval-Contreras *et al.*, 2017). They are most active between 4.0 and 4.5 pH, and are reduced between 5.5 and 8.0 pH, which explains their acid tolerance (Brzonkalik *et al.*, 2012). As a result of the facultative anaerobic nature of fungi, high gas (CO_2) concentrations enhance biomass without negatively impacting the growth rate (Brzonkalik *et al.*, 2012, Gacem and El Hadj-Khelil, 2016). Several factors promote fungus growth, including temperature and water activity, according to Milani (2013). The most common way mycotoxin enters animal feed is through mycotoxin-contaminated crops used during feed formulation and poor storage conditions. In particular, smallholder farmers experience significant economic losses when crops are destroyed due to mycotoxin contamination, which is why damaged crops are often used as animal feed. Consequently, animals are at risk for various diseases caused by mycotoxin-contaminated feed (Edwards, 2004).

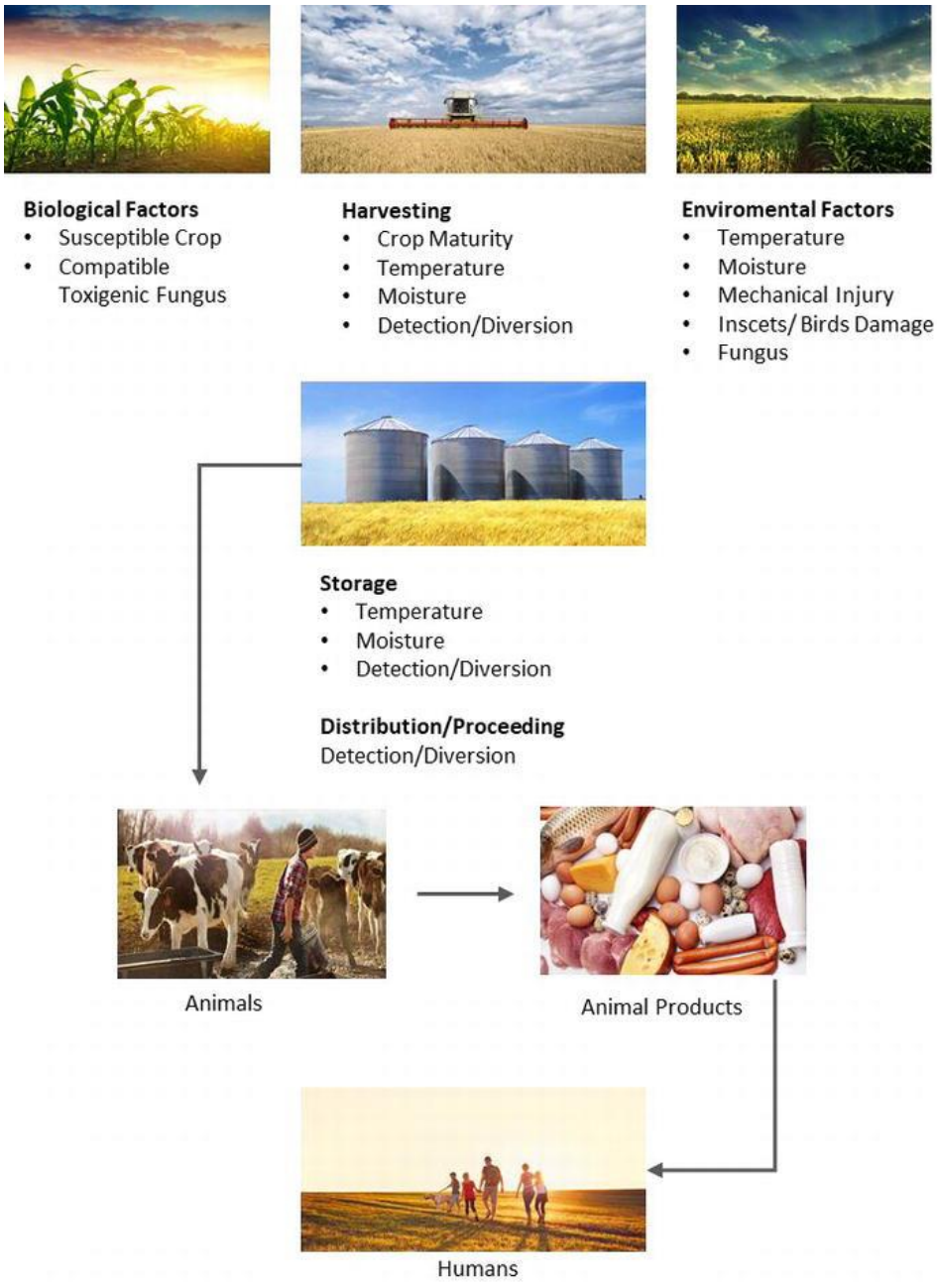

Figure 2. 7: Factors affecting mycotoxin occurrence in the food and feed chain.

Table 2. 4: The favourable temperature and water activity for the growth of mycotoxins

Mycotoxin	**Water activity**	**Temperature (°C)**
Aflatoxins	0.99	33
Ochratoxins	0.98	25–30
Fumonisins	0.9–0.995	15–30
Zearalenones	0.9–0.995	25
Deoxynivalenol	0.995	26–30
Citrinin	0.75–0.85	20–30

Adapted from: Milani (2013).

2.3.3.1.3 Effects of mycotoxins on pig production and meat quality

Pigs are the species most vulnerable to aflatoxins (Meissonnier *et al.*, 2008, Thieu *et al.*, 2008). Consequently, Dersjant-Li *et al.* (2003) pointed out that aflatoxins are immune-suppressors and have different effects on pigs, such as slower growth rates in young piglets and weaners, agalactia and abortion in sows. When exposed to aflatoxin, there is a consistent drop in feed conversion efficiency and decreased growth rate (7-10%) in pigs and poultry. Generally, feeding efficiency is less in pigs exposed to aflatoxins than in pigs that have not been exposed (Williams *et al.*, 2004). Also, as noted by Pu *et al.* (2021), aflatoxin exposure at 280 g/kg resulted in lower growth performance indicators such as average daily feed intake, average daily gain, and body weight.

Fumonisins are known also to affect the growth performance of pigs. Piglets consuming pure synthesised or naturally contaminated FB1 feed also experience a slight reduction in body weight and feed intake (Bouhet and Oswald, 2007). Marin *et al.* (2013) reported that fumonisins at a dose of 8 mg/kg affected piglets' weight differently according to their gender: the dose reduced male weight gain while the dose did not have an effect on female weight gain. The study by (Wan *et al.*, 2020a) fed piglets varying levels of fumonisins (7.2-25.1 mg/kg) found that average daily feed intake, average daily gain, and grain to feed ratio were adversely affected by the fumonisins. Also, the exposure of these fumonisins affected nitrogen retention.

Another class of mycotoxins that affects growth in pigs is trichothecenes. Pigs fed 1 to 3 mg/kg of deoxynivalenol reduce the growth rate (Marin *et al.*, 2013). Pigs receiving dietary deoxynivalenol consume less feed and gain less weight (Patience *et al.*, 2014). Deoxynivalenol reduces the weight gain and feed intake of pigs when used in naturally contaminated diets, and each extra mg/kg of DON lowers the gain by 8% (Dersjant-Li *et al.*, 2003). According to De Lucca and Walsh (2015), finishing pigs exposed to 18.53 mg/kg DON decreases the live weight, feed consumption and nutrient retention. Furthermore, Wellington *et al.* (2020) found that the consumption of DON adversely affected average daily feed intake, average daily gain, grain production and nitrogen retention. Accordingly, DON exposure reduces pigs' feed intake, negatively affecting their growth.

Components of animal feed, such as plants, can be contaminated by mycotoxins, leading to contaminated feed, meat, and meat products. In meat, the concentration of OTA varies between 10 - 90% and is determined by many factors, such as the content in feed, the feeding period, and the time of slaughter (Milićević *et al.*, 2008). Colour, pH, tenderness, and marbling are the

major aspects of measuring meat quality (Muchenje *et al.*, 2008). There is no comparative literature on the effect of mycotoxin-contamination on pork quality and carcass characteristics. **Table 2. 5** presents the effect that different mycotoxin species have on pigs' growth performance and haemo-biochemical parameters at different concentrations and time periods.

Table 2. 5: Effect of different mycotoxin species on growth performance and haemo-biochemical parameters.

Mycotoxin class	Model, exposure level, and period of study	Sample size	Origin of study	Main findings	Reference
Aflatoxin B1	Duroc×Landrace×Large white pigs treated with 5.3 and 372.8 µg/kg AFB1 for 42 days.	32	China	↓Numbers of neutrophils, monocyte, and total leukocyte (372.8 µg/kg). ↓Serum superoxide dismutase , catalase, and glutathione peroxidase. ↑Total liver superoxide dismutase No effect on serum concentration parameters	Fu *et al.* (2013)
	Duroc×Landrace×Yorkshire treated with 280 µg/kg AFB1 for 102 days.	14	China	↓Body weight, average daily feed intake and average daily gain. ↑ Superoxide dismutase ↓apparent total tract digestibility and damaged intestinal barrier integrity (related to the intestinal oxidative capacity)	Pu *et al.* (2021)
Deoxynivalenol	Pigs fed diets with 18.53 mg/kg for 11 weeks	48	Germany	↓Live weight ↓Feed consumption ↑ Nutrient retention	Goyarts and Dänicke (2005)
	3, 6 or 12 mg DON/kg	24	China	↓Serum total superoxide dismutase and glutathione peroxidase ↓Serum L-valine, glycine, L-serine, and L-glutamine ↑lymphocyte cell numbers	Wu *et al.* (2015)

				↑ alkaline phosphatase, blood urea nitrogen, alanine transaminase and aspartate aminotransferase	
	Finishing pigs fed with 1, 3, or 5 ppm for 42 days	200	Austria	↓Average daily feed intake and Average daily gain ↓gain:feed ↓ Nitrogen retention	Wellington *et al.* (2020)
	Weaned piglets fed diets with 1.9 mg/kg for 35 days.	36	USA	↓serum aspartate aminotransferase/alanine aminotransferase ↓blood urea nitrogen/creatinine ↓glucose ↓glutathione in jejunum	Holanda and Kim (2021)
	2.5 mg/kg for 28 days	120	Raleigh	↓ Body weight ↑ Alpha-lipoic acid ↑urea nitrogen/creatinine	Holanda *et al.* (2021)
Fumonisin	Crossed weaned piglets fed 8 mg FB1/kg for 28 days	20	France	↓ weight gain in males but had no effect in female ↑creatinine ↓specific antibody levels and mRNA expression level of IL-10 males	Marin *et al.* (2006)

	Weaned piglets fed at varying levels (7.2, 14.7, 21.9, 32.7, and 35.1 mg/kg) for 28 days.	350	USA	↓Average daily gain, average daily feed intake and gain: feed ratio ↑serum sphinganine-to-sphingosine ratio	Rao *et al.* (2020)
	2 mg/kg for 42 days	_	Austria	↑Serum sphinganine-to-sphingosine ratios	Masching *et al.* (2016)
Ochratoxin	TOPIGS-40 Weaned piglets fed 0.05 mg/kg for 33 days	12	France	↓total protein, albumin and nitric oxide in plasma, and interleukin-6 in the liver. ↑alanine aminotransferase and triglycerides in plasma and of superoxide dismutase in the liver.	Marin *et al.* (2006)
Zearalenone	Duroc×Landrace×Large white female weaned piglets, treated with 1.11 mg/kg ZEN for 28 days.	_	China	↑Genital organ weight ↓Serum superoxide dismutase ↓ No. of red blood cells and platelets.	Cheng *et al.* (2019)
	Duroc × Landrace× Large white female piglets treated with 1.0 mg/kg ZEN for 21 days.	32	China	↑length, width and area of vulva, the genital organ coefficient.	Su *et al.* (2018)
	1158.67 µg/kg for 21 days	24	China	↑Liver weight Caused cell damage on liver, uterus, and ovary of gilts (oxidative stress)	Zhang *et al.* (2021)

2.3.3.1.4 Biochemical and physiological parameters of mycotoxins

Biochemical parameters are sensitive indicators of animal health. They are an important metabolic profile test in animals (Oetzel, 2004). The biochemical profile evaluates the function of vital organs, measures electrolytes, and identifies circulating enzyme levels. According to (Magnus and Lali, 2009), serum biochemical parameters such as total protein, serum albumin, serum globulin, serum calcium, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), urea/BUN, and creatinine are critical for assessing animal health status. Also, one other important aspect that is important in biochemical parameters that relate to the health status of the animal which is either affected by the diet or their contamination, is the level of enzymes.

Consumption of large doses of aflatoxins causes acute toxicity that not only results in feed refusal and weight loss in pigs, but also causes changes in liver, kidney function, and also affects biochemical parameters (Phillips *et al.*, 1988). Consumption of aflatoxins by pigs results in increased alkaline phosphatase activity, cholinesterase activity, aspartate transaminase activity, and γ-glutamyltransferase decreased levels of albumin, urea nitrogen, cholesterol, total protein, magnesium, calcium, potassium, and phosphorus (Harvey *et al.*, 1991). In a study conducted by Fu *et al.* (2013) dietary aflatoxin B1 at 5.3 and 372 μg/kg to pigs decreased the serum superoxide dismutase (SOD), catalases (CAT) and glutathione peroxidases (GPx) though there were no effects on serum parameters. On the other hand, the exposure to 372 μg/kg decreased the number of neutrophils, monocytes, and total leukocytes.

Furthermore, aflatoxins reduce the ability of the RNA polymerase to transcribe DNA into mRNA in the nucleus (Yu *et al.*, 1996), thereby decreasing cell protein synthesis and increasing cell death (Zimmermann *et al.*, 2015). Aflatoxin B1's most noticeable impact is that it affects both cell-mediated immunity and has been proven to enhance inflammation (Seeboth *et al.*, 2012). In addition, aflatoxin B1 is carcinogenic and modulates immunity (Wild and Gong, 2010). As an outcome, AFB1 increases reactive oxygen species (ROS) and causes biomolecular oxidative damage in spleen mononuclear cells, however AFB1 and AFB2 combined have significantly higher pro-oxidant activity (Theumer *et al.*, 2010). Pu *et al.* (2021) also noted that exposure to aflatoxin B1 at 280 μg/kg decreases the intestinal oxidative capacity.

Deoxynivalenol causes changes in blood and serum biochemistry, as well as the systemic immune response in growing pigs (Swamy *et al.*, 2002, Accensi *et al.*, 2006). Animal studies have shown that dietary DON increases total immunoglobulin (Ig)A levels in the serum and interferes with the function of dendritic cells (Accensi *et al.*, 2006, Bimczok *et al.*, 2007). A

different study shows that the levels of IgG, IgM, and IgA secretion are significantly lower in murine lymphocytes treated with DON Berthiller *et al.* (2006). DON also regulates the particular immune response to ovalbumin immunization by increasing IgA and IgG levels against ovalbumin (Pinton *et al.*, 2009). It is also noted to increase the lymphocyte cell number, alkaline phosphatase, blood urea nitrogen, alanine amino transferase and aspartate aminotransferase (Wu *et al.*, 2015). In a different study by Holanda *et al.* (2021), dietary DON increases the ALA, urea nitrogen and creatinine. However, Holanda and Kim (2021) noted that at 1.9 mg/kg, dietary DON decreases some serum activities such as the blood urea nitrogen, and glucose.

The common biochemical mechanism underlying the harmful effects of mycotoxins in pigs involves oxidative stress. Oxidative stress occurs when the antioxidant capacity of cells/tissues is insufficient to protect against ROS (Sies, 1997). Consequently, excess ROS can directly target critical macromolecules, such as DNA, proteins, and lipids, and cause cell damage. The ROS-dependent cell death pathway can be triggered via necrosis or apoptosis if enough cell damage is incurred. Exposure to DON at varying concentrations (3, 6 and 12 mg/kg) decreased the total anti-oxidative capacity in the serum (Wu *et al.*, 2015) and it also decreased the glutathione in the jejunum affecting the total intestinal antioxidant capacity (Holanda and Kim, 2021).

The exposure of fumonisins, ochratoxins and zearalenone in pigs also tempers with serum activity and blood biochemistry. Fumonisins at either low or high concentrations decrease the sphinganine (SA) and sphingosine (SO) ratio (Rao *et al.*, 2020). Another study reported that dietary fumonisins at 8 mg/kg increases the creatine level. At the same time, it decreases the specific antibody level and reduces the mRna level of IL-10, suggesting the accumulation of the reactive oxygen species (Marin *et al.*, 2006). Dietary ochratoxins at 0.05 mg/kg decreases the total protein, albumin and nitrite oxide, while it increases the level of alanine aminotransferase and triglycerides in plasma and SOD (Marin *et al.*, 2006). Zearalenone at 1.11 mg/kg decreases serum SOD, the number of red blood cells and platelets (Cheng *et al.*, 2015). Another study reports that dietary ZEA at the concentration of 1 mg/kg increases IgA, IgG, IgM, as it also increases the BUN, CRE, AST and TBIL (Su *et al.*, 2018). Exposure to ZEA at 1158.67 ng L^{-1} is reported to increase the cell damage on the liver, uterus and ovary of gilt (Zhang *et al.*, 2021). There are limited studies that emphasize the effect of naturally intoxicated feed ingredients by mycotoxins on pig production.

2.4. Use of marula seed cake as an alternative protein source for pigs

2.4.1 The marula tree

Sclerocarya birrea (*Anacardiaceae*), commonly known as Marula tree (**Figure 2.8 (a)**), is a savannah tree belonging to the *Anacardiaceae* family. This plant produces pale yellow fruits of about 3-4 cm in diameter (**Figure 2.8 (c)),** with flesh that is both juicy and mucilaginous (Palgrave, 1977). The marula tree is native to Southern Africa. *S. birrea* is generally considered deciduous and mainly dioecious, though specific reports suggest that some monoecious trees may exist. Marulas are frequently dominant in communities and thus a keystone species in terms of community productivity.

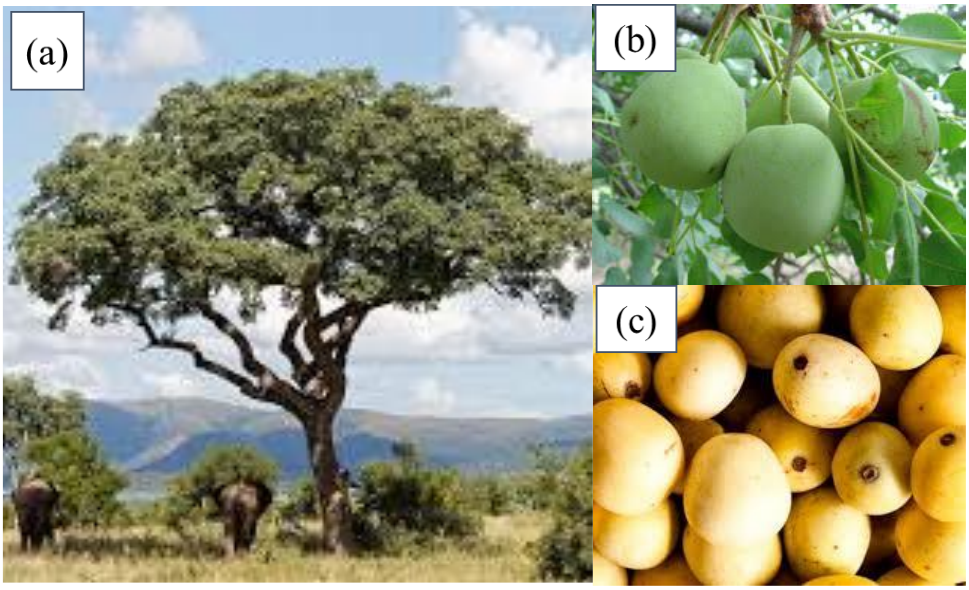

Figure 2. 8: (a) Marula fruit tree with the marula fruit in (b) unripe and (c) ripened state.

2.4.1.1 Classification, nomenclature, ecology and agronomy

Sclerocarya birrea, or Marula tree (A. Rich.) Subsp. caffra Hochst (Sond.) Kokwaro is a member of the *Anacardiaceae* family. The genus name *Sclerocarya* is derived from the Greek words "skleros", which means hard, and karyon, which means a nut, and refers to the hard stone of the fruit (Shone, 1979). The marula tree is a native fruit tree that is one of Africa's botanical treasures. The marula tree grows in warm, frost-free climates throughout Africa. It may grow in high-lying areas that experience very brief sub-zero temperatures during the winter (Gous *et al.*, 1988). It is found in 29 countries, throughout Eastern and Southern Africa. It occurs in 29 countries, distributed throughout most of SSA from 17° 15′ N in the Aïr Mountains of Niger to 31° 00′ S near Port Shepstone in South Africa (Chirwa *et al.*, 2007, Msukwa *et al.*, 2021, Jinga *et al.*, 2022).

The species occurs in open woodland and bush at medium to low altitudes. Trees of this variety reach heights of between 7 and 17m, have fissured grey bark, stout branches, and pale foliage, and stand leafless for some time in the year, particularly in winter. Insects are generally responsible for pollinating the flowers, and they usually begin blooming in September and end in November, before the fruit starts developing. Sprouting the marula seeds in river sand in spring will quickly produce a tree (Fujioka, 2021b). A variety of well-drained soils are favourable for the growth of the marula trees, mainly sandy soils or sandy loams (Hall *et al.*, 2002). However, despite its preference for well-drained soil and loam, the plant can also grow on rock.

2.4.1.2 Marula tree products, by-products and their uses

In rural communities, marula tree's fruit and seeds have long been used for nutrition (Shackleton, 2002, Hiwilepo-van Hal *et al.*, 2014). In addition to eating the fruit raw, it can also be made into jam, jelly, chewing gum, and pressed into juices (Leakey, 1999) or into alcoholic beverages (Gouwakinnou *et al.*, 2009). Known for being a good source of carbohydrates, protein, fat, vitamins, and minerals, and antioxidants in livestock feed (Gouwakinnou *et al.*, 2011), the seed cake has potential to replace the readily available feedstuff. Marula is an important indigenous tropical plant in several African countries (Petje, 2009, Bille *et al.*, 2013). Among its many benefits is its importance as food, medicine, and as a revenue source when marketed (Leakey and Akinnifesi, 2008). Marula has been noted to be a widely used species in converging protected areas in terms of game browsing (Gouwakinnou *et al.*, 2011) and within communal areas for fruit, wood carving, shade and making marula beer.

Phytochemicals present in the Marula tree (tannins, polyphenols, triterpenoids, phytosterols, and coumarins) are credited with its ethnomedical value (Ojewole *et al.*, 2010), and biological activities including antibacterial, antioxidant (Masoko *et al.*, 2008) and anti-inflammatory (Ndifossap *et al.*, 2010). Branches of *S. birrea* are chopped down by livestock owners during drought to provide nourishment for their animals (Mokgolodi *et al.*, 2011). The seeds are high in protein and fat and constitute an important emergency supplement (Thiong'o *et al.*, 2000). They also can be pressed to produce oil which is used for culinary and cosmetic purposes (Vermaak *et al.*, 2011) and in the manufacture of margarine, soap, and candles (Bergfeld *et al.*, 2011), leaving a by-product known as marula seed cake (MSC). The MSC, could potentially be a source of nutrients in animal feed that can be used to reduce feed costs and allow pig production to be intensified.

2.4.2.2 The nutritional composition of MSC

The nutritional composition of feed components is significant when assessing their potential of providing nutrients for feed formulation. For example, marula seed cake (**Figure 2.9 (b)**) is an agro-waste, a by-product of oil extraction of the marula seed nuts (**Figure 2.9 (b)**). Studies have been conducted on the nutritional composition or the nutritive value of MSC *(Mlambo et al.*, 2011b, Mdziniso *et al.*, 2016, Mthiyane and Mhlanga, 2017a, Malebana, 2018, Malebana *et al.*, 2018a, Mthiyane and Mhlanga, 2018a, Mthiyane and Hugo, 2019, Nkosi *et al.*, 2019). Though there are slight differences in the nutrient value of MSC in the reported studies, amongst other contributing factors leading to this variation, the chemical composition of MSC is significantly affected by the oil pressing process used to obtain the oil from the seed kernels (Malebana et al., 2018a).

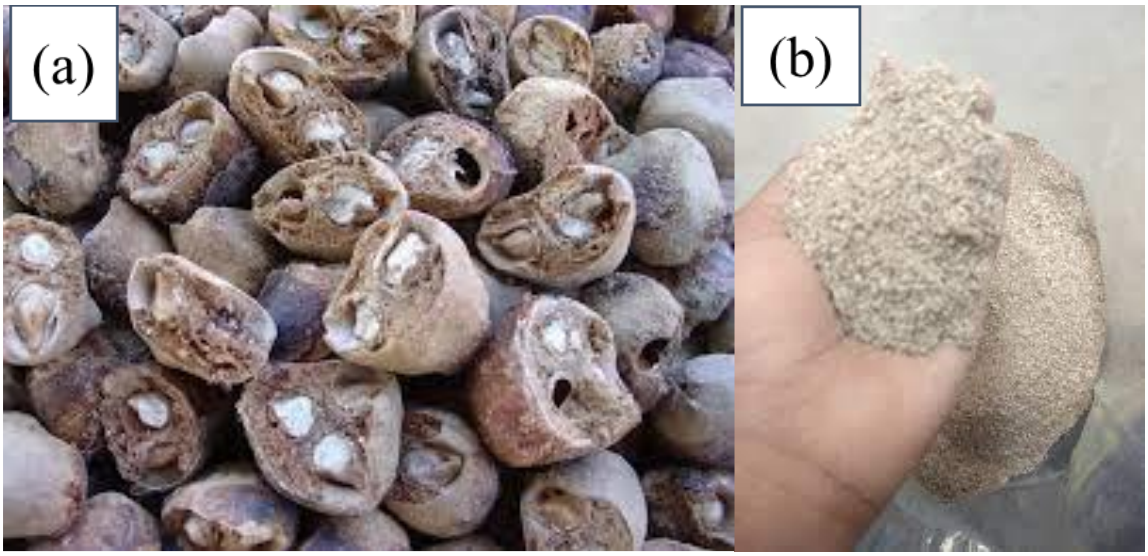

Figure 2. 9: (a) The marula nuts before the oil extraction and (b) the marula seed cake.

Notwithstanding, the overall chemical composition of MSC is equivalent to that of soybean meal, which is an ideal and widely utilized protein source in animal feed. The quality of animal protein sources is either defined by nitrogen value or crude protein content. The term "crude protein" (CP) refers to the nitrogen content of the feed, which contains both true protein and non-protein nitrogen, and its quality in animal feed is vital for growth and productivity (Abbasi *et al.*, 2018). Marula seed cake contains appreciable levels of crude protein (**Table 2.7**) varying from 362.2 to 472 g/kg CP DM (Mlambo *et al.*, 2011b, Mdziniso *et al.*, 2016, Mthiyane and Mhlanga, 2017a, Malebana, 2018, Malebana *et al.*, 2018a, Mthiyane and Hugo, 2019) that is higher and/or similar to other conventional plant-derived protein sources that are used in

livestock feeding, including urea and sunflower seed meal (Mlambo *et al.*, 2011b, Mthiyane and Mhlanga, 2017a, Malebana *et al.*, 2018a). In addition, the CP content of MSC surpasses other alternative protein sources, such as mucuna seed meal (260 g/kg DM) (Mthiyane *et al.*, 2018a) and *Melia azedarach* leaf meal (290.0 g/kg DM) (Mthiyane *et al.*, 2019). In relevance to its high protein content, it is promising to be suitable for all livestock species for maintenance, growth and production.

Moreover, MSC has a perceptible value of all essential amino acids than soybean meal **(Table 2.6)**. Notably, despite the low levels of lysine and methionine, which are growth-limiting amino-acids, lysine for non-ruminants, and methionine for growing ruminants as per nutrient requirements, may be efficient to supplement them in animal feed (Mthiyane and Mhlanga, 2017a, Malebana *et al.*, 2018a). Regardless of the low levels of lysine and methionine, MSC is high in sulphur amino acids, cyst(e)ine, arginine, and glutamic acid (Mthiyane and Mhlanga, 2017a). According to Colovic *et al.* (2018), sulphur amino acids have a role in producing intracellular antioxidants including glutathione and N-acetyl cysteine. Also, the crude fat, ether extract (EE), neutral detergent fibre (NDF), and acid detergent fibre (ADF) of MSC are more pronounced than that of SBM **(Table 2.6)**.

Also of importance, MSC has a considerable level of crude fat that ranges between 28.96 and 58.2 % DM (Mdziniso *et al.*, 2016, Mthiyane and Mhlanga, 2017a), which is exceptionally high in comparison to soybean meal in terms of dry matter of crude fat (Banaszkiewicz, 2011). The difference in the extract content of the MSC and SBM might be linked to the oil extraction techniques, MSC is cold-pressed while SBM is solvent extracted, and solvent extraction in SBM is 7% more efficient in oil extraction than cold press (Cakaloglu *et al.*, 2018). Studies reveal that either extract of MSC (289.6 – 394 g/kg DM) is of high values relative to conventional dietary protein sources, including that of SBM (Malebana *et al.*, 2018), and surpasses either extract of other alternative protein sources (Nkosi *et al.*, 2019).

Table 2. 6: Chemical, energy, amino acid, and mineral composition of marula seed cake versus soybean meal.

Chemical composition (g/kg DM)	**Marula Seed Cake**	**Soybean meal**
Dry Matter	901 – 966	879.1 - 906
Crude Protein	362.2 - 472.1	438 – 536
Crude Fat	289.6 – 582	55 – 300
Ether extract	289.6 – 632	5-40
Neutral Detergent Fibre	145.44 – 338	84.5-189
Acid Detergent Fibre	77.67 - 357.3	38.6-119
Ash	46.5 - 66.1	54.3-69
	Energy (MJ/kg DM)	
Gross Energy	28.49	-
Metabolisable Energy	1544.6	19.02
	Amino acid (% DM)	
Lysine	0.68 - 0.88	2.92-5.91
Methionine	0.44 - 0.79	0.6 - 1.55
Cysteine	0.97 - 1.45	0.66 - 0.75
Threonine	0.60 - 0.93	1.82 - 4.07
Isoleucine	1.29 - 1.77	1.76 - 2.47

Leucine	1.23 - 2.66	2.2 - 7.52
Phenylalanine	1.19 - 1.91	1.6 - 5.02
Histidine	0.61 - 1.32	1.0- 2.55
Arginine	5.39 - 7.63	2.45 - 3.43
Valanine	1.31 - 2.14	2.26 - 3.76
Proline	0.14 – 1.13	-
Glutamic acid	6.15 - 10.78	-
Mineral (g/kg DM)		
Macro minerals		
Sodium	0.10 - 1.39	0.18 – 1.1
Calcium	1.10 - 1.50	0.31 - 2.73
Phosphorus	9.27 - 13.20	6.37 - 7.43
Magnesium	5.5	2.72 - 3.10
Potasium	4.97 - 9.30	19.85 - 25.80
Micro minerals		
Iron	0.69	0.14
Copper	0.003	0.02
Zinc	0.06	0.06
Sulphur	5.92	4.59

Source: Banaszkiewicz (2011), Mlambo *et al*. (2011b), Mdziniso *et al*. (2016), Mthiyane and Mhlanga (2017a), Malebana (2018), Malebana *et al*. (2018a), Mthiyane and Mhlanga (2018a), Semwogerere *et al*. (2020).

When it comes to fatty acids, the most prevalent fatty acids in marula seed cake are predominantly monounsaturated fatty acids, followed by saturated fatty acids, and a minimal amounts of polyunsaturated fatty acids (Mthiyane and Mhlanga, 2017a, Malebana *et al*., 2018a, Mthiyane and Hugo, 2019). Therefore, the fatty acid profile of MSC is potentially better compared to that of SBM which is made up of PUFAs and almost equal proportions of the total saturated fatty acids and MUFAs (Malebana *et al*., 2018a). In addition, the fatty acid composition of MSC is equivalent to that of olive oil **(Table 2.7)**, which has numerous benefits for both human and animal health (Monfreda *et al*., 2012). According to Malebana *et al*. (2018), MSC has comparable neutral detergent fibre (145.44 – 338 g/kg DM) and acid detergent fibre (77.67 - 357.3 g/kg DM) contents with SBM, which is higher than that recorded for maize. Due to the comparability of MSC and the commonly utilized energy source in animal diets, this might mean that MSC may be able to perform a dual purpose in feeds, as both a dietary protein and an energy source, with minimal negative effect, if any, this includes limited detrimental impacts on nutritional digestibility. Furthermore, MSC has twice the gross energy content compared to SBM, owing to its high fibre and lipid content (Mdziniso *et al*., 2016, Malebana *et al*., 2018a).

The ash content signifies the total amount of minerals embedded in the feed (Ayoola *et al*., 2010). The range of ash content of MSC falls between 46.5 and 66.1 g/kg DM, which is lower than that of SBM (63.5 - 74.5 g/kg DM), still, it falls within the 10–100 g/kg DM threshold for feedstuff considered to be an excellent supplier of minerals (López-Alonso, 2012). In connection to the mineral content, MSC contains a significant amount of essential macro and micro minerals (Malebana *et al*., 2018a; Mthiyane and Mhlanga, 2017; Nkosi *et al*., 2019). The macro-minerals that occur in significant amounts in MSC are sodium (Na), calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K). In contrast, the macro-minerals are iron (Fe), copper (Cu), zinc (Zn), cobalt (Co), and sulphur (S). In addition, the mineral composition of MSC proves that it is an excellent source of calcium (1.10 - 1.50) on g/kg DM basis. Calcium is essential for the formation of bones, teeth, and eggshells, the transmission of nerve impulses, the decrease of capillary permeability, the control of metabolism, and the contraction of muscular characteristics in animals (McDonald *et al*., 2010).

Table 2. 7: Fatty acid of olive marula seed and olive oil composition.

Fatty acids (% DM)	Name	Marula Seed Cake	Olive oil
	Saturated		
C10:O	Capric	0.01 - 0.03	-
C12:O	Lauric acid	0.02	-
C14:O	Myristic	0.03 - 0.13	0.00-0.038
C15:O	Pentadecyclic acid	0.03	-
C16:O	Pamlitic acid	11.29 -13.57	9.49-18.98
C17:O	Margaric	0.125-0.2	0.00-0.12
C18:O	Stearic	6.00-7.66	0.16-4.15
C19:O	Nonadecanoic	0.03-0.07	-
C20:O	Arachidic	0.25-0.48	0.23-0.45
C21:O	Heneicosanoic	0.02-0.4	-
C22:O	Behenic acid	0.25	0.03-0.22
C23:O	Trycocylic acid	0.01	-
C24:O	Lignoceric acid	0.12-12	0.00-0.16
	Monounsaturated		
C14:1:	Myristoleic acid		-
C16:1C9	Palmitoleic	0.02-0.15	0.58-2.54
C17:1	Heptadecanoic acid	0.05	-
C18:1C9	Oleic acid	71.89-85.24	55.30-79.88
C18:1n9t	Elaidic acid	0.07	-
C20:1	Gadoleic acid	0.04	-
C22:1n9	Erucic	0.03	-
	Polyunsaturated		
C18:2C9,12	Linoleic	6.64-8.78	4.38-19.97
C20:2C9,12,15 (n=3)	a- Linoleic	0.04-0.344	-
C20:2C11,14 (n=6)	Eicosadienoic	0.028-0.032	-
C20:3C8,11,14(n=6)	Eicosatrienoic	0.098-0.122	-
C20: 3n3	Eiosatrienoic acid	0.12	-
C20:3n6	Dihomo-gamma-linolenic acid	0.03	-
C20:4n6	(Arachidonic acid)	-	0.23-0.45
C20:5n3	(Eicosapentaenoic acid)	0.36	-
	Total Fatty Acid Composition		
ΣSFA	Total Saturated Fatty Acids	17.94 – 34.85	9.91 – 24.12
ΣMUFA	Total Monousaturated Fatty Acids	72.1 - 85.58	55.88 – 82.42
ΣPUFA	Total Polyunsaturated Fatty Acids	7.316 – 9.428	4.61 – 20.42

Source: Mthiyane and Mhlanga (2017a), Malebana (2018), Malebana *et al*. (2018a), Adam *et al*. (2019), Mthiyane and Hugo (2019).

In a study by (Malebana *et al.*, 2018a), MSC showed to have a higher concentration of phosphorus, magnesium and copper compared to that with SBM. The quantity of micro-minerals in the MSC might be utilized to best advantage in formulating nutritionally balanced feedstuff. MSC's sulphur content (5.9 g/kg) is equivalent to that of canola meal (6.2 g/kg), a common dietary protein source in feeds, and greater than that of SBM (Spragg and Mailer, 2007, Semwogerere *et al.*, 2020). In simplicity, the relevance of sulphur levels of MSC and SBM means that the utilization of this alternative protein source in animal feeds may not jeopardize the abundance of essential amino acids, such as methionine, cysteine, and cysteine, hormonal production, and chondroitin synthesis (MacDonald *et al.*, 2019), and aid on glutathione synthesis since sulphur is a key precursor of this molecule (Mthiyane, 2006).

The quantity of minerals in MSC might be utilized to minimize mineral supplementation in diets (Mthiyane and Mhlanga, 2017a, Malebana *et al.*, 2018a). Although studies have proven MSC to be a protein-energy-rich feedstuff with a relatable amino acid profile, its mineral and vitamin content is not fully exploited or well-researched. Nonetheless, in terms of the nutritional content, MSC needs to be commercialized and utilized as a feed ingredient for ruminant and non-ruminant animals. In agreement with (Malebana *et al.*, 2018a), few supplementations will be needed to avoid essential nutrient deficiencies.

2.4.2.3 Anti-nutritional composition of MSC

As much as the studies on the chemical composition have demonstrated MSC to have great potential as a feedstuff, it has been found to contain a significant amount of anti-nutritional factors. According to Samtiya *et al.* (2020), anti-nutrients are one of the major key factors contributing to the limitation of the bioavailability of various components of animal feedstuffs. The presence of anti-nutritive compounds limits the digestion and absorption of protein, vitamins, and minerals, and may lead to the accumulation of toxic compounds (Khalid *et al.*, 2013, Woyengo and Nyachoti, 2013). The anti-nutritional composition of MSC has been characterized, it is known to contain tannins, saponins, phytate-phosphate, and oxalates and mycotoxins (Mthiyane and Mhlanga, 2017a, Malebana *et al.*, 2018a).The tannin content of MSC occurs at very low levels of 0.07 mg/100g DM, and the level of saponins in this alternative protein source also is present at significantly low levels of 21.21 mg/100g DM (Malebana, 2018). They are known to have almost no effect on livestock in such quantities, but when present in large concentrations, they suppress intestinal and ruminal ammonia production

(Westendarp, 2005, Piluzza *et al.*, 2014). Nevertheless, Malebana (2018) noted that due to the low levels of tannins and saponins in MSC, its utilization is not expected to compromise the digestion of protein, and is unlikely to cause haemolysis. This study further showed that the amount of phytate-phosphate and oxalates of MSC is 218.3 mg/100g DM and 15.03 mg/100g DM, respectively (Malebana *et al.*, 2018a). MSC has lower phytic acid (0.6 g/kg) values than SBM (Hassan *et al.*, 2011). Phytate-phosphates can form compounds with metals or proteins, reducing their bioavailability in the gut. At the same time, oxalates could have direct, and indirect effects on animal performance and health (Patel *et al.*, 2013, Nkosi *et al.*, 2019).

In a previous study Malebana *et al.* (2018a), noted that the presence of phytate-phosphate and oxalates in MSC could interfere with the bioavailability of nutrients, hence it would be advisable to supplement with additional essential minerals. Also, MSC is mainly contaminated by trichothecenes, Deoxynivalenol (DON), and T-2 toxins, which they occur in significant amounts of 66.31ppb (DON) and 68.75ppb (T-2) (Mthiyane and Mhlanga, 2017a). However, both DON, and T-2 toxins are present at acceptable levels according to the South African legislations on the maximum allowable levels of mycotoxins in animal feed (Njobeh *et al.*, 2012). However, the anti-nutritive composition of MSC is minimally investigated and requires to be further explored to better understand its impact to animal feed.

2.4.2.4 Productive responses of pigs to dietary marula seed cake

Mabena *et al.* (2022) reported the utilization of MSC (50, 100, 150, 200 g/ kg DM) to substitute SBM in Large White Landrace pigs for growth parameters, nutrient digestion, and carcass quality. In that study, dietary MSC had no impact on feed intake, while average daily gain were lowered at MSC levels more than 15%, resulting in poor feed conversion ratio. Furthermore, a nutrient digestibility trial was then conducted for 5 more days in that study. Protein digestibility was observed to be decreased when MSC levels exceeded 15%, whereas ether extract and fibre levels increased. Thereafter, the carcass parameters were also reported to detrimentally affect warm and cold carcass weights at MSC levels greater 15%, though on the other hand it improved meat redness and lightness.

On the reported study on the response in nitrogen (N) balance in slow-growing pigs fed on incremental levels of MSC (50, 100, 150, 200 g/kg DM) by Hlongwana et al. (2021b), nitrogen intake increased linearly with incremental levels of the dietary inclusion level of this alternative protein source. As MSC incorporation increased, so did nitrogen absorption, apparent

nitrogen digestibility, and nitrogen retention in pigs, reaching a maximum before decreasing. In addition, nitrogen utilization increased at a rate of 0.63 g for every 1 g in pigs fed with MSC-containing diets. Nevertheless, incorporating MSC in the diet of slow-growing pigs resulted in a linear reduction in total nitrogen excretion via urine and feces. Following that, urinary pH levels were measured, and MSC quadratically reduced the pH in these pigs. The study's findings by Hlongwana *et al*. (2021b) indicated that MSC incorporation in diets balanced for limiting amino acids caused the nitrogen balance reactions, and MSC reduces nitrogen excretion, potentially lowering ammonia volatilization, enabling it to be an alternate protein source for slow-growing pigs.

Also, research has been conducted on utilization of MSC by non-ruminant species which may potentially be used as a reference to piglets, such as broiler chicken (Mthiyane and Mhlanga, 2017b, 2018b), and Japanese quails (Mazizi *et al*., 2019b, 2020b). Reportedly, feeding MSC to Cobb-500 broilers chicken in comparison to control diets (SBM) negatively affected growth parameters (Mthiyane and Mhlanga, 2017b). Mthiyane and Mhlanga (2017a) noted that the body weight gain, feed consumption, and feed conversion efficiency of broiler chickens all dropped considerably as the incremental levels of MSC (5, 10, 15, and 20%). In relation to carcass parameters, (Mthiyane and Mhlanga, 2017b, 2018b) demonstrated that dietary MSC impacted broiler chicken growth parameters and dramatically reduced live weight at slaughter, plucked weight, dressed weight, and liver and neck weights.

Including MSC in broiler chicken detrimentally affects the production of Cobb-500 broilers (Mthiyane and Mhlanga, 2017b). According to these researchers, the consumption of oxidized lipids in MSC (Mthiyane and Mhlanga, 2017b) may have increased the absorption of lipid-derived radicals into the circulation, increasing oxidative reactions throughout multiple tissues of the chickens and, as a result, jeopardizing their productive performance and carcass characteristics. Another possible reason could be the intoxication by mycotoxins, DON and T-2 toxins. In animal nutrition, DON and T-2 toxins, are primarily reported to cause feed refusal in non-ruminants (Rotter and Oh, 1996, Rafai *et al*., 2000) which then affects other growth, and therefore carcass parameters.

In another study by Mthiyane and Mhlanga (2018b), dietary MSC (200 and 300 g/kg) decreased BWG, FCE, slaughter weight and weights of the heart, neck and feet in DL-methionine-supplemented (0.14%, 0.28% and 0.56%) broilers. Also, MSC decreased BWG, FI and slaughter weight in phytase-supplemented (0.01% and 0.02%) birds. Both DL-methionine and

phytase were not effective, and there were no significant MSC x DL-methionine or MSC x phytase interactions on any of the parameters measured, except for the MSC x phytase interaction for gizzard weight. As a result, MSC reduced broiler performance and carcass traits, which could not be improved by supplementing with DL-methionine or phytase. The problem of feeding MSC to non-ruminants is that they contain varying ANFs, which may exert greater detrimental effects than expected. Non-ruminant animals exhibit the greatest sensitivity to anti-nutritional factors while other species appear to have higher tolerance.

In a study where SBM was replaced by MSC (25%, 50% 75%, and 100%) in broiler Japanese quail grower and finisher, the inclusion of MSC did not detrimentally affect FI and FCE (Mazizi *et al.*, 2019b). In the same research, incorporating MSC to grower and finisher diets had no effect on growth performance as determined by total body mass, body mass increase, and empty carcass mass. As a consequence, the findings suggest that MSC may be used in place of SBM as a dietary protein source in broiler quail grower and finisher diets without impairing growth performance. The authors hypothesized that MSC lacked ANFs, which might have hampered digestion, absorption, and nutritional utilization. However, this speculation contradicts studies by Mthiyane and Mhlanga (2017b), and Malebana *et al*. (2018b) which reported the presence of anti-nutrients in MSC.

A reported study by Mazizi *et al*. (2020b) on feeding dietary MSC (25%, 50%, 75%, and 100%) in substitution to SBM affected meat quality parameters. The initial and final pH of meat from carcasses of quail-fed control diets (SBM) were decreased, although the meat was lighter and resulted to less red meat than counterparts on MSC-containing diets. However, MSC was reported to have no significant effects on thawing loss, cooking loss, and meat tenderness. On the proximate analysis meat also reported by Mazizi *et al*. (2020b), with an increase in dietary MSC, the ash level of the meat increased, but its CP and fat dropped. Moreover, the total saturated FA content of meat from birds fed 75% MSC was lower than that of other dietary treatments. Furthermore, meat from birds supplemented with 0% and 25% MSC diets had less oleic acid (OA) than the rest of the treatments. MSC has the potential to be used in quail diets without impairing the meat's physical and proximate characteristics. It may also be utilized to produce lean yet OA-rich meat, which may have health advantages for consumers.

The differences attained in the studies of broiler birds could be the gastro-intestinal morphology of the two different broilers as much as they are both non-ruminants. Another possible explanation could be that the hexane-mediated reduction of excess fat in the meal decreased

the dietary fat content to levels that did not conflict with food metabolism and absorption since the MSC utilized in the studies by Mazizi *et al*. (2019b) and Mazizi *et al*. (2020b) was hexane mediated. It can thus be concluded that defatting the MSC with hexane enhanced its aroma and taste, thereby possibly contributing positively to feed intake and nutrient utilization efficiency.

Utilizing MSC generated from cold pressing to substitute SBM in a study by Mthiyane and Mhlanga (2017b) and Mthiyane and Mhlanga (2018b) resulted in decreased broiler performance, unlike solvent extraction reported by Mazizi *et al*. (2019b) and Mazizi *et al*. (2020b). While cold pressing eliminates the majority of the lipid, it also leaves large amount of the fat and anti-nutrients in the meal, which may decrease feed intake due to higher calorie density and the potential of fat-induced rancidity (Mthiyane and Mhlanga, 2017b). Owing to the detrimental effects of MSC caused by the presence of mycotoxins, this suggests a further exploration of ameliorating agents to trash out the negative effects, ameliorating agents such as nano-biotechnology.

2.5 Nano-(Bio)Technology as a solution to the problem of mycotoxins in pigs

Nanotechnology is the creation of functional materials, devices, particles, and systems by manipulating matter, either length, breadth, or height, at a scale of approximately 1–100 nm (Laurent *et al*., 2008). At such a nanoscale, novel properties and functions occur because of size, which is exploited for application in pharmaceuticals and food/feed technologies. Nano-biotechnology is an emerging bioscience tactic used in agriculture, human health and nutrition. The potential application areas in animal science are growing at a slow pace in physiology, nutritional and biotechnology research (Boverhof *et al*., 2015).

Utilizing nanoparticles for medicine delivery comes from two main fundamental properties. Firstly, due of their tiny particle size, nanoparticles may pass through smaller capillaries and be taken up by cells, allowing for effective accumulation in the target areas. Second, the use of biodegradable ingredients for nanoparticle production allows for prolonged release within the target region over days or even weeks (Goryacheva *et al*., 2015). Nanoparticles increased the therapeutic efficiency as well as bioavailability.

In recent years, it was found that titanium, selenium, carbon-based, cerium, silver, gold, , or zinc oxide nanoparticles show unique antioxidant properties (Kovacic *et al*., 2010, Saravanakumar *et al*., 2015, Nelson *et al*., 2016). Furthermore, the green synthesis might

improve the antioxidant capabilities of metal nanoparticles (Sudha *et al*., 2017). Processes for producing nanoparticles using plant extracts are readily modular, less costly, and take a shorter period than the conventional route (Mittal *et al*., 2013, Saravanakumar *et al*., 2015, Singh *et al*., 2016). Green-synthesized nanoparticles are enriched in phyto-compounds or biochemicals, which results in higher donor activity (Swain *et al*., 2016b). These phyto-compounds encapsulated in nanoparticles can possibly biotransformation the mycotoxins throgh redox reaction and of electrophilic compounds via conjugation reactions by glutathione.

2.5.1 Type of nanoparticles (NPs)

Nanoparticles may be categorized according to their chemical properties as inorganic, organic, and carbon-based nanoparticles. Inorganic based nanoparticles are further classified into: a) Metal-based e.g. cadium, aluminium, copper, cobalt, gold, iron, silver, zinc and lead, and b) metal oxide nanoparticles are oxidised metals such as titanic oxide, silicon oxide, zinc oxide, magnetic iron-ore, iron oxide, aluminium oxide and cerium oxide. The inorganic nanoparticles contain inorganic nano-sized ingredients and are already licensed for feed use. Calcium dioxide, magnesium, silver, silicon, and nanoparticles for water purification, feed preservation, and antimicrobial packaging are all examples of nano-clay platelets (Bunglavan *et al.,* 2014, Al-Beitawi *et al*., 2017).

Organic nanoparticles are mainly molecules of proteins, fat, and sugar (Bunglavan *et al*., 2014). Organic nanoparticles may encapsulate nutrients and transport them into the bloodstream via the gastrointestinal tract, also known as nano-capsules. Organic nanoparticles are sub-divided into micelle, dendrimer, liposome, hybrid and compact polymetric (Jesus and Grazu, 2012). Therefore, these capsules are meant to provide the nutrients with improved bioavailability without altering the taste or bioavailability (FSAI, 2008). Encapsulated nanoparticles are utilized in feeds such as micelles, liposomes, and feed packaging systems as biosensors, identification markers, shelf-life extenders, and antimicrobials (Singh, 2016). Whether the nanoparticles are categorized according to the above-mentioned types, they are then synthesized by chemical, physical or by green synthesis.

2.5.1.1 Chemical and physical synthesis vs. green synthesis

Nanomaterials (NMs) can be synthesized via chemical, physical, or biological techniques. Physical and chemical synthesis techniques have been discovered to be highly costly, and potentially dangerous to the environment (Solomon, 2018). Chemical reducing agents, primarily organic and inorganic solvents, are required for the chemical-based synthesis of

nanomaterials. In aqueous or non-aqueous solutions, for the reduction of metal ions, several reducing agents such as sodium citrate, poly (ethylene glycol)-block copolymers, ascorbate, elemental hydrogen, tollens reagent, polyol process, N,N-dimethylformamide (DMF), and sodium borohydride (NaBH4), are used (Iravani *et al.*, 2014). Mostly, chemical-mediated nanoparticle synthesis techniques are performed at room temperature (Ghorbani, 2014).

As with chemical-based nanoparticle synthesis, chemical-based nanoparticles can be synthesized for a specific form, size, shape, composition, and orientation, making them suitable for an extensive range of research areas, including catalysis, data storage, drug delivery, imaging, and sensing. Furthermore, the operational parameters of chemical nanoparticle synthesis may be predicted precisely (Yu *et al.*, 2014). As a result of their nano size, NPs can induce inhalation abnormalities as well as other life - threatening conditions. Just sixty seconds of inhalation of air comprising NPs will quickly affect the lungs. Although chemical nanotechnology has improved living standards, it has also increased contamination, including atmospheric and water pollution; this nanotechnology-based problem is known as nano-pollution, and it is highly hazardous to animals and the environment (Shinde *et al.*, 2012).

Green nano-biotechnology is the synthesis of nanoparticles or nanomaterials utilizing biological processes such as plants, microorganisms, and viruses, or their by-products such as lipids and proteins, or using other biotechnological methods. Green nanoparticle synthesis makes use of environmentally sustainable, non-toxic, and healthy reagents. Since they are created in a single step, nanoparticles synthesized using biological approaches or green technologies offer a variety of advantages, including increased durability and adequate measurements (Basnet *et al.*, 2018).

Nanoparticles produced using green technology at times outperform those produced using chemical and physical processes. Green approaches, for example, avoid the use of costly chemicals, require less energy, and have ecologically friendly goods and by-products. Green chemistry theories are being used as a reference guide by researchers, scientists, chemical technologists, and chemists all around the world as they work to generate less dangerous chemicals and products (Baer *et al.*, 2008, Park *et al.*, 2011) Green nanobiotechnology, as a result, is a possible alternative technique for the manufacture of biocompatible stable nanomaterials (Haverkamp *et al.*, 2007). The standard approach for producing metallic nanoparticles by plants employs dried plant biomass as a bioreducing agent and metallic salt

as a substrate, correspondingly. Bottom-up synthesis of bio-based nanoparticles is used with the assistance of reducing and stabilizing agents.

Green synthesis of ZnO NPs is favoured over chemical and physical techniques because it is a more environmentally sustainable and cost-effective technique that does not require high temperatures, pressure, or harmful chemicals and eliminates toxic compound production (Basnet *et al.*, 2018). Plant-mediated bio-fabrication of ZnO nanoparticles has gained prominence over other types when large quantities are widely available, it includes secondary metabolites, reduces processing time for the preservation of bacterial and fungal cultures, and cross-contamination of plant extracts is negligible (Vijayakumar *et al.*, 2018, Kamran *et al.*, 2019).

2.5.2 Limitations of chemically and physically synthesized nanoparticles

Conventional chemical and physical methods for producing nanomaterials (NMs) can have major limitations, such as defective surface structure, low manufacturing quality, ridiculous costs, and high energy demands. Chemical synthesis methods typically require the usage of toxic chemicals, which have new hazardous properties toxicity has been reported in multiple nanoparticles due to size and dose, e.g., stabilization are poisonous and lead to non-eco-friendly by-products. Feeding pigs a high dosage of chemically synthesized ZnO causes in a large amount of unabsorbable Zn to be released into the environment, ultimately raising the chances of multiple dug resistance (Bednorz, 2013) and heavy metal contamination which brings toxicities to the environment (Wei *et al.*, 2010).

Chemically based NPs represent exogenous materials with their own physicochemical properties in organisms, and as a result, they may interfere with the standard physiological systems of embryos, maturing animals, and adults. NPs may also occasionally affect embryonic development, resulting in potentially fatal abnormalities. Not only do the chemicals that make up NPs react according to well-known processes, but the size of the NPs themselves gives certain unique features that undoubtedly interact with the organism's physical, chemical, and biological activities (Warad *et al.*, 2005).

Furthermore, NPs have been demonstrated to influence cardiac and vascular functioning by disrupting automatic equilibrium directly. The necessity for the biosynthesis of nanoparticles emerged as physical and chemical methods became more expensive. Chemical synthesis methods frequently result in the presence of some harmful chemicals absorbed on the surface, which may have an unfavourable effect on medical applications (Yu *et al.*, 2013). According

to (Wong-Pinto *et al.*, 2020), the mentioned is not an issue for biosynthesized nanoparticles via the green synthesis route.

2.5.3 Use of green materials as reducing agents for nanoparticle synthesis

The plant's ability to reduce is based on a variety of active compounds such as amino acids, polysaccharides, reducing sugars, polyphenols, proteins, and terpenoids. Plant extracts play a significant role in reducing the particle size of metal ions. They can serve as reducing and capping agents throughout the nanoparticle synthesis process (Mittal *et al.*, 2013). Specific plant organs, such as plant leaves, seeds, flowers, and roots can participate in bio-reduction (Iravani *et al.*, 2014, Duan *et al.*, 2015). The majority of researchers used plant extracts to achieve optimum productivity in nanoparticles growth such as, *Brassica oleracea* (Osuntokun *et al.*, 2019b), *Stigmaphyllon ovatum* (Elemike *et al.*, 2019b), and *Tulbaghia violaceae* (Lediga *et al.*, 2018), amongst others. Usually, a green or plant extract-induced reduction requires combining the extract with an aqueous metal reference solution at room temperature or specified temperature. Generally, the synthesis should be done in a short time.

In the "one-pot" synthesis procedure, several plants can be used to decrease and stabilize the metallic nanoparticles. Many scientists have used the green-synthesis approach to create metal/metal oxide nanoparticles from plant leaf extracts in order to explore their many applications further. Plants contain biomolecules (such as carbohydrates, proteins, and coenzymes) that have a high potential for converting metal salts into nanoparticles. Like other biosynthesis methods, gold and silver metal nanoparticles were originally explored in plant extract-assisted synthesis. It is thought that the origin of the plant extract influences the properties of the nanoparticles. Compared to other green synthesis methods, using plant extracts to make nanoparticles is simpler. In comparison to the more expensive and intricate process of microbial synthesis, processes for generating nanoparticles utilizing plant extracts are easily modular, less expensive, and require less time (Mittal *et al.*, 2013, Saravanakumar *et al.*, 2015, Singh *et al.*, 2016).

2.5.4 *Tulbaghia violaceae* and its use in green nanoparticles

Tulbaghia violacea is a native medicinal plant, commonly known as society garlic, wild garlic, sweet garlic, wild knoffel (Afrikaans) and itswele lomlambo (Xhosa) (Kubec *et al.*, 2002; Harris, 2004). *T violacea* is an evergreen, bulbous, fast growing perennial plant that can reach 0.5 m in height (**Figure 2.10**). It can grow quickly in most soils and tolerate prolonged drought, although the plant will flourish with regular watering (Harris, 2004).

Figure 2. 10: *Tulbaghia violacea* plant at a mature stage**.**

2.5.4.1 Classification, nomenclature, ecology and agronomic aspects

Tulbaghia violacea Harv. is a small bulbous herb belonging to Amaryllidaceae family (formerly: Alliaceae). It is indigenous to Eastern Cape, Gauteng, and KwaZulu–Natal provinces in South Africa, and is cultivated for medicinal, ornamental and culinary purposes (Chase *et al*., 2009). This has tubular mauve or light purple flowers found in umbels up to 20 at the tip of the slender stem/stalk. The plant is closely related to garlic (*A. sativum*) and is commonly known as wild garlic, society garlic or sweet garlic. It also has a garlic-like aroma when the leaves are bruised; hence the common name (Van Wyk and Gericke, 2000, Lyantagaye, 2011b). Depending on the environmental conditions, mature height ranges from 30 cm to 120 cm. The plant may be effectively developed in a tub and moved to a greenhouse or a frost-free location for the winter.

2.5.4.2 Sulphur content of its bulb and potential for mycotoxins biotransformation

Tulbaghia violacea is rich in sulphur-containing compounds. In most cases, these chemicals are responsible for the odors and medicinal capabilities of *Tulbaghia* species (Lyantagaye, 2011b). The distinctive odor of *Tulbaghia* is caused by a cysteine-derived amino acid called S-(methylthiomethyl) cysteine-4-oxide (marasmin), which is found in the cytoplasm (Lyantagaye, 2011b). When Tulbaghia tissue is bruized, a C–S lyase embedded in the cell

vacuoles interacts with marasmin, accelerating its degradation into thiosulfinate marasmicin (2,4,5,7-tetrathiaoctan-4-oxide) (Kubec *et al.*, 2002b, 2013b). The *Tulbaghia* spp. contains a lot more sulphur compounds (**Table 2.8**) that can be possibly used in the biotransformation of mycotoxins via redox reaction and of electrophilic compounds via conjugation reactions by glutathione.

Glutathione (GSH) is an endogenously generated tripeptide (glycine, cysteine, and glutamate) that operates in numerous enzyme systems in the body to aid in the detoxification of fat-soluble substances as well as an antioxidant, neutralizing free radicals (Prousky, 2008). *T. violaceae* contains cysteine amino acids, which function as a precursor for glutathione. Glutathione is a cysteine-containing (thiol) abundant in mammalian somatic cells and gametes. Glutathione is the immune system's primary detoxifier and the mother of all antioxidants (Hyman, 2010). The antioxidant resistance capability of the body is made up of enzymatic and non-enzymatic processes, the latter of which is predominantly reflected by glutathione (Luberda, 2005). It is involved in critical physiological processes that have substantial ramifications for many disease pathophysiologies, such as the maintenance of redox equilibrium, the removal of oxidative stress, the augmentation of metabolic detoxification, and the control of immune system activity.

Given the importance of oxidative stress in mycotoxin-induced disease, glutathione and glutathione precursors play an important role in therapy. GSH is synthesized from the three amino acid precursors in an ATP dependent two-step reaction the synthesis of glutathione in animals happens only in the cytosol (Wang and Ballatori, 1998, Noctor *et al.*, 2012). In all mammalian cells, GSH performs some critical physiological and metabolic activities, the most significant of which is the detoxification of free radicals via redox processes and of electrophilic substances via conjugation reactions (Wang and Ballatori, 1998). Animals, biodegrade glutathione conjugates, and the biodegradation happens extracellularly.

Table 2. 8: Chemical classes and representative sulphur compounds present in wild garlic

Class of compound	Name	Chemical name
Cysteine sulfoxide	Alliin	S-allyl-l-cysteine sulfoxide
	Methiin	S-methyl-l-cysteine sulfoxide
	Propiin	S-propyl cysteine sulfoxide
	Isoalliin	S-(trans-1-propenyl)-l-cysteine sulfoxide
	Ethiin	(SSRC)-S-ethylcysteine sulfoxide
	Marasmin	(RSRC)-S-(methylthiomethyl)cysteine-4-oxide
Thiosulfinate	Allicin	3-[(Prop-2-ene-1-sulfinyl)sulfanyl]prop-1-ene
	Marasmicin	S-(methylthiomethyl) (methylthio) methanethiosulfinate
		2,4,5,7-tetrathiaoctane-4-oxide
		2,4,5,7-tetrathiaoctane
		2,4,5,7-tetrathiaoctane-2,2-dioxide
		2,4,5,7-tetrathiaoctane-4,4-dioxide
		2,4,5,7-tetrathiaoctane-2,2,7,7-tetraoxide
Polysulfides		Dimethy disulphide
		Dimethy trisulfide (methyl methylthio), methyl, 2,4-dithiapentane
		(Ethylthio) acetic acid
		2-(methylthio) ethanol
		3-(methylthio)-propanenitrile

Sources: (Lyantagaye, 2011b).

The N-terminal cysteinyl moiety in GSH γ-linked to the carboxyl group of glutamate rather than the more usual -carboxyl peptide linkage, rendering GSH resistant to intracellular breakdown. The membrane-bound enzyme γ-glutamyl transpeptidase (γGT) is the only enzyme that removes the γ-glutamyl moiety from the GSH under physiological conditions. The first sequential breakdown products of GSH-conjugates are glutamate, then glycine, which is removed by dipeptidases. Both of the amino acids can, later on, be used for GSH synthesis (Lyantagaye, 2011b). The corresponding cysteine-conjugates can either be acetylated intracellularly at the amino group by N-acetyltransferases to form mercapturic acids (Nacetylcysteine S-conjugates) or cleaved by β-lyase (usually present in liver and kidneys to a mercaptan (Pickett and Lu, 1989). Glutathione S-transferases (GSTs) are a versatile protein family that plays critical functions in cellular defense against oxidative stress and toxic compounds metabolism. GSTs catalyze a wide range of reactions, including nucleophilic aromatic substitutions, Michael additions, isomerizations, hydroperoxide reduction, and the conjugation of several hydrophobic and electrophilic molecules with reduced glutathione (Jankova *et al.*, 2012). GSTs in the liver and other organs perform a well-documented function in toxin detoxification, oxidative stress response, and the manufacture of inflammatory mediators-leukotrienes in animals.

Physiologically, antioxidants protect cellular components from damage caused by oxidative processes involving free radicals. At relatively low concentrations, anti-oxidants can compete with other oxidizable substrates and prevent or delay the oxidation of the substrates by significant amounts substrates (Diplock, 1994). In recent years, research has shown that the production of oxidative stress and free radicals, namely ROS and RNS, plays a key role in the developing of a variety of illnesses, particularly cancer (Reuter *et al.*, 2010, Zuo *et al.*, 2015). In addition, antioxidants, primarily of natural origin, also have a protective effect against several mycotoxins (Sorrenti *et al.*, 2013).

The body reproduces its glutathione and can be destroyed by oxidative stress induced by mycotoxins. In the synthesis of GSH in a cell, GSH can only be synthesized by cells when cysteine is present as the rate-limiting substrate (Atmaca, 2004), and providing animals with thiols improves their GSH synthesis and its concentration (Badaloo *et al.*, 2002). Above all this context, it is evident that the s-containing compounds present in this plant can potentially eliminate the deleterious effect posed by the presence of mycotoxins in animals.

2.5.5 Use of zinc oxide and *T. violaceae* bulb extract-based nanoparticles in pig nutrition

Increasingly, nanotechnology is being utilized for utilizing nanoparticle-sized essential elements so that the animal's ability to absorb these elements is enhanced, improving production and health (Hussain *et al.*, 2020). Zinc is one of the most abundant trace elements in the animal body, and it should be added to the animal diets because it is not stored nor synthesized by the body (Swain *et al.*, 2016a). Among the most commonly used zinc supplements, zinc oxide has high antibacterial, antifungal, and growth-promoting properties (Yusof *et al.*, 2019). In addition to reducing zinc deficiency, it also lowers growth retardation (Rajendran and Mani, 2020). The size effect, however, improves the bioavailability of ZnO. Nano-sized ZnO increases bioavailability by enhancing the ionization of zinc. This section of the review aims to review the latest available literature on the effect of zinc oxide nanoparticles on animal health and production.

2.5.5.1 Effect of zinc oxide on productive performance and meat quality

Several studies have been conducted to explore the use of nano-ZnO on pig nutrition and performance (**Table 2.9**). Studies have shown zinc oxide nanoparticles to improve growth efficiency, boost feed consumption, and give economic advantages to weaned piglets (Yang and Sun, 2006, Wang *et al.*, 2018). Additionally, positive findings were obtained in dietary ZnO-N at high and low concentrations (5-60 mg/kg nano-ZnO), They improved the average daily feed intake and grain: feed ratio and after that increased the nutrient and energy utilization (Milani *et al.*, 2017).

According to Pei *et al.* (2019b), in weaned pigs, dietary supplementation with varied amounts of nano-ZnOs boosted average daily feed intake and average weight gain in ways comparable to or better than a high dosage of conventional ZnO. The response of the pigs to the supplementation of concentrations of nano-ZnOs, especially 450 mg/kg ZnO, improved average daily gain, average daily feed intake, and gain to feed ratio as efficient as a 3000 mg Zn/kg diet from conventional ZnO. Also different studies suggest that the utilization of nano-ZnO on weaned piglets enhanced the average daily gain and thereafter the slaughter weights (Sun et al., 2019, Mokone et al., 2022b). One of the biggest advantages of utilizing nano-ZnOs is that they reduce mineral excretion, which lowers the environmental challenges, one of the major challenges of feeding bulk zinc (Pei *et al.*, 2019b). There is a dearth of information on the effect of *T. violaceae* bulb extract-based nanoparticles on pig nutrition and pork quality, and the impact of zinc-oxide nanoparticles on pork quality.

Table 2. 9: Effect of nanoparticles on growth and haemo-biochemical parameters

Model and period of study	Number of animals	Nanoparticle dosage	Nanoparticles characterization	Main Findings	References
Weaned piglets for 45 days	96	Included in the diet	142 ± 15 nm	↑ zinc digestibility, serum growth hormone levels and carbonic anhydrase activity.	Li *et al.* (2016b)
Castrated males and females for 21 days	160	5, 30 or 60 mg/kg.	70 ± 38.6 nm	↑gain:feed ↑plasma Zn ↑nutrient and energy digestibility ↓Average daily feed intake	Milani *et al.* (2017)
Weaned piglets for 14 days	216	1200 mg/kg	30 nm	↑ plasma and tissue zinc ↑ mRNA expression of ZO-1 in ileal mucosa	Wang *et al.* (2017b)

Weaned piglets for 21 days	150	150, 300, or 450 mg/kg	71.61 nm	↑Average daily gain and Average daily feed intake ↑Zinc retention in the serum, heart, liver, spleen and kidney ↓zinc excretion	Pei *et al.* (2019b)
Weaned piglets for 28 days	216	0.3, 0.4, 0.5 or 0.6 g/kg.	25.0 nm	↑Average daily gain ↑ serum concentrations of alkaline phosphatase, (IgG) and superoxide dismutase. ↑ Immunoglobulin M (IgM) and the concentrations of superoxide dismutase and metallothionein in the liver	Sun *et al.* (2019)
Weaned piglets for 26 days	144	200, 300, 400, or 500 mg/kg	-	↑ intestinal antioxidant and immune capacity	Cui *et al.* (2021)
Weaned piglets for 82 days	30	200, 400 and 600 mg/kg.	50 nm	↑ weight gain ↑albumin ↑Total protein ↑Slaughter weights	Mokone *et al.* (2022b)

2.5.5.2 Effects of zinc oxide on biochemical and physiological parameters

Weaned piglets show several important problems, such as weak physiology and immune systems related to susceptibility to infections and diseases, especially diarrhoea and growth reduction. According to a recent study, ZnO affects the expression of proteins involved in glutathione metabolism and boosts the expression of anti-oxidative enzymes (Wang *et al*., 2009). Elevated GSH-Px activity may be an adaptive mechanism in response to increased oxidative stressors (Zhu *et al*., 2006).

Plasma zinc concentration was increased in pigs fed diets supplemented with 5, 30, and 60 mg/kg of nano-ZnO (Milani *et al*., 2017). Additionally, Pei *et al*. (2019b) reported Zn retention in the serum, heart, liver, spleen and kidney in pigs fed 150, 300 and 450 mg/kg nano-ZnO for 21 days, while it reduced the Zn excretion. In the same study, nano-ZnO had been reported to decrease the level of IgA IL-6 and TNF-alpha. According to (Li *et al*., 2016b), the inclusion of nano-ZnO in the diet of weaned piglets enhanced zinc digestibility, which then increased serum growth hormone levels. Mokone *et al*. (2022b) showed that the varying levels of nano-ZnO (200, 400 and 600 mg/kg) increased the serum levels of albumin and total protein.

Wang *et al*. (2017b), also reported that the supplementation of nano-ZnO at 1200 mg/kg increased the concentration of plasma zinc and also in tissues, also, they increase the mRNA expression of zonula occludens-1 (ZO-1) in ileal mucosa. Cui *et al*. (2021) reported an increase in intestinal antioxidant and immune capacity in pig-fed graded levels (200, 300, 400, 500 mg/kg) nano-ZnO. This suggests that one of the properties of ZnO nanoparticles is to enhance the antioxidative status of the animal. Now the properties that both T. violacea bulb and nano-ZnO have, open the room for further investigation of the effect of T. violacea bulb extract-based Phyto-encapsulated nanoparticles on pig production and health. There is a dearth of information on the effect of zinc-oxide and *T. violaceae* bulb extract-based nanoparticles on the impact on biochemical and physiological parameters.

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CHAPTER 3: SYNTHESIS OF ZINC OXIDE NANOPARTICLES USING EXTRACT OF *T. violacea* BULB

3.1 Introduction

Nanomaterials continue to gain scientific interest due to their nano range sizes, which confers some novel physical and chemical properties. In comparison to macro and micro materials, their increased surface area to volume ratio alters the mechanical, thermal, and catalytic properties of the material, thereby leading to increased dominance of the behaviour of atoms on the surface of the particles over those in the interior of the particles (Mathew *et al.*, 2010, Songsungkan and Chanthai, 2014). In addition to size, the shape (Madathiparambil Visalakshan *et al.*, 2020) and surface chemistry (Chen *et al.*, 2019a, Srijampa *et al.*, 2020) of engineered nanoparticles (ENPs) also play critical roles in defining ENP properties, with the latter factor interfacing with and regulating interactions between the ENP and the external environment particularly in biological systems (Huang *et al.*, 2021).

Classified broadly as organic and inorganic (including magnetic, noble metal, and semiconductor) ENPs, the latter is particularly of growing interest as they provide superior material properties and functional versatility (Xu *et al.*, 2006). Among the semiconductor nanoparticles, and specifically within the metal oxides, zinc oxide (ZnO) has gained much research attention and is considered to be one of the most promising materials (Aldeen *et al.*, 2022). This is due to its wide range of attractive properties such as chemical stability under ambient temperature, cost-effectiveness, wide band gap, and high surface-to-volume ratio (Jamdagni *et al.*, 2018). Hence, ZnO has found applications in wastewater treatment, bio-sensing, and optoelectronic devices (Murali and Sohn, 2018, Chen *et al.*, 2019b, Fouda *et al.*, 2018). In addition to this, it has been extensively applied in drug delivery, tissue engineering, agriculture and various biomedical fields due to its non-toxicity, chemical stability and ease of properties' modification (Iqbal *et al.*, 2021).

The synthesis of nanoparticles has conventionally been achieved by using chemical methods that involve the use of metallic precursors, stabilizing agents, and reducing (inorganic and organic) agents (e.g. sodium citrate, ascorbate, sodium borohydride ($NaBH_4$), elemental hydrogen, polyol process, toluene solvent, *N*,*N*-dimethylformamide (DMF) and poly(ethylene glycol)-block copolymers) (McGillicuddy *et al.*, 2017) to prevent the agglomeration of colloids (Zhang *et al.*, 2020). Unfortunately, the chemical method has become less favoured due to the

harmfulness and toxicity of chemicals to human health and the environment (Hussain *et al.*, 2016). In addition, chemically synthesized nanoparticles involve high material costs and, together, these disadvantages led to the emergence of the green chemistry (nanobiotechnology) approach to nanoparticle synthesis. The green approach to materials synthesis has the advantage of being biologically safe and environmentally friendly (Varma, 2012). It, therefore, results in the production of highly stable and easily studied nanoparticles (Gour and Jain, 2019).

The green strategy employs the principle of biomimicry in its exploitation of biomolecules (bioactive compounds) and metabolites from plant extracts, natural substances (vitamins, enzymes, amino acids, etc.) and microorganisms (bacteria, actinomycetes, yeasts, fungi, and algae) as reducing and capping agents in the bio-fabrication of nanoparticles for various applications (Mathew *et al.*, 2010, Gour and Jain, 2019). In particular, the utilization of plant extracts as sources of phytochemicals that serve as metal-reducing and capping agents (phyto-biosynthesis) has been demonstrated to be extremely practical and cheap. (Aisida *et al.*, 2020, Okeke *et al.*, 2020, Rastogi *et al.*, 2017). They also have great potential for expansion to a larger-scale generation of ENPs. In addition, plant-assisted nanoparticle synthesis kinetics is amply higher than in other biosynthetic approaches (Jadoun *et al.*, 2021). Plants are preferred to microbes for green synthesis of nanoparticles as they are non-pathogenic (Zhang *et al.*, 2020). Various parts of plants including extracts of fruit peels (Thi *et al.*, 2020), seeds (Shabaani *et al.*, 2020), flowers (Dobrucka and Długaszewska, 2016), roots (Raj and Jayalakshmy, 2015), stems (Joel and Badhusha, 2016) have been used to synthesize ZnO and other metal nanoparticles.

Hitherto limited studies have used *Tulbaghia violacea* bulb extracts in the biogenic synthesis of nanoparticles. *Tulbaghia violacea*, an indigenous plant widespread in Southern Africa (Bungu *et al.*, 2008), has similar secondary metabolites (mainly sulphur compounds) and biological activities as garlic (*Allium sativum*) (Aremu and Van Staden, 2013, Bungu *et al.*, 2008). It also contains numerous odour-forming compounds (Kubec *et al.*, 2002a, Kubec *et al.*, 2013a) and bioflavonoids (e.g. quercetin) (Hutchings, 1996), as well as flavonols, tannins, phlobatannins, proanthocyanidins, leucoanthocyanins, terpenoids, saponins, proteins, steroids, cardiac glycosides, coumarins, alkaloids, carbohydrates and anthocyanins that have potent antioxidant activities (Madike *et al.*, 2020, Takaidza *et al.*, 2018). Extracts from *T. violacea* have so far been utilized to fabricate polycaprolactone nanofibers (Madike *et al.*, 2020) as well as silver nanoparticles (Ledinga, 2019). However, no studies have reported the use of *T.*

violacea bulb extracts in the biogenic synthesis of ZnO nanoparticles. This study aimed to synthesize and characterize ZnO nanoparticles using extracts of *T. violacea*.

3.2 Materials and Methods

3.2.1 Plant collection and chemicals

T. violacae bulbs were harvested from the North-West University gardens at Mafikeng Campus and were properly identified. Zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$) and sodium hydroxide (NaOH) were purchased from Merck, Germany.

3.2.2 Preparation of aqueous extracts of *T. violacea*

The *T. violaceae* bulbs were cleaned with distilled water, the exterior of the bulbs was removed manually, and the bulbs were then dried at room temperature for 14 days. The dried plant bulb was cut into smaller pieces and dry blended to powdered form (**Figure 3.1**). An aqueous extract of the plant was prepared by adding 100 mL of distilled water to 1 g of bulb powder, and heated at 80 °C for 1 h with vigorous stirring (**Figure 3.1**), following a modified procedure (Osuntokun *et al.*, 2019a). It was allowed to cool to room temperature and filtered with no.1 Whatman filter paper with a pore size of 11 micrometres.

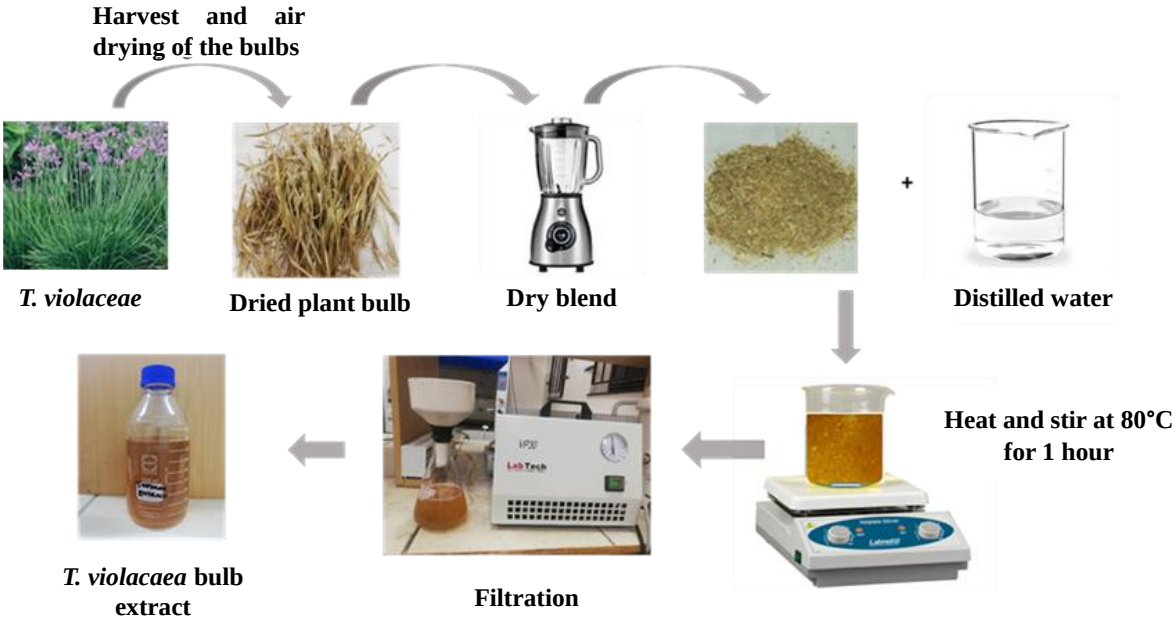

Figure 3. 1: Preparation of aqueous extract of *T. violacea* bulbs

3.2.3 Synthesis of *T. violacea* bulb extract-mediated zinc-oxide nanoparticles

ZnO nanoparticles were synthesised through a facile, green synthetic route following a modification of a reported method (Osuntokun *et al.*, 2019a). In a typical procedure, about 20 mL of the aqueous extract of *T. violacea* bulb extract was mixed with 0.05 M aqueous solution of $Zn(CH_3COO)_2 \cdot 2H_2O$, followed by the dropwise addition of 2.0 M sodium hydroxide solution to adjust the pH of the mixture to 12. The solution was continuously stirred for about 30 min, then transferred into a 100 mL conical flask and boiled at 80 °C until precipitates were formed, which marked the completion of the reaction (**Figure 3.2**). The solution was allowed to incubate at room temperature overnight, after which the formation of the nanoparticles could be visibly observed. The precipitates were collected by centrifuging at 4300 rpm for 30 min, and then washed twice with distilled water to remove unreacted metal salt. The precipitate was finally washed with 98% ethanol to isolate the pure product and then dried in the oven at 50 °C for 3 h to obtain a pale white powder which was further calcinated at 350 °C for 1 h to produce ZnO nanoparticles (**Figure 3.2**).

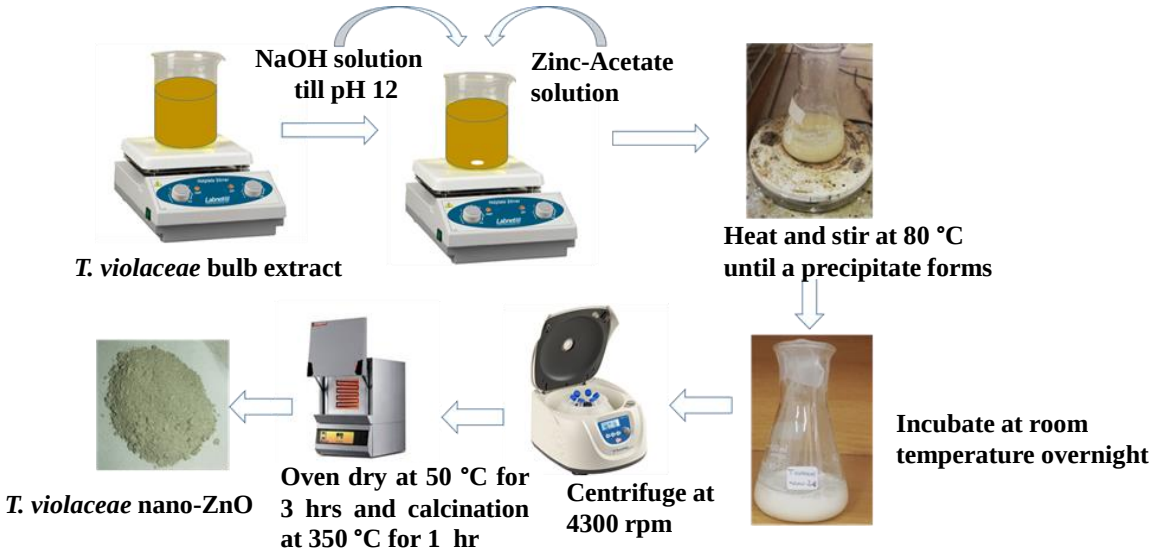

Figure 3. 2: The schematic presentation of the green synthesis of *T. violacea* nano-ZnO.

3.2.4 Conventional synthesis of ZnO Nanoparticles

The ZnO nanoparticles were also synthesized through a physical techniques presented (**Figure 3.3)**. In brief, 0.05 M of $Zn(CH_3COO)_2 \cdot 2H_2O$ was heated in a muffle furnace at 350 °C for 4 h, giving rise to a fine white powder labelled as conv. nano-ZnO.

Figure 3. 3: Conventional synthesis of ZnO nanoparticles

3.2.5 Phyto-encapsulation of *T. violaceae* Bulb Extract-based Zinc oxide Nanoparticles

The phytochemicals extracted from the *T. violacea* bulb using the method described above (**section 3.2.2**) were re-introduced to the green synthesized ZnO nanoparticles to phyto-encapsulate the nanoparticles. In brief, 2 g of *T. violacea* bulb extract-based zinc-oxide nanoparticles were added into 100 ml of *T. violacea* bulb extract in a 250 mL beaker, and stirred at 60 °C for 24 h to yield the phyto-encapsulated nano-ZnO.

3.2.6 Characterization of the nanoparticles

Advanced D8 x-ray diffraction (XRD) was done with an instrument from Bruker, equipped with a proportional counter using Cu Kα radiation (k = 1.5405 Å, nickel filter) was used to confirm the crystalline phase and was recorded in the range 2θ = 20 to 80°. Fourier transform-infrared (FTIR) spectra before calcination, and those of plant bulbs were recorded in the wavelength range of 400–4000 cm^{-1}, on a Cary 670 FTIR spectrometer (Agilent Technologies). External morphology of *T. violacea* ZnO nanoparticles was examined using a scanning electron microscope (SEM) Quanta FEG 250 under an acceleration voltage of 30 kV. The internal morphology and sizes of the nanoparticles were characterized using a TECNAI G2 (ACI) transmission electron microscope (TEM) with an accelerating voltage of 200 kV. The elemental composition of the nanoparticles was examined using energy-dispersive x-ray spectroscopy (EDX). The absorbance properties of synthesized *T. violacea* ZnO nanoparticles

were recorded from 200 to 800 nm using a Cary 30 UV-vis spectrophotometer (UV) (Agilent technologies). Photoluminescence (PL) analysis of *T. violacea* ZnO nanoparticles was recorded from 200 and 800 nm using a Perkin Elmer LS 45 fluorescence spectrometer.

3.3 Results and discussion

3.3.1 X-ray Diffraction (XRD) studies of *T. violacea* - ZnO nanoparticles and Conventional ZnO nanoparticles

The structural (XRD) and morphological (SEM and TEM) studies of the green synthesized ZnO and the **ones** prepared by the conventional approach **were** carried out **and** characterized. The XRD patterns of the *T. violacea* ZnO nanoparticles obtained after calcination at 350 °C for 1 h alongside the reference pattern from the standard file (JCPDS No. 00-036-145) are shown in **Figure (3.4)**. The patterns of the green synthesised ZnO nanoparticles showed distinguishable peaks at 2θ values of 31.82°, 34.46°, 36.30°, 47.59°, 56.67°, 62.91°, 66.41°, 68.00°, 69.31°, 72.55°, 76.98°, and 81.48°. The diffraction pattern matches well with the ZnO hexagonal wurtzite phase, lattice parameters a = b = 3.249 Å, c = 5.206 and corroborates previously reported findings (Ishwarya *et al*., 2018, Chan *et al*., 2021, Umamaheswari *et al*., 2021). Also, the conventionally synthesized ZnO nanoparticles which were obtained after calcinating zinc acetate dehydrate at 350 °C for 4 h are presented in **Figure (3.5)**. These particles follow the same reference pattern from the standard file (JCPDS No. 00-036-145) with the position of the peaks, appearing at the 2θ values of 31.87°, 34.53°, 36.36°, 56.71°, 62.98°, 66.48°, 69.20°, and 88.15°. The appearance of sharp peaks indicates that the ZnO nanoparticles were highly crystalline, while the average crystallite size of the nanoparticles was calculated using the Debye-Scherrer's Equation **(1):**

$$D=K\lambda/\beta\cos\theta \quad \textbf{(1)}$$

Where: D: is the crystallite size of ZnO in nm,

K: is the Scherrer shape factor (0.90),

λ: is the X-ray wavelength used (1.5406 Å),

β: is the full width at half maximum (FWHM) in radians, and

θ: is the Bragg diffraction angle in degrees.

Average particle sizes of *T. violacea* nano-ZnO were estimated as 28.69 nm, and the average particles size for the conventionally synthesized nanoparticles is 32.63 nm, with the particle size calculations shown in **Table (3.1) and (3.2)**. The larger crystalline size of the ZnO obtained from the conventional method might be attributed to the absence of a capping agent during the synthesis period and the high surface area of the particles, which led to increased growth.

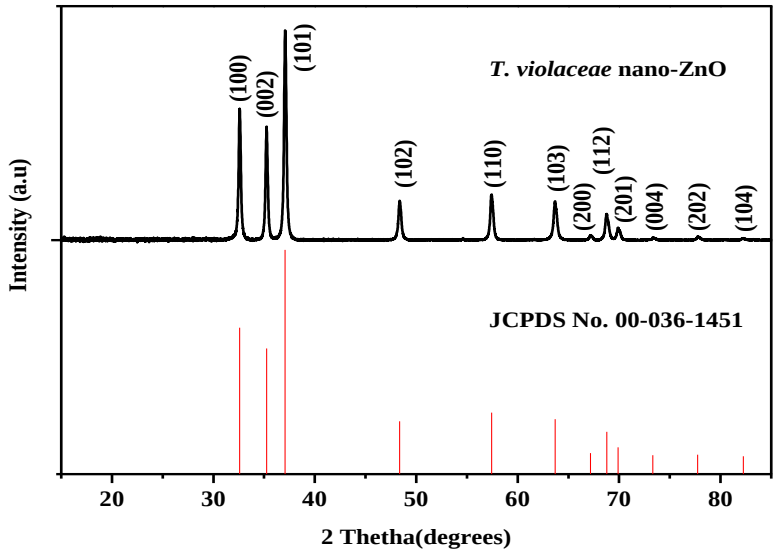

Figure 3. 4: XRD of *T. violacea* bulb-extract ZnO nanoparticles.

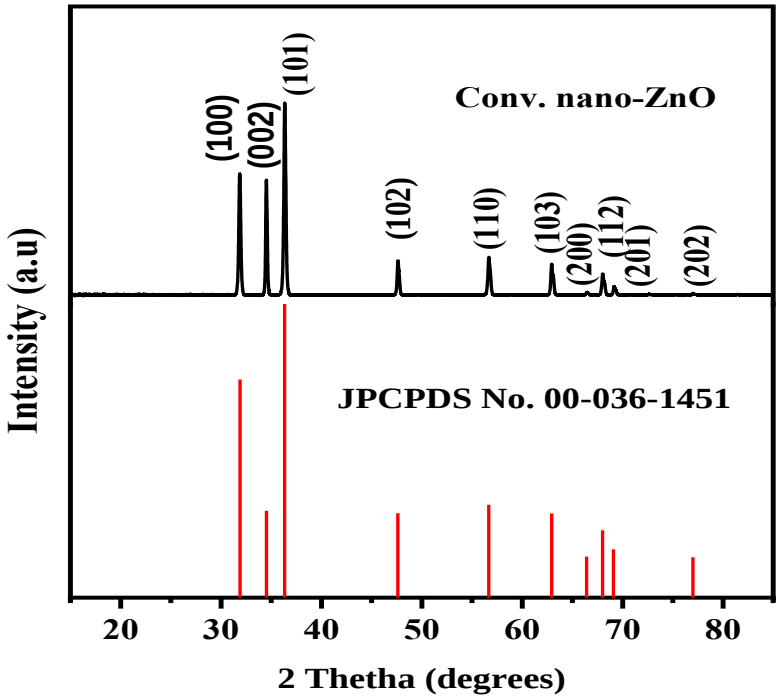

Figure 3. 5: XRD of conventionally synthesized ZnO nanoparticles

Table 3. 1: Calculations of the average particles size of the *T. violacea* nano-ZnO**.**

Centre (2Theta)	Width	Theta	D=Kλ/βcosθ	Average D(nm)
31,82458	0,22983	15,91229	35,94318803	28,6990892
34,47868	0,23083	17,23934	36,03506096	
36,31236	0,24681	18,15618	33,87446075	
47,61062	0,29431	23,80531	29,50297562	
56,67234	0,31426	28,33617	28,72072990	
62,92382	0,36921	31,46191	25,22545433	
66,45480	0,37264	33,2274	25,48580716	
68,03417	0,37722	34,017085	25,40817602	
69,17115	0,40342	34,585575	23,91940718	
77,05866	0,44393	38,52933	22,87563207	

Table 3. 2: Calculations of the average particles size of the conventional nano-ZnO.

Centre (2The`ta)	Width	Theta	D=Kλ/βcosθ	Average D(nm)
31,87106	0,23493	15,93553	35,16698143	32,63478204
34,52979	0,18677	17,264895	44,54208561	
36,35882	0,24003	18,17941	34,83592925	
56,70865	0,2462	28,354325	36,66661068	
62,97845	0,29238	31,489225	31,86335838	
66,48070	0,24403	33,24035	38,92323788	
68,06556	0,31686	34,03278	30,25388148	
69,20107	0,29745	34,600535	32,44681474	
88,14588	1,22668	44,07294	9,014138928	

3.3.2 Fourier transform infrared spectral (FTIR) studies of the *T. violacea* plant bulb extract

FTIR was employed to investigate the possible functional groups in the *T. violacea* plant bulb, and the spectrum is presented in **Figure (3.6)**. It shows a broad peak between 3000 and 3600 cm $^{-1}$, which could be assigned to the stretching vibration of the hydroxyl group (Das *et al.*, 2011). Two low-intensity peaks around 2918 and 2852 cm^{-1} corresponded to the symmetric and asymmetric, C–H of the aliphatic group (Divya *et al.*, 2017). A weak stretching peak around 2310 cm $^{-1}$ is attributed to the S-H or thiol group, and a slightly broad peak around 1603 cm $^{-1}$ is attributed to the O-H bending vibration. The band at 1320 and 1015 is associated with S=O thiol (sulfonate) group (Songsungkan and Chanthai, 2014). All the dominant functional groups present in this bulb are due to the major chemical characteristics of the plant, which are thiols and cysteine amino acids (Kubec *et al.*, 2002a, Lyantagaye, 2011a). The FTIR results revealed that band shifts in the wavenumber that are either raised or lowered from their convectional values. This shift in the wavenumber could be attributed to the presence of various functional groups in the extract (Umamaheswari *et al.*, 2021).

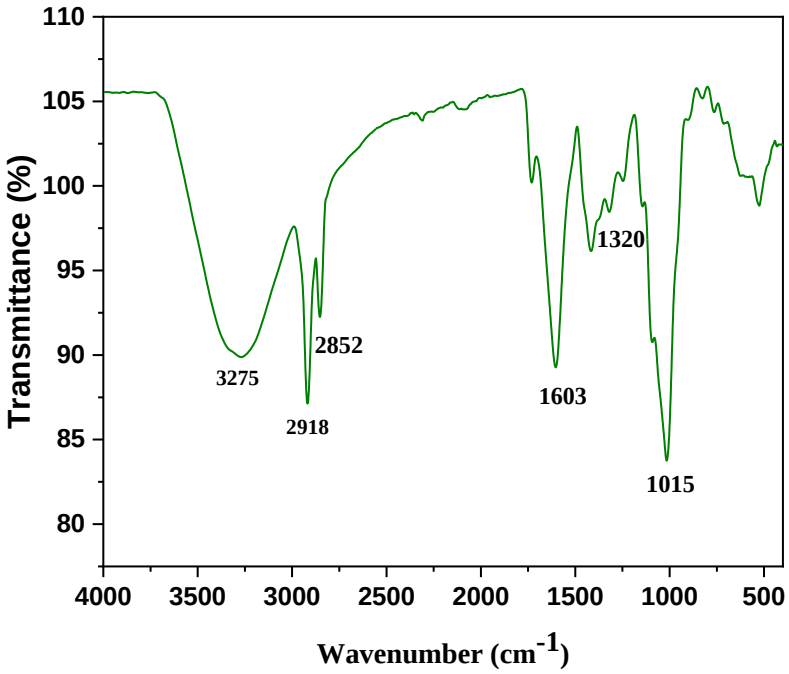

Figure 3. 6: Fourier transform infrared spectrum of *T. violacea* bulb.

3.3.3 Scanning electron microscopic (SEM) and Energy dispersive X-ray (EDX) studies of *T. violacea* ZnO nanoparticles

The SEM images of the green synthesized ZnO nanoparticles at different magnifications are presented in **Figure 3.7**(a) and (b). The images show that the particles were spherical and closely packed. Energy-dispersive X-ray spectroscopy (EDX) analysis presented in **Figure (3.7)** (c) revealed that the elemental composition of the nanoparticles was mainly Zn and O, with an atomic percentage composition similar to results reported in related studies *(Baruah et al.*, 2021, Khalafi *et al.*, 2019, Osuntokun *et al.*, 2019a). Also, the SEM has been performed on the conventionally synthesized nanoparticles, and the results are shown in **Figure (3.8)** (a) and (b) at different magnifications. The particles appeared to be spherical and corroborated the morphology of the particles obtained via the green route and similar literature report (Heng *et* al., 2011, Vanathi *et al.*, 2014, Yedurkar *et al.*, 2016). The observed carbon peak in the EDX spectra could well be attributed to the carbon tape used as part of the sample holder. The reaction product was found to be made up of high purity zinc nanoparticles (Dobrucka and Długaszewska, 2016).

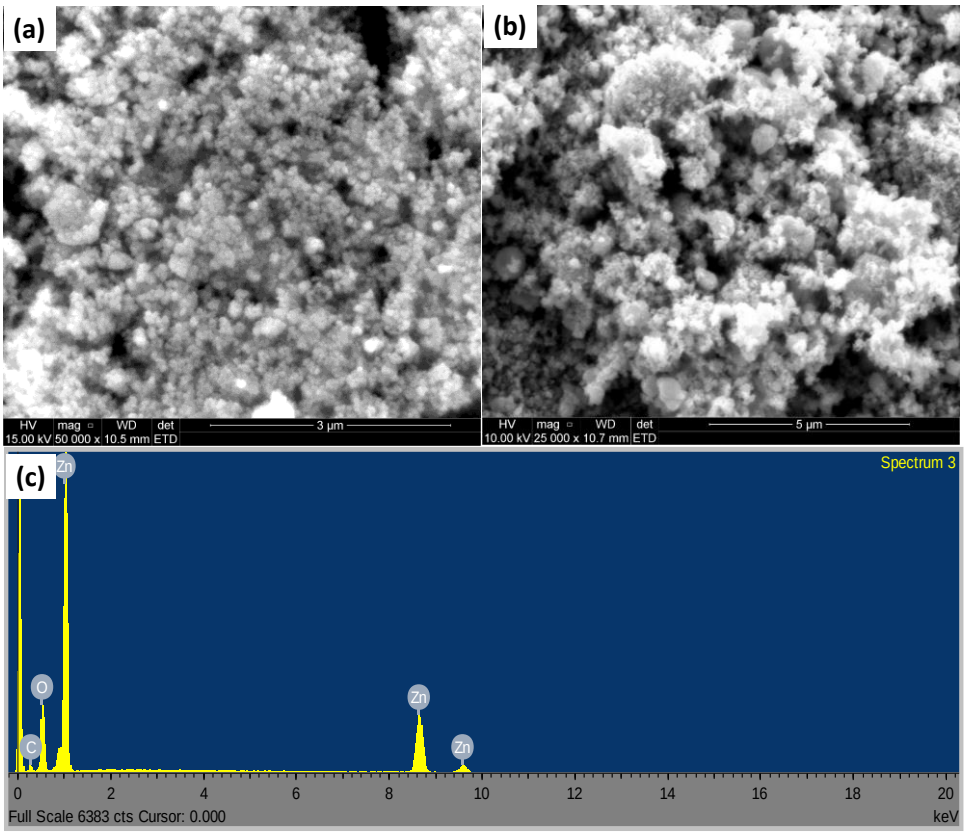

Figure 3. 7: SEM images at (a) low, and (b) high magnifications, (c) EDX spectrum of ZnO synthesized using the aqueous extract of *T. violacea* bulbs.

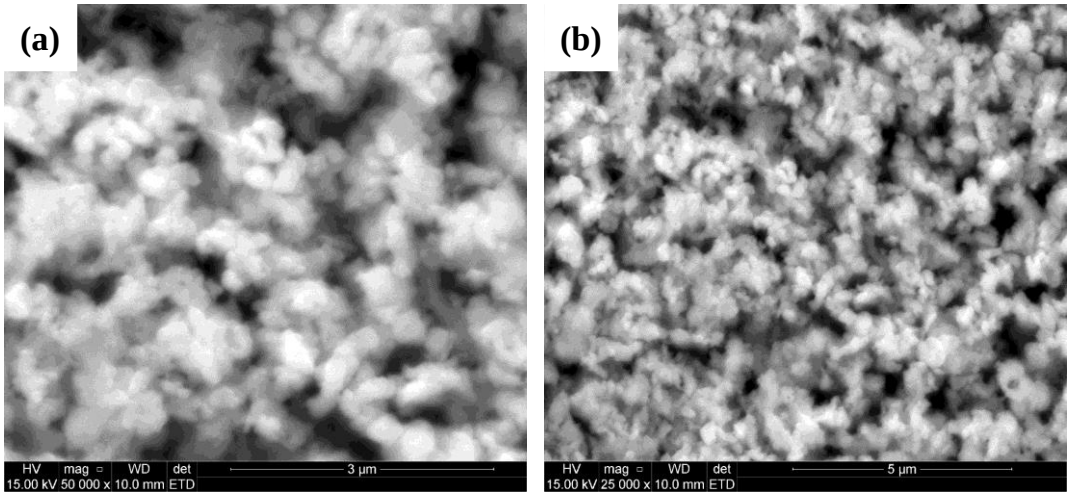

Figure 3. 8: SEM images at (a) low, (b) high magnification of conventionally synthesized ZnO nanoparticles

3.3.4 Elemental mapping studies of *T. violacea* ZnO nanoparticles

The elemental mapping micrographs are presented in **Figure 3.9**, which showed that component elements (Zn and O) were uniformly and evenly distributed across the entire nanoparticles (Ali *et al.*, 2018).

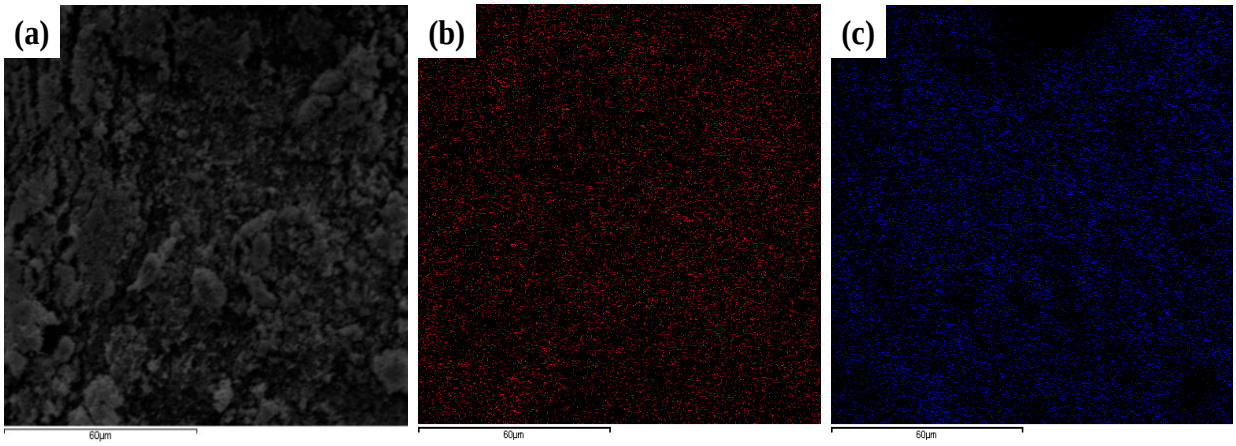

Figure 3. 9: (a) Elemental mapping of ZnO, (b) Zn, and (c) O elements of ZnO prepared using aqueous extract of *T. violacea* bulbs.

3.3.5 Transmission electron microscopic (TEM) studies of *T. violacea* bulb extract-based and conventional ZnO nanoparticles

The internal morphology of the ZnO nanoparticles was studied using TEM analysis, and the micrographs are presented in **Figure 3.10** (a) and (b) at different magnifications for samples obtained by green routes, and those of conventionally synthesized nanoparticles are shown in **Figure (3.11)** (a) and (b). The ZnO nanoparticles were of spherical morphology. Therefore, the observed dark areas could be the result of the agglomeration of the particles, and this observation has been corroborated by other reported studies involving the use of plant extracts (Vanathi *et al.*, 2014, Luque *et al.*, 2018, Elemike *et al.*, 2019a). **Figure (3.10)** (c) shows the particle size distribution histogram, highlighting that particle sizes range from 18.39 to 89.774 with an average of 45.26 nm. In the case of conventionally synthesized nanoparticles, the particle size and area distribution are shown in **Figure 3.11** (c). The particle size ranges from 19.29 to 98.84 nm with an average of 60.64 nm. The differences in the particle size were attributed by several factors, one being that the green synthesized nanoparticles had no capping agent or no modifier, and the surface of the particles were not capped hence the larger particle size of the *T. violacea* bulb extract-based zinc oxide nanoparticles, capping agents are known to enhance the particles size of nanoparticles (Phan and Nguyen, 2017).

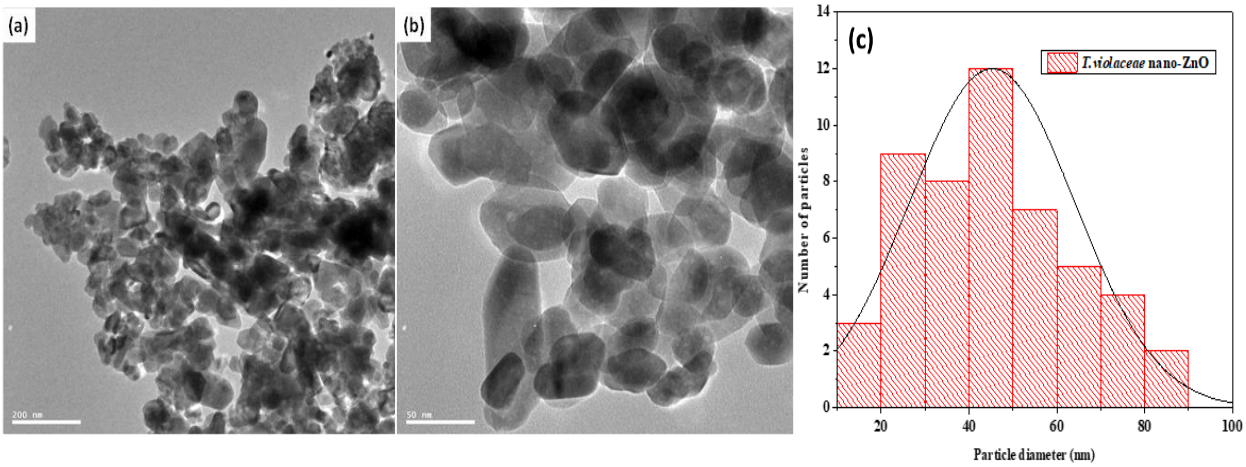

Figure 3. 10: TEM images at (a) low, and (b) high magnification, (c) Particle size distribution histogram of ZnO synthesized using the aqueous extract of *T. violacea* bulbs.

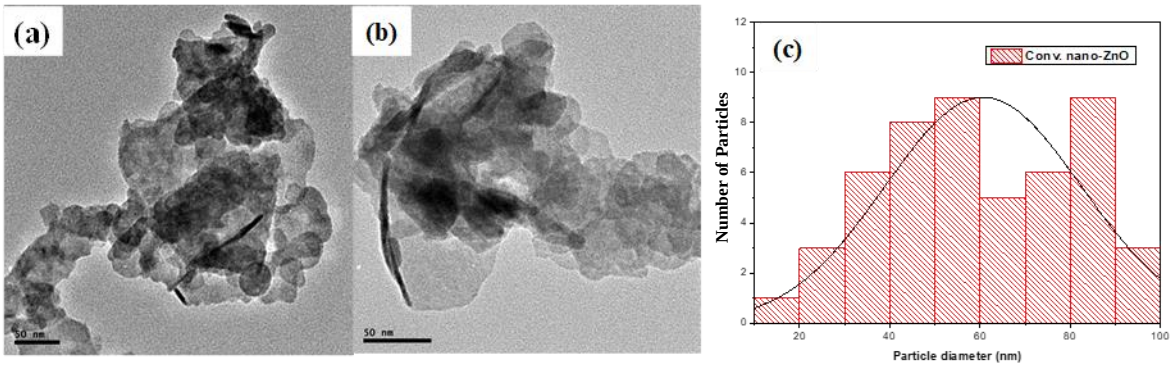

Figure 3. 11: TEM images at (a) low, and (b) high magnification, and (c) particle size distribution histogram of conventionally synthesized ZnO nanoparticles

3.3.6 UV-visible spectroscopy studies of *T. violacea* bulb extract-based biosynthesized ZnO nanoparticles

Semiconductor nanoparticles experience a change in optical property compared with their bulk counterpart due to quantum confinement. Therefore, the optical absorption spectra of ZnO nanoparticles were investigated at room temperature in the range 200–700 nm, as shown in **Fig. 3.12** (a). UV-visible absorption spectra of the biosynthesized ZnO nanoparticles shows maximum absorption peak at 273 nm. This corroborates with the earlier studies on the biosynthesis of zinc oxide nanoparticles using plant extract of *Vitex negundo L.* which confirmed the absorption of nanoparticles at 278 nm (Ambika and Sundrarajan, 2015). The optical band gap of the nanoparticles was then calculated from Tauc's relation shown in equation **(2)**:

$$\alpha h\nu = A(h\nu - Eg)^n \qquad (2)$$

Where, **α**: the absorption coefficient, **A**: constant, h: stands for Plank's constant, **v**: frequency of photon, **Eg**: denotes the optical band gap energy, and **n**: 1/2, which is for a direct band gap semiconductor.

A tangent is drawn on the spectra, and an extrapolation of the linear region of the plot of $(\alpha h\nu)^{1/2}$ on the ordinate axis against the energy of the photon (hv) on the abscissa axis produces the exact value of the band gap energy of the nanoparticles. Using equation **(2)** also, the optical band gap of the ZnO was obtained as 3.7 eV (**Fig: 3.12** (b)), which was shifted to the blue region compared to the band gap energy of ZnO bulk and corroborated with other related findings (Anbuvannan *et al.*, 2015). The shift was a consequence of the ZnO nanoparticulate size, which shifts the absorption maximum to a higher energy region.

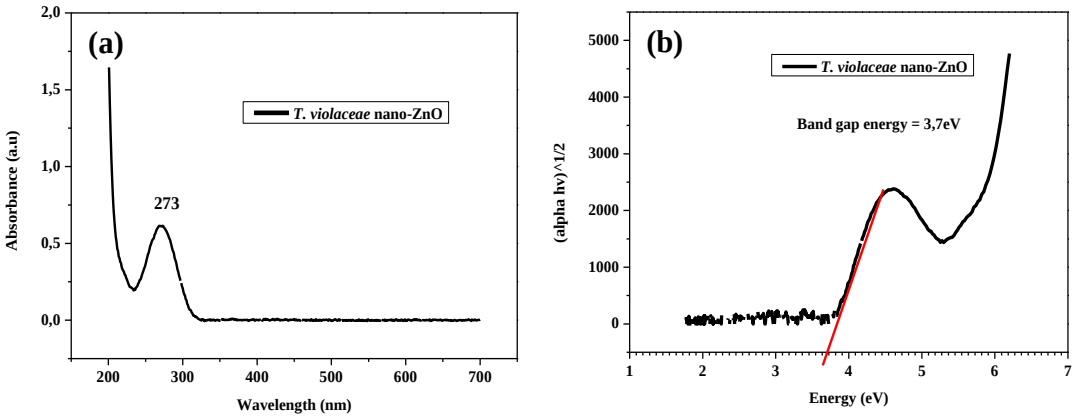

Figure 3. 12: (a) UV-visible spectrum and (b) band gap energy of nanoparticles.

3.3.7 Photoluminescence (PL) studies of *T. violacea* ZnO nanoparticles

Photoluminescence (PL) spectroscopy is an important tool used to study the optical properties of semiconducting materials. The room temperature PL spectrum of the ZnO nanoparticles is shown in **Fig. 3.13**. The spectrum was recorded over a wavelength range of 200 nm to 600 nm and showed an emission peak at 324 nm, which corresponds to the band edge emission of the nanoparticle. The peak occurred in the UV region, which could be attributed to the electronic transition from conduction band tail to valence band tail states, corroborating with a previous report (Osuntokun *et al.*, 2019a).

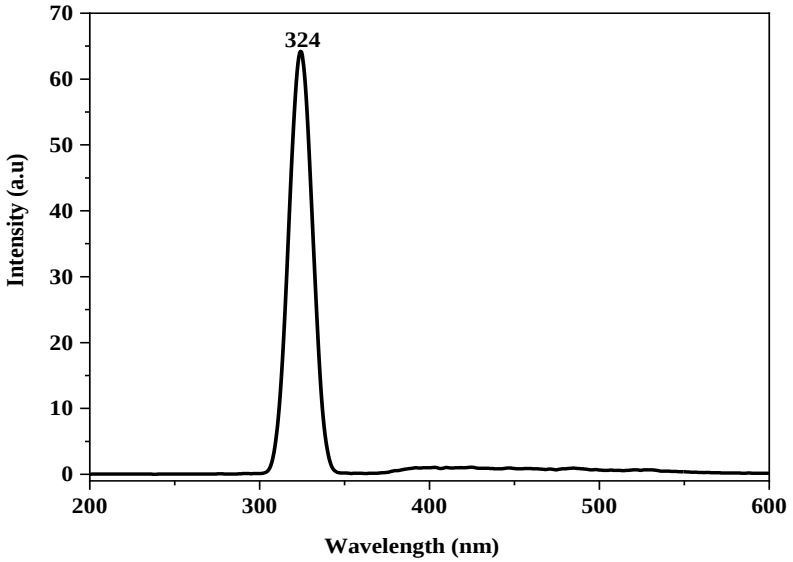

Figure 3. 13: Photoluminescence of *T. violacea* bulb -ZnO nanoparticles

3.4 Conclusion

ZnO nanoparticles have been prepared using the aqueous extract of *T. violaceae*, via calcination at 350 °C for 1 h in air to afford. Also, they were achieved through conventionally through the calcination of zinc acetate at 350 °C for 4 h. The prepared nanoparticles' optical, morphological, and structural properties were studied using UV-vis, photoluminescence, XRD, SEM, and TEM analysis. The XRD revealed that both the nanoparticles were of hexagonal crystalline phase, while FTIR studies indicated the functional groups in the plant that was responsible for the phyto-mediated synthesis. Morphological studies of the ZnO nanoparticles showed that they were both spherical-shaped nanoparticles. They were obtained with an average particle size of 45.26 nm for the green synthesized nanoparticle and 60.64 nm for the conventional ZnO nanoparticles, which also exhibited a high degree of agglomeration. The optical properties of the green synthesized nanoparticles showed a maximum absorption peak at 273 nm (an optical band gap of 3.7 eV) and an emission peak at 324 nm, which corresponded to the band edge emission of the nanoparticles.

3.5 References

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CHAPTER 4: EFFECTS OF ORAL ADMINISTRATION OF *T. violacea* EXTRACT-BASED GREEN ZNO NANOPARTICLES ON PERFORMACE, CARCASS CHARACTERISTICS, MEAT QUALITY, AND SERUM BIOCHEMISTRY IN PIGLETS FED DIETS SUPPLEMENTED WITH NATURALLY MYCOTOXIN-CONTAMINATED MARULA SEED CAKE

4.1 Introduction

The outbreak of the Covid-19 pandemic, along with an ever-increasing human population has intensified global food insecurity. The population growth which is predicted to double by 2050 in SSA will result in an increase in consumption and demand of animal protein, and a tremendous strain on animal production for the market for meat and other animal-derived food products will rapidly increase (Leridon, 2020a). Animal-derived protein (mostly from ruminants, poultry, and pigs) is important in the human diet due to its biological value. Per capita consumption of white meat (chicken and pork) in South Africa drastically increased from 21.48 kg in 2001 to 40.04 kg in 2017 (DAFF, 2017a). However, the high costs of feeds due to imported and expensive conventional protein sources like SBM limits pork production particularly by smallholder farmers in Southern Africa. This provides an opportunity to investigate of alternative protein sources available in SSA such as marula seed cake (MSC) as replacements for the expensive SBM in pig diets.

MSC, a by-product of oil extraction from marula seed kernels, is rich in almost all nutrients essential in pig production (Hlongwana *et al.*, 2021a). It possesses a remarkably high crude protein (CP) content (470 g/kg DM) (Mlambo *et al.*, 2011a, Mdziniso *et al.*, 2016). It is rich in all essential amino acids, similarly to SBM, except for lysine (Mariod and Abdelwahab, 2012, Mthiyane and Mhlanga, 2017a). Yet, due to its richness in CP and residual oil content, MSC is commonly afflicted by anti-nutritional factors, particularly the mycotoxins, to which pigs are most sensitive. Mycotoxins negatively affect body weight gain, feed intake, and feed conversion efficiency due to feed refusal, growth retardation, and even weight loss in pigs (Yang *et al.*, 2020a) through their depletion of antioxidant capacity and induction of oxidative stress (Mavrommatis *et al.*, 2021).

Multiple techniques have been used to facilitate the negative effect of mycotoxins. Dietary application of clay particles such as bentonites and zeolites is the most widely used technique (Dal Pozzo *et al.*, 2016). However, these clay absorbents are expensive and tend to bind minerals and vitamins in the diet (Bhatti *et al.*, 2018, Brown *et al.*, 2014). There is therefore a

need to investigate more effective strategies including nanotechnology. Zinc oxide (ZnO) nanoparticles (Nano-ZnO) possess antioxidant solid properties (Siripireddy and Mandal, 2017, Rajakumar *et al.*, 2018) and could therefore be a solution to the problem of mycotoxins. The objective of this experiment was therefore to investigate the ameliorative effects of orally administered *T. violaceae* bulb extract-fabricated Nano-ZnO against the toxicity of mycotoxins in Large White piglets fed diets supplemented with MSM.

4.2 Materials and methods

4.2.1 Study site and ethical approval

This study was conducted at the NWU Experimental Farm, Molelwane, located in Mafikeng, South Africa (25.85° S, 25.63° E) at an altitude of 1226m above sea level. The vegetation is semi-arid in Savannah, with an average annual rainfall of 450 mm. Temperatures range from 22 to 35° C in summer and 2 and 20°C during winter. This study was approved by the NWU AnimCare Research Committee (NWU-00801-21-A5).

4.2.2 Source and preparation of materials

A total of 60, weaned Large White piglets aged 4-weeks old were purchased from, PIC South Africa. The MSC was purchased from Maganu Production in KwaZulu-Natal Province, South Africa. Soya bean meal and all other feed ingredients were purchased from Simplegrow Agric Services (Pty) Ltd in Irene, Gauteng Province, South Africa. *T. violacea* bulbs were harvested from the grounds of NWU, Mafikeng Campus, South Africa. Zinc acetate and sodium hydroxide were purchased from Merck Chemicals, South Africa.

4.2.3 Housing and management of animals

Upon arrival, piglets were weighed, ear-tagged for easy identification, dewormed against internal parasites using 30 mg/kg Flubendazole for 10 consecutive days. Piglets were vaccinated for MMR and Meningitis, and for yellow fever during the study. They were individually housed in 1.5 x 0.7 x 1.5 m^3 concrete-floored and zinc-roofed pens, each equipped with a 30 cm x 30 cm x 35 cm stainless steel feeding trough and a nipple drinker. All piglets were raised under the same environmental conditions and facilities at room temperatures. Natural ventilation was allowed to reduce the risk of respiratory diseases. The piglets were maintained on natural light and continuous artificial light at night during the day.

The housing facility was cleaned thoroughly and disinfected with F10 disinfectant and fumigated a week before the commencement of the feeding trial. The area surrounding the experimental house was also cleaned. A footbath (F10 disinfectant solution) was placed at the

entrance to the pig house for biosecurity control. An acclimatization period of 2 weeks was allowed for piglets before data collection commenced. Feed and water were provided *ad libitum* during the 32-day experimental period and their quality was monitored daily.

4.2.4 Experimental design, diets and their preparation

The experiment used a completely randomized design (CRD) with 60 piglets (4-6 kg) randomly allocated to two (2) iso-energetic and iso-nitrogenous diets (**Table 4.1**) formulated by replacing SBM (Control; 0% MSC) (Group A) with MSC (Treatment; 20% MSC) (Group B), with the chemical composition of the experimental diets and that of MSC presented (**Table 4.2**). The animals were allocated into five treatment groups. Once-daily, Group A piglets (0% MSC) were orally drenched (gavaged) with the vehicle (25% ethanol in water; 25 mL ethanol: 75 mL water, v/v) whilst Group B piglets (20% MSC) were similarly orally drenched with 10 mL of: vehicle (25% ethanol) (Group B1), 50 mg/L of bulk ZnO in 25% ethanol (Group B2), 50 mg/L of conventionally-synthesized Nano-ZnO in 25% ethanol (Group B3), and 50 mg/L of green (*T. violaceae* bulb extract-fabricated) Nano-ZnO in 25% ethanol (from Chapter 3) (Group B4). Finally, all the piglets were orally drenched in the afternoon at 14h00 by manually and humanely holding each piglet and administering the respective solutions into the buccal cavity. The pen was an experimental unit, and each treatment was replicated 12 times.

Table 4. 1: Ingredient (g/kg diet) composition of experimental diets for weaning piglets.

Ingredients (kg)	0% MSC	20% MSC +			
	Vehicle	Vehicle	Bulk ZnO	Conventional Nano-ZnO	Green Nano-ZnO
Maize 7.50% CP	689.78	647.21	647.21	647.21	647.21
Wheat bran	48.13	100.00	100.00	100.00	100.00
Marula seed cake	-	200.00	200.00	200.00	200.00
Soya oilcake 47% CP	224.08	13.79	13.79	13.79	13.79
Limestone	10.824	11.592	11.592	11.592	11.592
MDCP 21%	8.586	8.501	8.501	8.501	8.501
Salt	5.894	5.988	5.988	5.988	5.988
L-lysine HCL	4.629	4.788	4.788	4.788	4.788
DL-Methionine	2.938	2.909	2.909	2.909	2.909
L-Threonine	1.859	1.960	1.960	1.960	1.960
L-Tryptophan	0.829	0.820	0.820	0.820	0.820
OptiPhos PLUS 5000	0.100	0.100	0.100	0.100	0.100
HOSTAZYM X	0.100	0.100	0.100	0.100	0.100
Std Swine weaner Px 2.2 kg	2.200	2.200	2.200	2.200	2.200
ENDOX Dry	0.042	0.042	0.042	0.042	0.042

OptiPhos PLUS 5000 is a light beige to brown granular form that contains 6-phytase (3.25% w/w), pregelatinised starch (1.5% w/w) and wheat meal (up to 100% w/w) . **HOSTAZYM X** is an additive that contains endo-1,4-beta-xylanase. **Std Swine weaner Px** is a standard weaner premix. **ENDOX Dry** is part of a complete antioxidant system designed to stabilize high fat feeds and protect fat-soluble vitamins in concentrated mineral premixes.

Table 4. 2: Nutrient composition (% DM) of MSC and experimental diets (as-fed basis) formulated for weaning piglets, and their mycotoxin status (ppb).

Components (% DM)	0% MSC	20% MSC +				MSC
	Vehicle	Vehicle	Bulk ZnO (50 mg/L)	Conventional nano-ZnO	Green synthesized nano-ZnO	
Dry Matter	91.57	91.98	91.98	91.98	91.98	95.62
Crude Protein	28.10	27.89	27.89	27.89	27.89	48.17
Organic Matter	95.02	94.98	94.98	94.98	94.98	93.97
Moisture	8.43	8.02	8.02	8.02	8.02	4.38
Ether Extract	3.86	10.27	10.27	10.27	10.27	37.69
Ash	4.98	5.02	5.02	5.02	5.02	6.03
Neutral Detergent Fibre	12.66	18.37	18.37	18.37	18.37	5.41
Acid Detergent Fibre	8.12	12.10	12.10	12.10	12.10	5.30
Acid Detergent Lignin	1.24	3.50	3.50	3.50	3.50	2.15
Mycotoxin status (ppb)						
Aflatoxin B1	103.62	528.42	528.42	528.42	528.42	386.7
Aflatoxin B2	3556.53	6500.53	6500.53	6500.53	6500.53	1244.53
Aflatoxin G1	11922.13	2539.73	2539.73	2539.73	2539.73	2966.93
Aflatoxin G2	3380.60	1241.20	1241.20	1241.20	1241.20	974.2
TOTAL	18962.89	10809.89	10809.89	10809.89	10809.89	5572.37

0% MSC = diet formulated with no MSC; 20% MSC = diet formulated with 200 g/kg MSC substituting commercial dietary protein sources; ZnO = bulk ZnO; Nano-ZnO = conventionally (physically) synthesised ZnO nanoparticles; green Nano-ZnO = *T. violaceae* bulb extract-mediated ZnO nanopartic1

4.2.3 Chemical analysis of MSC and the experimental diets

Dry matter (DM) of MSC and the experimental diets was determined by weighing approximately 1 g of each sample (in 3 replicates) in pre-weighed crucibles and drying them in an oven at 105 °C for 12 hours. The samples were then cooled for 30 min in a desiccator and weighed to obtain the weight differences. The weight loss was measured as the moisture content and the DM was calculated as the difference between the initial weight and the moisture content. In determining the OM, the dried (moisture-free) samples were burnt in a muffle furnace at 550 °C for 5 hours. Burnt samples were then put in a desiccator for 30 min, weighed and the differences between the initial weight and the final weight was calculated. The loss in weight was measured as OM content and the residue as ash.

Crude protein (CP) content was determined following the standard macro-Kjeldahl method (AOAC, 2005: method no. 984.13). Total nitrogen content was determined by the standard macro- Kjeldahl method and converted to crude protein by multiplying the percentage N content by a factor of 6.25. Firstly, two blanks were prepared in the following way: 1 weighed paper was added to each digestion tube along with 2 Kjeldahl tablets. 25 ml of Sulphuric acid (98%) was then added and the blanks were suspended after the tubes were gently swirled. Using an analytical balance, 0.5 g of the sample was weighed on a nitrogen-free paper. The samples were placed including the paper in a digestion tube and 2 Kjeldahl tablets were added. Also, 25 ml of 98% sulphuric acid was added, then suspended. The samples were then digested for 60 to 90 minutes and were allowed to cool at room temperature before distillation. The distillation followed the digestion process which was coupled by dilution, boric acid titration, neutralization and distillation and titration. In-depth, the digestion solution was diluted with 70 ml of distilled water, 60 ml of 4% boric acid was added, along with drops of indicator, and then 70 ml of 45% sodium hydroxide was added to the digestion solution. The whole mixture was then distilled for 4 minutes. The receiving vessel was titrated with N Sulphuric acid to determine the amount used to neutralize ammonia. Then the following formula was used to determine % N, which was therefore used to calculate % CP:

$$N\% = \frac{(\text{Consumption} - \text{Blank}) \times \text{AcidN} \times 0.014}{\text{Weight of sample}} \times 100$$

$$CP\% = N\% \times 6.25$$

The method used to determine crude fat or ether extraction of MSC and the experimental diets uses petroleum ether as an extracting solvent, where the extracted compounds are predominantly triacylglycerides. In conducting this analysis, a labelled filter bag was placed on a digital weighing balance, and was zeroed. For each sample, about 1-2 g was weighed and recorded as (WB1B). The filter bags were heat-sealed and closed within 4mm of the top to encapsulate the sample. A blank bag was included in this technique to measure effect of this extraction on a bag weight. The sample bags were then oven-dried for 2 hours, after oven-dry they were cooled in a desiccant pouch, weighed and recorded as (WB2B). The samples were then put into sample holder or carousel and placed in an extractor. The extraction time was then selected, and extraction proceeded according instrument's instructions. When the extraction process was complete, the samples were placed in the oven for 15-30 min, then cooled in a desiccant pouch and weighed as (WB3B). Now, the following formula was used to calculate % Crude fat:

$$\% \text{ Crude Fat} = \frac{100\ (WB2\ B - WB3B)}{WB1B}$$

The fibre was determined using the ANKOM 2000 Fibre analyser (ANKOM Technology, New York) by measuring the acid detergent fibre (ADF), neutral detergent fibre (NDF), and the acid detergent lignin (ADL) contents of MSC and experimental diets. In preparation for analysis, 0.45 to 0.55 g of each sample was weighed (in triplicates) directly into ANKOM filter bags and sealed with the hot sealer. One blank bag (without the sample) was included for correctional factor determination. For NDF analysis, the neutral detergent solution was prepared by adding 60g of neutral detergent solution concentrate to 10 ml Triethylene glycol in 1L distilled water. Samples were then put inside the bag suspender which was placed into the ANKOM vessel. The neutral detergent solution was then added into the vessel to cover the bags in the suspender and 4ml of alpha-amylase plus 20g of sodium sulphite were added. The heat (>70°C - 100°C) button was turned on and the samples were agitated for 75 min to extract the non-cell wall components. After the extraction, the agitation and heat were turned off, and the hot solution was drained out before the lid was opened. The samples were then removed and rinsed with water (>70 °C - 100°C) with 4ml of alpha-amylase. The samples were dry-pressed gently with a paper towel then rinsed with enough acetone to cover the samples in a glass beaker for 3-5 min. The samples were then oven-dried at 105 °C for 2-4 hours, after which they were cooled in a desiccator at room temperature for 30 min and then weighed. The % NDF was then calculated using the following formula:

$$\% \text{ NDF (as-received basis)} = \frac{(W3 - (W1 \times C1)) \times 100}{W2}$$

Where: W1 = Bag tare weight, W2 = Sample weight, W3 = Dried weight of bag with fibre after extraction process, and C1 = Blank bag correction.

For ADF determination, the acid detergent solution was prepared by adding 4g of acid detergent concentrate to 140 ml of 98% HCl in 1L distilled water. Then 100 mL/bag of the acid detergent solution was added in the fibre analyser vessel, closed and agitated under heat (>70°C - 100°C) for 60 min. After extraction, agitation and heat were turned off, the hot solution was drained before the lid was opened. In three cycles, the samples were then rinsed with water (> 70 °C) and agitated for 5 min until the water was at neutral pH. Bags were then removed and gently drained of excess water, put in 250 mL beakers and acetone added to cover the bags which were then soaked for 3 to 5 min. The bags were then removed from acetone and placed on a wire screen to air-dry at 105°C for 2-4 hours. After that, the bags were removed from oven, placed into a collapsible desiccant directly and flattened to remove air. The bags were then cooled at ambient temperature and weighed, then the following formula was used to calculate %ADF:

$$\% \text{ ADF (as-received basis)} = \frac{(W3 - (W1 \times C1)) \times 100}{W2}$$

Where: W1 = Bag tare weight, W2 = Sample weight, W3 = Dried weight of bag with fibre after extraction process, and C1 = Blank bag correction factor.

For ADL determination, the same samples left after ADF determination were put in a 250 mL beaker and 98% HCl was added to cover the bags which were soaked for 5 min and then %ADL calculated

$$\% \text{ ADL (as-received basis)} = \frac{(W3 - (W1 \times C1)) \times 100}{W2}$$

Where: W1 = Bag tare weight, W2 = Sample weight, W3 = Dried weight of bag with lignin after extraction process, and C1 = Blank bag correction factor.

Mycotoxin analysis was also performed using high performance liquid chromatography-mass spectrophotometry (HPLC-MS) and the Vicam Myco6in1®LC/MS/MS method available at the Animal Health laboratory at North-West University. Four classes of aflatoxins (AFB1, AFB2, AFG1, and AFG2) were determined. In analysing mycotoxins, the procedure prescribed by the manufacturer (EASI-EXTRACT® rBiopharm, Darmstadt, Germany) for the extraction and cleaning of mycotoxins with IAC was followed. In this test, monoclonal antibodies are used, which is why it is very specific to the target toxins. As well as being precise, the test is also sensitive, rapid, and easy to conduct. However, the cost of using this technology limits its use (Şenyuva and Gilbert, 2010, Razzazi-Fazeli and Reiter, 2011). The solvent that was used for this extraction was 80% methanol. In this experiment, the extracts were applied to columns, and the mycotoxins were used as antigens bound to antibodies present in the column. After passing the column through the HPLC apparatus, the mycotoxins were eluted using HPLC grade methanol for determining the concentration of mycotoxins.

This study was conducted using the Shimadzu Prominence UFLC liquid chromatography system (Kyoto, Japan) Each of the IAC extracts was dissolved in 500 µL of HPLC grade acetonitrile according to the method prescribed by Ekwomadu *et al*. (2021). In the HPLC system, an aliquot of (25 µL) was dissolved in 250 µL of O-phthaldialdehyde solution (OPA from Sigma) and injected within minutes, since OPA was unstable. Within a short period of time, derivatized analogs can break down rapidly into nonfluorescent compounds. In this analysis, samples were run at 1 mL per minute ($min^{-}1$) while recording retention times. In the mobile phase, methanol and sodium dihydrogen phosphate (80/20, v/v) were used with 335 nm excitation and 440 nm emission wavelengths. During aflatoxins analysis, a coring cell was used as an electrochemical cell (CoBrA cell) (Dr. Weber Consulting, Mannheim, Germany) for derivatization of aflatoxins at 365 nm and 440 nm, respectively. In the mobile phase, we used 20:20:60 v/v/v of methanol, acetonitrile, and water (v/v/v) containing 119 mg potassium bromide (KBr) and 350 µL nitric acid ($4MHNO_3$). Mycotoxins were assessed by plotting the calibration curves from the area of peak of standards and the concentration of standards based on the chromatograms obtained and the mycotoxin standards used.

4.2.6 Measurements

4.2.6.1 Growth performance

Feed offered and feed left per animal were weighed using a weighing scale, recorded and feed intake was calculated as the difference between feed offered and refusals. Body weights were measured by weighing each piglet weekly using a scale. Then body weight gain was obtained by calculating the difference in live weights between the initial weight and the new weight for each animal. Feed conversion efficiency was calculated by dividing the body weight gain (g/day) by the feed intake (g/day). The following formulae were used for the calculation of performance parameters:

1. Daily Feed Intake (g/day) = Feed offered (g) – feed refusals (g) / number of days
2. Daily Body weight gain (g/day) = New body weight (g) – initial body weight (g) / number of days
3. Feed Conversion Efficiency = Body weight gain (g/day) / Feed intake (g/day)

4.2.6.2 Carcass and meat quality

On day 33 of the experiment, all animals were transported for slaughter at a local abattoir (Benade Abattoir, Mafikeng, North-West Province, South Africa). After 12 hours of feed restriction, they were stunned and exsanguinated while suspended. Carcasses were placed in a dehairer at 62 ℃ for 5 min, and the remaining hair was removed using a knife and flame. Carcasses were then eviscerated, split and hot carcass measurements taken before they were placed in a chiller set at -80 ℃ for 24 hr. After chilling, cold carcass measurements were performed and all the *Longissimus dorsi* muscle was collected from the back of each piglet for meat quality analysis. The *L. dorsi* muscle was then transferred into a refrigerator and examined for meat quality traits. For preservation purposes, muscle samples were vacuum-packed and deep-frozen at -80 ℃.

4.2.6.2.1 Carcass measurements

After slaughter, carcasses were manually eviscerated and individually weighed to determine hot carcass weights (HCW). The carcasses were then chilled in a cold room for 24 hours and reweighed to determine cold carcass weight (CCW). Carcass cuts (fore-leg, shoulder, hind-leg, ham, and belly) were measured using a digital balance (Explorer EX224, 0.01 g readability (2 decimal places) supplied by OHAUS Corp, Parsippany, NJ, USA), and rib and back lengths were measured using a tape.

4.2.6.2.2 Meat pH, temperature and color

Meat pH_{45min} and pH_{24h} were post-moterm measured on the *L. dorsi* muscle using a Corning Model 4 pH-temperature meter (Corning Glass Works, Medfield, MA) equipped with an Ingold spear-type electrode (Ingold Messtechnik AG, Udorf, Switzerland) (Stanford *et al.*, 2003). After every 20 measurements, the pH meter was calibrated with standard solutions. Meat color (L^* = lightness, a^* = redness, and b^* = yellowness) was measured by using a Minolta color-guide (BYK-Gardener GmbH, Geretsried, Germany), with a 20 mm diameter measurement area. The color meter was calibrated before measurements and every after 10 samples using the zero and white calibration as recommended by the manufacturer. Colour recording was done on the surface of a freshly cut slice of all the *L. dorsi* after being allowed to bloom for 1 hour on a polystyrene tray at 4 °C.

4.2.6.2.3 Thawing and cooking loss

Thawing loss was measured and calculated from the *L. dorsi* as a percentage of weight loss before and after thawing. Thawed samples (1 cm thick and 70 ± 5 g) were cut perpendicularly to muscle direction. Then all samples were hanged in a vertical chiller for 12 hours as described by Ali *et al.* (2016) and the liquid was collected in cups placed under the hangers. Thawing loss was calculated as follows:

$$\text{Thawing loss (\%)} = \frac{frozen\ sample\ weight(g) - final\ weght\ after\ thawing(g)}{frozen\ sample\ weight\ (g)} \text{x } 100$$

After thawing, the samples were re-weighed and placed in silver trays at 100 °C for 1 hour in an oven for cooking loss. They were pre-heated to 72 °C for 45 min and then cooked after which they were cooled at room temperature (± 20 °C) for 30 minutes. The internal temperature was measured using an analog thermometer (75°C). The samples were then re-weighed and used to calculate cooking loss using the following formula:

$$\text{Cooking loss (\%)} = \frac{weight\ before\ cooking(g) - weight\ after\ cooking(g)}{weight\ before\ cooking(g)} \times 100$$

4.2.6.2.4 Water holding capacity and drip loss

Water holding capacity was determined according to a procedure by Kristensen and Purslow (2001). A *L. dorsi* muscle sample (0.5 ± 0.05 g) from each piglet was placed on filter paper and pressed using a 60 kg weight for 5 min in a centrifuge tube with filter units, they were

heated for 20 min at 80 ℃, and then cooled for 10 min. Samples were then centrifuged at 2,000 *g* for 10 min at 4 ℃ and WHC was calculated as the change of sample weight.

$$WHC\ (\%) = \frac{initia\ weight - weight\ after}{initial\ weight} \text{x } 100$$

To evaluate drip loss, a slice of 2-cm thickness (100 ± 5 g) of *L. dorsi* muscle was placed into a polypropylene bag (Dongbang Co., Korea), packaged by vacuum, and then stored for 24 hours at 4 ℃. Drip loss was calculated as the difference in weight of samples. After evaluating drip loss, the same samples were used to evaluate cooking loss by cooking them for 40 min at 70 ℃ in a water bath, and then cooled to room temperature (Cho *et al*., 2015).

$$Drip\ loss(\%) = \frac{initial\ weight\ of\ sample - final\ weight\ of\ sample}{initial\ weight\ of\ sample} \text{x } 100$$

4.2.6.3 Serum biochemistry

4.2.6.3.1 Preparation

At day 32 of the feeding trial, 10 mL of blood was drawn from the jugular vein using sterile disposable syringes and needle into non-heparinized tubes. Blood samples were incubated for 2 hours and subsequently centrifuged at 3000 x g for 15 min and serum stored in 1.5 mL Eppendorf tubes at -20 ^{o}C for further essay.

4.2.6.3.2 Biochemical parameters

Clotted blood (collected in red tubes) was centrifuged to generate serum for biochemical analysis: total nitrogen, glucose, urea, creatinine, calcium, albumin, cholesterol, globulin, amylase, phosphorus, blood urea nitrogen/creatinine (BUN/CREA), albumin/globulin (ALB/GLOB), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphate (ALKP) were analyzed using an automated IDEXX Vet Test Chemistry Analyser (IDEXX Laboratories Inc).

4.2.6.4 Statistical analysis

All data with repeated measurements (feed intake, body weight gain and feed conversion efficiency) was analyzed using the GLM procedure of Minitab (2000) version 13 according to the following statistical model:

$Y_{ij} = \mu + D_i + W_j + (D \times W)_{ij} + E_{ij}$

Where: Y_{ij} = dependent variable, μ = population mean, D_i = effect of diet, W_j = effect of week, $(D \times W)_{ij}$ = effect of interaction between diet and week, and E_{ij} = random error associated with observation ij, assumed to be typically and, independently distributed.

In a CRD, data on overall growth performance, haemo-biochemical parameters, internal organs, carcass traits, and meat quality parameters were analysed using the GLM procedure of Minitab (2000) version 13 according to the following linear statistical model:

$$Y_i = \mu + D_i + E_i$$

Where: Y_i = dependent variable, μ = population mean, D_i = effect of diet, and E_i = random error associated with observation i, assumed to be normally and independently distributed.

For all statistical tests, significance was declared at $P < 0.05$. Where statistically different, least-square means were compared using Tukey's test.

4.3 Results

4.3.1 Growth performance

The effect of oral administration of *T. violacea* bulb extract-mediated Nano-ZnO on weekly feed intake (FI), body weight gain (BWG), and feed conversion efficiency (FCE) in weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated MSC is presented in **Table 4.3**. The results showed that dietary incorporation of 20% MSC had lower BWG and FCE that the control treatment, whilst bulk ZnO and both the conventional and green forms of Nano-ZnO elicited further deleterious effects, with the latter form showing the worst effects, on these parameters ($P < 0.001$). Meanwhile, dietary inclusion of 20% MSC produced pigs with higher weekly FI than the control treatment, whilst bulk ZnO and Nano-ZnO's conventionally and green forms had poor production of this parameter, with green Nano-ZnO showing the worst effect ($P < 0.001$). On the other hand, the data showed a significant effect of the week on BWG, FCE and FI ($P < 0.001$) with both BWG and FCE being highest in Week 1, declining in Week 2, rebounding in Week 3, and linearly declining thereafter with lowest values in Week 5 whilst, in contrast, FI linearly increased from Week 1 to Week 5. This was evidenced by a significant Treatment x Week interaction for BWG ($P < 0.05$), FCE ($P < 0.01$) and FI ($P < 0.001$).

The effect of oral administration of *T. violacea* phyto-mediated Nano-ZnO on overall FI, BWG and FCE of weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated MSC is presented in **Table 4.4**. The results demonstrated that dietary inclusion of 20% MSC had no effect on FI, as was the administration of bulk and conventionally-synthesized Nano-ZnO, whilst green Nano-ZnO decreased this parameter ($P < 0.001$). Similarly, dietary inclusion of 20% MSC had no effect on BWG whilst, on the other hand, administration of bulk ZnO as well as conventionally-synthesized and green Nano-ZnO, had lower performance than the control diet on this parameter, with the green Nano-ZnO showing the worst effect ($P < 0.001$). Further, dietary inclusion of 20% MSC had no effect on FCE, similarly to the impact of bulk ZnO and conventionally-synthesized Nano-ZnO, whilst green Nano-ZnO has a detrimental impact on this parameter ($P<0.05$).

Table 4. 3: Effect of oral administration of *T. violaceae* bulb extract-mediated Nano-ZnO on weekly feed intake (g/day), body weight gain (g/day) and feed conversion efficiency (BWG/FI) in weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

Week	Diet	Body Weight Gain (g/day)	Feed Intake (g/day)	Feed Conversion Efficiency
1	0% MSC + Vehicle	740.48^{a}	508.93^{a}	1.45^{a}
	20% MSC + Vehicle	784.42^{ac}	635.69^{a}	1.24^{ac}
	20% MSC + ZnO (50 mg/L)	686.90^{bc}	540.80^{a}	1.28^{ac}
	20% MSC + Nano-ZnO (50 mg/L)	750.65^{bc}	527.73^{a}	1.44^{ac}
	20% MSC + Green Nano-ZnO (50 mg/L)	195.24^{b}	286.69^{b}	0.68^{bc}
2	0% MSC + Vehicle	372.62^{a}	727.65^{a}	0.52^{a}
	20% MSC + Vehicle	290.91^{ac}	728.92^{a}	0.40^{ac}
	20% MSC + ZnO (50 mg/L)	207.14^{bc}	686.39^{a}	0.30^{ac}
	20% MSC + Nano-ZnO (50 mg/L)	207.79^{bc}	675.09^{a}	0.31^{ac}
	20% MSC + Green Nano-ZnO (50 mg/L)	309.53^{b}	507.86^{b}	0.62^{bc}
3	0% MSC + Vehicle	615.47^{a}	801.24^{a}	0.77^{a}
	20% MSC + Vehicle	558.44^{ac}	852.45^{a}	0.66^{ac}
	20% MSC + ZnO (50 mg/L)	284.33^{bc}	727.38^{a}	0.40^{ac}
	20% MSC + Nano-ZnO (50 mg/L)	342.86^{bc}	697.86^{a}	0.49^{ac}

	20% MSC + Green Nano-ZnO (50 mg/L)	176.19[b]	438.45[b]	0.39[bc]
4	0% MSC + Vehicle	478.57[a]	961.46[a]	0.50[a]
	20% MSC + Vehicle	218.17[ac]	942.38[a]	0.23[ac]
	20% MSC + ZnO (50 mg/L)	200.00[bc]	866.01[a]	0.23[ac]
	20% MSC + Nano-ZnO (50 mg/L)	181.82[bc]	814.13[a]	0.22[ac]
	20% MSC + Green Nano-ZnO (50 mg/L)	71.43[b]	449.50[b]	0.16[bc]
5	0% MSC + Vehicle	491.67[a]	1200.00[a]	0.41[a]
	20% MSC + Vehicle	306.82[ac]	1200.00[a]	0.26[ac]
	20% MSC + ZnO (50 mg/L)	250.00[bc]	1200.00[a]	0.21[ac]
	20% MSC + Nano-ZnO (50 mg/L)	90.91[bc]	1200.00[a]	0.08[ac]
	20% MSC + Green Nano-ZnO (50 mg/L)	-58.33[b]	600.00[b]	-0.10[bc]
Pooled SEM		22.11	4.76	0.03
Effect	**Diet**	P<0.001	P<0.001	P<0.001
	Week	P<0.001	P<0.001	P=0.001
	Diet X Week	P<0.05	P<0.001	P<0.01

[a,b,c] Within row means with different superscripts are significantly different ($P < 0.05$); SEM = standard error of the mean.

Table 4. 4: Effect of oral administration of *T. violaceae* bulb extract-mediated Nano-ZnO on overall feed intake (g/day), body weight gain (g/day) and feed conversion efficiency in weaned Large White piglets.

Parameter	Diets					SEM	P-value
	0% MSC Vehicle	20% MSC Vehicle	20% MSC + ZnO (50 mg/L)	20% MSC + Nano-ZnO (50 mg/L)	20% MSC + Green Nano-ZnO (50 mg/L)		
Overall daily feed intake (g/day)	841.21[a]	869.83[a]	797.41[a]	775.24[a]	451.35[b]	9.03	P<0.001
Overall daily body weight gain (g/day)	548.91[a]	439.75[ac]	330.99[bc]	320.64[bc]	148.72[b]	12.31	P<0.001
Feed conversion efficiency	0.74[a]	0.57[ab]	0.49[ac]	0.52[a]	0.38[bc]	0.02	P<0.05

[a,b,c] Within row means with the different superscripts are significantly different (P < 0.05); SEM- standard error of mean.

4.3.2 Carcass and meat quality

4.3.2.1 Carcass parameters

The effect of oral administration of *T. violacea* bulb extract-mediated Nano-ZnO on the hot carcass (HCW) and cold carcass (CCW) weights, as well as weights of fore-leg, shoulder, hind-leg, ham, belly, and rib and back length of weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated MSC is presented in **Table 4.5**. The results showed that dietary incorporation of 20% MSC had lower HCW ($P < 0.001$), CCW ($P < 0.001$), foreleg weight ($P = 0.001$), and hind leg weight ($P < 0.001$) than the control, whilst bulk ZnO and both conventional and green forms of Nano-ZnO elicited further deleterious effects, with the latter form showing worst effects, on these parameters. In contrast, dietary inclusion of 20% MSC did not affect the weights of the shoulder, ham, belly, and rib and back lengths whilst bulk ZnO as well as conventionally-synthesized and green Nano-ZnO, in particular the latter, had depressive effects on shoulder weight ($P = 0.001$) and ham weight ($P < 0.01$). Similar to the effect of 20% MSC inclusion, bulk ZnO had no effect on belly weight whilst conventionally-synthesized and green Nano-ZnO decreased this parameter, with green Nano-ZnO showing a more depressive effect ($P < 0.01$). Also, similarly to the effect of 20% MSc inclusion, both bulk ZnO and conventionally-synthesized Nano-ZnO had no impact on rib length, and back length while green Nano-ZnO decreased these parameters ($P < 0.05$ and $P < 0.01$, respectively).

Table 4. 5: Effect of oral administration of *T. violaceae* bulb extract-mediated green ZnO nanoparticles on carcass parameters in weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

Parameters	**Diets**					**SEM**	**P-value**
	0% MSC	**20% MSC**	**20% MSC +**				
Solution [ZnO]	**Vehicle**	**Vehicle**	**ZnO (50 mg/L)**	**Nano-ZnO (50 mg/L)**	**Green Nano-ZnO (50 mg/L)**		
Hot carcass weight (g)	1782^{a}	1354^{b}	1311^{bc}	1212^{bc}	903^{c}	0.33	P<0.001
Cold carcass weight (g)	1713^{a}	1324^{b}	1262^{bc}	1152^{bc}	833^{c}	0.33	P<0.001
Foreleg weight (g)	775.13^{a}	583.09^{b}	602.90^{b}	540.33^{b}	392.50^{b}	16.23	P=0.001
Shoulder weight (g)	555.61^{a}	481.68^{ab}	413.35^{bd}	422.00^{ad}	258.08^{cd}	13.21	P=0.001
Hind leg weight (g)	707.78^{a}	443.73^{b}	520.35^{b}	419.15^{b}	315.00^{b}	15.06	P<0.001
Ham weight (g)	1079.50^{a}	796.35^{ad}	776.90^{bd}	753.15^{cb}	447.00^{be}	29.31	P<0.01
Belly weight (g)	445.28^{a}	341.75^{a}	360.30^{ac}	278.50^{bc}	192.75^{bc}	13.90	P<0.01
Rib length (cm)	22.78^{a}	22.20^{ab}	22.70^{a}	21.70^{ab}	18.33^{b}	0.29	P<0.05
Back length (cm)	60.11^{a}	55.40^{a}	57.10^{a}	54.70^{a}	40.17^{b}	0.94	P<0.01

^{a,b,c,d,e} Within row means with different superscripts are significantly different ($P < 0.05$); SEM = standard error of the mean.

4.3.2.2 Meat quality

The effect of oral administration of *T. violacea* bulb extract-mediated Nano-ZnO on meat pH, temperature and color (L, a, b) measured 45 min and 24 hours after slaughter in weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated MSC at 20% inclusion rate is presented in **Table 4.6**. Neither 20% MSC inclusion nor administration of ZnO (both bulk and nanoparticle forms) had any effect on meat pH, temperature and colour at 45 min and 24 hours after slaughter ($P>0.05$). It was only meat temperature that was decreased ($P<0.001$) and meat yellowness that was increased ($P < 0.05$) after 24 hours of cold storage.

The effect of oral administration of *T. violacea* bulb extract-mediated Nano-ZnO on water holding capacity (WHC), cooking loss, drip loss and thawing loss of meat from weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated MSC at 20% inclusion rate is presented in **Table 4.7**. The results indicate that there was no effect of inclusion of 20% MSC nor ZnO (both bulk and Nano-ZnO) on all parameters ($P>0.05$), except for WHC, which was decreased ($P < 0.01$) by green Nano-ZnO ($P<0.01$).

Table 4. 6: Effect of oral administration of *T. violaceae* bulb extract-mediated green ZnO nanoparticles on meat colour in weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

	Treatment	pH	Temp. (°C)	L*(lightness)	a*(redness)	b*(yellowness)
45 min	0% MSC + Vehicle	6.021	16.76[a]	45.341	7.094	1.217[a]
	20% MSC + Vehicle	5.709	16.78[a]	48.602	6.361	2.501[a]
	20% MSC + ZnO	5.995	15.98[a]	47.608	7.168	2.161[a]
	20% MSC + Nano-ZnO	6.122	16.58[a]	45.547	7.756	2.639[a]
	20% MSC + Green nano-ZnO	6.235	16.60[a]	46.487	8.968	3.193[a]
24 hr	0% MSC + Vehicle	5.931	11.44[b]	49.094	7.170	3.147[b]
	20% MSC + Vehicle	5.934	13.03[b]	44.840	7.064	2.859[b]
	20% MSC + ZnO	5.957	11.16[b]	50.053	7.463	3.541[b]
	20% MSC + Nano-ZnO	5.955	11.53[b]	50.070	6.693	2.941[b]
	20% MSC + Green nano-ZnO	5.980	14.32[b]	48.993	7.538	3.240[b]
	SEM	0.03	0.20	0.57	0.16	0.16
Effects	Time	P>0.05	P<0.001	P>0.05	P>0.05	P<0.05
	Treatment	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
	Time x Treatment	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05

[a,b] Within row means with different superscripts are significantly different ($P < 0.05$); SEM = standard error of the mean.

Table 4. 7: Effect of oral administration of *T. violaceae* bulb extract-mediated green ZnO nanoparticles on water-holding capacity, drip loss, cooking loss, and thawing loss of weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

Parameter	**Diets**					**SEM**	**P-value**
	0% MSC	**20% MSC**	**20% MSC +**				
Solution [ZnO]	**Vehicle**	**Vehicle**	**ZnO (50 mg/L)**	**Nano-ZnO (50 mg/L)**	**Green Nano-ZnO (50 mg/L)**		
Water holding capacity (%)	34.95^{ab}	32.59^{ab}	22.78^{ab}	41.87^{a}	19.92^{b}	1.26	P<0.01
Drip loss (%)	3.45	4.16	3.53	3.73	3.91	0.12	P>0.05
Thawing loss (%)	11.52	13.33	13.89	14.56	13.44	13.44	P>0.05
Cooking loss (%)	61.62	62.43	61.93	60.65	60.54	0.62	P>0.05

^{a,b} Within row means with different superscripts are significantly different ($P < 0.05$); SEM = standard error of the mean.

4.3.3 Serum parameters

The effect of oral administration of *T. violacea* bulb extract-mediated Nano-ZnO on serum biochemical parameters in weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated MSC is presented in **Table 4.8**. Neither dietary inclusion of 20% MSC nor oral administration of ZnO (both bulk and Nano-ZnO) had any effect on serum biochemical parameters ($P>0.05$), except for albumin ($P <0.05$), cholesterol ($P <0.05$), and amylase ($P = 0.001$) all of which were decreased by oral administration of green Nano-ZnO.

Table 4. 8: Effect of oral administration of *T. violacea* bulb extract-mediated green ZnO nanoparticles on serum biochemical parameters in weaned Large White piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake.

Parameter	**Diets**					**SEM**	**P-value**
	0% MSC	**20% MSC**	**20% MSC +**				
Solution [ZnO]	**Vehicle**	**Vehicle**	**ZnO (50 mg/L)**	**Nano-ZnO (50 mg/L)**	**Green Nano-ZnO (50 mg/L)**		
Glucose (mmol/L)	2.44[a]	1.77[a]	1.64[a]	1.86[a]	1.03[a]	0.12	P>0.05
Symmetric dimethylarginine (µg/dL)	46.00[a]	45.20[a]	42.10[a]	39.70[a]	31.67[a]	1.56	P>0.05
Creatinine (µmol/L)	89.33[a]	100.50[a]	88.90[a]	91.60[a]	63.00[a]	3.50	P>0.05
Urea (mmol/L)	3.41[a]	3.31[a]	2.86[a]	3.09[a]	3.05[a]	0.14	P>0.05
Blood urea nitrogen/creatinine	9.56[a]	8.20[a]	9.50[a]	9.90[a]	14.17[a]	0.54	P>0.05
Phosphorus (mmol/L)	4.76[a]	5.06[a]	4.88[a]	4.25[a]	4.59[a]	0.10	P>0.05
Calcium (mmol/L)	1.87[a]	1.88[a]	1.84[a]	1.61[a]	1.35[a]	0.06	P>0.05
Total protein (g/L)	63.78[a]	53.00[a]	57.00[a]	52.00[a]	33.33[a]	2.40	P>0.05
Albumin (g/L)	25.33[a]	19.90[ac]	19.70[ac]	16.00[ac]	12.33[bc]	0.81	P<0.05
Globulin (g/L)	39.22[a]	33.10[a]	31.30[a]	31.80[a]	21.17[a]	1.48	P>0.05

Albumin/Globulin	0.66[a]	0.64[a]	0.66[a]	1.13[a]	0.63[a]	0.09	P>0.05
Alanine aminotransferase (U/L)	76.89[a]	77.20[a]	90.80[a]	67.50[a]	61.30[a]	3.59	P>0.05
Alkaline phosphatase (U/L)	82.00[a]	97.20[a]	107.70[a]	88.00[a]	56.50[a]	4.52	P>0.05
γ-glutamyl transferase (U/L)	27.67[a]	31.10[a]	23.20[a]	24.60[a]	23.33[a]	1.56	P>0.05
Total bilirubin (µmol/L)	8.18[a]	6.27[a]	4.86[a]	3.65[a]	2.25[a]	0.52	P>0.05
Cholesterol (mmol/L)	1.72[ab]	2.69[a]	2.23[ab]	2.00[ab]	1.03[b]	0.11	P<0.05
Amylase (U/L)	769.78[a]	715.00[a]	519.50[ab]	463.50[ab]	232.00[b]	25.76	P=0.001
Lipase (U/L)	27.44[a]	21.50[a]	24.30[a]	20.10[a]	19.67[a]	1.92	P>0.05

[a,b] Within row means with different superscripts are significantly different ($P < 0.05$); SEM = standard error of the mean.

4.4 Discussion

The objective of this study was to investigate the ameliorative effects of orally administered *T. violacea* bulb extract-fabricated Nano-ZnO against the toxicity of mycotoxins in Large White piglets fed diets supplemented with MSC, an alternative protein source with a high CP content (470 g/kg DM) (Mlambo *et al.*, 2011a, Mdziniso *et al.*, 2016) and an essential amino acids composition similar to SBM, except for lysine (Mariod and Abdelwahab, 2012, Mthiyane and Mhlanga, 2017a). Whilst it is nutritionally of high value, MSC is commonly afflicted by anti-nutritional factors, particularly mycotoxins, to which pigs are most sensitive. Mycotoxins negatively affect body weight gain, feed intake, and feed conversion efficiency due to feed refusal, growth retardation, and even weight loss (Yang *et al.*, 2020a). They also induce oxidative stress through their depletion of cellular and tissue antioxidants (Mavrommatis *et al.*, 2021). Indeed, feeding diets supplemented with MSC (0 to 20%) was shown to depress growth performance, FI and FCE in broiler chickens (Mthiyane and Mhlanga, 2017a, 2018a). For this reason, this study sought to investigate green Nano-ZnO as a strategy to ameliorate the toxicity of mycotoxins in MSC supplemented diets for piglets.

Whilst dietary incorporation of 20% MSC resulted in lower weekly BWG and FCE than the control, results from this study demonstrated 20% MSC inclusion to have caused no effect on overall BWG and FCE. If anything, the inclusion of 20% MSC in the diet increased weekly FI whilst it had no effect on overall FI. Overall, these results suggest that dietary incorporation of 20% MSC did not cause significant deleterious effects on performance parameters in the weaning piglets, as had been expected. These data corroborate findings from studies with Japanese quails (Mazizi *et al.*, 2019a, 2020a) and pigs whereby dietary MSC inclusion caused no deleterious effects. The data suggest that whilst MSC showed evidence of having quite high levels of mycotoxins (particularly aflatoxin B1 and aflatoxin B2) **(Table 4.2)**, the dietary levels of these secondary metabolites should not be cause for great concern as they did not cause significant deleterious effects in the piglets. In fact the data showed the SBM-containing diet to be the one that was bedevilled by high total mycotoxin infestation arising from high levels of aflatoxin G1 and aflatoxin G2 **(Table 4.2)**. These results are quite interesting in that they vindicate MSC and suggest that there was no need for an ameliorative strategy in the form of green Nano-ZnO in the first place. Indeed, in contrast to previous observations of Nano-ZnO enhancing growth performance in weaned piglets (Zhao *et al.*, 2014, Milani *et al.*, 2016, Wang *et al.*, 2017a), oral administration of ZnO-based ameliorants mainly green Nano-ZnO caused very deleterious effects on performance parameters in this study. The use of green Nano-ZnO

in this study was associated with toxicity. Nano-ZnO has indeed been shown to induce toxicity in various biological systems (Song *et al.*, 2010, Giovanni *et al.*, 2015). These nanoparticles have been demonstrated to cause higher toxicity than other metallic oxide nanoparticles (Cho *et al.*, 2013, Watson *et al.*, 2014) most probably arising from their ion-shedding ability and induction of oxidative stress (Liu *et al.*, 2017). It may be this induced oxidative stress that led to decreased FI in piglets fed diets supplemented with Nano-ZnO (Milani *et al.*, 2016).

In this study, dietary incorporation of 20% MSC had lower HCW, CCW, foreleg weight and hind-leg weight, than the control. These results corroborate the previous finding whereby dietary inclusion of 15% MSC had lower warm and cold carcass weights in pigs (Mabena *et al.*, 2022). However, the oral administration of ZnO nanoparticles detrimentally affected the hot and cold carcass parameters, similarly to what was observed regarding the performance parameters. This study also showed no effects of dietary 20% MSC inclusion nor or oral administration of ZnO (both bulk and nanoparticle forms) on meat pH, temperature, colour at 45 min and 24 hours after slaughter, and drip thawing loss and cooking loss. Instead, only meat temperature was decreased and meat yellowness increased after 24 hours of cold storage, as well as WHC which was decreased by oral administration of green Nano-ZnO. These results further vindicate MSC and, instead, indicate its potential utility as an alternative to SBM. They are in agreement with the findings (Mazizi *et al.*, 2020a, Mabena *et al.*, 2022). The deleterious effects of green Nano-ZnO are in line with their observed deleterious effects on performance, as discussed above. Current results, however, do not corroborate the findings of Mabena *et al.* (2022) who reported decreased meat ultimate pH in pigs fed diets with 20% MSC.

Lastly, the results of the current study showed that neither dietary inclusion of 20% MSC nor oral administration of ZnO (both bulk and Nano-ZnO) had any effect on serum biochemical parameters, except for albumin, cholesterol, and amylase, all of which were lowered by oral administration of green Nano-ZnO. According to Ali *et al.* (2012), as biochemical changes emerge from abnormal conditions in the body, serum biochemical indices are used to evaluate health status and discover subclinical disorders as. Serum albumin is the main protein in blood plasma responsible for controlling blood volume by regulating the blood's colloid osmotic pressure (Pei *et al.*, 2019a). It is a marker of the animal's nutritional status (Keller, 2019) and its reduction by green Nano-ZnO is undesirable. However, current data corroborate a previous study in which Nano-ZnO decreased serum albumin in weaning piglets fed diets supplemented with 200 mg/kg nano-ZnO (Mokone *et al.*, 2022a). On the other hand, serum cholesterol has been associated with the advancement of coronary disease; hence its reduction by green Nano-

ZnO is highly desirable. The current study further corroborates previous studies whereby a significant decrease in plasma cholesterol was observed in broiler chickens fed diets with high amounts of zinc (Herzig *et al*. (2009) and in another study when Nano-ZnO was fed to broiler chickens (Hussan *et al*., 2022). Serum amylase is associated with lipid diarrhea, weight loss, and malnutrition as a result of advanced chronic pancreatitis (Flood *et al*., 1978, Ebisawa *et al*., 2007, Waljee *et al*., 2009).

4.5 Conclusion

Marula seed cake does not detrimentally affect growth, carcass parameters, some meat quality and could be utilized as an alternative protein source in weanling piglets. On the other hand, the application of marula seed cake and green Nano-ZnO detrimentally affected growth, carcass parameters, serum parameters, except for meat quality. Therefore, incorporating 20% MSC can be utilized in pig production and there is no need for any ameliorative strategy in the form of green Nano-ZnO.

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CHAPTER 5: GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATION

5.1 General discussion

Despite having the world's highest population growth rate, Sub-Saharan Africa continues to represent a significant share, with one in four people in this region suffering from food insecurity due to low agricultural production (Prosekov and Ivanova, 2018). As a result of the contribution of pigs toward food security and nutrition, pork will continue to be in high demand to feed this rapidly expanding human population (Godfray *et al.*, 2010). However, pig production is plagued by the lack of affordable feed resources in this region. As a result, resource-limited farmers or rather smallholder farmers are compelled to use imported and expensive commercial feedstuffs that are largely unaffordable and in short supply (Mthiyane and Mhlanga, 2017a, Chivandi *et al.*, 2012), hence the use of alternative protein sources like marula seed cake. Marula seed cake is a - dense agro-waste rich in protein and essential amino acids for pig production. Its nutrient value is comparable to conventional protein sources. However, this alternative protein source is contaminated by mycotoxins and that requires the search for ameliorative agents.

ZnO nanoparticles, which were used as potential ameliorative agents, had been prepared using the aqueous extract of *T. violaceae*, via calcination at 350 °C for 1 h in air to afford, while the conventionally synthesised ZnO was achieved through and the calcination of zinc acetate at 350 °C for 4 h. All the main characterization techniques proved that both the nanoparticle were of zinc oxide. The particle sizes for both green and conventional nanoparticles (45.26 and 60.64 nm) were within the size range of nanoparticles. Incorporating only 20% marula seed cake did not detrimentally affect growth, carcass parameters, meat quality and could be utilized as an alternative protein source in weaned Large-White piglets. However, the application of marula seed cake and green Nano-ZnO and conventional Nano-ZnO detrimentally affected pig's growth, carcass parameters, serum parameters, except for meat quality, of which the opposite was expected.

5.2 Conclusion

This study showed the ability of *T.violacea* as a stabilizing agent in the biosynthesis of zinc oxide nanoparticles and the effect of phyto-encapsulated *T.violacea* bulb-extract nano-ZnO orally gavaged in pig fed diets containing naturally-mycotoxin contaminated marula seed cake. MSC doesn't detrimentally affect the growth, carcass parameters, meat quality, and health status of the

animals. However, the opposite was observed with the inclusion of MSC coupled with green nano-ZnO. This concludes that the toxins in MSC are minimal and should be utilized on their own in pig production without the ameliorative agents.

5.3 Contribution to knowledge

The study on the oral *Tulbaghia violacea* extract-based nano-ZnO administration in piglets fed diets supplemented with naturally mycotoxin-contaminated marula seed cake, is a multi-disciplinary study that emphasized the use of green nanobiotechnology and the use of alternative protein source in animal nutrition. This study reported the first time synthesis of *T. violacea* ZnO nanoparticles and has been published in peer-reviewed journals. Also, it reported the use of alternative protein source, marula seed cake in pig nutrition for weaned piglets. Now, with all the knowledge acquired from this dissertation, scientists will find more studies from this study about phyto-encapsulation to enhance the embodiment of knowledge around this matter. The animal feed companies will be aware of this alternative protein source and formulate cheaper diets to the market. Lastly, the small-holder farmers will now be aware of cheaper feeds that will help them enhance profitability, and subsequently enhance food security in the sub-Saharan Africa.

5.4 Recommendation

The use of MSC in the replacement of soybean meal demonstrated its best ability in pig production and its commercialization should be considered in the market. However, the oral administration of green nano-ZnO at 50 mg/L showed detrimental effects in piglets fed natural- mycotoxin contaminated MSC. Now, future studies should investigate varying concentrations (low to high) of green nano-ZnO and their cytotoxic potential. Also, the effects vary *T. violacea* as a foraged meal at different pig production stages.