An *in vitro* investigation into the potential benefits of *Psychotria zombamontana* as a poultry feed additive

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DECLARATION

I declare that the thesis/dissertation, which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed ……..

Date **25/11/2018**……..

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Abstract

Poultry feed is one of the main constraints, after disease, in poultry production for smallholder farmers in Africa. It also acts as the first step of the food safety chain in the "farm to fork" model. With the poultry industry being the largest individual agricultural industry in South Africa, the nature of the microorganisms present in the feed is of great importance. Problematic microorganisms may lead to an increased feed conversion ratio of livestock, illness or disease in livestock, or even disease in humans.

The primary aim of this study was to evaluate the activity of *Psychotria zombamontana* (Rubiaceae) against problematic fungi and bacteria that may be associated with poultry feed. This species was selected following promising preliminary antifungal results. Additionally, further biological activities that may be beneficial to the chickens when added to the feed were evaluated, including antioxidant and anti-inflammatory activity. Acetone leaf extracts of *P. zombamontana* showed promising activity against all microorganisms tested as well as very high antioxidant activity and anti-inflammatory activity.

An effort was made to isolate the active compounds through bioassay-guided fractionation. Two active sub-fractions were obtained from the acetone leaf extract of *P. zombamontana*. Both sub-fractions were found to have antimicrobial activity, and have an effect on cytokine production. One of the sub-fractions also displayed promising activity against 15-lipoxygenase, an enzyme implicated in inflammation. Compounds within the two sub-fractions were identified using gas chromatography-mass spectroscopy (GC-MS). Results of this analysis

suggested that the sub-fractions comprised a number of compounds, but only the major compounds were reported as many were present in negligible amounts. Some of the compounds identified were reportedly inactive, while the rest have previously been investigated and shown to have activities which correlated with the activity observed for the *P. zombamontana* extract during this study.

The extract and sub-fractions were also investigated for cytotoxicity for preliminary evidence of safe ingestion of plant material or compound by poultry. Results show that the extract and sub-fractions were relatively non-cytotoxic and therefore may be safe for ingestion, but *in vivo* studies are necessary to confirm this.

These findings, coupled with the promising antimicrobial, antioxidant and anti-inflammatory activity, suggest that *P. zombamontana* could be a highly beneficial addition to poultry feed. It may be possible for the plant material to be used by smallholder farmers as a form of alternative control of fungal contaminants. Furthermore, a functional product may be formulated using this plant for use by commercial farmers. This encourages further studies *in vivo* to more closely investigate the effects of the crude plant material as well as pure compounds on poultry when added to feed. Additionally, further studies should aim to observe the curative effect of the extract or compounds on artificially infected poultry, as well as any possible preservative effect the material may have on the feed.

Keywords: alternative control, anti-inflammatory, antimicrobial, antioxidant, cytotoxicity, feed additive, poultry, *Psychotria zombamontana*.

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List of abbreviations, acronyms and symbols:

ABTS	-	2, 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AGP	-	Antibiotic growth promoter
ARC		Agricultural Research Council
Conc.	-	Concentration
DCM	-	Dichloromethane
DMSO	-	Dimethyl sulphoxide
DPPH	-	2,2-Diphenyl-1-picrylhydrazyl
FCS	-	Foetal calf serum
GC-MS		Gas chromatography-mass spectrometry
IC	-	Inhibitory concentration
IL	-	Interleukin
INT	-	Iodonitrotetrazolium chloride
LC	-	Lethal concentration
LOX	-	Lipoxygenase
LPS	-	Lipopolysaccharide
MEM	-	Minimum essential medium
MIC	-	Minimum Inhibitory Concentration
NMR	-	Nuclear Magnetic Resonance
NO	-	Nitric oxide
PBS	-	Phosphate buffered saline
PDA	-	Potato Dextrose Agar
SC	-	Solvent control
SD	-	Standard deviation
SF	-	Sub-fraction
SI	-	Selectivity index
TLC	-	Thin Layer Chromatography
TNF	-	Tumour necrosis factor

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1. General Introduction

1.1 Background and motivation

Poultry feed may act as a carrier for many microorganisms; it also acts as the first step of the food safety chain in the "farm to fork" model (Crump et al. 2002; Maciorowski et al. 2007). The nature of the microorganisms present in the feed is therefore of great importance, as problematic microorganisms may lead to an increased feed conversion ratio of livestock, illness or disease in livestock, or even disease in humans (Crump et al. 2002; Maciorowski et al. 2007). The problematic microflora present in poultry feed may consist of fungi and bacteria, with each group having specific negative effects on the feed, or the birds which consume it.

Fungi may invade and grow on poultry feed in storage. Some will only pose a problem through causing rancidity or just due to the deteriorating effect they have on the feed, but certain species may produce mycotoxins. Mycotoxins are secondary metabolites produced by fungi that are detrimental to human and animal health, and species from the *Fusarium*, *Aspergillus* and *Penicillium* genera are the most common mycotoxin producers (Houssou et al. 2009; Yiannikouris and Jouany 2002). Mycotoxins may decrease the feed intake of animals and have a negative effect on animal performance, therefore causing a decrease in production of animal products (Yiannikouris and Jouany 2002). Some examples of mycotoxins that cause problems in poultry feed include trichothecenes, aflatoxins, ochratoxin A and fumonisins (Maciorowski et al. 2007).

Specific storage methods are used in an effort to reduce invasion of feed by mycotoxigenic fungi and subsequently decrease the amount of mycotoxins present in the feed (Yiannikouris and Jouany 2002). Other methods of reducing mycotoxins include control of feedstuffs added in the production of animal feed, for example only using high quality feedstuffs from selected farms (Pettersson 2004). One may argue that if the feed or feedstuff is already contaminated with mycotoxigenic fungi, these methods may not be very effective. The addition of feed additives is also used as a control method; these additives include adsorbents, which absorb and retain toxins; antioxidants, which act in alleviating the effect of the toxins; and enzymes, which may break down specific toxins (Pettersson 2004).

One of the most important poultry feed additives are antibiotic growth promoters (AGPs). These substances modify the intestinal microflora, targeting Gram-positive bacteria usually

associated with poor health and performance (Bedford 2000). The addition of AGPs to feed started in the 1950's and has since become controversial due to the build-up of resistance of bacterial pathogens to antimicrobials. These resistant strains of bacteria may render specific antibiotics useless for therapeutic use and other antibiotics must then be used to control these pathogens. Not only do these resistant bacteria cause a problem in animal health, but they can potentially cause major problems in human health due to the ability of bacteria to transfer resistant genes to human pathogens via conjugation (Castanon 2007; Verraes et al. 2013).

Many plant extracts have shown antifungal activity against a wide range of fungi, as well as antibacterial activity (Nascimento et al. 2000; Wilson et al. 1997). Such antimicrobial activity, paired with the fact that the general public may more readily accept plant material as a natural additive, makes plants an attractive alternative to AGPs (Ferket 2004; Toghyani et al. 2010). In this research project, leaf extracts from *Psychotria zombamontana* (Kuntze) E.M.A. Petit were investigated for activity which could prove useful if the plant was to be used as a feed additive.

The choice of *P. zombamontana* as a research focus in this study was influenced by the fact that many of the species in the genus *Psychotria* produce a variety of bioactive compounds, including compounds with antimicrobial and antiviral activity (Matsuura et al. 2013). For example, *Psychotria hawaiiensis* (Gray) Fosberg has shown antifungal activity, mainly against human pathogenic fungi, but no inhibition was shown against *Aspergillus* species (Locher et al. 1995). Extracts from *P. zombamontana* have previously exhibited activity against mycobacteria (Aro et al. 2015). This species is indigenous to South Africa (van Wyk and van Wyk 2013), and further study on it was additionally motivated by promising results obtained in a study of its activity against *Fusarium* spp. (Querl 2016).

1.2 Justification for the Study

The inhibition of the growth of fungi such as *Fusarium* spp. may reduce the production of mycotoxins in poultry feed and the diseases associated with these mycotoxins. Additionally, the inhibition of growth of disease-causing organisms such as *Aspergillus* spp. may reduce the incidence of aspergillosis and other infections in poultry. Furthermore, if the extracts improve the feed conversion ratio of poultry, they may be used as a possible substitute for AGPs.

Psychotria zombamontana extracts have previously shown antifungal activity against some fungi implicated in producing mycotoxins and disease (Querl 2016). It is important to confirm such antifungal activity *in vitro* against a wider range of fungal species causing contamination of feed as well as fungal infections in poultry. Other useful biological activities such as antioxidant and anti-inflammatory activity would increase the value of the potential plant-based feed supplement. Identification of the active compounds of the species would be useful for further standardization studies to enable quantification of chemical markers in the feed. Hence, this study incorporates isolation and identification of active compounds from the *P. zombamontana* leaf extract.

1.3 Aim

The aim of this study was to investigate the activity of *P. zombamontana* against common fungal and bacterial pathogens associated with poultry feed contamination as well as diseases in poultry. An additional aim was to investigate whether the extract has any antioxidant or anti-inflammatory activity, which may provide supplementary beneficial health effects to poultry when added to poultry feed.

1.4 Objectives

The specific objectives of the study were to:

- investigate the antibacterial and antifungal activity of leaf extracts of *P. zombamontana* against common bacterial and fungal pathogens affecting poultry;
- screen the extracts for cytotoxicity;
- screen the extracts for antioxidant and anti-inflammatory activity; and,
- identify active components in *P. zombamontana* leaf extracts.

1.5 Structure of dissertation

Chapter 2. A review of the difficulties faced by poultry farmers with regard to problematic microorganisms associated with poultry feed and the current control methods available.

Chapter 3. This chapter comprises a review of the challenges faced by poultry farmers with regard to problematic microorganisms associated with poultry feed. Background information on feed additives with an emphasis on plant material as alternative additives is provided. Furthermore, a brief introduction on *Psychotria* species is given, focusing on their uses and bioactivity.

Chapter 4. This chapter reports on additional activity (antioxidant and anti-inflammatory) exhibited by *P. zombamontana* which could prove beneficial to poultry if the plant was supplemented into the feed. The *in vitro* cytotoxicity of the plant is also investigated.

Chapter 5. Based on the activity observed in Chapter 3 and 4, this chapter focuses on the isolation and identification of active compounds present within the leaf extracts of *P. zombamontana*.

Chapter 6. Using the results obtained from the previous chapters in this study, this chapter focuses on the significance of the results obtained. Suggestions regarding future work are also made.

2. Literature Review

2.1 Poultry feed in South Africa

Poultry feed usually consists of a few main ingredients, that is wheat (*Triticum aestivum* L.) (18–55%), maize (*Zea mays* L.) (11–48%), and soya (*Glycine max* (L.) Merr.) meal (19–25%). The feed may also contain a variety of minor components such as barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), fish bone meal, vitamins, and inorganic salts (Bouvarel et al. 2009). Feed availability is one of the main constraints, after disease, in poultry production for smallholder farmers in Africa (Bell 2009). This is partly due to scavenging being a significant source of feed intake by free roaming flocks, which make up a large portion of poultry farming practice among smallholder farmers in developing countries (Bell 2009; Jensen 1996).

Besides smallholder farmers, poultry feed is also an important part of the commercial poultry industry and should command the same attention as the industry itself (Beg et al. 2006). The poultry meat industry is a significant agricultural industry in South Africa, being the largest individual agricultural industry in the country with a gross value of R40 billion (Esterhuizen 2016). The farmers supporting this industry produced around 1.3 billion tons of meat collectively in 2016 (Esterhuizen 2016). With such a large industry depending on continuous production of poultry meat, regulation of poultry feed is a crucial factor in the prevention of losses. Quality control of this feed is important to ensure low mortality of the birds; this is especially relevant in the case of possible mycotoxin contamination of the feed by fungi. One such example is *Aspergillus flavus*, and the associated mycotoxin that it produces, aflatoxin. The fact that this fungal species is the most common storage fungal contaminant globally, coupled with the potential of this species to produce mycotoxins, has resulted in *A. flavus* being deemed the main culprit for huge economic losses in poultry production (Beg et al. 2006).

2.2 Fungi and mycotoxins associated with poultry feed

The presence of fungi in poultry feed is common and is mainly due to the raw materials used in feed production. Contamination of the raw materials by fungi occurs in both the pre-harvest (field fungi) and post-harvest periods (storage fungi) (Dalcero et al. 1998). Contamination is particularly widespread in tropical countries in which poultry production is growing rapidly

(Okoli et al. 2006; Van den Berghe et al. 1990). Of the fungal genera contaminating poultry feed, species of *Fusarium*, *Aspergillus* and *Penicillium* are usually the most common (Dalcero et al. 1998; Okoli et al. 2006; Saleemi et al. 2010). Species from the above-mentioned genera are also known for their ability to potentially produce mycotoxins (Amadi and Adeniyi 2009).

Mycotoxins are secondary metabolites of some filamentous fungal species. They are low molecular weight natural compounds, which can be harmful to both livestock and humans (Zain 2011). The occurrence of these toxins is more frequent in hot, humid climates, as these conditions favour the growth of the mycotoxin-producing fungi (Zain 2011). Exposure to mycotoxins may occur through dermal and inhalation routes, but ingestion is most common. Sufficient exposure via any of these routes may result in mycotoxicoses (Zain 2011).

Some mycotoxins are not only important due to their direct negative effects but may also interact with specific diseases, increasing the susceptibility of poultry toward these diseases. One example is the interaction previously observed by Hamilton and Harris (1971) between aflatoxins and *Candida albicans*. Contamination of poultry feed by mycotoxins is the cause of significant losses in poultry production in both smallholder and industrial farming systems (Beg et al. 2006).

2.2.1 *Fusarium* spp.

The *Fusarium* genus includes plant pathogenic species, which are among some of the most studied plant pathogens. Several of the species within this genus cause disease on some of the most economically significant crops and food/feed grains. In addition to this, *Fusarium* species have the ability to potentially produce a wide array of mycotoxins, including fumonisins, trichothecenes and zearalenone (Glenn 2007).

Fumonisins are produced mainly by *F. verticillioides* and *F. proliferatum* (Oliveira et al. 2006). These species are found very often on maize, which is one of the main components of poultry feed (Labuda et al. 2003). The presence of fumonisins in poultry feed may result in several negative effects, including impaired disease immunity, diarrhoea, hepatotoxicity, decreases in the weight of the liver, spleen and bursa of Fabricius, decrease in total body weight and poor feed conversion (Richard et al. 2003; Labuda et al. 2003). Zearalenone is produced by a number of species of *Fusarium*, particularly by *F. graminearum*. Zearalenone affects the urogenital system. The effect it causes is oestrogenic and produces a condition known as hyperoestrogenism in pigs, but it has also been reported to affect poultry (Oliveira et al. 2006).

Fusarium graminearum is also known for its production of vomitoxin (also known as deoxynivalenol, part of the trichothenes group of toxins). While this toxin is quite deleterious to the health of mammals, chickens seem to be much more resistant to the harmful effects (Moran et al. 1982).

2.2.2 *Aspergillus* spp.

The *Aspergillus* genus is an extremely important one, especially in terms of agriculture. Species from this genus may cause problems in both plant and animal production. For example, some species are primarily plant pathogens, but may potentially produce mycotoxins, which can harm animals which ingest them. Additionally, some species in this genus have been known to cause aspergillosis in animals, an important mycotic disease (Akan et al. 2002). Some of the more important species in this genus with regard to poultry farming include *A. fumigatus, A. flavus* and *A. parasiticus*.

Aspergillosis is a threat to the health of poultry flocks and it seems to be more prevalent in the younger chicks (Sajid et al. 2006). The prevalence of aspergillosis in younger chicks is thought to be due to the immaturity of phagocytes, although environmental factors may also play a role (Bennett 1988). In severe cases of aspergillosis, infected birds die overnight even though appearing healthy the previous day. In less acute cases of the disease, sick birds become weary accompanied by a rise in body temperature. The birds lose their appetite and prefer darkness after which paralysis of legs and wings may also develop. The birds cough and sneeze frequently, a slimy discharge develops from the nose and beak, and the eyelids swell and exhibit thick yellow deposits. Symptoms may also include seizures, lameness, convulsions, dehydration, depression and hind limb paresis. In the later stages of aspergillosis the birds develop diarrhoea, after which they waste in condition and most generally die within a week (Arné et al. 2011; Asthana 1944).

As mentioned previously, some species within this genus, such as *A. flavus*, have the ability to produce aflatoxins. These toxins have direct negative impacts on the health of poultry, which include failure to gain weight, liver damage, decreased haemoglobin, weakness and a decrease in egg production and feed efficiency, even at low levels of exposure (Brown and Abrams 1965; Doerr et al. 1983; Smith and Hamilton 1970). In addition to direct impacts on the health of chickens, aflatoxicosis increases the susceptibility of the birds to specific pathogens (Hamilton and Harris 1971).

2.2.3 *Candida* spp.

Candida spp. are a genus of yeasts, some of which are pathogenic towards animals and humans. *Candida albicans* is known to infect the skin, nails, intestine, mouth and various other areas in humans (Wyatt and Hamilton 1975). This yeast causes crop mycosis in chickens (Wyatt and Hamilton 1975). Chickens have been identified as carriers of *C. albicans* and it has been observed that older birds are more likely to act as carriers of the fungus (Jordan 1953).

Candida albicans has in fact been acknowledged as a regular inhabitant of the avian gastrointestinal system, and candidiasis is generally considered to be an opportunistic disease. Therefore any factors that may suppress the immune system of the birds will then predispose them to the disease, which explains the interaction observed between aflatoxicosis and candidiasis (Velasco 2000; Hamilton and Harris 1971).

2.2.4 *Cryptococcus* spp.

Cryptococcosis, caused by the yeast *Cryptococcus* spp., is a disease affecting both humans and animals, which mainly manifests as meningitis in humans. This disease is actually considered rare in pet birds, and although the yeast has been frequently isolated from pigeon droppings worldwide it has been found that these birds do not play an active role in the dissemination of this disease (Abou-Gabal and Atia 1978; Velasco 2000). That being said, though this disease does not seem to be a common occurrence in poultry husbandry, the symptoms of this specific disease in infected chickens are quite debilitating. They include the development of lesions consisting of granulomas and necrosis. These lesions form in the liver, lungs, spleen and intestines (Bauck 1994; Velasco 2000). Therefore, due to the importance of *Cryptococcus* species in public health as well as the potential of species like *C. neoformans* to negatively affect production, this genus of fungi is one worth noting. Species from this specific genus do not seem to be commonly associated with poultry feed, but are very prevalent within the litter and dust contained in poultry housing (Okiki and Ogbimi 2017).

2.3 Important bacterial pathogens in poultry

Bacterial pathogens in poultry cause more alarm due to their ability to spread to human hosts via zoonosis, rather than the effect they may have on production. With species such as those

within the *Salmonella* genus acquiring multi-drug resistance, it is no wonder that these pathogens are a cause for concern (Usera et al. 2002).

2.3.1 *Escherichia coli*

While *E. coli* is usually a harmless bacterial species commonly forming part of the microflora within the digestive tract, some strains of *E. coli* cause disease in human hosts, resulting in diseases such as haemorrhagic colitis and haemolytic uremic syndrome (Doyle and Schoeni 1987). Certain strains of *E. coli*, namely avian pathogenic *E. coli* (APEC), cause extraintestinal diseases in chickens such as aerosacculitis, polyserositis and septicaemia (Dho-Moulin and Fairbrother 1999). These extraintestinal diseases caused by the APEC strains may result in large economic losses, and many of these strains possess antibiotic resistance increasing the difficulty of control (Dho-Moulin and Fairbrother 1999; Gross 1994).

2.3.2 *Salmonella* spp.

Poultry is known to be the greatest reservoir of *Salmonella* species worldwide (Buxton 1957; Quist 1963). *Salmonella* spp. may spread to humans via zoonosis, which greatly increases the importance of this pathogen, especially due to salmonellosis in humans being a very important food-borne disease. In fact, *Salmonella*, along with *Campylobacter*, accounts for over 90% of reported cases of bacterial food poisoning worldwide, and poultry or poultry products have been implicated in most of the food-borne illnesses caused by these bacteria (Thorns 2000).

The significance of salmonellosis in poultry has been recognized since the early 1920's, as it normally manifests as an acute disease in birds less than one month of age, causing mortality rates of up to 80% or higher (Biester and Schwarte 1959). The disease is only significant to the birds themselves at a young age as older birds seem to show no symptoms of infection, yet they may act as long-term carriers of the disease (Quist 1963). It is believed that the presence of the disease in a breeding flock will most likely lead to problems such as increased mortality in chicks, decreased hatchability and fertility, as well as reduced egg production. This disease may also stunt growth and cause debilitation, increasing the susceptibility of the birds to other diseases (Quist 1963).

2.4 Feed additives

Feed additives are additional components mixed into the poultry feed that do not convey any nutritional benefit towards the birds, but may serve alternative purposes, for example growth promotion (Singh et al. 2015).

2.4.1 Antibiotics

Antibiotics have been used for many years for therapeutic reasons, but more recently they have been used as growth promoters of animals due to the interaction these substances have with the intestinal microflora (Castanon 2007; Dibner and Richards 2005). Though describing these substances as "growth promoters" is inaccurate (they do not act in the same manner as anabolic steroids, which do in fact promote growth), these substances act by allowing animals to fully express their genetic potential for growth in the absence of inhibition (Ferket 2004). Nevertheless, the subtherapeutic use of these substances meant a drastic increase in their presence in agriculture. With this increase in use came concern about the development of antibiotic resistance in animal microflora, and the transfer of this resistance to human microflora (Castanon 2007).

This concern led to a ban of the subtherapeutic use of antimicrobials in agriculture in Europe, starting with Sweden in 1986 (Cogliani et al. 2011). The ban of antibiotic growth promoters (AGPs) caused an impact on animal production due to the increase in pathogenic load faced by the animals, which led to increased interest in the development of alternative methods in an effort to compensate for this. Some of these alternative methods included changes in husbandry, such as increasing sanitation in an effort to reduce pathogen load, adjusting diet digestibility and enzyme supplementation, and adding acidifiers to lower pH and reduce growth of enteric microflora (Ferket 2004). Herbs have been used to increase the palatability of human food for centuries and many of these herbs as well as other plant extracts have been recognised for their significant health benefits (Ferket 2004). Some of these benefits include appetite stimulation, antioxidant protection and antimicrobial ability, as well as several others. It is thought that these antimicrobial plant-based compounds could be used to replace some AGPs (Ferket 2004).

2.4.2 Mycotoxin adsorbents

Mycotoxin adsorbents are non-nutritive substances added to feed that function in reducing the uptake of mycotoxins and distribution to major organs. Various substances have been used for

this purpose, with the most commonly used group of mycotoxin adsorbents being aluminium silicates (Binder 2007). While some of these substances do tend to reduce the uptake of mycotoxins, they are very specific and in most cases one type of adsorbent will only be effective against one specific mycotoxin (Binder 2007). Furthermore, investigations by Avantaggiato et al. (2004, 2005) showed that most commercially available mycotoxin adsorbents failed to sequester *Fusarium* mycotoxins *in vitro*. This is a problem, as addition of adsorbents to feed will most probably only be effective against a small number of mycotoxins, and may have no effect on any *Fusarium* mycotoxins present.

2.4.3 Plant material as an alternative feed additive

Some of the beneficial effects conveyed by active plant substances when added to the animal diet may include a better feed conversion ratio, increased immune response and antioxidant, antibacterial and anthelminthic actions, as well as improvements in digestive enzyme secretion (Jamroz et al. 2005). Several studies have been carried out to evaluate the efficacy of specific plants as alternative "growth promoters" in poultry feed. Some of these studies will be discussed to further illuminate the potential of plant material to act as an alternative to AGPs.

In a study carried out by Landy et al. (2011a), *Azadirachta indica* A. Juss (neem) was investigated for its effect on the immunity, performance and carcass traits of broiler chickens. This was done by using 192 day-old broiler chicks, separated randomly into four treatments, with each treatment comprising four replicate pens of 12 chicks. The treatments used consisted of a basal diet (control), basal diet and 4.5 mg flavophospholipol/kg (positive control) or basal diet and 7 or 12 g neem fruit powder/kg. Body weights of the chicks were then determined on days 1, 14, 28 and 42. Feed intake was determined at the same periods and used to calculate feed conversion ratio. Two birds per replicate were slaughtered on day 42 and used to determine carcass and organ weights. Antibody titres of influenza, Newcastle viruses and sheep red blood cells were also measured. The addition of flavophospholipol to the basal diet significantly increased the final body weight of broilers at 42 days of age compared to the other treatments. The highest feed conversion ratio seen on day 42 was in birds with diets supplemented with 7g/kg neem. The supplementation of 7g/kg neem also yielded the highest antibody titres against sheep red blood cells and influenza virus. None of the treatments seemed to have an effect on the carcass traits and internal organ weights. It was stated that supplementing the broiler diet with 7g/kg of neem would have a favourable effect on the immune response of the birds without any adverse effect on growth performance (Landy et al. 2011a).

Ghazalah and Ali (2008) evaluated the efficacy of *Rosmarinus officinalis* L. (rosemary) as a growth promoter in broiler diets, as well as its effect on the immunity and performance of the birds. This was done by adding rosemary leaf meal (RLM) to either grower (7-28 day) or finisher (29-49 day) diets in three different concentrations (0.5, 1 and 2%). Two hundred day-old Arbor Acres chicks were used and these chicks were divided equally into four treatments with each treatment consisting of five replicates of 10 chicks. Mortality was recorded daily and at days 28 and 49, live bodyweight and feed intake were determined. At 50 days, blood was taken from birds to determine plasma levels and several other characteristics. Carcass characteristics were also determined and the immune response to sheep red blood cells was measured. Additionally, the differences in lymphoid organ weights including the thymus and spleen were determined. It was found that chicks which had been fed 0.5 % RLM exhibited higher body weights, greater weight gain, and better feed conversion ratio during the experimental period compared to the control. Additionally these birds exhibited better physical properties in terms of the chicken meat, increased plasma total protein, albumin and globulin with a decrease in glucose, total lipids and cholesterol content. Furthermore, immune response against sheep red blood cells was also improved and the percentage of the lymphoid organs was higher in comparison to the controls. In contrast to this, feeding chicks RLM in concentrations higher than 0.5% resulted in decreased growth and digestibility of most nutrients. Therefore, it was concluded that low levels of dietary RLM may be used in broiler diets to safely promote growth as well as potentially impart beneficial elements to the consumer (Ghazalah and Ali 2008).

A study done by Landy et al. (2011b) investigated the efficacy of *Echinacea purpurea* L. (purple coneflower) as a substitute for antibiotic growth promoters on performance, carcass characteristics and immune response in broiler chickens. In this study, 336 day-old broiler chicks (Ross 308) were randomly assigned to seven treatments, with each treatment group consisting of four replicates and 12 chicks per replicate. The seven treatments consisted of: basal diet (control), basal diet and 4.5 mg flavophospholipol/kg (positive control), basal diet and dried powdered aerial parts of *E. purpurea* (referred to as EP1) continuously (5 g/kg), basal diet and EP1 continuously (10 g/kg), basal diet and ethanolic extract of *E. purpurea* (referred to as EP2) continuously (0.25 g/kg), basal diet and EP1 with 3-days application followed by 11 application free days intermittently (5 g/kg), and finally basal diet and EP1 with 3-days application followed by 11 application free days intermittently (10 g/kg). Blood samples were taken on the 28th and 31st day and used to evaluate immune responses against sheep red blood

cells and Newcastle virus. The results of the study showed that the use of the 5 g/kg EP1 continuously led to the highest daily feed intake, daily weight gain and immune response against both sheep red blood cell and Newcastle virus in comparison to the other treatment groups. Additionally the intermittent application of 10 g/kg EP1 had a significant effect during the grower period on daily weight gain, feed conversion ratio and immune responses against sheep red blood cells. Carcass traits were not affected in any of the treatment groups except the intermittent groups, where the percentage weight of the small intestine was lower. In conclusion, this study showed that the application of 5 g/kg dried aerial parts of *E. purpurea* in broiler diets could significantly improve performance and immune responses of chicks.

Studies done by Hernandez et al. (2004) where a combined essential oil extract of *Piper nigrum* L. (pepper), *Cinnamomum verum* J.Presl (cinnamon), and *Origanum vulgare* L. (oregano), and a combined extract of *Salvia officinalis* L. (sage), *Thymus vulgaris* L. (thyme), and rosemary were investigated for their effects when added to broiler feed. Addition of plant material may also be beneficial to chicken health via increases in daily growth, improvement in apparent ileal and whole-tract digestibility of nutrients, as well as improvements in faecal composition and extract digestibility (Hernandez et al. 2004).

2.5 *Psychotria* spp.

2.5.1 Taxonomy and distribution

Psychotria, belonging to the family Rubiaceae, is a large genus of predominantly woody plants. It consists of around 2 000 species, making it one of the world's largest genera of woody plants, and the largest genus within the Rubiaceae family (Davis et al. 2001). There is high taxonomic complexity within the group and recent systematic studies have shown that *Psychotria* is either paraphyletic or polyphyletic (Davis et al. 2001).

Species of *Psychotria* are most commonly found in pantropically wet to seasonal forests (Paul et al. 2009). Between the species, the growth forms are generally similar, comprising small trees and shrubs. They are obligate outcrossing species, pollinated by insects, and the genus is usually responsible for a large proportion of species and stems in the understories of many tropical forests (Paul et al. 2009).

Psychotria zombamontana (Kuntze) E.M.A. Petit is a woody plant indigenous to South Africa. The leaves are leathery and the margin is frequently wavy (Figure 2.1). On the under-side of the leaves domatia can be seen at the base of semi-main veins. The plant is said to have bacterial nodules on the leaves. The flowers are small and white and the fruit are ovoid drupes (van Wyk and van Wyk 2013).

Figure 2.1 *Psychotria zombamontana* (Photo: B. Querl).

2.5.2 Uses and biological activity

Bioactive compounds are often isolated from species within the Rubiaceae family and this family is thought to have considerable chemical potential (Matsuura et al. 2013). The *Psychotria* genus has earned the title as a 'hot' genus with regard to bioactivity with many of the species showing different activities (Matsuura et al. 2013).

Jayasinghe et al. (2002) found that *Psychotria gardneri* (Thwaites) Hooker and *Psychotria stenophylla* (Thwaites) Hooker showed a wide spectrum of antimicrobial activity when using the disc diffusion method. Specifically *P. gardneri* displayed activity against *Saccharomyces cerevisiae*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus* and *Aspergillus niger*. *Psychotria stenophylla* showed activity against *Ustilago maydis*, *E. coli*, *M. luteus*, *B. subtilis*, *B. cereus* and *A. niger*. Extracts from *Psychotria nigra* L. were also tested,

however, these displayed less activity than *P. gardneri* and *P. stenophylla*, specifically against *M. luteus*, *E. coli*, *S. cerevisiae* and *B. cereus* (Jayasinghe et al. 2002).

In another study, Khan et al. (2001) found that *Psychotria microlabastra* L. (Sphalm) possesses a wide spectrum of antibacterial activity. This was shown in a disc diffusion assay, where methanol extracts of the leaves, stem bark and root bark were tested against 26 different species of bacteria. The plant showed activity against all tested bacteria (Khan et al. 2001). *Psychotria colorata* (Willd. ex R. & S.) Muell. Arg. was found to possess analgesic activity by Elisabetsky et al. (1995) using the formalin, writhing and tail-flick methods. This plant was chosen for testing due to the use of its leaves and flowers by Amazonian Caboclos as pain killers, as discovered during an ethnopharmacological survey. *Psychotria myriantha* Müll.Arg. has displayed antichemotactic activity (inhibition of chemotaxis, the ability of some microorganisms to move in a certain direction corresponding to the gradient of an increasing or decreasing concentration of a specific chemical) (Simoes-Pires et al 2006).

The fruit of *Psychotria capensis* (Eckl.) Vatke is used in the OR Tambo district of the Eastern Cape to treat vomiting and diarrhoea (Bisi-Johnson et al. 2010). Bioactivity has been previously described in the extracts of this plant, namely antimycobacterial activity (Aro et al. 2015). Anti-elastase activity has also been found in extracts from *P. capensis*, meaning that this plant also shows activity against the process of aging of the skin (Ndlovu 2013). In an MSc study done by Kafua (2010), the author reported promising antifungal activity of extracts from *P. capensis*. These results were confirmed in further investigations by Querl (2016), which also revealed promising activity of *P. zombamontana* acetone extracts against *F. verticillioides*, *F. graminearum* and *A. flavus.* The only other bioactivity recorded from extracts of *P. zombamontana* is antimycobacterial activity. A lack of cytotoxicity was also reported, unlike *P. capensis*, which was found to be cytotoxic (Aro et al. 2015). Besides these few studies, there seems to be a scarcity of any other information regarding this plant. *Psychotria zombamontana* was chosen for this study due to the abundance of bioactivity present within the *Psychotria* genus as well as the promising antifungal activity displayed by *P. zombamontana* during preliminary studies.

2.6 Conclusion

In summary, poultry feed is a major component of the poultry industry in South Africa, and it may act as a constraint to production if quality control of the feed is not optimal. This feed is highly vulnerable to contamination by microorganisms, some of which may only cause slight

deterioration in nutritional value. However, there are certain species of fungi whose colonization of feed may lead to mycotoxicoses and other conditions in poultry, negatively affecting production. Currently, mycotoxin adsorbents and AGPs are in use to combat these effects, but both come with disadvantages, such as the specificity of adsorbents and the contribution to antibiotic resistance by AGPs. Plants are an underutilised resource, brimming with biological activity which may aid in providing a solution to this problem. Many plants have displayed activity against problematic fungal species, and may have beneficial effects when added to animal feed. *Psychotria* is a genus of the Rubiaceae well known for biological activity and *P. zombamontana* has previously shown promising antifungal activity against species from the *Fusarium* and *Aspergillus* genera. This plant was therefore investigated for its efficacy as a potential feed additive for poultry, in terms of beneficial biological activity on poultry health and efficacy against common problematic fungi.

3. Antimicrobial activity of crude acetone leaf extracts of *Psychotria zombamontana* against microorganisms implicated in causing disease in poultry

3.1 Introduction

With poultry being such a large industry in South Africa, relying on the continuous production of poultry meat, farmers are under tremendous pressure to deliver, and large losses may be catastrophic. Some species of fungi play a notable role in some of the losses experienced, with species from *Aspergillus* known to cause aspergillosis, an important mycotic disease that can lead to death of chickens overnight (Akan et al. 2002; Asthana 1944). Other species such as *Candida albicans* and *Cryptococcus neoformans* may act as opportunistic pathogens of the birds, and some fungal species such as *Fusarium verticillioides* and *Aspergillus flavus* may produce mycotoxins in the feed. Presence of these mycotoxins in the feed may lead to various symptoms, ranging from impaired disease immunity and weakness, to liver damage (Hamilton and Harris 1971; Labuda et al. 2003; Oliveira et al. 2006).

Bacterial pathogens in poultry cause alarm mainly due to their ability to spread to human hosts via zoonosis (Usera et al. 2002). For example certain strains of *Escherichia coli*, usually a harmless bacterial species commonly found within the digestive tract, may cause disease in human hosts resulting in conditions such as haemorrhagic colitis and haemolytic uremic syndrome (Doyle and Schoeni 1987). Additionally, strains of *E. coli* belonging to avian pathogenic *E. coli* (APEC), can cause aerosacculitis, polyserositis and septicaemia in chickens (Dho-Moulin and Fairbrother 1999).

Significant bacterial pathogens associated with poultry include *Salmonella* species, and poultry are the greatest reservoir of *Salmonella* species worldwide (Buxton 1957; Quist 1963). *Salmonella* spp. may spread to humans via zoonosis, which greatly increases the importance of this pathogen (Thorns 2000). With species within the *Salmonella* genus acquiring multi-drug resistance, it is no wonder that these pathogens are a great cause for concern (Usera et al. 2002). Furthermore, salmonellosis is a notable disease in young chickens, usually manifesting as an acute disease in birds less than one month of age, sometimes causing mortality rates of up to 80% or higher (Biester and Schwarte 1959; Quist 1963).

There are solutions available commercially for problematic microorganisms, such as adsorbents for mycotoxigenic fungi as well as antibiotic growth promoters (AGPs). Although

these methods of control can be effective, they both come with disadvantages, such as the specificity observed in adsorbents and the effect AGPs can have on antibiotic resistance (Binder 2007; Castanon 2007). Plants have previously shown activity against a wide range of bacteria and fungi, and therefore would be an ideal starting point in the search for alternatives to antibiotics as new pathogen control methods (Nascimento et al. 2000; Wilson et al. 1997). More specifically, a genus from the Rubiaceae family, *Psychotria*, has gained attention due to the high amount of bioactivity present within the genus (Matsuura et al. 2013). *Psychotria zombamontana* (Kuntze) E.M.A. Petit is an understudied species within this genus, which has shown promising antifungal activity against species of *Fusarium* and *Aspergillus* during preliminary testing (Querl 2016).

Psychotria is a large genus of predominantly woody plants, consisting of around 2 000 species. This makes it one of the world's largest genera of woody plants, and the largest genus within the Rubiaceae family (Davis et al. 2001). Species of *Psychotria* are most commonly found in pantropically wet to seasonal forests and are usually responsible for a large proportion of species and stems in the understories (Davis et al. 2001; Paul et al. 2009). *Psychotria zombamontana* is a woody plant indigenous to South Africa (van Wyk and van Wyk 2013). Antifungal activity has been observed in acetone leaf extracts of this plant against species from both *Fusarium* and *Aspergillus* during preliminary studies (Querl 2016).

In this study, acetone leaf extracts of *P. zombamontana* were investigated for antimicrobial activity against common problematic fungi and bacteria associated with poultry feed. Acetone was the extracting solvent of choice due to its ability to extract a wide range of polar and non-polar compounds and low toxicity to test microorganisms in antifungal assays (Eloff 1998a).

3.2 Materials and Methods

3.2.1 Collection of plant material

Leaves of *P. zombamontana* were collected from the Manie van der Schijff Botanical Garden at the University of Pretoria, Pretoria, South Africa during the month of April (summer) in the year 2017. Voucher specimens were prepared and deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria (voucher number: PRU122248). The collected leaves were dried at room temperature, ground to a fine powder using an MF10 grinder (IKA China) with a 1 mm diameter sieve element and stored in sealed jars in the dark until extraction.

3.2.2 Extraction

Acetone extracts were prepared using the dried plant material with 10 ml acetone added per 1 g of dried leaf material. The acetone plant mixture was sonicated for 30 minutes, then filtered through Whatman No 1 filter paper using a Büchner funnel. Thereafter, additional acetone was added to the sediment resulting from the filtration and this mixture was sonicated for 30 min and then filtered. This process was repeated until the mixture remained colourless after sonication. The supernatants were then pooled together and dried under a fume hood. Dried extract was stored in a glass jar and refrigerated at 4°C until further use.

3.2.3 Antifungal screening of extracts

3.2.3.1 Culturing fungal strains

The fungi chosen for this study were mostly field isolates of fungal species commonly associated with poultry feed and consisted of both filamentous fungi and yeasts. The filamentous fungi chosen were obtained from the Agricultural Research Council – Biosystematics division, Roodeplaat, South Africa and included *Fusarium verticillioides* (PPRI 7259), *Fusarium oxysporum* (PPRI 8287), *Fusarium graminearum* (PPRI 7723) and *Aspergillus flavus* (PPRI 3954). The yeasts chosen included *Candida albicans* (ATCC 10231) and a clinical isolate of *Cryptococcus neoformans* obtained from the Department of Veterinary Tropical Diseases, isolated from a Gouldian finch. Fungal cultures were maintained on Potato Dextrose Agar (PDA). Filamentous fungi were incubated at 28°C, while yeasts were incubated at 37°C for approximately five days to allow for fungal growth. Once cultures had grown, plates were stored at 4°C until further use.

3.2.3.2 Microdilution assay

The microdilution assay was carried out in 96-well plates in a similar manner to that performed by Eloff (1998b) but with slight modifications, these include the use of Sabouraud Dextrose broth during the serial dilution instead of water. During this experiment, fungal broth was prepared for each fungus, by inoculating each culture in Sabouraud Dextrose broth and incubating on a shaker at 28°C for 7 days. The broth from each culture was then adjusted to a turbidity equivalent to a 0.5 McFarland standard ($1.5x10^8$ cfu/ml) using a spectrophotometer. Stock solutions of 32 mg/ml were prepared in acetone for the plant extract.

For the assay, 100 µl of Sabouraud Dextrose broth was pipetted into all wells of a 96-well plate, and thereafter 100 µl of extract, or acetone as the negative (solvent) control and amphotericin B as the positive control were added to wells of the first row in the manner displayed below in Figure 3.1. The extract solutions, solvent control (acetone) and positive control were then serially diluted (50%) with the broth, by pipetting 100 µl from row A to row B, then 100 µl from row B to row C and so on, until 100 µl was eventually taken from row H and discarded. In the next step, 100 µl of each fungal broth was added to each well in their respective column e.g. wells 1, 2 and 3 of rows A-H (no fungi were added to the sterility controls). Finally, 40 µl of 0.4 mg/ml *p*-iodonitrotetrazolium chloride (INT) was added to every well on the plate including sterility control wells. Plates were covered and sealed before incubating plates containing filamentous fungi at 28°C and yeasts at 37°C for 48 h. The minimum inhibitory concentration (MIC) was determined using the colour change of the indicator. The entire experiment was repeated twice, with three replicates in each experiment.

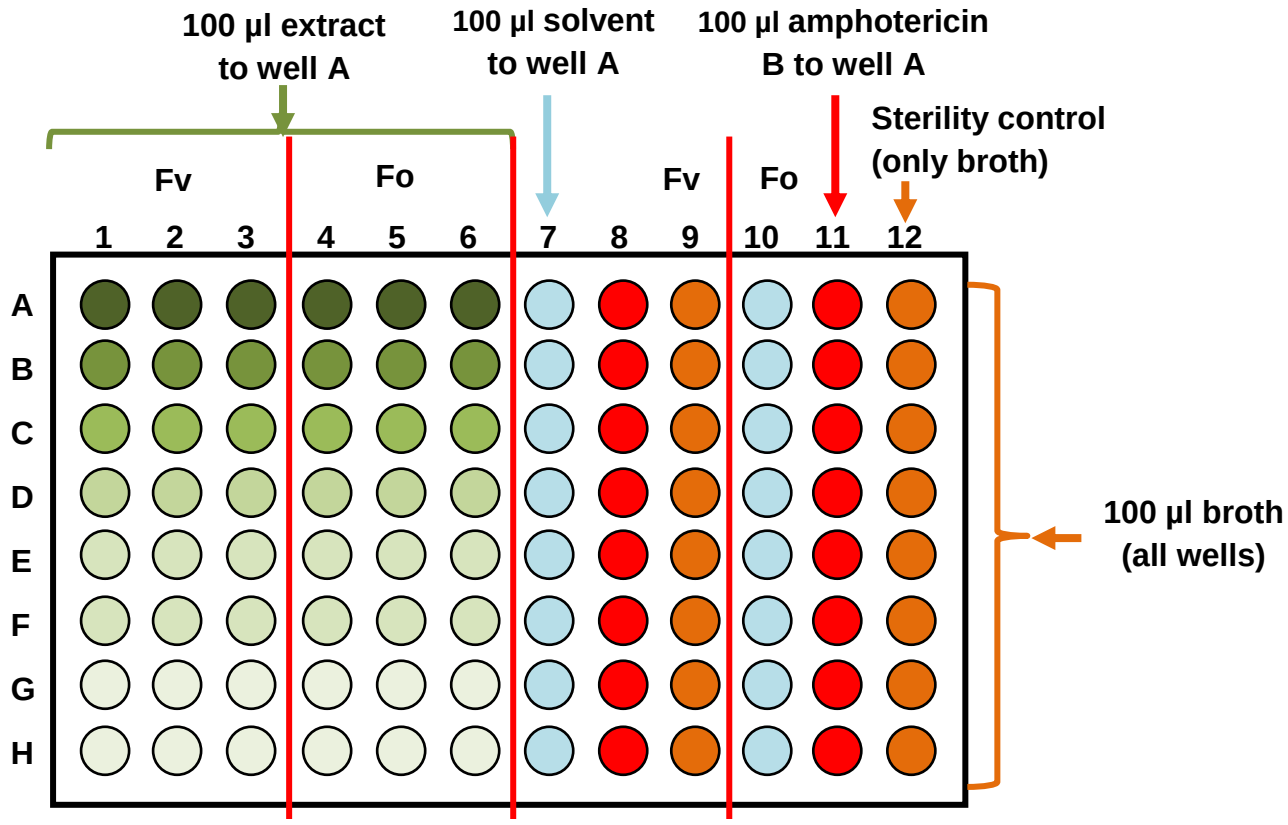


Figure 3.1 Simplified diagram of the microdilution assay. Fv: *Fusarium verticillioides*; Fo: *Fusarium oxysporum*.

3.2.4 Antibacterial screening of extracts

3.2.4.1 Culturing bacterial strains

Bacteria chosen for this study included field isolates and ATCC strains of species commonly associated with poultry. The species chosen comprised *Escherichia coli* (field isolate B3427/16 and ATCC strain 25922), *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis, ATCC 13076) and a clinical isolate of *Salmonella enterica* subsp. *enterica* serovar Gallinarum (*S.* Gallinarum, isolated from a chicken, obtained from the collection of the Department of Veterinary Tropical Diseases). The cultures were maintained on Mueller Hinton agar and incubated at a temperature of 37°C for 24 h before storing at 4°C until further use.

3.2.4.2 Microdilution assay

The microdilution assay was carried out in 96-well plates in a similar manner to that described in section 3.2.3.2. During this experiment, bacterial broth was prepared for each bacterium, by growing each culture in Mueller Hinton broth on a shaker for 24 hours. The broth from each culture was then adjusted to a concentration equivalent to a 0.5 McFarland standard (equivalent to $1.5x10^8$ cfu/ml) using a spectrophotometer. Stock solutions of 32 mg/ml were prepared in acetone for the plant extract.

For the assay, 100 µl of Mueller Hinton broth was pipetted into all wells of a 96-well plate, and thereafter 100 µl of extract, acetone (negative/ solvent control) or gentamicin (positive control) were added to the first row in the manner displayed above in Figure 3.1. The extract solutions, acetone and gentamicin were then serially diluted (50%) with the broth, by pipetting 100 µl from row A to row B, then 100 µl from row B to row C and so on, until 100 µl was eventually taken from row H and discarded. In the next step, 100 µl of each bacterial broth was added to each well in their respective column e.g. wells 1, 2 and 3 of rows A-H (no bacteria were added to the sterility controls). Finally, 40 µl of 0.4 mg/ml INT were added to every well on the plate including sterility control wells. Plates were covered and sealed before incubation at 37°C for 24 hours. The MIC was determined using the colour change of the INT indicator. The entire experiment was repeated twice, with three replicates in each experiment.

3.3 Results and Discussion

3.3.1 Antifungal screening

The crude acetone leaf extract of *P. zombamontana* exhibited promising activity against the fungi tested in the microdilution assay. The positive control seemed to have little effect on the *Fusarium* species, but showed activity within its working range against *A. flavus*, *C. albicans* and *C. neoformans*. MIC values (Table 3.1) were recorded as the border between the colour change of light pink to dark pink, as this is the concentration where fungal growth was inhibited, while wells lacking the red colour indicate fungicidal activity (Fig. 3.2).

Table 3.2. MIC values of the crude acetone leaf extract of *P. zombamontana* against fungi associated with poultry and poultry feed.

	MIC (mg/ml)	
Fungi	***P. zombamontana***	**Amphotericin B**
Fusarium verticillioides (PPRI 7259)	0.5000	2.5000
Fusarium oxysporum (PPRI 8287)	1.0000	1.2500
Fusarium graminearum (PPRI 7723)	0.5000	0.6125
Aspergillus flavus (PPRI 3954)	0.5000	0.0031
Candida albicans (ATCC 10231)	0.5000	0.0125
Cryptococcus neoformans	0.5000	0.0125

SD = 0

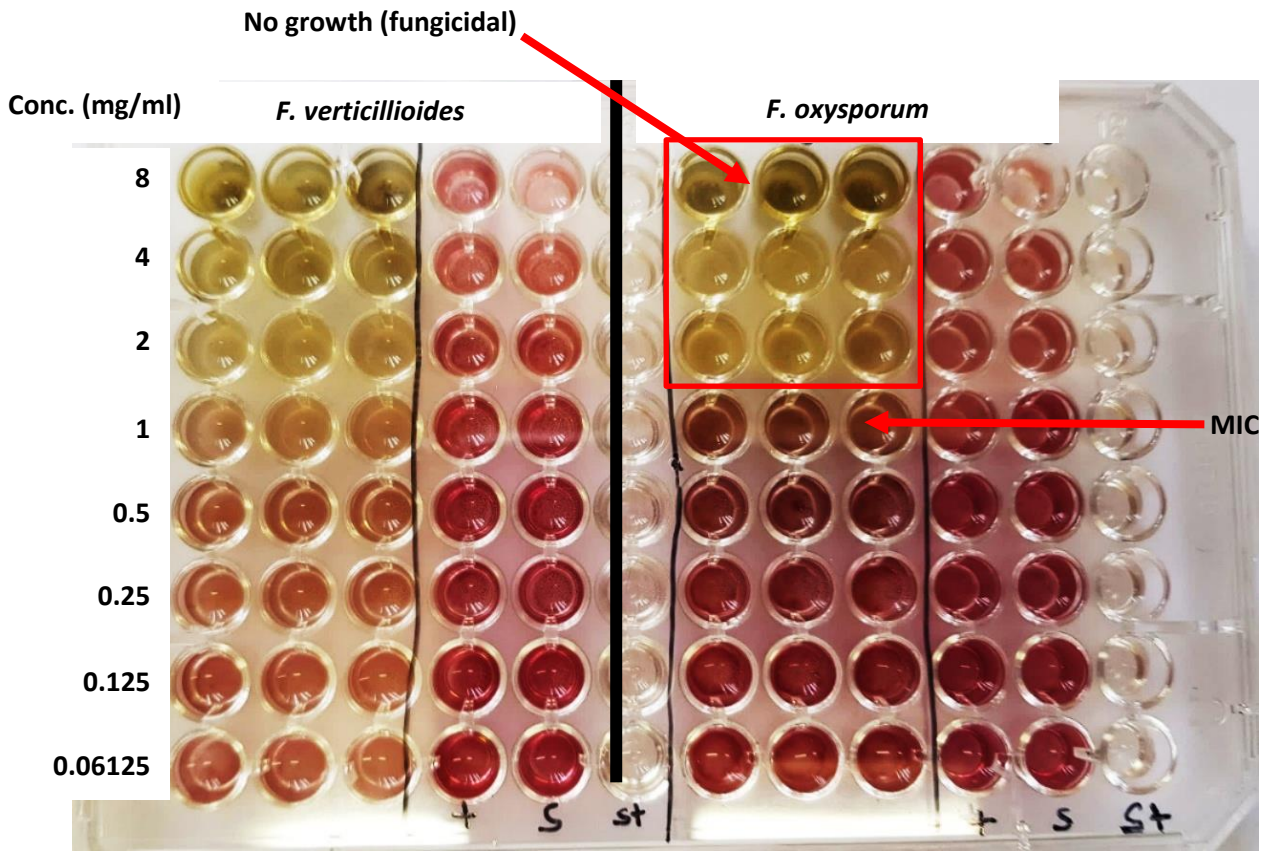


Figure 3.2. Microdilution assay in a 96-well plate for the crude *P. zombamontana* acetone leaf extract against *F. verticillioides* and *F. oxysporum*.

According to a classification based on MIC results of a microdilution assay after 48 hours proposed by Souza et al. (2007), strong inhibitors display an MIC of ≤ 0.5 mg/ml; moderate inhibitors display an MIC of between 0.6 and 1.5 mg/ml and weak inhibitors display an MIC of > 1.6 mg/ml. The crude extract of *P. zombamontana* may then be classed as a strong inhibitor of *F. verticillioides*, *F. graminearum*, *A. flavus*, *C. albicans* and *C. neoformans*, while it acts as a moderate inhibitor of *F. oxysporum*.

In comparison to previous antifungal studies on *Psychotria* species, the activity of *P. zombamontana* is very promising. Kafua (2010) tested the activity of methanol, dichloromethane (DCM) and acetone leaf extracts of *Psychotria capensis* (Eckl.) Vatke against different species of *Fusarium* including *F. verticillioides*, *F. graminearum* and *F. oxysporum*. During this study, Kafua (2010) utilized the microdilution technique, and results showed that the acetone extract of *P. capensis* was most active against all fungi tested. *Psychotria capensis* acetone extract displayed an MIC of 1.5 mg/ml against *F. verticillioides*, 3.2 mg/ml against *F. oxysporum* and 6.25 mg/ml against *F. graminearum*. *Psychotria capensis* exhibited MIC values higher than 1.6 mg/ml, against *F. oxysporum* and *F. graminearum* and can therefore be classed

as was a weak inhibitor against these species. It can be noted that the crude acetone extract of *P. zombamontana* displayed better antifungal activity when compared to the crude acetone extract of *P. capensis*, especially with regard to *F. oxysporum* and *F. graminearum.*

Agripino et al. (2004) performed a study where ethanol leaf extracts of *Psychotria mapoureoides* DC. and *Psychotria nuda* (Cham. & Schltdl.) Wawra were tested for antimicrobial activity. The organisms tested included *C. albicans*, *E. coli* and *Salmonella aureus.* In this study, extracts of both species were found to be inactive against all organisms tested (Agripino et al. 2004). Another study, carried out by Giang et al. (2007), tested the antimicrobial activity of butanol, ethanol, chloroform and hexane fractions originating from a methanol extract of *Psychotria reevesii* Wall. against a number of microorganisms which included *C. albicans* and *E. coli*. All fractions seemed to display no activity against *C. albicans* or *E. coli*, though they did show activity against some of the other microorganisms tested (Agripino et al. 2004).

A study performed by Locher et al. (1995) found *Psychotria hawaiiensis* (A.Gray) Fosberg to be active against *Epidermophyton floccosum.* In the study mentioned, Locher et al. (1995) determined the antifungal activity of several plant extracts, including the acetonitrile bark extract of *P. hawaiiensis* in a microdilution assay. *Psychotria hawaiiensis* had an MIC of 20 µg/ml against *E. floccosum* but did not display any inhibition of *A. flavus* and *A. niger* (Locher et al. 1995). Jayasinghe et al. (2002) investigated the antifungal activity of *Psychotria gardneri* (Thwaites) Hooker and *Psychotria stenophylla* (Thwaites) Hooker against *A. niger* using a disc diffusion assay, for which both *P. gardneri* and *P. stenophylla* displayed activity, albeit low. *Psychotria nigra* L. did not show any activity against the fungus at all (Jayasinghe et al. 2002). *Psychotria nilgiriensis* Deb. & Gang has also been shown to display antifungal activity. In a study by Devadoss et al. (2012), root and fruit extracts of this plant showed activity against *Aspergillus niger* and *A. fumigatus* in an agar well diffusion assay, similar to that used by Bobbarala et al. (2009).

A study done by Rosas-Burgos (2009) tested extracts of *Baccharis glutinosa* Pers. and *Ambrosia confertiflora* DC. for activity against *A. flavus*, *A. parasiticus* and *F. verticillioides*. This study used the agar infusion method to determine the MIC_{50} of each extract toward each fungus. It was found that the MIC_{50} of the ethyl acetate extract of *B. glutinosa* was 0.3 mg/ml for *F. verticillioides* and *A. flavus* (Rosas-Burgos 2009). The MIC of *P. zombamontana* for *F.*

verticillioides is therefore slightly lower than that displayed by the *B. glutinosa*, but this value was slightly higher against *A. flavus*.

Thembo et al. (2010) conducted a study investigating the antifungal activity of extracts from *Tagetes minuta* L., *Lippia javanica* (Burm.f.) Spreng., *Amaranthus spinosus* L. and *Vigna unguiculata* (L.) Walp.. All extracts tested showed no antifungal activity against *A. flavus*, when tested using a microdilution assay. However, in this same study the methanol and hexane extracts of *A. spinosus* displayed MIC of <0.5 mg/ml against *F. verticillioides* (Thembo et al. 2010), which was similar to that displayed by *P. zombamontana* in this study. This illustrates how great the variation in activity between different plant extracts may be against different species of fungi.

3.3.2 Antibacterial screening

The acetone extract of *P. zombamontana* displayed promising activity against the bacteria tested in this study. The MIC values obtained from the microdilution assay against both the field isolates and ATCC strains of species from *Escherichia* and *Salmonella* ranged from 0.125 to 0.5 mg/ml (Table 3.2; Figure 3.3). From the proposed scale described previously (section 3.3.1), *P. zombamontana* had good antibacterial activity, acting as a strong inhibitor against all bacteria tested.

Table 3.2. MIC values observed for the crude acetone leaf extract of *P. zombamontana* against bacteria associated with poultry.

	MIC (mg/ml)	
Bacteria	***P. zombamontana***	**Gentamicin**
Escherichia coli (B3427/16)	0.5000	0.0613
Salmonella Gallinarum	0.2500	0.5000
Escherichia coli (ATCC 25922)	0.1250	0.0078
Salmonella Enteritidis (ATCC 13076)	0.1250	0.0156

SD = 0

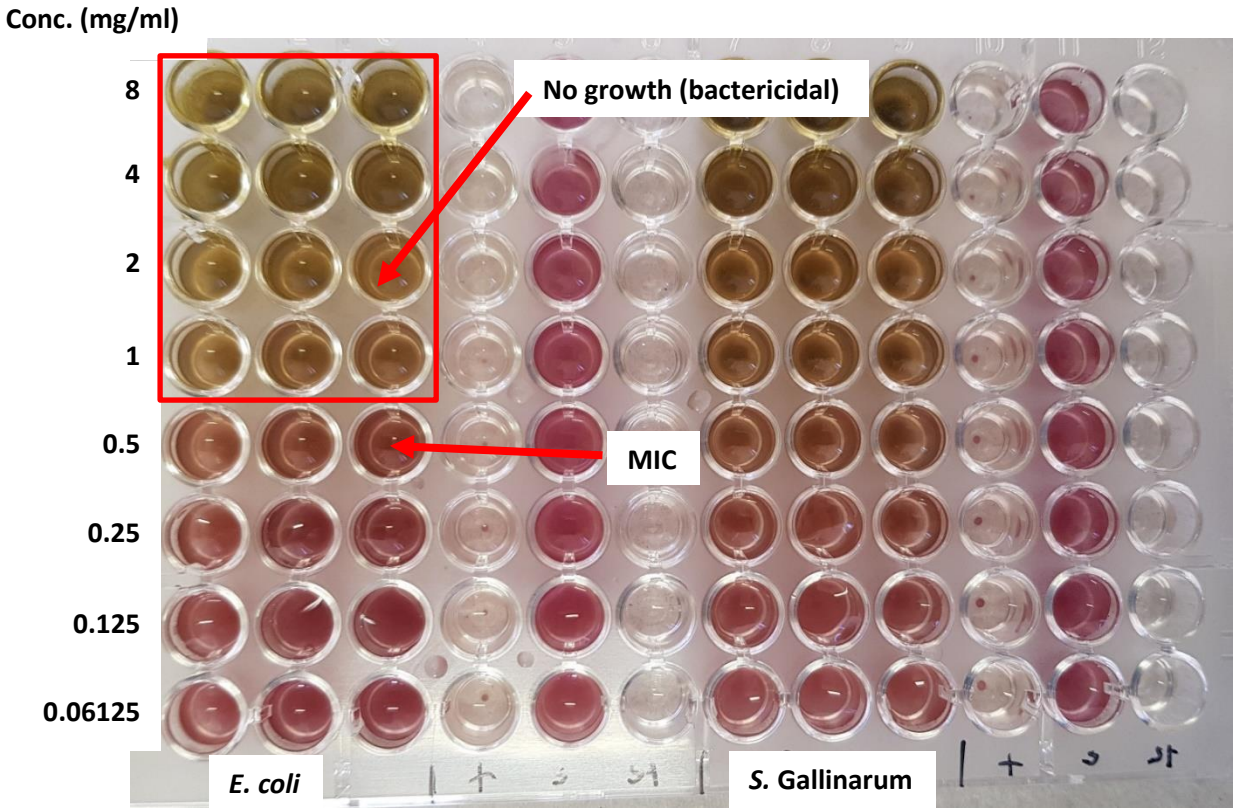


Figure 3.3. Microdilution assay in a 96-well plate for the crude *P. zombamontana* acetone leaf extract against *E. coli* (B3427/16) and *S.* Gallinarum (field isolate).

Other species within the *Psychotria* genus have also shown both antibacterial activity in previous studies. For example, a study done by Jayasinghe et al. (2002) found that *P. gardneri* and *P. stenophylla* had a wide spectrum of antibacterial activity. The methanol bark extract of *P. gardneri* and the DCM bark extract of *P. stenophylla*, exhibited activity against *E. coli*, *Bacillus subtilis* and *Bacillus cereus*. *Psychotria nigra* was also found to display activity but slightly less, with the bark methanol extract only showing inhibition against *E. coli* and *B. cereus*. A study by Razafintsalama et al. (2017) tested methanol inflorescence extracts of *Psychotria bridsoniae* A.P.Davis & Govaerts and methanol stem (with leaves and fruit) extracts of *Psychotria oreotrephes* (Bremek.) A.P.Davis & Govaerts for antibacterial activity against a variety of bacteria. Both species displayed activity against some of the bacteria, but did not show any activity against *E. coli* or *S.* Enteritidis. Additionally, *Psychotria microlabastra* L. (Sphalm) was observed to possess a wide spectrum of antibacterial activity during a study by Khan et al. (2001). Methanol extracts of the leaves, stem bark and root bark were tested against 26 species of bacteria, including species from *Escherichia*, *Salmonella*,

Bacillus, *Klebsiella* and *Staphylococcus* using a disc diffusion assay. The plant exhibited activity against all bacteria tested (Khan et al. 2001).

Previous studies have shown other plant extracts possess inhibitory activity against both *E. coli* and *Salmonella* species. A study done by Aboaba et al. (2006) evaluated the antibacterial activity of aqueous and ethanol extracts of *Entada africana* Guill. et Perr. (bark), *Terminalia avicennoides* Guill. and Perr. (bark), *Mitragyna stipulosa* (DC) O. Kuntze (bark) and *Lannea acida* A. Rich (stem bark) against ten strains of *E coli* 0157:H7. The results of this experiment revealed that the extracts displayed variable antibacterial activity, and some did not display inhibition at all. Minimum inhibitory concentrations of active extracts ranged from 1.65 mg/ml to 50 mg/ml, with the ethanol extract of *E. africana* displaying the most activity and inhibiting the growth of all ten strains (Aboaba et al. 2006). Another study, carried out by Doughari et al. (2007) investigated activity of organic and aqueous leaf extracts of *Balanites aegyptiaca* L. Drel. and *Moringa oleifera* Lam. against *Salmonella typhi*. It was found that the ethanol extracts of both plants were more active compared to extracts prepared with the other solvents, and that *B. aegyptiaca* was more active against the bacteria, with an MIC of 6.5 mg/ml while *M. oleifera* had an MIC of 8 mg/ml. While these plants mentioned above undoubtedly display activity, it is interesting to note the greater activity displayed by *P. zombamontana* during this study.

The antimicrobial activity exhibited by *P. zombamontana* is due to active compounds or groups of compounds within the extract. With *Psychotria* being such a large genus, it is difficult to assume what compounds are specifically responsible for the activity observed during this study. That said, previous studies involving isolation of active compounds from this genus may aid in assuming which class of compound is most likely responsible. For example, Liu et al. (2016) isolated an indole alkaloid from *Psychotria pilifera* Hutch., namely 16,17,19,20–tetrahydro–2,16–dehydro–18–deoxyisostrychnine. This alkaloid was found to be extremely active against *E. coli*, displaying an MIC of 0.781 µg/ml. The acetone fruit extract of *Psychotria nilgiriensis* Deb & M.G.Gangop. was observed by Devadoss et al. (2013) to have activity against *E. coli* using a disk diffusion assay. Phytochemical screening reported a high concentration of alkaloids in the fruit extract. It was also reported by Hadi and Bremner (2001) that alkaloids present in *Psychotria malayana* Jack were antimicrobial in nature. While it cannot be said for certain which compound is responsible for the activity of *P. zombamontana*, it is possible given the above information, that the antimicrobial activity of the extract is due to the presence of alkaloids.

3.4 Conclusion

This study signifies the first report of antifungal and antibacterial activity of extracts from *P. zombamontana* using the microdilution method. Results reported in this chapter display the promising potential of *P. zombamontana* as an antimicrobial agent. This information therefore increases the feasibility of *P. zombamontana* having potential for development as an antimicrobial additive for poultry feed. Although the inhibition shown by *P. zombamontana* was observed to be relatively strong against all microbes tested, it is possible that inhibition may be stronger if the extract was fractionated, or if antimicrobial compounds were isolated.

4. *Psychotria zombamontana* as a poultry feed additive: *In vitro* safety and further possible beneficial effects

4.1 Introduction

Psychotria zombamontana (Kuntze) E.M.A. Petit belongs to a family recognised for having rich biological activity, the Rubiaceae (Matsuura et al. 2013). Previously in this study, leaf extracts of this plant were investigated for their efficacy as a control agent for problematic fungi and bacteria related to poultry and their feed. The high levels of bioactivity encouraged further study into additional potential beneficial effects of the plant material on the health of the poultry while simultaneously controlling feed contamination. In other words, the presence of *P. zombamontana* in poultry feed may provide benefits beyond merely antimicrobial activity. For example, the plant may possess antioxidant and possibly anti-inflammatory activities, both of which would have positive effects on the health of poultry.

Antioxidant activity is an important factor to consider as it may prevent or reduce oxidative stress in poultry. This process has been linked to a mechanism of biological damage in animals, and been reported to affect poultry growth as the cause of several pathologies including exudative diathesis (Dam and Glavind 1938; Goldstein and Scott 1956; Noguchi et al. 1973; Van and Ferrans 1976). The addition of this plant material to poultry feed could therefore play a part in disease prevention in birds as well as increased longevity for layers (Padayatty et al. 2003).

Oxidative rancidity (OR) may also occur in poultry meat, and this is known to be one of the main causes of deterioration, which in turn may cause a decrease in nutritional value, flavour, texture and appearance in the meat, as well as causing foul odours (Fellenberg and Speisky 2006; Valenzuela and Nieto 1996). Valenzuela and Nieto (1996) stated that supplementation with antioxidant substances in the diet may function to prevent or delay OR of animal meat.

It is very possible that *P. zombamontana* possesses such antioxidant activity as previous studies have illuminated these effects in many other plant species including those within the *Psychotria* genus. Fragoso et al. (2008) evaluated the antioxidant activity of the methanolic crude leaf extract of *Psychotria umbellata* Vell. as well as a monoterpene indole alkaloid, psychollatine, produced in the leaves of the species. In this study the extract and psychollatine were found to display both antioxidant activity and antimutagenic activity, with the extract displaying notable

OH- scavenging activity, even more so than psychollatine. This may be due to the high amount of flavonoids present within the leaves, as these substances can increase antioxidant properties of extracts (Rice-Evans et al. 1996; Fragoso et al. 2008).

Anti-inflammatory activity has been widely reported in many plant-derived compounds (Calixto et al. 2004). Several *Psychotria* species have been reported to possess this activity. For example, *Psychotria octosulcata* Talbot was found to display anti-inflammatory properties during chronic and acute *in vivo* assays by Mariyammal and Kavimani (2013). The authors found that methanolic leaf extracts of *P. octosulcata* reduced anti-inflammatory response during the paw oedema method (acute) and the cotton pellet granuloma pouch method (chronic) in albino rats. There have also been reports of analgesic and antihyperalgesic activity displayed by species such as *Psychotria colorata* (Willd. ex R. & S.) Muell. Arg. and *Psychotria sarmentosa* Blume (Elisabetsky et al. 1995; Ratnasooriya and Dharmasiri 2012). Inflammation is a complex process, usually involving various mediators, such as cytokines (derived from macrophage cells) (Yeşilada et al. 1997). It is therefore necessary to perform more than one test to determine the presence and scale of activity.

Cytokine modulation is another activity that some extracts may possess, and induction and inhibition of certain cytokines may have specific effects on the immune system and inflammatory response. These cytokines represent groups of specific substances (most of which are multifunctional in nature) that play a part in a large proportion of the inflammatory response (Calixto et al. 2004). Cytokines can usually be divided into pro-inflammatory and anti-inflammatory classes, with the pro-inflammatory cytokines usually responsible for initiating or amplifying inflammation, while anti-inflammatory cytokines work to antagonise these effects (Dinarello 2000; Opal and Depallo 2000). It has been found that modulation of these cytokines may be affected by certain inflammatory diseases, for example sepsis. These diseases cause an imbalance in the cytokine network, as well as excessive recruitment of leukocytes to the sites of inflammation (Calixto et al. 2004; Proudfoot et al. 2003). Hence, plant extracts with the ability to modulate cytokine production are desirable as they may function to counteract the imbalances caused by inflammatory diseases, benefiting the affected organism.

Before the plant may benefit poultry in any way one must be sure that it is safe for ingestion, and one preliminary way to do this is by observing whether the plant has cytotoxic activity. Though plants are seen as a natural alternative in the eye of the public and therefore believed to be safer, there are plants known to display strong cytotoxic activity, immediately deeming

them unsafe for consumption. Several species from *Psychotria* have been reported to be cytotoxic, though this activity was evaluated mainly on cancer cells such as rat hepatoma cell lines and lymphocytic leukaemia cells (Roth et al. 1986; Lee et al. 1988). These species include *P. forsteriana* and *P. serpens*, which were both found to display cytotoxicity. In this case it is advantageous as cytotoxicity towards the specific cancer cell lines tested indicates that these plants have anticancer activity (Roth et al. 1986; Lee et al. 1988). Although the examples given previously represent a beneficial form of cytotoxicity, the presence of this activity is not always desired. This is because cytotoxicity, or more specifically, basal cytotoxicity represents the adverse effects which have resulted via impairment of cell functions common to all cells in an organism (Ekwall 1995). Cellular toxicity readings are useful as a preliminary guideline since basal cell functions usually support organ-specific cell function (Castano and Gómez-Lechón 2005). That said, these *in vitro* studies may not fully encompass the scope of toxicity of a specific substance. *In vivo* testing is therefore required in the target organisms to obtain a true representation of the toxicity of a substance.

The aim of this chapter was to investigate the antioxidant, anti-inflammatory activity of *P. zombamontana* as well as to define the *in vitro* toxicity of the crude extract.

4.2 Materials and Methods

4.2.1 Plant collection and preparation of extracts

Collection of material from the plants was carried out at the Manie van der Schijff Botanical Garden at the University of Pretoria, Pretoria, South Africa in April (summer) 2017. Preparation of extracts was carried out using acetone, in the same manner described in the previous chapter (section 3.2.2).

4.2.2 Antioxidant assays

4.2.2.1 DPPH assay

Antioxidant activity of acetone leaf extracts of *P. zombamontana* was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Brand-Williams et al. (1995) with modification to a 96-well microplate format proposed by Ahmed et al. (2014). A stock solution of plant extract was prepared by adding DMSO to dried plant extract up to a concentration of 10 mg/ml. During this assay, 100 µl of sterile distilled water was pipetted into 96-well

microplates, and 100 µl plant extract dissolved in methanol was pipetted into the first column and then serially diluted. Finally, 100 µl of DPPH was added to each well of the microplates and the plates were incubated at room temperature for 30 min in the dark. After incubation absorbance was read at 517 nm using an Epoch™ microplate spectrophotometer (Biotek U.S.A). Ascorbic acid (vitamin C) and Trolox (a vitamin E analogue) were used as positive controls during this experiment. The entire assay was repeated twice, using replicates of three.

4.2.2.2 ABTS assay

The free radical decolourization assay (Re et al., 1999; Ahmed et al., 2014) with modification to a 96-well microplate format was carried out as an additional test for antioxidant activity, using 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). For this assay a stock solution of plant extract was prepared by adding DMSO to dried plant extract up to a concentration of 10 mg/ml. This assay was carried out by adding 100 µl of sterile distilled water into each well of the microplates, 100 µl of plant extract was then added to the first column and serially diluted. The same was done for the positive controls, ascorbic acid and Trolox. Thereafter, 100 µl of ABTS was added and the mixture was allowed to react for 6 min, after which absorbance was read at a wavelength of 735 nm using an Epoch™ microplate spectrophotometer (Biotek U.S.A). The entire assay was repeated twice, using replicates of three.

4.2.2.3 Phenolic content

Phenolic content of the extract was measured to confirm any antioxidant activity present, as high phenolic content seems to correlate with high antioxidant activity (Iniyavan et al. 2012). This was done using the Folin-Ciocalteu method, similar to that described by Makkar (2003) but with slight modifications to incorporate a 96-well microplate format. Gallic acid was used as the standard to determine total phenolic content. Stock solution of the plant extract was prepared using 50% methanol up to a concentration of 2 mg/ml. During this assay 50 µl of 50% methanol plant extract was transferred first to five test tubes, one test tube per replicate. Next, 950 µl of sterile distilled water was added to each of the tubes followed by 500 µl of 1N Folin-Ciocalteu reagent and then 2.5 ml of 2% sodium carbonate (Na_2CO_3); this was done in the absence of light. Following this 200 µl was taken from each replicate and transferred to a microplate, using five wells per replicate. That is, replicate one was contained in column 1, cells A to E, replicate two was contained in column 2, cells A to E and so on. A blank was prepared in the same manner as described for the extract, but with 50 µl of 50% methanol in

place of the extract. Different concentrations of gallic acid were prepared between 0 mg/ml and 150 mg/ml. The microplate was then incubated at room temperature for 40 min in the dark and then immediately taken to an Epoch™ microplate spectrophotometer (Biotek U.S.A) where absorbance was read at 750 nm. Using the readings obtained from the samples in conjunction with absorbance readings for the different gallic acid concentrations, total phenolics were calculated and expressed as gallic acid equivalents per gram dry weight (GAE/gDW).

4.2.3 Anti-inflammatory assays

4.2.3.1 Lipoxygenase inhibition assay

The lipoxygenase inhibitory activity was measured in a similar manner to that described by Lyckander and Malterud (1992). The assay was carried out in a borate buffer (0.2 M, pH 9.00) and measured at 234 nm after addition of 15-lipoxygenase (15-LOX), using linoleic acid (134 µM) as a substrate. The final enzyme concentration was 167 U/ml. Test samples were prepared as 10 mg/ml solutions using dimethyl sulfoxide (DMSO) (final DMSO concentration of 1.6%) and quercetin served as the positive control. The enzyme solution was stored on ice. Controls were measured at intervals throughout the experimental period to ensure that the enzyme activity was constant. The IC_{50} values were determined using linear regression and substituting values into the graph equation. The absorbance at 234 nm was measured using an Epoch™ microplate spectrophotometer (Biotek U.S.A).

4.2.3.2 Cytokine analysis

For this assay a kit obtained from BD Biosciences (U.S.A) was used. Prior to the assay, stock solutions of 50 mg/ml of extract was prepared using acetone. In this assay human leukaemia monocytic cell line THP-1 was cultured in RPMI-1640 medium supplemented with 10% FCS and 1% PSF. The cell suspension (supplemented with 0.10 µg/ml phorbol 12-myristate 13-acetate) was prepared and seeded into 96-well microplates, at a density of 2×10^5 cells/ml and then incubated for 72 h at 37 °C in a 5% CO_2. Media was then removed and the cells were washed with PBS, then lipopolysaccharide (LPS, 100 µl of 2 µg/ml solution) which had been prepared in complete medium was added to the wells to induce an inflammatory response. After one hour the cells were then treated with 100 µl of extract and positive control (quercetin) at a concentration of 50 µg/ml, and the cells were then incubated for 48 h under the prementioned conditions. Thereafter, the supernatant was removed from the wells and stored at -70°C. The amount and type of cytokines induced were then analysed using a cytometric

bead array kit, namely the BD™ CBA Human Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA, U.S.A, 2018) and a BD Accuri™ C6 Plus flow cytometer with C6 software (BD Biosciences, San Jose, CA, USA, 2018). There are several different types of cytokines; those detected by the prementioned kit include interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12p70 (IL-12p70) and Tumour Necrosis Factor (TNF-α). Some of these cytokines have different roles acting as pro-inflammatory agents and some acting as anti-inflammatory agents, with some even showing dual functions. Therefore, the use of this assay may help to give an idea on the cytokine modulating effects of a sample and whether those effects are beneficial or not.

4.2.4 Cytotoxicity assays

The tetrazolium-based colorimetric (MTT) assay was carried out similarly to that described by Mosmann (1983) and modified by McGaw et al. (2007), where DMSO is used to dissolve the formazan crystals instead of acid-isopropanol. This assay performed to evaluate the cytotoxicity of the crude plant extracts against Vero African green monkey kidney cells. Cells were maintained in minimal essential medium (MEM) supplemented with 0.1% gentamicin and 5% foetal calf serum.

Confluent monolayer cultures were used to prepare cell suspensions which were plated at predetermined cell densities of $1x10^5$ cells/ml into each well of a 96-well microplate, excluding columns 1 and 12 which only contained MEM. Plates were incubated for 24 h at 37°C in a 5% CO_2 incubator, so that the cells could attach to the wells. Stock solutions of extract were made using acetone, and dilutions were prepared in MEM. Doxorubicin was used as the positive control. The MEM was removed from the prepared plates and replaced with 200 µl of the extract at differing concentrations in triplicate. Following this, the plates were incubated at 37°C in a 5% CO_2 incubator for a period of 48 h, and the wells were then rinsed using 200 µl phosphate buffered saline (PBS) and 100 µl of fresh medium was then dispensed into the wells. MTT dissolved in PBS (30 µl of a 5 mg/ml solution) was added to each well and incubated for 4 h at 37°C. The medium was then removed and the MTT formazan crystals were dissolved in 50 µl DMSO. Absorbance was measured immediately on a ChroMate 4300 microplate reader (Awareness Technology, Inc. U.S.A) at a wavelength of 540 nm and 630 nm. The LC_{50} values were obtained by calculating the concentration of extract that resulted in a 50% reduction in absorbance compared to untreated cells.

4.3 Results and Discussion

4.3.1 Antioxidant assays

The results of the antioxidant assays showed that *P. zombamontana* displayed impressive activity in both assays. A guideline proposed by Efferth and Kuete (2010) considers samples with IC_{50} values lower than 50 µg/ml to have high antioxidant capacity. The crude extract displayed values well below that threshold during both the ABTS and DPPH assays (Table 4.1).

Table 4.1 IC_{50} values of crude leaf extracts of *Psychotria zombamontana* in the DPPH and ABTS assays.

Sample	DPPH		ABTS	
	IC_{50} (µg/ml)	**SD**	**IC_{50} (µg/ml)**	**SD**
P. zombamontana	3.387	0.138	1.616	0.087
Ascorbic acid	2.680	0.215	0.842	0.257
Trolox	0.679	0.257	3.618	0.632

In comparison to other studies on *Psychotria* species, this activity is definitely notable. Formagio et al. (2014) performed a study in which extracts of *Psychotria carthagenensis* Jacq., *Psychotria leiocarpa* Cham. & Schltdl., *Psychotria capillacea* (Müll.Arg.) Standl. and *Psychotria deflexa* DC. were tested for antioxidant activity using the DPPH and ABTS assays. The results of this study showed that *P. carthagenensis* and *P. capillacea* displayed the best activity, with IC_{50} values of 16.92 ± 4.58 µg/ml and 30.05 ± 6.22 µg/ml, respectively, during the DPPH assay, and 92.5% ± 7.43 and 87.34% ± 8.32 free radical scavenging ability, respectively, during the ABTS assay (Formagio et al. 2014). While the free radical scavenging ability displayed by both species was quite high, the IC_{50} displayed by *P. zombamontana* during the DPPH assay in this study is still much higher in comparison. Iniyavan et al. (2012) evaluated the antioxidant activity of the fruit, leaves and stem of *Psychotria nilgiriensis* using several different extractants. The study found that the acetone extract showed the best activity in the DPPH assay, with the fruit, stem and leaf extract displaying IC_{50} values of 20 µg/ml, 30.9 µg/ml and 103.3 µg/ml, respectively. While these values are still very promising, the

acetone leaf extract of *P. zombamontana* is undoubtedly much more potent with regard to antioxidant activity.

The total phenolic content of the crude acetone extract was observed to be 17.273 ± 0.957 mgGAE/gDW. Other species within the *Psychotria* genus have been reported to possess much higher values. For example, *Psychotria capillacea* (Müll.Arg.) Standl. was reported to have a phenolic content of 148.42 ± 4.69 mgGAE/gDW by Formagio et al. (2014). The same study also investigated other species of *Psychotria* and the lowest value was observed in *Psychotria carthagenensis* Jacq. which still had 40 mgGEA/gDW. Even though the value displayed by *P. zombamontana* is not as high as those previously observed in other *Psychotria* spp. the value obtained is still substantial and so correlates with the antioxidant activity displayed in the DPPH and ABTS assays. It may be said therefore that the high phenolic content contributes to the high antioxidant activity possessed by the crude extract of *P. zombamontana*. The presence of high antioxidant activity in the crude extract is promising, as this beneficial activity could be useful in poultry feed if leaf material from *P. zombamontana* were added, by protecting the feed against the effects of oxidants. As mentioned previously, antioxidant activity could most importantly be beneficial to both the health of the birds and the quality of the poultry meat produced (Padayatty 2003; Valenzuela and Nieto 1996).

4.3.2 Anti-inflammatory assays

Results from the LOX assay carried out suggest that the crude extract of *P. zombamontana* does have activity against this inflammation-related enzyme. The extract displayed an IC_{50} value of 5.57 µg/ml, which is promising considering the value for the positive control, quercetin, which was 12.09 µg/ml. *Psychotria zombamontana* appears to display anti-inflammatory activity at a very low concentration. This is beneficial, not only due to the usual effects of anti-inflammatory agents, but because this may increase the chance of *P. zombamontana* acting as a suitable alternative to antibiotic growth promoters (AGPs). Niewold (2007) suggested that the true mechanism of AGPs may be via anti-inflammatory action. It was stated that catabolic mediators produced by inflammatory cells in the intestine may have a negative effect on beneficial microflora. Hence inhibition of the production and excretion of catabolic mediators may be responsible for the growth promoting effects of AGPs (Niewold 2007). If this is the case, then the use of *P. zombamontana* in the feed may be very advantageous, acting as a growth promoter without risking the development of antibiotic resistance.

The crude extract of *P. zombamontana* was observed to have an effect on cytokine production (Table 4.3).

Table 4.2 Induction or inhibition of cytokines from macrophages by the crude acetone leaf extract of *P. zombamontana.*

Cytokine	Cell only (pg/ml)	Cell + LPS (pg/ml)	Cell + 50 µg/ml *P. zombamontana* (pg/ml)	Quercetin (pg/ml)
Interleukin 12p40	0.00	0.18	1.01	0.25
Tumour necrosis factor	36.91	4 892.50	14 694.14	24 698.15
Interleukin 10	0.14	2.82	1.97	1.91
Interleukin 6	0.00	603.43	1 950.58	2 903.06
Interleukin 1 beta	20.04	290.20	518.01	1 369.51
Interleukin 8	30 414.56	16 140.67	6 209.86	6 297.89

The presence of *P. zombamontana* seemed to induce the production of interleukin 6 (IL-6), interleukin 1 beta (IL-1β), and the tumour necrosis factor (TNF), to a much lesser extent than quercetin; all three of these cytokines are pro-inflammatory cytokines (Wang et al. 1999; Fassbender et al. 1998). Interleukin 12p40 (IL-12p40) was also induced by *P. zombamontana*, to a slightly greater extent compared to quercetin; IL-12p40 is also known as a pro-inflammatory cytokine (Fassbender et al. 1998). Additionally, the presence of *P. zombamontana* inhibited the production of interleukin 8 (IL-8), a cytokine responsible for inducing chemotaxis of defence cells toward the site of infection (Luster 1998). This effect is actually advantageous, due to the influence inflammatory diseases have on excessive leukocyte recruitment; antagonists of IL-8 are seen to be anti-inflammatory agents (Proudfoot et al. 2003). The presence of *P. zombamontana* also induced the production of interleukin-10 (IL-10) an anti-inflammatory cytokine which functions in limiting and terminating inflammatory response (Moore et al. 2001). Although some of the effects caused by the extract in this assay may not seem ideal, due to the complexity of the inflammatory pathway it is difficult to truly state the implication of these effects regarding whether or not they are truly beneficial.

Other *Psychotria* species have been tested for anti-inflammatory activity. A study done by Saha et al. (2004) tested the inhibitory activity of leaf and stem methanol extracts of *Psychotria rostrata* Blume on nitric oxide (NO) production in cells induced with LPS and IFN-γ. In this study, leaf and stem methanol extracts of *P. rostrata* did reduce production of NO, but this was due to the cytotoxic effect of the plant extract. There is not much information available on the activity of other *Psychotria* species.

From previous studies, another species of *Psychotria* which has been observed to effect cytokine modulation is *Psychotria acuminata* Benth. Glinski et al. (1995) reported that a specific green pigment originating from *P. acuminata*, pheophorbide *a*, caused an inhibition in the binding of tumour necrosis factor-alpha and IL-8 to cell surface receptors. Strangely enough this activity was dependent on the simultaneous presence of light, a common phenomenon in pheophorbides according to Glinski et al. (1995).

Other species within the Rubiaceae family have also been reported to modulate cytokine production. For instance, *Oldenlandia affinis* (Roem. & Schult.) DC. was found to significantly inhibit the production of TNF-α. Nworu et al. (2017) found that treating macrophages (induced with LPS) with 5-100 µg/ml of a cyclotide-rich fraction obtained from *O. affinis* significantly reduced production of TNF- α. Additionally, the same fraction was reported to display an IC_{50} of 49.53 µg/ml in an inducible NO assay, confirming the anti-inflammatory activity of the fraction (Nworu et al. 2017). Another Rubiaceae species, *Ixora coccinea* L., was reported by Upadhyay et al. (2013) to decrease production of certain pro-inflammatory cytokines. In this study, methanol leaf extracts of *I. coccinea* were observed to have an inhibitory effect on the production of TNF-α, IL-6 and IL-1β in macrophage cells treated with LPS. The methanol extracts also reduced NO production, and acted by inhibiting cyclooxygenase 2 (COX-2) mediated prostaglandin E2 (PGE2) production in macrophage cells induced with LPS (Upadhyay et al. 2013).

Even well-known herbs have been reported to display activity with regard to cytokine production. A study done by Mueller et al. (2010) reported the benefits of diets rich in herbs, as a result of their findings. During their investigation, DMSO extracts of several common culinary herbs, including thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.), sage (*Salvia officinalis* L.), basil (*Ocimum basilicum* L.), ginger (*Zingiber officinale* Roscoe) and marjoram (*Origanum majorana* L.) were tested for their effect on cytokine production. Though some of these extracts did not show any significant effect, sage was found to improve the anti-

inflammatory profile of the cytokines. In addition, some of the other plant products they tested, for example nutmeg, was a potent inhibitor of TNF-α, IL-6 and IL-10 production (Mueller et al. 2010).

In another study, *Phyllanthus amarus* Schumach. & Thonn. was investigated for anti-inflammatory activity by Kiemer et al. (2003). In this study, hexane and ethanol/water extracts of *P. amarus* were used to investigate the effect of the plant on several cytokines including TNF, IL-1β and IL-10, as well as its effect on NO induction. It was found that both extracts inhibited the production of TNF, IL-1β and IL-10, and inhibited the expression of NO. This activity is quite promising, and demonstrates an example of the spectrum of activity that would have been extremely beneficial in this study. Perhaps isolation of individual active compounds may improve the effect they have on cytokine production, in that antagonistic compounds may be separated and removed, reducing negative or enhancing positive effects.

4.3.3 Cytotoxicity assays

The results of the cytotoxicity screening for the crude extract showed that the crude acetone extract of *P. zombamontana* had some toxicity, but according to Efferth and Kuete (2010) a crude extract is only considered to have *in vitro* cytotoxicity if the LC_{50} after incubation for 48 h is less than 20 µg/ml (Table 4.3). However, based on the value obtained for the cytotoxicity assay, the selectivity index (SI) of the crude extract is 0.0966, which is much less than 1.0, indicating that the cytotoxicity effect was greater than the antimicrobial activity. Though the extract itself could be deemed non-cytotoxic according to the proposed threshold, using it in concentrations which cause inhibition of microbes may lead to negative effects on the poultry.

Table 4.3 Cytotoxicity (LC_{50} value) of the crude acetone leaf extract of *P. zombamontana* against Vero monkey kidney cells.

Sample	LC_{50} (µg/ml)	SD
P. zombamontana	43.4500	0.0084
Doxorubicin	5.4200	0.0064

Although the crude extract had some toxic effects on the cells and displayed a low SI, it may be possible for the leaf meal to be added in small doses, enough to convey benefits on the health of the birds without causing toxicity. Another point to note is that once metabolised by the birds, the material may not be toxic at all, but on the other hand, toxicity may increase. A true

representation of the effect that adding leaf material of *P. zombamontana* into the feed would have on the birds can only be observed via additional studies *in vivo*.

Another solution to lower toxicity would be to isolate the active fractions or even compounds present within the extract as mixtures are more likely to contain toxicity than individual constituents (Cowan 1999). These may still possess beneficial activity, without causing toxicity, as in some cases the compounds responsible for toxicity may be removed during the process of isolation.

4.4 Conclusion

The crude extract of *P. zombamontana* was observed to have strong antioxidant activity and anti-inflammatory activity and relatively low cytotoxicity. This plant is therefore a promising option as an alternative feed additive, with the potential to reduce problematic microorganisms within the feed while protecting the birds from oxidative damage as well as potentially enhancing the immune response via cytokine modulation. With that said, future work must be carried out to investigate the effects of this plant *in vivo*, if use of the crude plant material is to be investigated further.

5. Isolation and identification of active components from *Psychotria zombamontana*

5.1 Introduction

Plants are known to possess bioactivity of a wide variety, from antioxidant activity to antimicrobial activity and almost everything in between (Kasote et al. 2015, Nascimento et al. 2000; Wilson et al. 1997). This activity is due to the presence of active compounds within the respective plant species. When testing a plant for activity, the first step usually involves preparation of a crude extract, and testing that crude extract for the presence of specific activities. The crude extract in question contains many compounds, as any compound that is soluble in the selected solvent will have been extracted. This presence of abundant compounds can usually lead to interesting results, in that these compounds may work synergistically with one another, or some compounds may even antagonise the active compound (Freeman et al. 2010; Rates 2001, Schinor et al. 2007).

It is therefore desirable to isolate the active compounds in hopes of possibly increasing their specific activity. This process is also an important step in identification and characterisation of known and unknown compounds, which in turn is a critical step toward standardisation for product formulation. Another advantage of isolation is the possible removal of toxic compounds, as mixtures are more likely to contain toxic constituents (Cowan 1999). Occasionally, crude extracts can display high toxicity, rendering them unsuitable for use *in vivo*. This toxicity may be due to specific compound(s) within the extract, which may not be responsible for the beneficial activity of the extract. This way, compounds may be selectively isolated via bioassay-guided separation, effectively separating out the toxic compound/s while the beneficial compounds are isolated. It would then be possible to use the isolated compounds in further *in vivo* testing and later in product formulation.

Henceforth the next step, after investigating the activity of a crude extract, is to isolate and identify the active compounds responsible. Isolation is critical for determination of the structure and identity of the active compounds, and is important for future synthesis as well as comparative studies. Therefore, the purpose of this chapter was to attempt to isolate active constituents from the *Psychotria zombamontana* (Kuntze) E.M.A. Petit crude extract and investigate the relative activity and toxicity of these constituents.

5.2 Materials and Methods

5.2.1 Fractionation of extracts and isolation of active compounds

Isolation of active components was carried out using bioassay-guided fractionation whereafter each fractionation step, fractions were investigated for antimicrobial activity using microdilution assays. Similar active fractions identified using thin layer chromatography (TLC) were then combined and used for further fractionation.

5.2.2 Bulk extraction and solvent-solvent fractionation (SSF)

Plant material was collected as described previously (section 3.2.1). Bulk extraction was carried out using 160 g of ground, dried plant material. The dried material was placed in a large glass container, and acetone was added to a level where the material was sufficiently covered. The glass container was left for a period of 72 h, with the mixture being agitated periodically, after which the extract was dried using a rotary evaporator (rotavapor). SSF was used to separate the obtained extract into broad groups of chemical compounds based on their polarity. SSF was conducted in a separating funnel with non-polar solvents, and then more polar solvents were used. In this case SSF of the crude extract started by dissolving the extract in some hexane (only the soluble components of the extract dissolved in hexane) extract and components that did not dissolve were kept aside for further use. Next, the dissolved extract was added to the separating funnel with 2 L of hexane and 2 L water, the funnel was shaken. Once a clear separation had formed between the two solvents (Figure 5.1), the water was eluted and kept to one side and the hexane was eluted and taken to the rotavapor to dry. The water was poured back into the funnel and chloroform was added to the dried extract (extract which did not dissolve in chloroform was kept aside) and the dissolved extract was added to the funnel with 2 L of chloroform. The funnel was shaken and once a clear separation formed between the two solvents, the chloroform fraction was removed and dried using the rotavapor. This process was repeated for butanol, and finally water. The fractions obtained were tested for antifungal activity using the microdilution assay.

Figure 5.1 Fractionation of the *Psychotria zombamontana* extract with chloroform (bottom layer) and water (top layer).

5.2.3 Antifungal activity of fractions

Fractions were tested for antifungal activity using the microdilution assay as described in Section 3.2.3.2. The fractions were tested for activity against *F. verticillioides* and *C. albicans* as these were the best representatives of the two fungal morphologies used in this study. During this assay, concentrations of 10 mg/ml of each fraction were prepared using 10% DMSO, while the positive control used was amphotericin B and 10% DMSO was used as the negative control.

5.2.4 Column chromatography

Before starting with column chromatography, fractions obtained were analysed using TLC to determine if any could be pooled together due to similarities in compounds. The fractions were spotted on to TLC plates and developed using HEA (hexane:ethanol:ammonium hydroxide 90:9:1) and EMW (ethyl acetate:methanol:water 40:5.4:4.6) solvent systems.

Fractions which displayed promising antifungal activity against *F. verticillioides* and *C. albicans* were used in further fractionation. The fractions chosen were hexane and chloroform, these fractions were the most active and were pooled together due to their similarity (observed during TLC analysis). An open column was packed with 1 L of silica gel 60 and used to further

fractionate the 1.16 g of the pooled hexane and chloroform fractions previously obtained. Solvent systems were chosen based on the separation performance observed during TLC. These were used as the mobile phase during column chromatography for gradient elution of solvents with increasing polarity. The solvents used included hexane, dichloromethane (DCM) and methanol. Mixtures of the solvents were prepared for the isolation as follows, starting with non-polar (in volumes of 500 ml):

1. 100% hexane
2. 97:3 hexane:DCM
3. 95:5 hexane:DCM
4. 90:10 hexane:DCM
5. 85:15 hexane:DCM
6. 80:70 hexane:DCM
7. 70:60 hexane:DCM

↓

8. 100% DCM
9. 97:3 DCM:methanol etc..

Sub-fractions obtained from column chromatography were then analysed via bioautography.

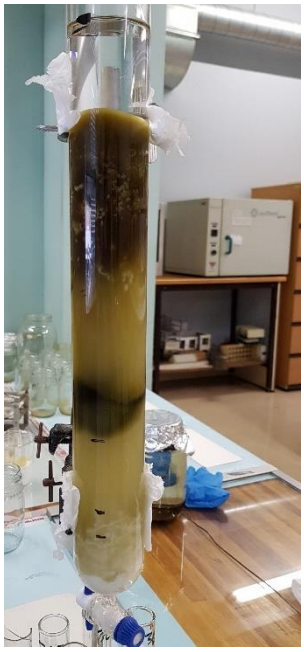


Figure 5.2 Silica gel chromatography of the pooled non-polar fractions obtained from SSF of the *P. zombamontana* acetone leaf extract.

5.2.5 Bioautography of sub-fractions

Antimicrobial activity of sub-fractions isolated from column chromatography was investigated using bioautography. Broth suspensions used during this method were prepared by culturing *F. verticillioides*, *C. albicans* and *E. coli* as described in Sections 3.2.3.1 and 3.2.4.1) and adjusting the optical density of those cultures to 0.5 McFarland standard ($1.5x10^8$ cfu/ml) using a spectrophotometer. In this method, sub-fractions were spotted onto TLC plates and eluted using suitable solvent systems. The solvent systems used consisted of hexane:ethanol:ammonium hydroxide (90:9:1) (HEA), chloroform:ethyl acetate:formic acid (5:4:1) (CEF) and ethyl acetate:methanol:water (40:5.4:4.6) (EMW). The HEA solvent system was used for sub-fractions which were non-polar in nature, while CEF was used for the moderately polar and EMW used for the very polar sub-fractions. Once the plates developed, they were sprayed with a suspension of fungal or bacterial broth. The TLC plate was then placed in a closed chamber to maintain humidity and incubated for 24 h at 28°C for *F. verticillioides* and 37°C for *E. coli* and *C. albicans*. TLC plates were then sprayed with a solution of INT (2 mg/ml) and incubated for 3 h, after which the plates were checked for the presence of inhibition zones (clear areas where microbial activity is not present to reduce INT to the red formazan product). Two fractions observed to display inhibition zones were investigated further.

5.2.6 Compound identification and structure elucidation

One-dimensional Nuclear Magnetic Resonance spectroscopy (1-D NMR) was carried out by Dr Mamoalosi Selepe (Department of Chemistry, University of Pretoria) using a 500 MHz apparatus (Bruker) to determine whether the isolated fractions were sufficiently pure for identification. Due to the results obtained from the 1-D NMR and the non-polar nature of the sub-fractions, both were investigated further using gas chromatography-mass spectrometry (GC-MS) in an attempt to identify the most abundant compounds present. GC-MS analysis was carried out by Dr Yvette Naude (Department of Chemistry, University of Pretoria) using a LECO Pegasus 4D GC-TOFMS (LECO Africa (Pty) Ltd., Kempton Park, South Africa) with a Rxi-5SilMS GC column (30 m x 0.25 mm ID x 0.2 µm film thickness) (Restek, Bellefonte, PA, USA). The following parameters were used:

- Injection volume 1 µl, splitless injection, splitless time set at 30s
- GC inlet 250°C
- GC oven temperature programme 40°C (hold for 3 min) at 10°C/min to 300°C (hold for 5 min).

- Carrier gas used was UHP Helium (Afrox, South Africa) at 1 ml/min set at constant flow mode
- Mass acquisition range 40-550 Da
- MS transfer line temperature 280°C
- Ion source temperature 230°C
- MS solvent delay 5 min
- Electron energy 70 eV in the electron ionisation mode (EI+)
- Data acquisition rate 10 spectra/ s
- Detector voltage 1750 V.

The resulting data was combined and interpreted, and only compounds corresponding to major peaks which showed high similarity (above 90%) were recorded.

5.2.7 Anti-inflammatory activity of sub-fractions

The sub-fractions with antimicrobial activity were also investigated for anti-inflammatory activity to detect whether the use of these would accrue any benefits beyond antimicrobial activity. Anti-inflammatory activity was tested using the same methods as described in Section 4.2.3). LOX and cytokine assays were carried out as previously described.

5.2.8 Cytotoxicity of active sub-fractions

Due to the toxicity observed for the crude extract of *P. zombamontana*, the active sub-fractions were also tested for cytotoxicity to determine whether the compounds responsible for the antimicrobial activity were also responsible for the toxicity or if the toxic compounds had been successfully separated out. The assay was carried out as described in Section 4.2.4) on Vero monkey kidney cells.

5.3 Results and Discussion

The bulk extraction of 160 g of dried material yielded 21.92 g of dried extract (13.7% yield), which was then used in SSF. The results of the microdilution assay using the fractions obtained from SSF showed that each of the four fractions possessed some measure of antifungal activity against both fungi tested. It is also worth noting that all fractions tested, save for the water

fraction, displayed higher activity against *F. verticillioides* than the crude extract, with the more non-polar fractions showing a higher level of activity (Table 5.1; Fig. 5.3). This, interestingly enough, correlates with preliminary antifungal tests on the crude extract of this plant, where no activity was observed when methods that favour polar active compounds were employed (Querl 2016). Diffusion methods were used previously (i.e. disc diffusion and agar well diffusion) and the results of these assays suggested that the extract of *P. zombamontana* possessed no antifungal activity. Janssen et al. (1987) stated that assays requiring the diffusion of the active compounds were more suited to polar active compounds, and not suitable for non-polar ones.

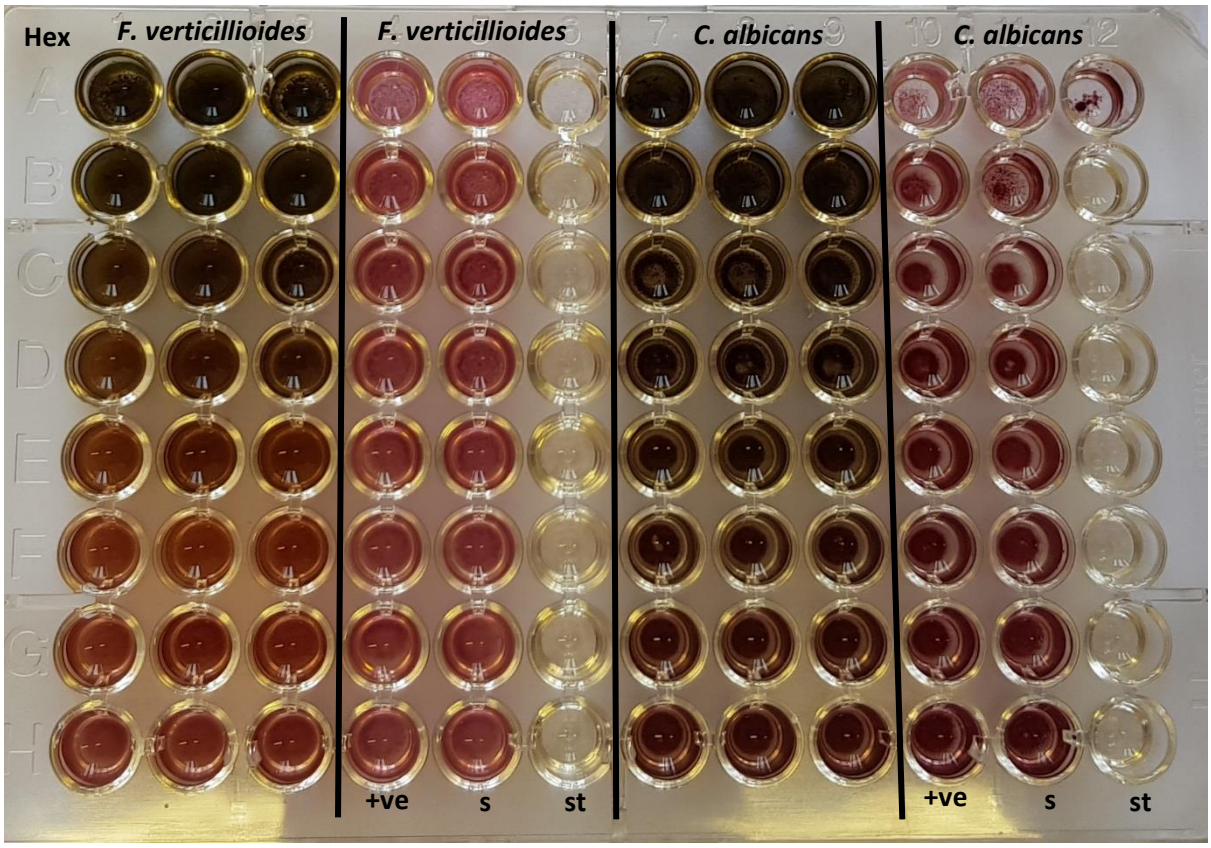


Figure 5.3 MIC assay of the hexane fraction against *F. verticillioides* and *C. albicans*.

Table 5.1 MIC values for each fraction against *F. verticillioides* and *C. albicans*.

	MIC (mg/ml)	
Fraction	***F. verticillioides***	***C. albicans***
Water	0.6250	0.6250
Butanol	0.3130	0.3130
Chloroform	0.1560	0.3130
Hexane	0.0781	0.3130
Amphotericin B	2.5000	0.0125

SD = 0

As stated previously, the non-polar fractions were the most active, and so these fractions were taken to the next step in the isolation process, namely column chromatography. Before moving to the column, thin layer chromatography (TLC) of the fractions was carried out to determine any similarities in compounds present. The results showed that the hexane and chloroform fractions were very similar (Figure 5.4), and hence the two fractions were pooled together for use in the silica column chromatography.

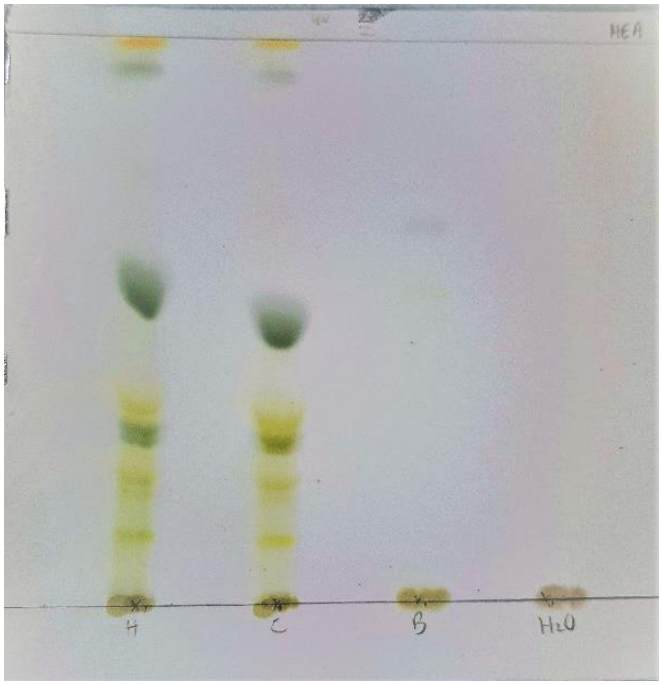


Figure 5.4 TLC of the fractions using the HEA solvent system, showing that the two non-polar fractions, hexane and chloroform, contain similar compounds. H: hexane; C: chloroform; B: butanol.

After separating the pooled non-polar fractions, using the silica gel column, 23 sub-fractions were obtained and tested against *F. verticillioides*, *C. albicans* and *E. coli* using the bioautography method. Of the 23 sub-fractions, two were active against both fungi tested, namely sub-fraction two (SF2) and sub-fraction seven (SF7) (Fig. 5.5-5.7). Both sub-fractions displayed activity against *F. verticillioides*, *C. albicans* and *E. coli*, as seen by the inhibition zones indicated in Figures 5.5-5.7 below. From the position of the active compounds in each bioautogram, it appears that the same compounds were active against all three of the test organisms, hence there was a lack of specificity of the antimicrobial activity.

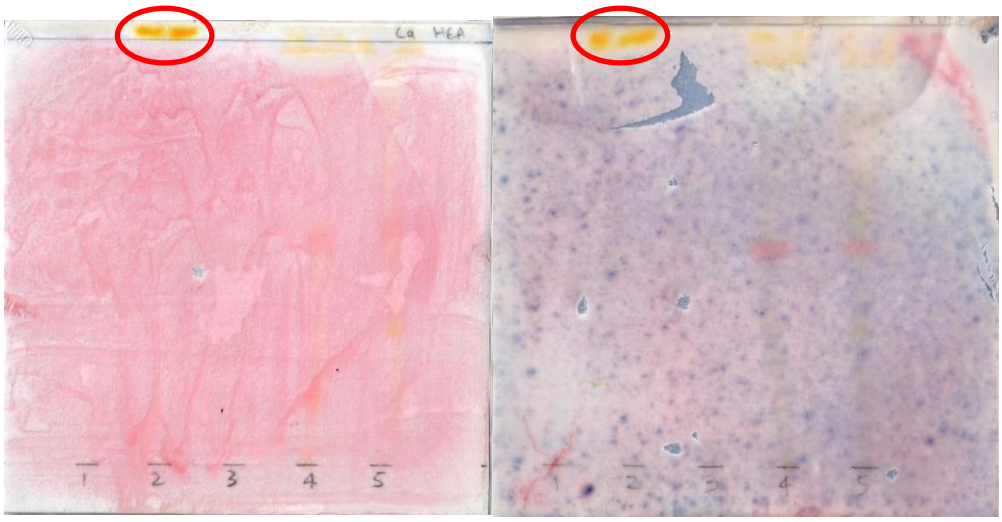


Figure 5.5 Bioautogram of sub-fractions 1-5, with *C. albicans* (left) and *F. verticillioides* (right). The clear zones marked represent inhibition zones and therefore the presence of an active compound.

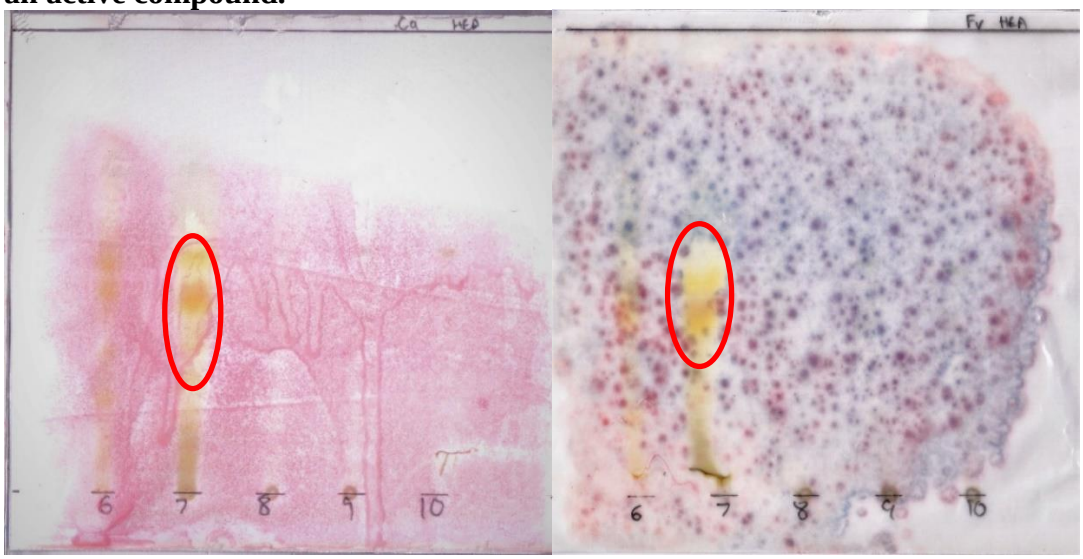


Figure 5.6 Bioautogram of sub-fractions 6-10, with *C. albicans* (left) and *F. verticillioides* (right). The clear zones marked represent inhibition zones and therefore the presence of an active compound.

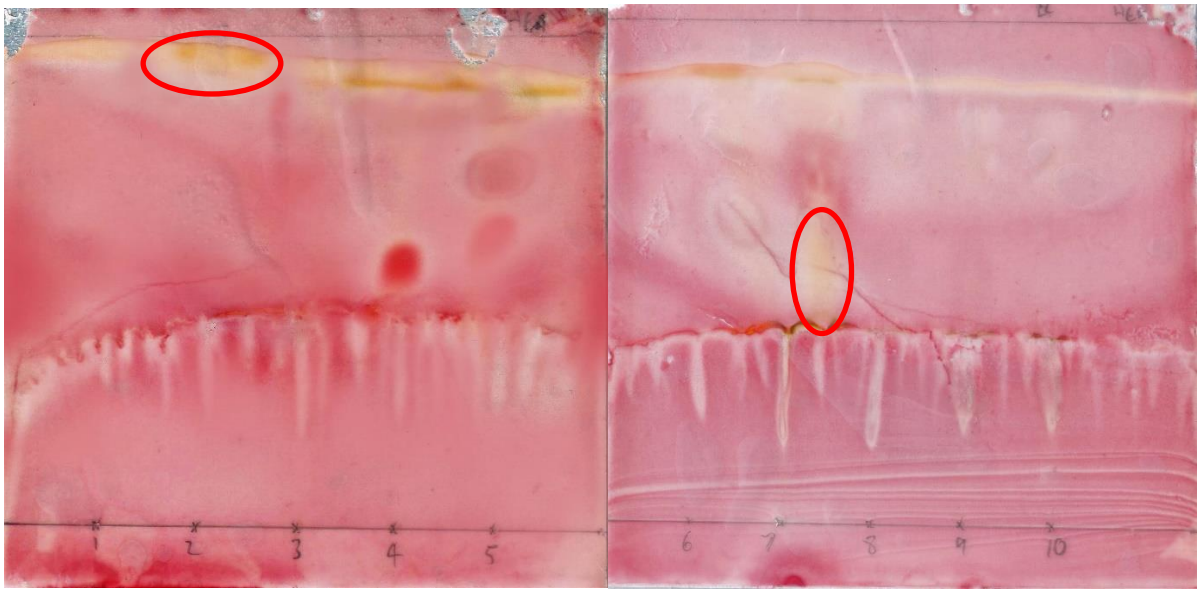


Figure 5.7 Bioautogram of sub-fractions 1-5 (left), and sub-fraction 6-10 (right) against *E. coli*. The clear zones marked represent inhibition zones and therefore the presence of an active compound.

Sub-fractions 2 and 7 were further investigated due to the activity displayed by both. Although it was desirable to further separate the two sub-fractions, difficulties were experienced with regard to the low mass of the sub-fractions obtained, 193.2 mg for SF-2 and 60 mg for SF-7. Additionally, finding suitable solvent systems for separation of these sub-fractions proved to be very difficult after several attempts using TLC and various eluents. This resulted in the decision to assay the sub-fractions using additional bioassays to investigate their cytotoxicity and anti-inflammatory activity. The sub-fractions were then analysed using methods capable of detecting several compounds in a sample (GC-MS) to identify the mixture of compounds present.

With regard to the anti-inflammatory assays, the results obtained for the LOX assay showed that SF-7 possessed good anti-inflammatory activity, similar to the crude extract with an IC_{50} value of 16.59 µg/ml, but SF-2 did not seem to have any effect. The positive control, quercetin, had an IC_{50} value of 12.09 µg/ml. The value obtained for SF-7 is very promising, and indicates that the active compounds present within the fraction are both antimicrobial and anti-inflammatory. It was initially anticipated that if anti-inflammatory activity were present in either fraction, it would be higher, due to the removal of potentially antagonistic compounds. It is not so in this case, as the activity for the sub-fraction was actually slightly lower, meaning

that the compounds in the crude extract are possibly working synergistically to produce higher activity.

Cytokine analysis showed that both sub-fractions affected cytokine production (Table 5.2). Both sub-fractions caused an increase in IL-12p40, and SF-2 caused an increase in IL-10, to a greater extent that that observed for quercetin. Both IL-12p40 and IL-10 are anti-inflammatory cytokines. Both sub-fractions also caused increases in TNF, IL-6 and IL-1β. While these are usually identified as proinflammatory cytokines, their induction may not necessarily be problematic. This is because, as stated previously, the inflammatory response is highly complex, with mediators usually playing more than one role in the process. These assays were carried out to investigate the effect of the sub-fractions on cytokine production. More investigation on these effects is required, both *in vitro* and *in vivo* to truly determine their specific implications.

Table 5.2 Induction or inhibition of cytokines released by macrophages following exposure to the crude acetone extract of *P. zombamontana.*

Cytokine	Cell only (pg/ml)	Cell + LPS (pg/ml)	Cell + 100 mg/ml SF-2 (pg/ml)	Cell + 100 mg/ml SF-7 (pg/ml)	Quercetin (pg/ml)
Interleukin 12p40	0.00	0.18	0.76	0.95	0.25
Tumour necrosis factor	36.91	4 892.50	19 749.73	11 808.79	24 698.15
Interleukin 10	0.14	2.82	3.12	1.28	1.91
Interleukin 6	0.00	603.43	3 064.41	953.53	2 903.06
Interleukin 1 beta	20.04	290.20	585.74	295.08	1 369.51
Interleukin 8	30 414.56	16 140.67	6 658.51	8 111.56	6 297.89

Cytotoxicity assays were carried out to determine the safety of the sub-fractions *in vitro*, in the hope that the toxicity displayed by SF-2 and SF-7 would be lower than that observed for the crude extract (Table 5.4). The results of the assay suggest that both sub-fractions display very low toxicities, much lower than the thresholds discussed in the previous chapter. It is therefore possible to state that neither sub-fraction has *in vitro* cytotoxicity. This outcome is very beneficial, as the low cytotoxicity shown by both fractions encourages further investigation into their use as a natural product. The selectivity index (SI) was not determined for the sub-

fractions, this was due to the low yield obtained for both. Bioautography was therefore chosen as the sole method for antimicrobial assays, resulting in a lack of MIC values.

Table 5.3 LC_{50} values observed for SF-2 and SF-7 during cytotoxicity assays.

Sample	LC_{50} (mg/ml)	SD
Sub-fraction 2	96.0220	12.4510
Sub-fraction 7	85.1310	0.1220
Doxorubicin	0.0054	0.0064

In an attempt to identify the compounds present in both sub-fractions, one-dimensional NMR was first carried out to determine which method of compound analysis would be most suitable. Results of this revealed that the sub-fractions were very non-polar and that GC-MS would be a promising option for compound identification.

GC-MS was carried out to determine the chemical composition of both SF-2 and SF-7, and results of the experiment were interpreted using a library in which peaks are compared to known compounds and their percentage similarity as well as percentage area (abundance) are displayed. Both sub-fractions contained quite a number of compounds, hence only the compounds with both the highest percentage area as well as percentage similarity were recorded. Most of the resulting identified compounds were very non-polar, correlating with previous results and providing an explanation for the poor separation during TLC (Table 5.4 and Table 5.5). The graphs obtained from the GC-MS displayed some peaks that were almost superimposed onto one another, indicating the difficulty in separating those specific compounds (Figure 5.8 & 5.9). This finding correlates with the difficulty experienced in separation during TLC analysis.

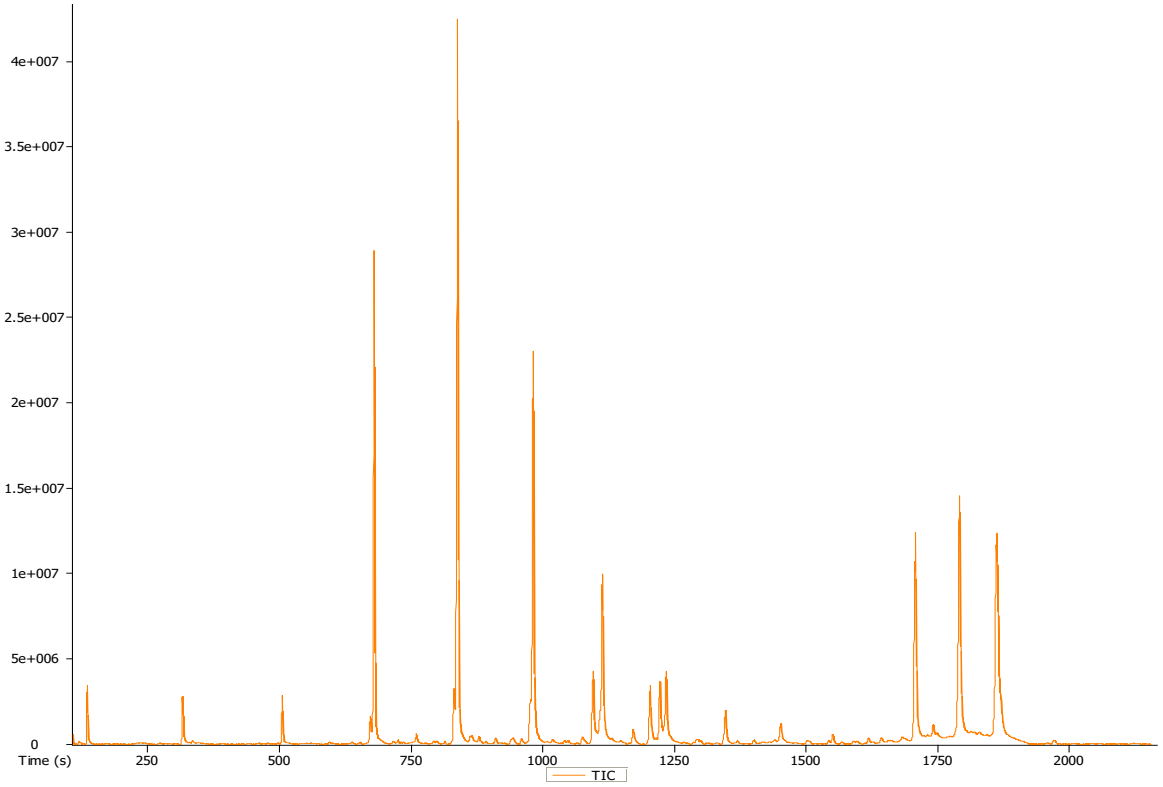


Figure 5.8 Chromatogram obtained from GC-MS of SF-2.

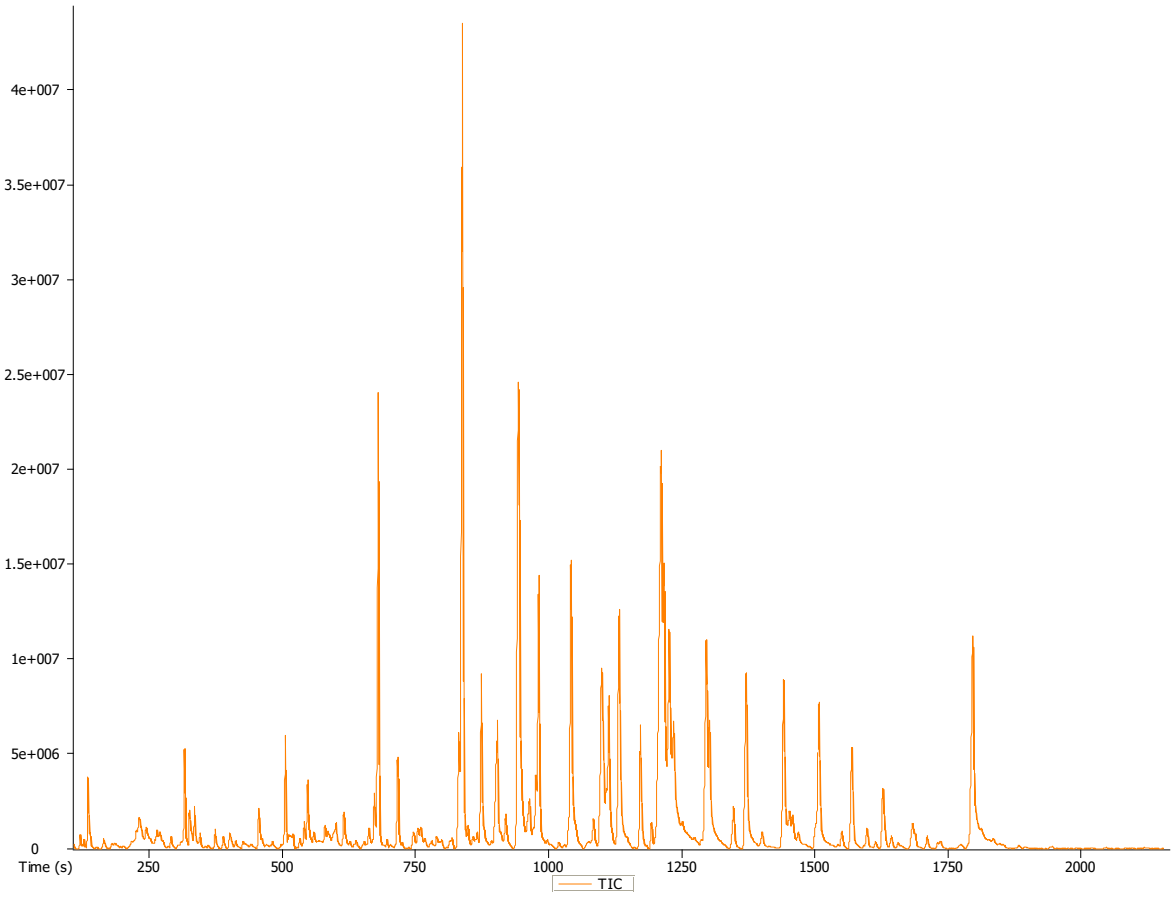


Figure 5.9 Chromatogram obtained from GC-MS of SF-7.

Table 5.4 List of compounds identified from SF-2 and their respective structures.

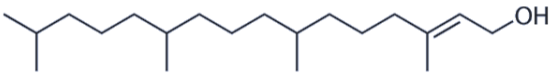
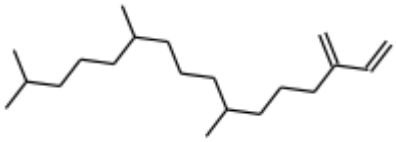
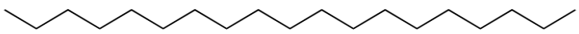
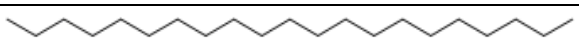
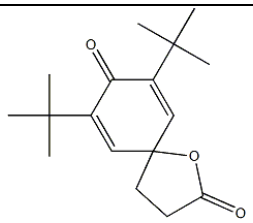
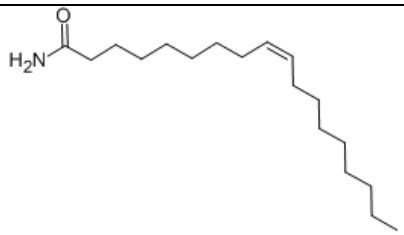
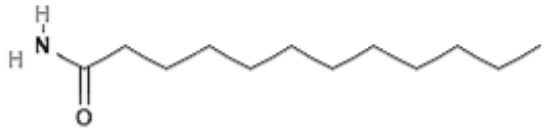
Compound	Area (%)	Similarity (%)	Structure
Hexadecane	5.75	95.00	
Nonadecane	9.40	94.90	
Heneicosane	5.76	95.70	
Tetracosane	1.44	95.00	
Phytol	3.69	92.10	
Neophytadiene	4.74	93.30	

Table 5.5 List of compounds identified from SF-7 and their respective structures.

Compound	Area (%)	Similarity (%)	Structure
Hexadecane	2.51	94.70	
Nonadecane	5.51	94.70	
Heneicosane	1.68	94.30	
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1.13	94.70	
Oleamide	4.52	90.00	

Dodecanamide	2.00	9.13	
Octadecanoic acid	1.98	92.70	

The compounds listed in Table 5.4 and 5.5 are known compounds, most of which have been isolated from other plant species.

Nonadecane, found in both sub-fractions, has previously been reported by Dandekar et al. (2015) to display high antioxidant activity, and this may explain the high activity displayed by the extract during the DPPH and ABTS assays in the previous chapter. This compound has also been reported to have antidiabetic activity by Hamidi et al. (2012) which is not within the scope of this project, but nonetheless is quite interesting. Tetracosane, found in SF-2, was also reported to have antioxidant activity, further supporting the results obtained from the antioxidant assays of the crude extract (Dandekar et al. 2015). Phytol, found in SF-2, has been reported to display antimicrobial, antifungal, anti-inflammatory, anticancer, diuretic and antimalarial activity (Dandekar et al. 2015). Additionally, octadecanoic acid found in SF-7 is reported to have antibacterial and antifungal activity (Dandekar et al. 2015). The presence of these compounds therefore explains the antibacterial and antifungal activity displayed by the extract. The compound 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione does not seem to possess any activity, but is commonly found on the surface of plants (Adeosun et al. 2013). Heneicosane also does not seem to display activity; this compound is a volatile oil constituent (Verma et al. 2011). Oleamide, identified in SF-7 is reported to be an endogenous sleep-inducing compound, first isolated from cats, but has been reported in plant species, such as *Ziziphus jujuba* Mill., as a possible chemo-preventative against Alzheimers' disease (Cravatt et al. 1995; Heo et al. 2003).

Most of the compounds identified from the two sub-fractions have no record of being isolated from any other species within the *Psychotria* genus (Yang et al. 2016). Octadecanoic acid (stearic acid) is the only exception, having been previously isolated from *Psychotria hainanensis* H.L.Li by Li et al. (2011). However, some of the compounds identified have been isolated from other species within the Rubiaceae family. Phytol for example was isolated from *Morinda lucida* Benth by Elufioye et al. (2015) and was identified to be the compound

responsible for the anticholinesterase activity possessed by the plant. Tetracosane has also been isolated from a species within the Rubiaceae, namely *Gardenia jasminoides* J.Ellis and this compound was the only alkane isolated from an essential oil originating from the flower of the species (Obuzor and Nwaokolo 2010).

5.4 Conclusion

Both sub-fractions obtained in this study display bioactivity, and have cytotoxicity values much higher than the recommended threshold. This suggests that these would be safe to use, and hence impart their specific activities without causing harm to poultry. Compounds identified from these sub-fractions are known compounds which have previously been isolated from other plant species. The compounds identified serve to confirm the activity observed in the crude extract and their non-polar nature grants an explanation for the difficulties faced during separation. With this in mind, these two sub-fractions make excellent candidates as alternative poultry feed additives. Furthermore, the antimicrobial activity coupled with the anti-inflammatory activity displayed by the sub-fractions suggests that they may also act as possible alternatives to antibiotic growth promoters.

Although these two bioactive sub-fractions are not cytotoxic, before being deemed completely safe, *in vivo* studies must first be carried out. These are necessary to investigate the effects of long-term ingestion of these products and hence should be considered in future work.

6. General discussion and conclusion

This study marks the first attempt to characterise the antifungal, antibacterial, antioxidant and anti-inflammatory activity of *Psychotria zombamontana* (Kuntze) E.M.A. Petit, as well as the first successful attempt at identifying active components. The aim of this research study was to investigate the potential of *P. zombamontana* as an alternative feed additive.

Based on the findings of this study, *P. zombamontana* is a plant possessing broad activity, with the crude extract displaying promising activity in all assays used. The crude extract showed strong activity against all microorganisms tested (MIC values of 0.125-1.0 mg/ml), as well as potent antioxidant activity together with a high phenolic content, and finally the extract was also observed to have anti-inflammatory efficacy.

Two sub-fractions were isolated from the crude extract and several compounds were identified within them. These sub-fractions were investigated for their antimicrobial activity using the bioautography method, and both displayed activity against *Fusarium verticillioides*, *Candida albicans* and *Escherichia coli*. Additionally, one sub-fraction showed anti-inflammatory activity in the LOX assay, and both sub-fractions had an effect on cytokine production. The consequences that these effects on cytokine production may have on poultry is unclear, and can only be confirmed during further *in vivo* testing. Finally, both sub-fractions were tested for cytotoxic activity, and both sub-fractions had toxicity well above threshold levels implying low cytotoxicity. Therefore, the active sub-fractions obtained from *P. zombamontana* showed antimicrobial and anti-inflammatory activity and were non-cytotoxic.

Identification of constituent compounds within the obtained sub-factions was achieved using GC-MS. Results of this analysis suggested that both sub-fractions contained a number of compounds, but only those contributing to major peaks coupled with a high similarity percentage were chosen. From the compounds chosen, several were long-chain alkanes, explaining the non-polar nature of the sub-fractions and difficulty in separating these related compounds. Some of the compounds were previously found by other researchers to be inactive, while others had certain useful properties, including antioxidant and antimicrobial activities.

The activity observed for *P. zombamontana* makes it a potential candidate as an alternative poultry feed additive and growth promoting agent. As well as the obvious benefits of antimicrobial activity, the antioxidant and anti-inflammatory activity displayed by the crude

extract could help in preventing oxidative damage to poultry meat, increasing shelf life, and additionally contributing to growth promotion of the birds (Valenzuela and Nieto 1996; Niewold 2007). The crude extract also displayed cytotoxicity above the described threshold values, deeming it non-cytotoxic. These findings are positive, increasing the possibility of small-holder farmers having their own natural product to aid in poultry farming. The farmers could potentially grow these plants as a crop, harvest material from the plants and add the ground plant material to their poultry feed prior to storage. Before this could become a reality however, further testing is necessary. It must be stated that slight cytotoxicity and a low selectivity index was displayed, and it is unclear whether this toxicity will be a significant factor once the plant material is metabolised by the birds. Hence further testing on the use of the crude material *in vivo* is needed.

Psychotria zombamontana could also contribute toward the search for alternative feed additives through the formulation of a refined commercial product. The first step in this process is to isolate and identify the compounds responsible for the desired activity. Unfortunately, complete isolation of active compounds from *P. zombamontana* was not achieved due to difficulties experienced with the yield of the sub-fractions obtained, as well as finding a suitable solvent system for separation. While individual compounds were not isolated, two active sub-fractions were obtained and some of these compounds identified within those sub-fractions have known activities which correlate with those displayed by both the sub-fractions and the crude extract.

This study also contributes toward certain Sustainable Development Goals (SDGs) as outlined by the United Nations. These specific goals include Goal 2: Zero Hunger, Goal 3: Good Health and Well Being and Goal 12: Responsible Consumption and Production. *Psychotria zombamontana* contributes toward Goal 2 by potentially reducing the losses faced by smallholder and subsistence farmers, which may increase their production of food and subsequently improve their livelihood. The use of *P. zombamontana* as a growth promoter could potentially reduce the use of antibiotic growth promoters, thereby reducing the incidence of antibiotic resistance, contributing toward Goal 3. Finally, a potential reduction in the use of chemical control agents, due to the use of *P. zombamontana* by commercial farmers, could contribute towards Goal 12 (United Nations Sustainable Development Goals 2018).

Further research outside the scope of this study will need to include the effect of the plant extract on poultry *in vivo* as well as efficacy of the plant additive in decreasing the

contamination levels of feed with mycotoxigenic and/or disease-causing microorganisms. Furthermore, the efficacy of the supplement in preventing or curing diseases in poultry caused by common contaminating fungi or bacteria may be determined. Future work should also focus on further study of the sub-fractions and active components, and their use *in vivo* through live poultry trials. Attention should also be directed towards the formulation of a product using the identified active constituents. Furthermore, activity was observed in some of the more polar sub-fractions and, though to a lesser degree, there may be additional beneficial compounds present which may contribute to a more comprehensive beneficial formulation.

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